MITOCHONDRIAL PROTEIN BIOSYNTHESIS IN CULTURED RAT HEPATOCYTES: CYTOCHROME C OXIDASE

by

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A THESIS

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ABBREVIATIONS

CAP	Chloramphenicol
CHI	Cycloheximide
DCIP	2,6-dichlorophenolindophenol
DNA	Deoxyribonucleic acid
DPM	Disintegrations per minute
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis(B amino-ethyl ether) N,N'-tetraacetic acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
mRNA	Messenger ribonucleic acid
PBS	Phosphate buffered saline
PMS	Phenazine methosulfate
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
TRIS	Tris (hydroxymethyl) aminomethane
TX-100	Triton X-100
tRNA	Transfer ribonucleic acid
x g	times gravity

I. INTRODUCTION

The biosynthesis of functional mitochondria involves the cooperation of mitochondrial and extra-mitochondrial genetic systems. The cytoplasmic genetic system is responsible for most of the proteins present in mitochondria, while the mitochondrial genetic system is responsible for relatively few polypeptides (1) which are mostly hydrophobic and tightly associated with the inner mitochondrial membrane (2). Three of these mitochondrially synthesized proteins have been shown to be associated with cytochrome c oxidase in several systems including yeast (Saccharomyces cerevisiae) (3,4), Neurospora (1) and Xenopus laevis oocytes (5). In yeast, five other mitochondrially synthesized products have been associated with oligomycin-sensitive ATPase and cytochrome b, while the rest of the mitochondrial genome codes for rRNA and tRNA components (6). The mitochondrial protein products account for only 5-15% of the mitochondrial protein mass but are essential for the proper function of the organelle. The ability of these two genetic systems to work together is of great interest, not only in the assembly of mitochondria in general, but more specifically, in the formation of genetic-mosaic gene products.

One of these genetic-mosaic proteins, cytochrome c oxidase (E.C. 1.9.3.1.), is a multi-subunit enzyme complex that functions as the terminal oxygen acceptor of the respiratory chain in the inner mitochondrial membrane. The functional complex is dependent on both mitochondrial and cytoplasmic protein synthesis, and in addition to polypeptides from each of these sources, contains two molecules of heme a, two atoms of copper and phospholipids. Recent work (7) confirms that the complex spans the inner mitochondrial membrane and is assymetrically arranged.

Most of the work localizing the synthesis of the cytochrome c oxidase subunits has been done on lower eukaryotes as mentioned above by using various inhibitors of protein synthesis. CHI and emetine have been shown to specifically inhibit cytoplasmic protein synthesis (8), while CAP is a specific inhibitor of mitochondrial protein synthesis (9). By manipulating the protein synthesis of cells with these inhibitors in the presence of radioactively labeled amino acids, mitochondrial or cytoplasmic subunits in yeast can be specifically labeled. Such studies have shown that the three largest subunits of cytochrome c oxidase (I-III) are labeled when cytoplasmic ribosomes are blocked with CHI. This indicates they are probably products of the mitochondrial genome. They are not labeled when mitochondrial protein synthesis is blocked by CAP. However, under those conditions and in mitochondrial mutants (petites) lacking mitochondrial DNA, only the four smallest subunits (IV-VII) are synthesized. Recent work on the seven subunit yeast enzyme complex demonstrates the existence of a polyprotein precursor to all four cytoplasmically translated subunits (10). The precursor, which is synthesized in the cytoplasm, appears to be associated with the inner mitochondrial membrane before processing (11) and has several peptide sequences in addition to those found in the final products.

In mammalian systems, conflicting results have been obtained regarding the number of cytochrome c oxidase subunits. In the study of the beef heart enzyme, Eytan (12) found six subunits upon SDS-polyacrylamide gel electrophoresis, while Capaldi (13) found seven using a gel electrophoresis system which afforded greater resolution. The organization of the enzyme complex was investigated and in both

laboratories was found to extend through the inner mitochondrial membrane. Subunits II and III are exposed on the cytoplasmic side, subunits II, III, IV, V and VII on the matrix side and subunits I and VI are located within the membrane (numbering system of Capaldi).

Using freshly isolated rat liver cells, Ries, Hundt and Kadenbach (14) immunoprecipitated a cytoplasmically synthesized putative precursor to cytochrome c oxidase with rabbit antibody immunoglobulins. The double-diffusion analysis using anti-cytochrome c oxidase antibody raised against rat liver cytochrome c oxidase showed immunological relationships between cytochrome c oxidase, mitochondria, microsomes and cytosol. The SDS-polyacrylamide gel electrophoresis of both purified and immunoprecipitated cytochrome c oxidase revealed the presence of seven bands that have molecular weights similar to those described by Capaldi for beef heart cytochrome c oxidase. Subunit seven was split into three components. They also presented evidence that in isolated rat liver cells, the cytoplasmically synthesized protein is a precursor to subunits IV-VII in agreement with the yeast studies previously mentioned (10,11). This leaves subunits I-III as possible products of mitochondrial synthesis.

