Formation of Yeast Mitochondria. I. Kinetics of Amino Acid Incorporation during Derepression*

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ABSTRACT: We have studied the kinetics of incorporation *in vivo* of isotopically labeled phenylalanine into various subcellular and submitochondrial fractions of *Saccharomyces cerevisiae*. Both pulse and pulse-chase techniques were employed, alone and in conjunction with studies on the effect of the selective drugs chloramphenicol, cycloheximide, and antimycin A. One of the mitochondrial fractions, containing approximately 10% of the total mitochondrial protein, was found to incorporate phenylalanine as rapidly as did the cytoplasmic ribosomes and to export labeled material to other fractions. Incorporation at this site was chloramphenicol sensitive.

However, it appeared that the protein(s) synthesized at this chloramphenicol-sensitive site could account for only a small proportion of the protein even in the incorporating fraction itself. These results therefore suggest that only a small amount of protein is made within the mitochondrion; the majority of the proteins of this organelle is of cytoplasmic origin.

Studies over the past decade on a wide variety of organisms have led to the conclusion that mitochondria contain a system for the transmission and expression of genetic information distinct from that constituted by nucleus and cytoplasm. Furthermore, isolated mitochondria are now known to be capable of incorporating labeled amino acids into at least some of the insoluble proteins of the mitochondrial membranes. This incorporation is sensitive to chloramphenicol and insensitive to cycloheximide, while the converse appears to be true for cytoplasmic protein synthesis *in vitro*. For reviews of these and related topics, see Roodyn and Wilkie (1968) and Borst *et al.* (1967).

Such studies *in vitro* suffer, however, from some rather severe limitations. Incorporation rates into proteins are generally quite low, usually an order of magnitude less than those observed with the same particle fraction of intact cells. The danger of some of the observed effects because of contaminating bacterial or microsomal components imposes some rather stringent limitations of technique. The actual concentration of amino acids and cofactors (citric acid cycle substrates, purine nucleoside triphosphates, etc.) required *in vitro* may bear little resemblance to those existing around and inside the mitochondria in the intracellular milieu. Finally, and perhaps most importantly, the use of highly purified mitochondria of necessity eliminates any possibility of studying the flux of stoichiometric or regulatory materials either "inward" from nucleus plus cytoplasm into mitochondria, or "outward" from the latter to the former. For these reasons, we have chosen to extend our studies on the extent and limits of mitochondriogenesis to an investigation of the incorporation of radioactive amino acids into intact yeast cells and their derived subcellular and submitochondrial fractions.

This organism offers the following advantages. (1) It is a relatively small, simple, single-celled eukaryote capable of elaborating mitochondria of canonical structure and function. (2) It is characterized by a rapid generation time and capable of growth on simple and defined media; therefore, cells, at any stage in their growth cycle, as well as mitochondria and other subcellular fractions and organelles, can be obtained rapidly, conveniently, and in large amounts. Labeled precursors such as amino acids are taken up rapidly and their rate of incorporation into various products can be followed expediently. (3) It permits the use of well-defined nuclear and cytoplasmic genotypes for the performance of genetic crosses and the determination of the influence of genetic conditions on mitochondriogenesis (Sherman and Slonimski, 1964; Mackler *et al.*, 1965; Negrotti and Wilkie, 1968; Thomas and Wilkie, 1968a,b; Linnane *et al.*, 1968). (4) Its growth, and the development of its mitochondria, are subject to physiological regulation; for instance, when yeast is grown on 1% glucose, a period of time can be elected where aerobic glycolysis is replaced by terminal oxidation of ethanol (and acetate) as a carbon and energy source. Coincident with this change (release from glucose repression) there occurs little increase in total cell mass or protein but a rapid elaboration of functionally and structurally competent mitochondria, as well as of certain cytoplasmic enzymes (Ephrussi *et al.*, 1956; Linnane, 1965; Polakis

* Contribution No. 1627 from the Chemical Laboratory, Indiana University, Bloomington, Indiana 47401. *Received May 23, 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 Institute of Health during 1967–1968 is gratefully acknowledged.

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

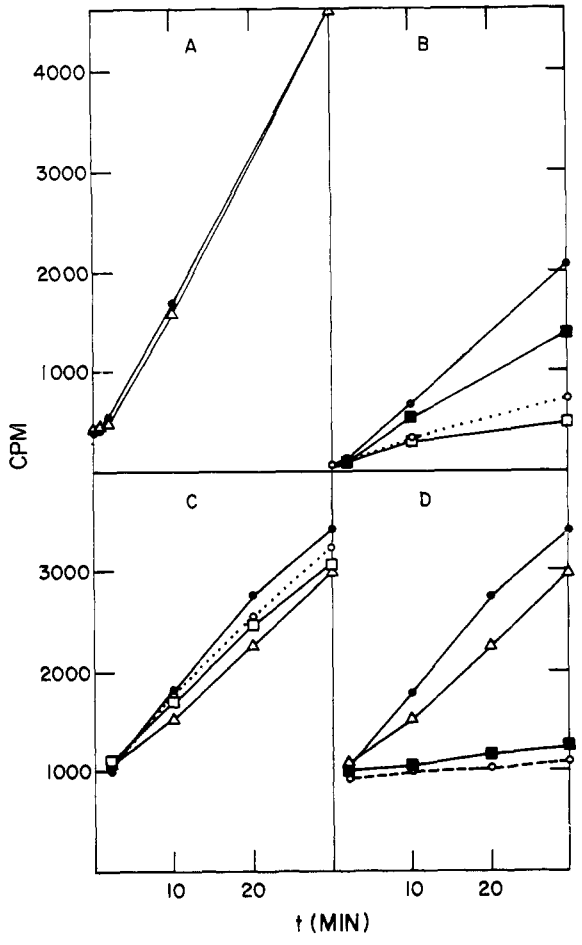


FIGURE 1: Effect of chloramphenicol and cycloheximide on L-[^{14}C]phenylalanine incorporation. Aliquots (25 ml) of cells were mixed with 1.6 μCi of labeled amino acid (80 μM in the cycloheximide experiments (B), 162.5 μM (A, C, D)) and at t = 0 the drugs were added. At the times indicated, 1-ml samples were pipetted into 10 ml of cold 5% trichloroacetic acid. After 1.5 hr at 0°, the suspensions were filtered through Millipore filters, washed, dried, and counted in a Beckman Widebeta counter. (A) Effect of chloramphenicol on repressed cells (12 hr in 5% glucose): (—Δ—) 4 mg/ml of chloramphenicol. (—●—) Control. (B) Effect of cycloheximide on derepressing cells (12 hr in 1% glucose; oxygen uptake = 75). (—■—) 1 μg/ml, (···○···) 2 μg/ml, (–□–) 3 μg/ml, and (—●—) control. (C) Effect of chloramphenicol on derepressing cells (12 hr in 1% glucose) (oxygen uptake = 115). (···○···) 1 mg/ml, (—□—) 2 mg/ml, (—Δ—) 4 mg/ml, and (—●—) control. (D) Effect of chloramphenicol plus cycloheximide on depressing cells (oxygen uptake = 75). (—Δ—) 4 mg/ml of chloramphenicol, (—■—) 5 μg/ml of cycloheximide, (---○---) 4 mg/ml of chloramphenicol + 5 μg/ml of cycloheximide, and (—●—) control.

et al., 1965; Polakis and Bartley, 1966; Witt *et al.*, 1966; Jayaraman *et al.*, 1966; Utter *et al.*, 1967).

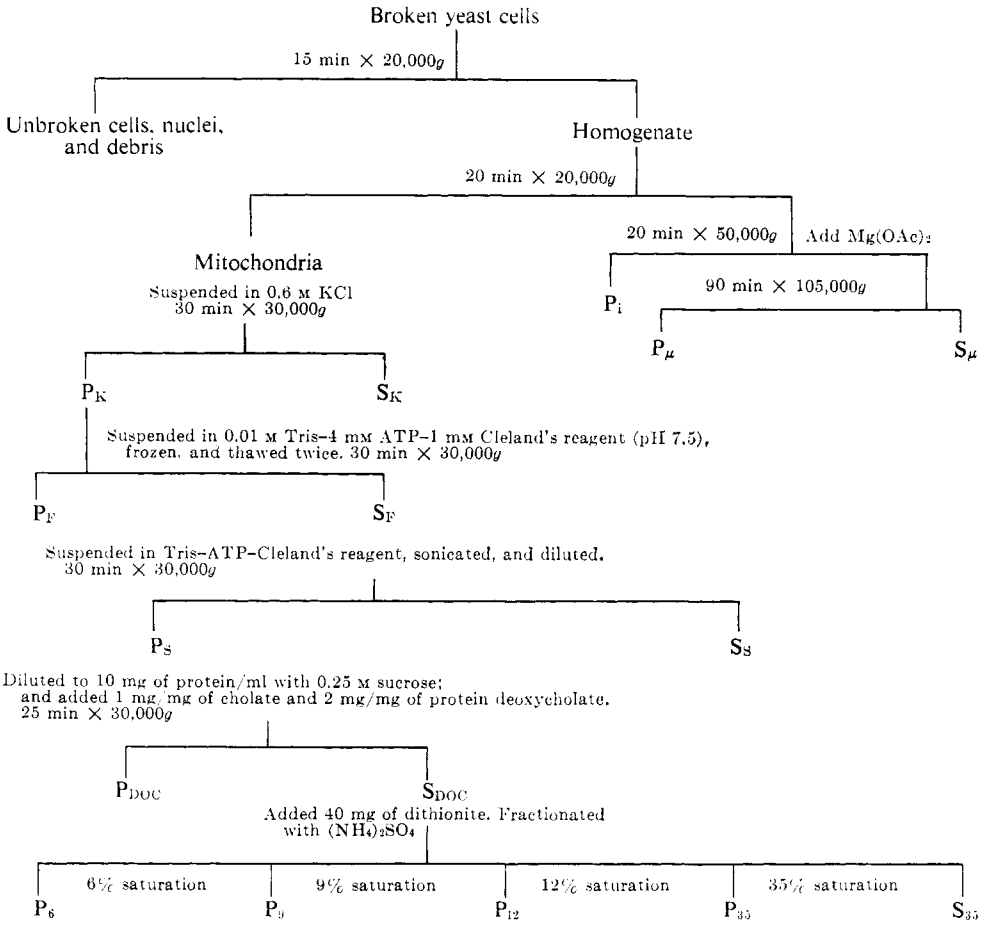
In the study reported here we have investigated the synthesis of mitochondrial proteins during this derepression to determine the limits of the independence of these synthetic events from those in the cytoplasm, and to provide information on the products of mitochondrial synthesis. Other studies, both *in vivo* (Haldar *et al.*, 1966, 1967; Beattie *et al.*, 1966) and *in vitro* (Roodyn *et al.*, 1961; Beattie *et al.*, 1967; Neupert *et al.*, 1967; Kadenbach, 1967a), had already provided evidence that the main products form part of the inner membrane system, but had not determined whether all the proteins of this membrane share the same origin, nor whether any other mitochondrial proteins are synthesized by the particle. We have approached the problem by a variety of means, all employing incorporation of radioactive amino acids and designed to test the following hypothesis. We propose that the initial products of mitochondrial protein synthesis will have the following characteristics. (1) On exposure of cells to radioactive amino acids these products will become rapidly labeled with an initial increase in specific radioactivity perhaps comparable with that of the microsomal fraction of the cytoplasm, and higher than that of other mitochondrial proteins. (2) After exposure to a short pulse, followed by a chase with unlabeled amino acids, their radioactivity will remain constant, or decrease and appear in other fractions; and (3) their synthesis will be inhibited by chloramphenicol but not by cycloheximide. Lacking a technique that provides separation of individual proteins, we have attempted instead to find a defined submitochondrial fraction exhibiting some or all of these characteristics.

Results and Discussion

Preliminary Experiments. Linnane and his collaborators (Linnane *et al.*, 1967; Huang *et al.*, 1966; Clark-Walker and Linnane, 1966, 1967) have reported that yeast cells are capable of normal growth in the presence of chloramphenicol as long as glycolysis is the predominant metabolic pattern but that they fail to produce cytochromes *a*, a_3, and *b* and therefore are incapable of respiration. We have made a detailed study of the derepression of mitochondrial development, respiratory activity, and the levels of a number of characteristic mitochondrial enzymes and the effect of chloramphenicol and cycloheximide on these processes (Jayaraman *et al.*, 1966; Mahler *et al.*, 1968; Henson *et al.*, 1968; South and Mahler, 1968). Under our particular conditions, with 1% glucose as the carbon and energy source, aerobic glycolysis is the predominant metabolic pattern during the first 11-hr growth.[1] During this, the exponential phase, mitochondrial development and activity are severely curtailed due to glucose repression. Chloramphenicol (at concentrations $\leq$4 mg/ml) has no effect on growth rate (as measured by OD_{600}, or cell protein) and little on enzyme activity. After 11 hr the glucose concentration drops to <0.1% (Henson *et al.*, 1968) and the cells go into early stationary phase, during which there is a decrease in growth rate to $\leq$20% of that in exponential phase accompanied by a significant augmentation of the activity of a large number of mitochondrial enzymes, of the amount of cytochromes, and of mitochondrial structures with an attendant switch-

[1] The particular medium used, as well as the response curves relating oxygen uptake and the activity of a number of derepressible enzymes to growth rate and glucose concentration (Mahler *et al.*, 1968; South and Mahler, 1968; Henson *et al.*, 1968), ensures that the cells are in a state of continuously increasing, rather than one of fluctuating, respiratory activity (Ohaniance and Chaix, 1964).

SCHEME I: Fractionation Scheme Used in These Experiments.[a]



[a] By means of this scheme, the following marker enzymes are concentrated (both by total and specific activities) in the fractions indicated: cytochrome oxidase and antimycin-sensitive DPNH–cytochrome *c* reductase in the mitochondria; TPNHc cytochrome *c* reductase in P_μ (with considerable activity also in P_i); DPNH–ferricyanide reductase and L-malate dehydrogenase in both mitochondria and S_μ. The mitochondrial fraction contains both intact and damaged mitochondria, but its enzymatic activities are equivalent to those observed with the more homogeneous mitochondrial preparations obtained from spheroplast lysates, and it is relatively free of other membranous components, at least in derepressed cells. Most mitochondrial fragments and a variety of other membranes are concentrated in P_i. Cytochrome oxidase and antimycin-sensitive DPNH–cytochrome *c* reductase remain associated with particulate fractions through P_S. The bulk of the mitochondrial L-malate dehydrogenase is liberated into S_{FT} and the bulk of the mitochondrial ATPase into S_S. From their difference spectra, P_{12} is rich in cytochromes *b* and c_1, and P_{35} in cytochromes *a* and a_3. DOC = deoxycholate.

over to respiratory metabolism. This derepression is virtually complete in 6 hr at 30° and can conveniently be monitored by any of the parameters just cited, including the ability of cells to take up oxygen: this capability, here called oxygen uptake,[2] rises from a value of <50 μmoles/min per mg of cell protein at t = 11 hr to ~250 μmoles/min per mg of protein by t = 17 hr.[1] Derepression is inhibited in characteristic fashion by both chloramphenicol (added at any $t = \leq 11$ hr) and cycloheximide (added at t = 11 hr) (Mahler *et al.*, 1968; Henson *et al.*, 1968). Hence some stage, at least, of this process requires active protein synthesis by two separate systems and is not likely to be due to enzyme activation, *e.g.*, the attachment of prosthetic groups to their respective apoproteins, or of soluble proteins to an insoluble matrix.

The effect of the addition of chloramphenicol and cycloheximide on protein synthesis by whole cells, as measured by the incorporation of labeled phenylalanine, is shown in Figure 1. Repressed cells, grown for 12 hr in 5% glucose (Clark-Walker and Linnane, 1967), exhibited no inhibition of incorporation by chloramphenicol at 4 mg/ml (Figure 1A), in accord with the absence of any effect of the inhibitor on growth rate. With derepressing cells, however (12 hr on 1% glucose, oxygen uptake ≃ 100 μmoles/min per mg of protein), chloramphenicol inhibited the rate of protein synthesis by 22% (Figure 1B), while cycloheximide at concentrations of 5 μg/ml inhibited it by 91% (Figure 1C). Both inhibitors together reduced incorporation to 6.1% of the control (Figure 1D). Since the effect of both drugs together is greater than that of either alone, each agent probably

[2] Oxygen uptake is measured in micromoles per minute per milligram of cell protein at 30°.

TABLE I: Distribution of Phenylalanine Label at Isotopic Equilibrium.[a]

Homogenate	Total cpm[a] 4,340,000	% Recov (100)	
S_μ (cytoplasmic, soluble)	1,810,000	41.6	
P_μ (microsomes)	400,000	9.2	
P_i (mitochondrial fragments, etc.)	1,280,000	29.4	
Mitochondria (Mit)	551,000	12.7	(100)
$\Sigma(S_\mu + P_\mu + P_i +$ Mit)	4,041,000	92.9	
S_K (KCl extract)	86,800		15.7
P_K (residue)	476,000		86.3
S_F (extract after freeze–thaw)	50,000		9.1
P_F (residue)	372,000		67
S_S (extract after sonication)	76,400		13.8
P_S (residue)	316,000		52.3
$\Sigma(P_S + S_K + S_F + S_S)$	528,000		90.6
S_{DOC} (bil salt extract)[b]	212,000		(100)
P_6 (precipitate with 6% $(NH_4)_2SO_4$)	62,300		29.5
P_9 (precipitate with 9% $(NH_4)_2SO_4$)	20,600		9.7
P_{12} (precipitate with 12% $(NH_4)_2SO_4$)	26,800		12.5
P_{35} (precipitate with 35% $(NH_4)_2SO_4$)	34,900		16.4
S_{35} (soluble in 35% $(NH_4)_2SO_4$)	9,460		4.5
$\Sigma(P_6 + P_9 + P_{12} + P_{35} + S_{35})$	153,200		72.6

[a] Samples were labeled 2 hr with L-[^{14}C]phenylalanine and fractionated as in Scheme I. The values shown are averages for eight fractionations. Since the isotope distribution between various fractions does not change significantly between 30 and 120 min, we assume that isotopic steady state has been attained by that time. [b] DOC = deoxycholate.

inhibits a different protein-synthesizing system, thus confirming the results obtained *in vitro* (Siegel and Sisler, 1965; Wintersberger, 1965; de Kloet, 1965, 1966; Linnane *et al.*, 1967; Mahler *et al.*, 1967; Lamb *et al.*, 1968). The total inhibition exerted by the two inhibitors acting in concert is less than that expected by arithmetical summation, which suggests that the two systems may not be quite as independent from each other as the results *in vitro* predicted. These experiments, together with the response curves of release from glucose repression (Henson *et al.*, 1968; Mahler *et al.*, 1968; South and Mahler, 1968), provide the rationale for the use of these two drugs with cells during derepression.

Several previous studies indicated that the structural proteins of the inner membrane might be synthesized by the particles themselves (Roodyn *et al.*, 1961; Haldar *et al.*, 1967; Beattie *et al.*, 1967), and that these proteins accounted for a sizable portion of the insoluble proteins of the particle (Green *et al.*, 1961; Richardson *et al.*, 1963; Kopaczyk *et al.*, 1966; Woodward and Munkres, 1967). We therefore first attempted to isolate a structural protein fraction from inner membrane using a fractionation scheme based upon experiments by Sharp (1966) which indicated that treatment of mitochondria with the nonionic detergent, Trition X-100, preferentially solubilized outer membranes (see Materials and Methods). In this study, 200 μCi of ^{3}H-reconstituted protein hydrolysate was added to 250-ml aliquots of yeast grown 12.6 hr on 1% glucose. Five minutes later, either chloramphenicol or cycloheximide was introduced and incorporation was continued for 15 or 45 min. Cycloheximide caused a profound inhibition of synthesis of all five classes of mitochondrial proteins (P_K, P_{ou}, S_{ou}, P_{in}, and S_{in}; for definitions, see Materials and Methods), while the synthesis of all except perhaps one fraction was relatively unaffected by chloramphenicol. Thus this system was not sufficiently sensitive to permit detection of fractions susceptible to selective inhibition by the two drugs.

Kinetics of Labeling and Experimental Design. These preliminary experiments indicated that mitochondrial protein synthesis proper accounts for only a relatively small proportion of the total mitochondrial protein. In studying the problem further, we divided the mitochondrial fraction into a total of 13 subfractions by means of a modification of a procedure published by Richardson *et al.* (1964) and Kopaczyk *et al.* (1966) for preparing structural protein. It employed KCl extraction, followed in turn, by freezing and thawing and by sonification in Tris–ATP–Cleland's reagent to remove readily solubilizable proteins (about 43% of total) from the mitochondria. The residue was then suspended in cholate plus deoxycholate (1 and 2 mg per mg of protein, respectively). That portion which remained in solution after 25 min at 30,000*g* (about 70% of the total insoluble protein remaining after freezing and sonication) was further fractionated with $(NH_4)_2SO_4$ (Scheme I, Materials and Methods, and Table I).

For incorporation studies, phenylalanine was substituted for the labeled protein hydrolysate. This amino acid was found to be incorporated into both mitochondrial and nonmitochondrial fractions of derepressing cells without significant lag. When its addition at t = 0 min was followed by a chase with a 100-fold excess of cold phenylalanine added at 0.75, 1.5, or 3 min and maintained for 15 min thereafter, the rates of incorporation (cpm incorporated $\times$ min^{-1}) during this 15-min chase period were 1.13, 0.95, and 1.01% of the pulse rate for mitochondria and 1.06, 1.15, and 1.14% of the pulse rate for the supernatant. Therefore since none were significantly different from the theoretical rate of 1.00%, the "cold" chase was fully effective.

In order to obtain maximal sensitivity, cells were first

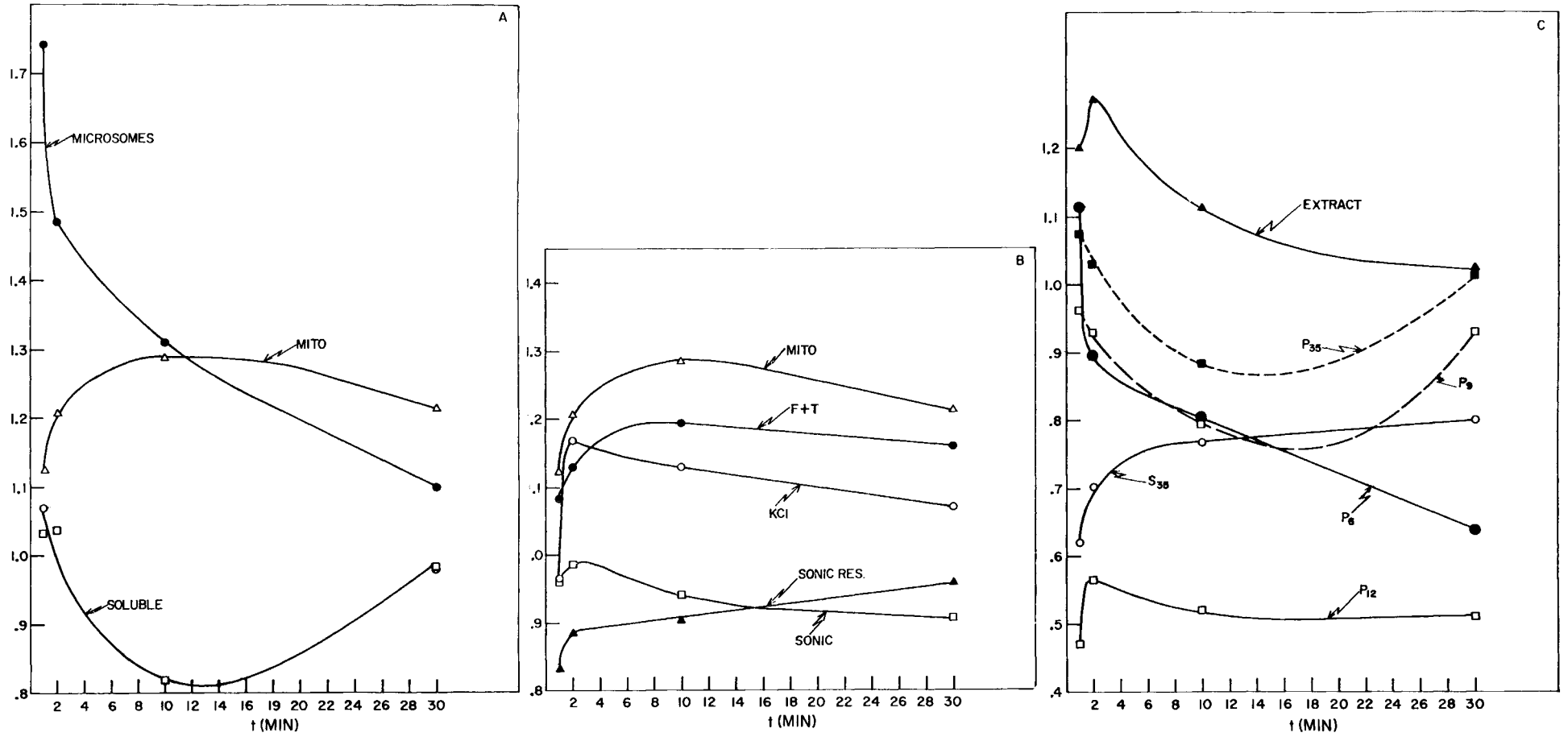


FIGURE 2: Distribution of activities in subcellular and submitochondrial fractions (Scheme I) during continuous incorporation. The data are presented as the ratio of the $^3H/^{14}C$ values for a fraction (T/C) divided by the $^3H/^{14}C$ value for the homogenate for any one time. (A) Subcellular fractions: (—●—) microsomes, P_μ; (—□—) soluble, S_μ; (—△—) mitochondria; and (—○—) membranes, P_i. (B) Easily solubilized mitochondrial fractions: (—○—) KCl extract, S_K; (—● —) frozen and thawed extract, S_F; (—□—) extract after sonication, S_S; (—▲—) residue after sonication, P_S; (—△—) mitochondria. (C) Fractions solubilized by bile salts and precipitated by $(NH_4)_2SO_4$: (—●—) P_6 (6%); (—□—) P_9 (9%); (—□—) P_{12} (12%); (—■—) P_{35} (35%); (—○—) soluble in 35%, S_{35}; (—▲—) S_{DOC} (total extract). The left ordinate is $(T/C)/(T_{(Hom)}/C_{(Hom)})$.

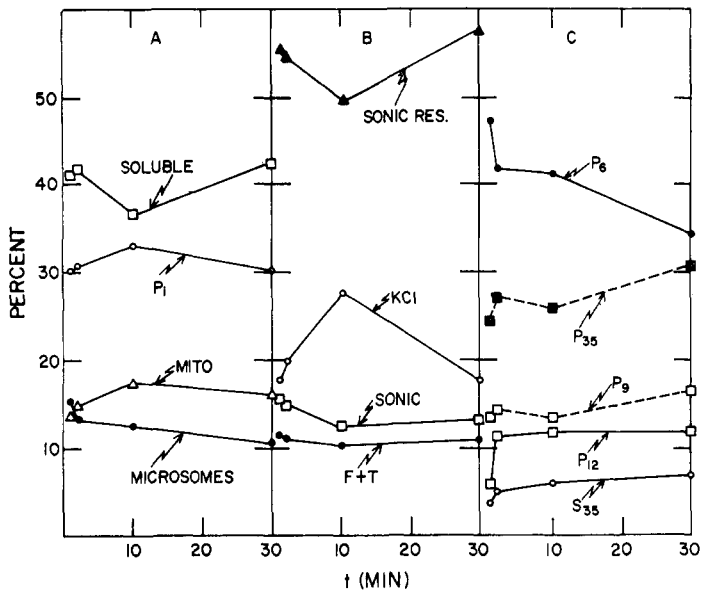


FIGURE 3: Distribution of L-[^{3}H]-phenylalanine during a 30-min labeling period. Cells labeled for various times with ^{3}H-labeled amino acid and then mixed with cells labeled for 2 hr with L-[^{14}C]phenylalanine. This mixture was fractionated by Scheme I. The percentages shown were obtained by taking the ratio of the total tritium counts incorporated by a fraction (normalized to an average ^{14}C value for that fraction) to the sum of the counts incorporated by the fractions making up the homogenate (A), the mitochondria (B), or a cholate–deoxycholate-soluble fraction of the mitochondria (C). For designations, see Table I and Figure 2.

labeled with L-[^{3}H]phenylalanine during the experiment proper, then mixed with equal aliquots of other cells labeled with L-[^{14}C]phenylalanine for 2 hr for kinetic, and for either 5 or 20 min for inhibition studies, and fractionated according to Scheme I. Results are expressed in terms of ^{3}H/^{14}C ratios or as the ratio of this ^{3}H/^{14}C value for a sample point to that for a control. The ^{3}H/^{14}C values are equivalent to specific radioactivities calculated on a protein basis: they correct the data for random variations in cell breakage, sample loss on plating, etc.; and ^{14}C counts are, in general, a better means for determining protein concentration than are the micromethods available. The standard deviation for each set of 17 fractions (whole homogenate plus 16 subfractions) was determined for each experiment by comparing the ^{3}H/^{14}C ratios of each set from cells labeled for 2 or 10 min for kinetic studies and 5 or 20 min for inhibition studies with both [^{3}H]- and L-[^{14}C]phenylalanine. For the kinetic experiments, with 2- and 10-min points, the standard deviation in each set was 20% of the average value. Significantly, the largest deviations from the average occurred with the fractions obtained after solubilization with cholate–deoxycholate (6 of these 12 fractions had ^{3}H/^{14}C ratios more than 1.5 standard deviations from the average). While all of the counts had been corrected for quenching by the three-channel method of Hendler (1964), apparently the chloroform used as the standard quencher in determining the corrections did not behave quite like the detergent-treated mitochondrial material. However this type of error should be constant for a given fraction and should therefore disappear for the ratio of two values from the *same* fraction. Indeed, when the 2-min ^{3}H/^{14}C ratios were divided by the 10-min values, a normal population of double ratios was obtained with 6 of the 17 values falling between one and two standard deviations, and with none falling more than two standard deviations from the average. The standard deviation of the double ratio (0.097) was only 12% of the average value, and the variances were about one-tenth of those for the ^{3}H/^{14}C values. For this reason, much of the data is presented as the ratio of the ^{3}H/^{14}C ratios for any sample point to that of an appropriate control for that particular mitochondrial fraction.

Kinetics of Labeling. CONTINUOUS INCORPORATION (Figure 2). Kinetics of labeling have long been used to identify precursor–product relationships in protein synthesis. For continuous incorporation studies, we allowed cells grown to the later stages of derepression (t = 13.5 hr, oxygen uptake = 180) to incorporate L-[^{3}H]phenylalanine for 1, 2, 10, or 30 min. Incorporation into the total homogenate was linear over the 30-min period, with the mitochondrial fraction at isotopic steady state accounting for some 13% of the homogenate value (Table I). To obtain the data of Figure 2, the ^{3}H/^{14}C ratio for each fraction was divided by the corresponding ratio for the homogenate in order to aid in the ready identification of those fractions which are potential sites of protein synthesis. A horizontal straight line with a value of unity indicates a fraction with incorporation kinetics that do not deviate significantly from those of the average of the whole cell, *i.e.*, one in which export and import remain balanced throughout the time of interest. Positive slopes indicate import, negative ones, export.[3]

As in similar experiments with other cells (Keller *et al.*, 1954; Littlefield *et al.*, 1955; Rabinovitz and Olson, 1956; Haldar *et al.*, 1967), P_μ, the microsomal fraction, is a prime candidate for the site of cytoplasmic protein synthesis in yeast (Hauge and Halverson, 1962). It exhibits a very rapid rate of initial labeling, faster than that of any other fraction, followed by a gradual loss of label coincident with export to other components. Of all the submitochondrial fractions investigated, only P_6, the most insoluble fraction of the membrane proteins

[3] While the shape of the curve for each fraction is correct, each value on the curve (and also each slope) must be multiplied by some unknown constant. For instance, the specific activities of the detergent-treated fractions of the mitochondria are lower than that of the supernatant from which they were derived.

TABLE II: Effect of a Standard Chase and a Chase in the Presence of Inhibitors following a 1-min Pulse with L-[^{3}H]Phenylalanine.[a]

	Std Chase/ Theor Chase	% *P*	Chloramphenicol in Chase/ Chase	% *P*	Cycloheximide in Chase/ Chase	% *P*
Homogenate	1.07		0.824	8.8	0.716	1.2
S_μ (soluble)	1.07		1.35	0.2	0.790	4.4
P_μ (microsomes)	0.774	3.4	1.10		0.968	
Mitochondria	1.15		1.01		0.868	
S_F (freeze–thaw)	1.18	9.2	1.13		0.793	
P_S (sonic)	1.22	4.0	1.10		0.917	
S_{DOC} (bile salt)	1.03		1.72	<0.05	1.04	
P_6	0.760	2.6	1.89	<0.05	1.10	
P_9	0.945		2.47	<0.05	1.49	<0.05
P_{12}	2.05	<0.05	1.47	<0.05	2.06	<0.05
P_{35}	1.45	<0.05	1.89	<0.05	2.13	<0.05
S_{35}	1.70	<0.05	2.50	<0.05	2.40	<0.05

[a] The ^{3}H/^{14}C values for each sample point have been divided by those for a corresponding fraction of a control. Thus the ratios for a chase in the presence of 2 mg/ml of chloramphenicol or 0.625 μg/ml of cycloheximide have been divided by those for a standard chase without inhibitor. The theoretical chase values were calculated on the basis of those for a 1-min pulse making the assumption that 100-fold dilution of the label by unlabeled amino acid results in a decrease in ^{3}H incorporation to 0.010 the initial rate. The probability, *P*, that a value is not different from 1 was calculated using a standard deviation of 0.097. This same standard deviation can be used to calculate the probability that any fraction in the table is significantly different from any other, *i.e.*, that the double ratio for P_6 is different from that for the homogenate. Values for *P* which were greater than 10% are not indicated.

of the particle, shows an analogous labeling pattern. There is evidence for rapid import into certain submitochondrial fractions (P_S and S_{35}), as well as of flux through (*i.e.*, both import and export) the soluble portion of the cytoplasm (S_μ), and through most of the easily solubilized submitochondrial fractions. These conclusions are strengthened by an examination of the rate of change of the *total* activities in the various fractions (Figure 3).

Although providing clear indication of import by many mitochondrial components, the data do not permit a decision as to its source. The two likely candidates are P_6, and the microsomes, P_μ. Even at the shortest time tested, however, P_6 represents only 1.6% of the protein label of the whole homogenate, while P_μ and S_μ account for 15.4 and 41.1%, respectively (Figure 2). From similar experiments, Beattie *et al.* (1966) concluded that at least some of the soluble mitochondrial proteins were exported into the mitochondria from the cytoplasm.

Kinetics of Labeling. PULSE-CHASE STUDIES. Cells were pulsed for 1 min with labeled phenylalanine and then mixed with a 100-fold excess of unlabeled phenylalanine, either alone or in the presence of 2 mg/ml of chloramphenicol or 0.625 μg/ml of cycloheximide, and allowed to grow for an additional 29 min (*cf.* Diagram I; Materials and Methods). The rate of incorporation during a 1-min pulse period was used to calculate the amount of incorporation expected at the end of the 29-min chase period, with the assumption (see preceding section) that incorporation during the chase should proceed at 0.010 times the pulse rate. Table II shows the ratio of the *actual* ^{3}H/^{14}C value over the *calculated* value for this parameter for each fraction, as well as the level of significance of the deviations of this calculated double ratio from unity, the value expected if incorporation into each fraction did proceed at the theoretical rate. The expectation is that a fraction such as P_μ, the microsomes, which synthesizes and exports proteins to other parts of the cell, will have a double ratio significantly less than unity, while a fraction which imports proteins will have a double ratio greater than unity. The results are in good agreement with those of the continuous-labeling studies. P_μ and P_6 are the only fractions showing significant export while various insoluble submitochondrial fractions all show significant import.

The presence of cycloheximide during the chase period had no effect on export from either P_μ or P_6. Therefore the presence of this inhibitor does not prevent release of nascent protein to other parts of the cell. Since it is specific for cytoplasmic protein synthesis, it would not be expected to affect protein transport from P_6, the mitochondrial synthesis site. Similarly, the presence of chloramphenicol during the chase would not be expected to have any effect on the export of proteins from the microsomes, and no change was found in P_μ. Chloramphenicol does cause a change in P_6, however, from a pattern of export in its absence to one of import in its presence. We interpret this as an indication that the amount of protein synthesized by and attached to the P_6 particles is small. When the rate of synthesis by this frac-

TABLE III: Effect of 1 μg/ml of Antimycin A on a 10-min Pulse and on a Chase following a 1-min Pulse.[a]

	Anti (10 min)/10-min Pulse	Chase + Anti/1-min Pulse
Homogenate	0.130	0.634
S_μ (cytoplasmic, soluble)	0.236	0.576
P_μ (microsomes)	0.181	0.636
P_i (mitochondrial fragments etc.)	0.110	0.546
Mitochondria	0.0844	0.675
S_K (KCl extract)	0.0816	0.631
S_F (extract after freeze–thaw)	0.0779	0.636
S_S (extract after sonication)	0.0744	0.509
P_s (residue)	0.0719	0.635
S_{DOC} (bile salt extract)	0.133	0.651
P_6 (precipitate with 6% $(NH_4)_2SO_4$)	0.198	0.565
P_9 (precipitate with 9% $(NH_4)_2SO_4$)	0.144	0.720
P_{12} (precipitate with $(NH_4)_2SO_4$)	0.171	0.904
P_{35} (precipitate with $(NH_4)_2SO_4$)	0.207	1.01
S_{35} (soluble in 35% $(NH_4)_2SO_4$)	0.181	1.07

[a] The values in this table were obtained by dividing the $^3H/^{14}C$ values for the antimycin samples by those for the corresponding fractions from either a 10- or a 1-min control pulse. The standard deviation to be used for evaluating probability functions is 0.097. Anti = antimycin A.

tion is stopped or slowed by means of chloramphenicol, it becomes apparent that the major portion, even of its proteins is imported from the cytoplasm.

Effect of Blockage of Respiration. ANTIMYCIN. When antimycin A (at a concentration of 1 μg/ml) is added to a suspension of yeast cells, energy production as measured by oxygen uptake stops immediately (or at least within the response time of a Gilson oxygraph). Therefore, this compound can provide a means for studying the energy requirements for protein transfer in our system. As illustrated in Table III, cells preincubated with this drug for 2 min before a 10-min pulse and fractionated as described in the previous experiments, incorporated L-[^{3}H]phenylalanine at approximately 10% of the control rate. However, when antimycin was added following a 1-min pulse period together with a 100-fold excess of cold amino acids, after an additional 29-min growth most of the cellular fractions had lost activity, and therefore their proteins had presumably become degraded to acid-soluble materials. These data therefore provide information required for the calculation of turnover rates. The fact that neither P_μ nor P_6 appears to have lost a greater proportion of the phenylalanine incorporated than the bulk of the cellular material tends to imply that protein export from these fractions also requires energy. Interestingly, degradation does not appear to have occurred in the P_{12}, P_{35}, or S_{35} mitochondrial fractions (*cf.* Wheeldon and Lehninger, 1966).

Effects of Inhibitors. PULSE LABELING. Additional clues concerning the site of mitochondrial protein synthesis can be derived from studies on the effects of selective drugs on incorporation into various fractions. We therefore preincubated cells with drugs for 2 min, pulsed them with L-[^{3}H]phenylalanine for an additional 5 or 20 min, and mixed them with samples labeled for the corresponding period with the ^{14}C-labeled amino acid in the absence of drug (Diagram II; Materials and Methods). Short pulse times were used to minimize aberrations due to the action of cycloheximide (de Kloet, 1966; Fukuhara, 1965) or chloramphenicol (Maaloe and Kjeldgaard, 1966) on RNA synthesis. Furthermore the sensitivity to chloramphenicol was tested in the presence of cycloheximide. Since the bulk of the protein synthesis in these cells is ribosomal and, hence, sensitive to cycloheximide, this paradigm should greatly reduce the noise level, so that chloramphenicol inhibition, which effects only a relatively small proportion of the total protein, can be rendered more visible and its differential effect on various fractions, if such exists, established.

Seventeen fractions were again obtained from each sample. Double ratios were calculated as described earlier to provide estimates of significance and to eliminate any residual quench variations. The standard deviation of the double ratio (20-min $^3H/^{14}C$)/(5-min $^3H/^{14}C$) was 11% of the average value, while the standard deviations for the 20- and 5-min $^3H/^{14}C$ ratios were 13 and 16% of the average, respectively. The absolute value of the standard deviation of the double ratio, 0.17, valid for all double ratios calculated in this experiment, was used to assess the significance of all the data in Table IV.

Effect of Inhibitors. LABELING PATTERNS. If the most insoluble protein fraction of the mitochondria is indeed the site of mitochondrial protein synthesis, amino acid incorporation into this fraction in the presence of chloramphenicol plus cycloheximide should be significantly lower than that in the presence of cycloheximide alone. As Table IV shows, the least soluble fraction did indeed behave in this fashion. This effect was seen in both the sample labeled for 5 min and the one labeled for 20 min. In a 5-min pulse, chloramphenicol exerted a significant inhibition on this fraction even when compared with a control with no inhibitor added. Several other mitochondrial fractions also showed significant inhibition by chloramphenicol under these conditions but only the least soluble was inhibited to a level significantly lower than the average cell protein.

Nearly all of the fractions including the soluble portion of the cytoplasm showed a suggestive, though statistically not highly significant, inhibition by chloramphenicol. Because of the known specificity of this inhibitor *in vitro*, this *in vivo* inhibition, if real, indicates that either (1) some proteins synthesized within the mitochondria

are exported to all parts of the cell, or (2) when mitochondrial protein synthesis is inhibited, a signal is transmitted from the mitochondria to alter cytoplasmic protein synthesis. Since the extent of the informational capacity of mitochondrial DNA, as well as its expression, appear limited to relatively few proteins (Tewari *et al.*, 1966; Borst *et al.*, 1967; Roodyn and Wilkie, 1968; Mehrotra and Mahler, 1968), the first explanation seems unlikely. We have no information on whether the hypothetical signal affects all of cytoplasmic protein synthesis or only those proteins that are to be exported from the microsomes to the mitochondria.

Conclusions

In order to derive meaningful conclusions concerning sites of synthesis from some of the isotope experiments described here certain conditions must be satisfied (Kuehl, 1967). (a) Cell fractions must be obtained without loss, gain, or redistribution of label; (b) added amino acids must reach the pools surrounding the synthesis site simultaneously and dilution by these preexisting pools must not be grossly different; and (c) any hypothetical movement from a synthesis to a utilization site must be relatively slow compared with the rate of synthesis itself. The details of the fractionation procedure used, enzyme distribution data (Henson *et al.*, 1968), and the absolute energy requirement for both incorporation and decay make it almost certain that condition a is fulfilled. That condition b is met is indicated by the absence of a lag in either incorporation or chase. Some of the parameters concerning c will be discussed in the next paragraph.

With these provisos these labeling experiments provide evidence that two fractions, namely, the microsomes and P_6, the most insoluble of the mitochondrial subfractions, can qualify on kinetic grounds as sites of protein synthesis and export. These two fractions not only resemble one another in continuous-labeling and pulse-chase experiments but they differ radically from all other fractions. Our interpretation is that these two fractions represent two independent sites of protein synthesis, P_6 being comparable with the mitochondrial protein synthesis site observed in incorporation studies *in vitro*. A possible alternative explanation postulates that the microsomal fraction is the only site of protein synthesis and that this fraction, with only a very short transport time intervening, directly furnishes all the proteins of the insoluble protein fraction or, in other words, that P_6 merely represents a redistribution center. Two lines of evidence tend to rule out this hypothesis, however. (1) To date, there is no evidence that easily solubilized mitochondrial proteins, synthesized by the ribosomes, must become first and rapidly attached to an insoluble component as an essential step in the transport process. In studies on the mechanism of this transport *in vitro* Kadenbach (1967a) reported that the rate of transfer of proteins from microsomes to mitochondria was slow compared with microsomal protein synthesis, and that the proteins transferred were found directly in the easily solubilized fraction of the mitochondria. Similarly he (1967b), Freeman *et al.* (1967), and González-Cadavid and Campbell (1967) all found that cytochrome *c*, synthesized by a component of the microsomal fraction, was transported only relatively slowly into the mitochondria. (2) In our own experiments, the most insoluble mitochondrial fraction was also the one most sensitive to chloramphenicol. Inhibition by chloramphenicol is one of the characteristic properties of the mitochondrial protein-synthesizing system *in vitro* (Wintersberger, 1965; Campbell *et al.*, 1966; Linnane *et al.*, 1967; Mahler *et al.*, 1967; Lamb *et al.*, 1968) and mitochondriogenesis is blocked by chloramphenicol *in vivo* (Clark-Walker and Linnane, 1967).

The rate and extent of synthesis we have been observing in P_6 can account for only a small proportion of all the proteins required for mitochondrial biogenesis. In *vitro* studies have provided some evidence that the major product of mitochondrial incorporation is found in a "structural" protein fraction (Roodyn *et al.*, 1961; Roodyn, 1962; Truman, 1964; Wheeldon and Lehninger, 1966; Beattie *et al.*, 1967; Neupert *et al.*, 1967). In our hands, the total protein in this fraction itself can account for only 11% of the mitochondrial protein at isotopic steady state (Table I); and from pulse-chase experiments with chloramphenicol and pulse-labeling studies in the presence of either chloramphenicol or cycloheximide, it is likely that the major portion of even this fraction is synthesized in the cytoplasm. Although we have not attempted to estimate the amount or destination of protein exported from P_6, it is not likely that it can provide for the majority of other mitochondrial proteins. On the other hand we estimate that the initial rate of amino acid incorporation into the mitochondrion is quite high (see also Jayaraman *et al.*, 1966), of the same order of magnitude as the rate of incorporation by microsomes.

Despite the small total amount of protein produced by the mitochondrial system, however, it is also apparent that the mitochondrial and cytoplasmic systems are closely linked. As already discussed in the results section, while chloramphenicol has no effect on phenylalanine incorporation into repressed cells (and no nonspecific effect on the oxygen uptake or enzymatic activities of depressed cells even after a 25-min preincubation), chloramphenicol added to depressing cells reduces the phenylalanine incorporation of *all* fractions and thus of total cellular synthesis by some 20%. Further insight is provided by enzyme data from which it is apparent that activities of enzymes of cytoplasmic origin, but resident at the time of assay both in the mitochondria and the cytoplasm (*e.g.*, L-malate dehydrogenase, NADH: ferricyanide reductase), are affected by the drug (Mahler *et al.*, 1968; Henson *et al.*, 1968). For these reasons, we propose that instead of the exclusively structural role previously ascribed to it, the product(s) of mitochondrial synthesis also fulfills a regulatory function, perhaps concerned with the integration of mitochondrial proteins, regardless of origin, a process which in turn either directly or indirectly affects the rate and extent of all protein synthesis during derepression. It should also be mentioned that the electrophoretic pattern on acrylamide gels of this insoluble fraction is heterogeneous and complex (P. Perlman and H. R. Mahler, manuscript in preparation), thus confirming the heterogeneity

TABLE IV: Effect of Chloramphenicol and Cycloheximide on L-[^{3}H]Phenylalanine Incorporation during a 5- or 20-min Pulse.[a]

	5 min				20 min			
	Chloramphenicol/Con	% *P*	Chloramphenicol + Cycloheximide/Cycloheximide	% *P*	Chloramphenicol/Con	% *P*	Chloramphenicol + Cycloheximide/Cycloheximide	% *P*
Homogenate	0.684	7.8	0.867		0.771		0.826	
S_μ	0.734		0.813		0.732		0.868	
P_μ	0.824		0.848		0.807		0.921	
P_i	0.674	7.0	0.728		0.717		0.798	
Mitochondria	0.730		0.654	5.6	0.717		0.800	
S_K	0.754				0.767		0.855	
S_F	0.741		0.732		0.773		0.712	
S_S	0.580	4.4	0.668	6.7	0.673		0.720	
P_S	0.497	0.6	0.611	3.6	0.619	3.8	0.693	8.6
P_{DOC}	0.666	6.4	0.807		0.804		0.896	
D_{DOC}	0.700	9.2	0.800		0.732		0.519	1.2
P_3	0.501	1.0	0.516	1.2	0.804		0.509	1.2
P_6	0.938		0.603	3.2	0.604	3.2	0.647	5.2
P_9	0.833		0.599	3.0	0.594	3.0	0.597	3.0
P_{18}	0.737		0.697	9.0	0.729		0.774	

	5 min[b]		20 min[b]	
	Cycloheximide/Con	Cycloheximide + Chloramphenicol/Chloramphenicol	Cycloheximide/Con	Cycloheximide + Chloramphenicol/Chloramphenicol
Homogenate	0.315	0.399	0.226	0.242
S_μ	0.279	0.309	0.169	0.200
P_μ	0.498	0.513	0.378	0.431
P_i	0.345	0.372	0.240	0.267
Mitochondria	0.301	0.270	0.136	0.152
S_K	0.255		0.123	0.137
S_F	0.370	0.366	0.154	0.169
S_S	0.221	0.255	0.169	0.181
P_S	0.337	0.414	0.152	0.171

P_{LOC}	0.434	0.525	0.191	0.213
S_{DOC}	0.369	0.422	0.218	0.155
P_3	0.350	0.366	0.312	0.197
P_6	0.386	0.277	0.210	0.225
P_9	0.430	0.310	0.253	0.254
P_{18}	0.351	0.332	0.256	0.272

[a] The numbers shown are the ratios of the $^3H/^{14}C$ values of the samples indicated. The probability that this double ratio is significantly different from 1 was calculated on the basis of a standard deviation of 0.17. The fractionation scheme used differed from Scheme I in the concentration of mitochondrial protein during detergent treatment (15.1 mg/ml) and in the concentrations of $(NH_4)_2SO_4$ used to fractionate the detergent-soluble material. [b] Values for per cent *P* in these columns were all <0.05%. Con = control.

of the kinetics of its synthesis, and that its analysis during double-labeling experiments similar to those just described form part of our current endeavor. None of our experiments are inconsistent with a model that postulates that not only all of the easily solubilizable (matrix space and easily detachable membrane bound) proteins, but also many of those linked tightly to the inner membrane of the mitochondria, including certain ones located in and associated with the "structural protein" fraction, are actually of cytoplasmic origin. By analogy with cytochrome *c* it might then be anticipated that these proteins are coded for by nuclear genes (Sherman *et al.*, 1966). Suggestive evidence for this contention is provided by the observation that the structural protein fraction of particles isolated from cytoplasmic (ρ^-) respiratory-deficient mutant cells, which are presumably deficient in functional mitochondrial DNA (Tewari *et al.*, 1966; Corneo *et al.*, 1966; Moustacchi and Williamson, 1966; Mounolou *et al.*, 1966; Mehrotra and Mahler, 1968) cultured under conditions designed to minimize repression by glucose, exhibit an electrophoretic pattern indistinguishable from that of the wild type (ρ^+) except for the absence of a single band (P. Perlman and H. R. Mahler, manuscript in preparation). Very similar conclusions have also been reported independently by Work *et al.* (1968) and Tuppy *et al.* (1968).

Materials and Methods

Fractionation by Bile Salts. KINETIC STUDIES (see Diagram I). *S. cerevisiae* was grown on 1% glucose in a semisynthetic medium in a 10-l., New Brunswick Microferm. At 13.5 hr, 250-ml aliquots were transferred into 1-l. flasks, 200 μCi of L-[^{3}H]phenylalanine (10 μM) was added, and the mixtures (flasks 1–4) were incubated with shaking at 30° for 1, 2, 10, or 30 min. Incorporation was stopped by pouring the cells over crushed ice. The samples were harvested and each was mixed with 12 g of carrier yeast and with 41 ml of yeast labeled for 2 hr with 4 μCi of L-[^{14}C]phenylalanine (80 μM) (flask 8). The oxygen uptake of the labeled cells was 180 mμmoles of O_2/min per mg; that of the carrier 226.

PULSE-CHASE STUDIES (see Diagram I). Three aliquots (250 ml, flasks 5–7) of the cells used in the kinetic studies were mixed with 200 μCi of L-[^{3}H]phenylalanine (10 μM). One minute later, a 100-fold excess of unlabeled phenylalanine (flask 5) or phenylalanine plus 2 mg/ml of chloramphenicol (flask 6) or 0.625 μg of cycloheximide (flask 7) was added and incorporation was continued for 30 min. These samples were harvested and also combined with 12 g of carrier and 41 ml of ^{14}C-labeled cells (flask 8).

INHIBITOR SENSITIVITY STUDIES (see Diagram II). *S. cerevisiae* was grown for 11.5 hr as above, then 250-ml aliquots of the yeast were transferred to 1-l. flasks. When the oxygen uptake of the cells had reached approximately 78, they were preincubated in either 2% methanol (flasks 17 and 18), 2% methanol plus 4 mg/ml of chloramphenicol (flasks 15 and 16), 2% methanol plus 5 μg/ml of cycloheximide (flasks 13 and 14), or 2% methanol plus 4 mg/ml of chloramphenicol and 5 μg/ml of cycloheximide (flasks 11 and 12) and then allowed to incorporate

DIAGRAM I

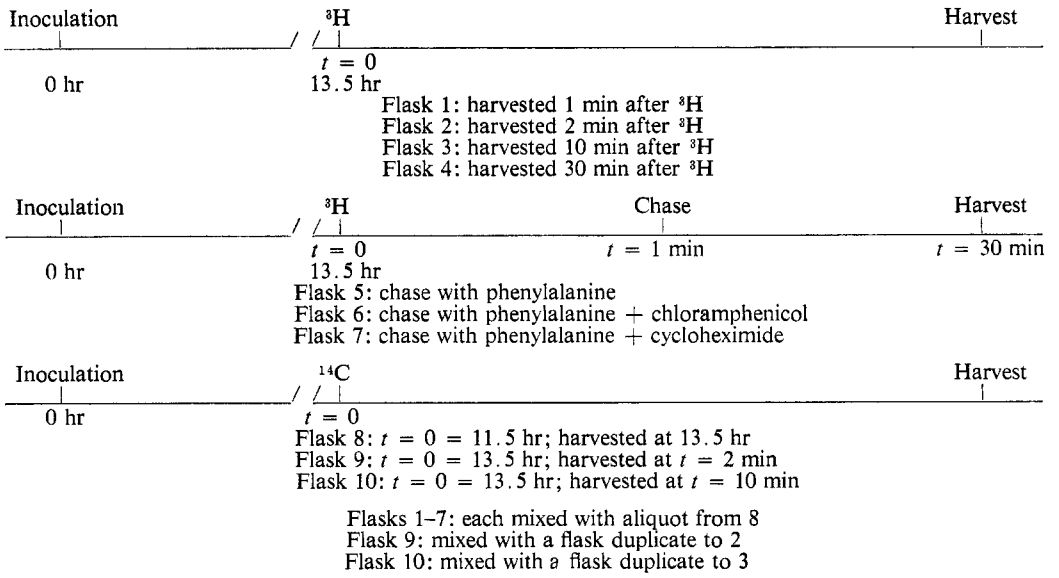
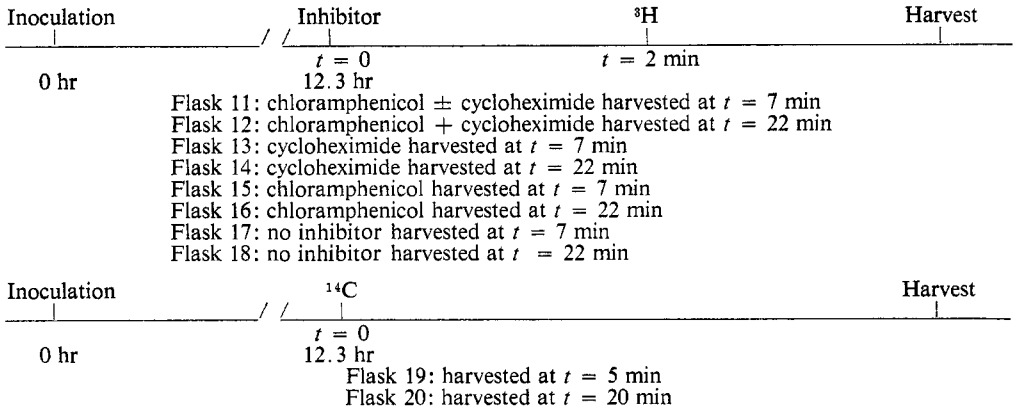


DIAGRAM II



175 μCi of L-[^{3}H]phenylalanine (10 μM) for either 5 or 20 min. The samples were harvested and mixed with 41 ml of cells labeled either 5 or 20 min with 9 μCi of L-[^{14}C]phenylalanine (10 μM) (flasks 19 and 20) and with 11.5 g of carrier yeast (oxygen uptake 214).

STATISTICS. The data were calculated in terms of the ratio of L-[^{3}H]- to L-[^{14}C]phenylalanine incorporation since the ^{14}C value could be used to correct the ^{3}H data for incomplete breakage of the cells and for experimental variations between the samples such as losses in plating. Data for statistical treatment were provided by the control samples in the inhibitor studies and by fractionation of two extra samples containing cells labeled for 2 or 10 min with 200 μCi of L-[^{3}H]phenylalanine (10 μM) mixed with cells labeled for the same time period with 33 μCi of L-[^{14}C]phenylalanine (1.1 and 1.5 μM) in the kinetic and pulse-chase studies (P = probability; see also Table I).

FRACTIONATION SCHEME KINETIC AND PULSE-CHASE STUDIES (Scheme I). The labeled samples were suspended to 20 ml in 1 M sorbitol, 0.1 M Tris, and 0.02 M EDTA (pH 8), containing 0.2% bovine serum albumin, mixed with 20 ml of glass beads, and shaken 30 sec in a Braun cell homogenizer. Once the homogenate had been decanted, the beads were washed with the same buffer. After the suspension had been diluted to 35 ml with this wash fluid, the final pH was approximately 7.1. This mixture was centrifuged for 15 min at 2000g to remove cell debris and then 20 min at 20,000g to sediment the mitochondria. The supernatant from the treatment was diluted 5 to 9 with water, made 0.87 M in $Mg(OAc)_2$, and centrifuged 20 min at 50,000g to give the fraction P_1 and 90 min at 105,000g to give S_μ and P_μ.

The mitochondria themselves were washed once with 12.5 ml of 1 M sorbitol and then suspended to 12.5 ml in 0.6 N KCl. After 5 min at room temperature, these solutions were centrifuged at 30,000g for 30 min and the pellets were suspended to 12.5 ml in 0.01 M Tris, 4 mM ATP, and 1 mM Cleland's reagent (pH 7.5) and frozen and thawed twice. The material sedimenting after

30 min at 30,000*g* was suspended to 2 ml with the same Tris–EDTA–Cleland's reagent buffer, sonicated for 60 sec (8 A, setting 4, small probe) on a Branson Sonifier, diluted to 12.5 ml with Tris–ATP–Cleland's reagent, and again centrifuged 30 min at 30,000*g*. This pellet was suspended in 0.25 M sucrose to give 19 mg of mitochondrial protein/ml and treated with 2 mg/mg of deoxycholate and 1 mg/mg of cholate. The supernatant after 25 min at 30,000*g* was mixed with dithionite (40 mg/ml) and made 6% saturated in $(NH_4)_2SO_4$. The supernatant after approximately 10-min standing and after 10-min centrifugation at 8000*g* was, in turn, made 9% saturated in $(NH_4)_2SO_4$, then 12, 15, and 35% as indicated in Scheme I. The material was left overnight at 4° at the 12% $(NH_4)_2SO_4$ step.

INHIBITOR SENSITIVITY STUDIES. The fractionation scheme used in this experiment differed from Scheme I only in that the sample for cholate–deoxycholate treatment contained 15.1 mg/ml of mitochondrial protein and that $(NH_4)_2SO_4$ cuts were made at 3, 6, 9, and 18%. The samples stood overnight at 4° in 6% $(NH_4)_2SO_4$.

COUNTING. The fractions in Scheme I were plated and washed by the techniques of Mans and Novelli (1961) and counted in a Packard Tri-Carb scintillation counter. Quench corrections were obtained by the method of Hendler (1964) using hexadecane with chloroform as a quencher to construct standard curves.

Fractionation by Triton X-100. Mitochondria were isolated from 250-ml aliquots of yeast cultures, as described in the preceding fractionation scheme. After being washed once in 1 M sorbitol–0.2% bovine serum albumin, the organelles were suspended in 20 ml of water and stripped of readily soluble proteins by being frozen and thawed twice in an ethanol–Dry-Ice bath, centrifuged 25 min at 30,000*g*, suspended in 10 ml of 0.6 N KCl, incubated at room temperature for 5 min, and, in turn, centrifuged 25 min at 30,000*g*. The residue was suspended in 5 ml of 1% Triton X-100 in 0.25 M sucrose–0.01 M Tris (pH 7.5) and incubated 30 min at 0°. That portion of this suspension which was sedimented by centrifugation for 20 min at 30,000*g* was suspended in 4 ml of a 1% sodium dodecyl sulfate, 0.25 M sucrose, and 0.01 M Tris buffer (pH 7.5), and sonicated 30 sec with a Branson Sonifier. The solution was then reduced with 1 drop of saturated dithionite, brought to 15% saturation with $(NH_4)_2SO_4$, and centrifuged 20 min at 30,000*g* to give S_{in} and P_{in}. The supernatant from the Triton extraction was also brought to 15% saturation with $(NH_4)_2SO_4$ and centrifuged 20 min at 30,000*g* to give P_{ou} and S_{ou}.

Protein was determined by the method of Lowry *et al.* (1951).

GROWTH MEDIA. Cells for these experiments were grown in a semisynthetic medium containing 1% glucose and 2 g/l. of Difco-Certified Bacto yeast extract, 1 g/l. of $(NH_4)_2SO_4$ and KH_2PO_4, and 0.5 g/l. of NaCl and $MgSO_4$.

MATERIALS. The glass beads used in these studies were Glasperlen (0.45–0.50 mm) from B. Braun. The L-[^{3}H]phenylalanine and the L-[^{14}C]phenylalanine were both purchased from Schwarz BioResearch, Inc., the specific activity of the former being 1.0 Ci/mmole and of the latter 315–355 mCi/mmole. Both were mixed with Sigma Grade DL-phenylalanine to give the molarities of L-phenylalanine indicated in the Methods section. The reconstituted [^{3}H]protein hydrolysate was purchased from Schwarz BioResearch. Other reagents and their sources were: Cleland's reagent (dithiothreitol), A grade from Calbiochem; Trizma base, reagent grade from Sigma; ATP, grade 2 from Sigma; Triton X-100 (alkylphenoxypolyethoxyethanol) from Rohm and Haas; cholic acid from Matheson Coleman and Bell (recrystallized from 70% ethanol); sodium deoxycholate (Mann Analyzed) from Mann Research Laboratory; and antimycin A from Sigma. The chloramphenicol used was a gift of Parke Davis and Co.; the cycloheximide, a gift of Upjohn Research Laboratories.

Acknowledgment

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

References

Beattie, D. S., Basford, R. E., and Koritz, S. B. (1966), *Biochemistry 5*, 926.

Beattie, D. S., Basford, R. E., and Koritz, S. B. (1967), *Biochemistry 6*, 3099.

Borst, P., Kroon, A. M., and Ruttenberg, G. J. C. M. (1967), *in* Genetic Elements: Properties and Function, Shugar, D., Ed., New York, N. Y., Academic, p 81.

Campbell, M. K., Mahler, H. R., Moore, W. J., and Tewari, S. (1966), *Biochemistry 5*, 1174.

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.

Corneo, G., Moore, C., Sanadi, D. R., Grossman, L. I., and Marmur, J. (1966), *Science 151*, 687.

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

de Kloet, S. R. (1966), *Biochem. J. 99*, 566.

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

Freeman, K. B., Haldar, D., and Work, T. S. (1967), *Biochem. J. 105*, 947.

Fukuhara, H. (1965), *Biochem. Biophys. Res. Commun. 18*, 153.

González-Cadavid, N. F., and Campbell, P. N. (1967), *Biochem. J. 105*, 443.

Green, D. E., Tisdale, H. D., Criddle, R. S., Chen, P. Y., and Bock, R. M. (1961), *Biochem. Biophys. Res. Commun. 5*, 109.

Haldar, D., Freeman, K., and Work, T. S. (1966), *Nature 211*, 9.

Haldar, D., Freeman, K., and Work, T. S. (1967), *Biochem. J. 102*, 684.

Hauge, J. G., and Halverson, H. O. (1962), *Biochim. Biophys. Acta 61*, 101.

Hendler, R. W. (1964), *Anal. Biochem. 7*, 110.
Henson, C. P., Perlman, P., Weber, C. N., and Mahler, H. R. (1968), *Biochemistry 7*, 4445 (this issue; following paper).
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.
Kadenbach, B. (1967a), *Biochim. Biophys. Acta 134*, 430.
Kadenbach, B. (1967b), *Biochim. Biophys. Acta 138*, 651.
Keller, E. B., Zamecnik, P. C., and Loftfield, J. W. (1954), *J. Histochem. Cytochem. 2*, 378.
Kopaczyk, K., Perdue, J., and Green, D. E. (1966), *Arch. Biochem. Biophys. 115*, 215.
Kuehl, L. (1967), *J. Biol. Chem. 242*, 2199.
Lamb, A. J., Clark-Walker, G. D., and Linnane, A. W. (1968), *Biochim. Biophys. Acta 161*, 415.
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., Saunders, G. W., Gingold, E. B., and Lukins, H. B. (1968), *Proc. Natl. Acad. Sci. U. S. 59*, 903.
Littlefield, J. W., Keller, E. B., Gross, J., and Zamecnik, P. C. (1955), *J. Biol. Chem. 217*, 111.
Lowry, O., Rosebrough, J. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Maaloe, O., and Kjeldgaard, N. O. (1966), Control of Macromolecular Synthesis, New York, N. Y., Benjamin, p 127.
Mackler, B., Douglas, H. C., Will, S., Hawthorne, D. C., and Mahler, H. R. (1965), *Biochemistry 4*, 2016.
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.
Mans, R. J., and Novelli, G. D. (1961), *Arch. Biochem. Biophys. 94*, 48.
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.
Moustacchi, E., and Williamson, D. H. (1966), *Biochem. Biophys. Res. Commun. 23*, 56.
Negrotti, T., and Wilkie, D. (1968), *Biochim. Biophys. Acta 153*, 341.
Neupert, W., Brdiczka, D., and Bücher, Th. (1967) *Biochem. Biophys. Res. Commun. 27*, 488.
Ohaniance, L., and Chaix, P. (1964), *Biochim. Biophys. Acta 90*, 221.
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.
Rabinovitz, M., and Olson, M. E. (1956), *Exptl. Cell Res. 10*, 747.
Richardson, S. H., Hultin, H. O., and Fleischer, S. (1964), *Arch. Biochem. Biophys. 105*, 254.
Richardson, S. H., Hultin, H. O., and Green, D. E. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 821.
Roodyn, D. B. (1962), *Biochem. J. 85*, 177.
Roodyn, D. B., Suttie, J. W., and Work, T. S. (1961), *Biochem. J. 80*, 9.
Roodyn, D. B., and Wilkie, D. (1968), The Biogenesis of Mitochondria, London, Methuen.
Sharp, C. W. (1966), Ph.D. Dissertation, Indiana University, Bloomington, Ind.
Sherman, F., and Slonimski, P. P. (1964), *Biochim. Biophys. Acta 90*, 1.
Sherman, F., Stewart, J. W., Margoliash, E., Parker, J., and Campbell, W. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 1498.
Siegel, M. R., and Sisler, H. D. (1965), *Biochim. Biophys. Acta 103*, 558.
South, D., and Mahler, H. R. (1968), *Nature 218*, 1226.
Tewari, K. K., Voetsch, W., Mahler, H. R., and Mackler, B. (1966), *J. Mol. Biol. 20*, 453.
Thomas, D. Y., and Wilkie, D. (1968a), *Biochem. Biophys. Res. Commun. 30*, 368.
Thomas, D. Y., and Wilkie, D. (1968b), *Genet. Res. 11*, 33.
Truman, D. E. S. (1964), *Biochem. J. 91*, 59.
Tuppy, H., Swetly, P., and Wolff, I. (1968), *European J. Biochem. 5*, 339.
Utter, M. F., Duell, E. A., and Bernofsky, C. (1967), *in* Aspects of Yeast Metabolism, Mills, R. K., Ed., Oxford, Blackwell, p 197.
Wheeldon, L. W., and Lehninger, A. L. (1966), *Biochemistry 5*, 3533.
Wintersberger, E. (1965), *Biochem. Z. 341*, 409.
Witt, I., Kronau, R., and Holzer, H. (1966), *Biochim. Biophys. Acta 118*, 522.
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.