Cytochrome c oxidase has been purified from rat liver mitochondria (15) and was demonstrated to have six polypeptide components on SDS-polyacrylamide gel electrophoresis. However, this electrophoresis system was not adequate to provide complete separation of subunits II and III (13). With this inadequacy in resolution considered, the immunoprecipitation study supports the idea of mitochondrial synthesis of the larger subunits and contributes to the body of evidence that isolated mitochondria can synthesize some of their

own polypeptide components (16). Further considerations on the use of isolated mitochondria should be noted here. First, the purity of the mitochondrial preparation can have significant consequences if microsomal or bacterial contamination exist. Secondly, since products of both mitochondrial and cytoplasmic synthesis are required for the functional enzyme complex, unrecognized regulatory mechanisms may operate to couple the two systems of protein synthesis, and this could be disrupted by isolating the mitochondria from the cytoplasm. Also, the lack of cytoplasmic products in this system may prevent the incorporation of some mitochondrially produced subunits.

Several studies have demonstrated the usefulness of isolated hepatocytes as a system that can be manipulated for the investigation of mitochondrial protein synthesis over a period of several hours (17,18), but no work has been done specifically on cytochrome c oxidase. The detection of a minimum of 7-8 mitochondrially-synthesized proteins on SDS-polyacrylamide gel electrophoresis was reported (17), but it was also demonstrated that freshly isolated hepatocytes have disaggregated polysomes and impaired functions that recover over a period of culture (19). Thus the reported number of mitochondrially-translated peptides might be low due to the disruption of hepatocyte function immediately after isolation in addition to inadequate electrophoretic resolution of the gel electrophoresis system used (13).

The use of cultured mammalian cell lines in the study of mitochondrial protein synthesis has grown, and many aspects of the system itself and labeling procedures have been investigated. In general, findings from electrophoresis and autoradiography confirm the stability of the end products of mitochondrial protein synthesis (20).

There are also indications that cytoplasmically made proteins play a role in the stabilization of mitochondrially synthesized enzyme subunits, perhaps by the association of the two types of subunits permitting their integration into the membrane (21). In regard to the number of mitochondrial products, Hare, Ching and Attardi (22) demonstrated in HeLa cells the CHI resistent, CAP sensitive synthesis of 15 polypeptides as resolved by SDS-urea polyacrylamide gel electrophoresis. The application of purified human placental cytochrome c oxidase to the gel system resulted in the resolution of seven subunits that correspond to the subunits of beef heart cytochrome c oxidase. Three of the 15 mitochondrially synthesized polypeptides co-purified with human placental cytochrome c oxidase. These labeled polypeptides were found to migrate in the gel system with the three largest subunits of the complex. These results extend the genetic mosaic model of cytochrome c oxidase biosynthesis to mammalian systems. While important, this study used a tumor derived continuous cell line which could conceivably vary from normal human cells in regard to cytochrome oxidase synthesis. Also the isolated cytochrome c oxidase was only 50 percent pure (22).

This summary has briefly described work that has been done in the area of mitochondrial protein synthesis and more specifically, the synthesis of the subunits of cytochrome c oxidase. A system for the primary culture of rat hepatocytes has been developed in an attempt to clearly demonstrate which subunits of the enzyme complex are products of mitochondrial protein synthesis and which are cytoplasmic products in the mammalian cell. Freshly isolated hepatocytes and cultured hepatocytes are compared with regard to the stability of mitochondrial

protein synthesis, the number of mitochondrial gene products, and the specific activity of various enzymes. In addition, the question of a cytoplasmic precursor to cytochrome c oxidase subunits IV-VII is examined in this mammalian system. A better understanding of the general protein synthesizing capacity of mitochondria and the enzyme activity in cultured hepatocytes is gained, as well as specific information on the mitochondrial synthesis of cytochrome c oxidase subunits which will contribute to the knowledge of genetic mosaic gene products. This study represents the first attempt to evaluate the synthesis of mitochondrial polypeptides in primary cultures.

II. MATERIALS AND METHODS

A. Materials.

Hanks Balanced Salt Solution (10X), L-15 media, penicillin and streptomycin were obtained from Gibco. Fetal bovine serum and 0.5% trypan blue were purchased from Flow Laboratories. Ampholines were obtained from LKB (a gift from Dr. Kabat's laboratory). Electrophoresis purity grade acrylamide and bis-acrylamide for isoelectric focusing were purchased from Bio-Rad Laboratories. (^{35}S)-methionine (1000 Ci/mmole) and En^{3}Hance were obtained from New England Nuclear. The following were obtained from Sigma: emetine, CHI, CAP, collagenase (type IV), HEPES, Tris, heparin, cytochrome c (type VI), insulin, bovine serum albumin (Fraction V), PMS, DCIP. Gentamicin was purchased from Microbiological Associates. Other chemicals used were reagent grade or better.

Filters (0.45 um pore size) for sterile filtration and precipitate collection were obtained from Nalge Company and Millipore Corporation, respectively. Nylon 900 mesh/cm^2 (Nitex HC3-253) was purchased from TETKO (Elmford, NY). The 2000 mesh/cm^2 nylon was a gift of Dr. Howard Mason's laboratory.

B. Solutions, Buffers and Media.

Phosphate buffered saline:	145 mM NaCl, 2.6 mM KH_2PO_4, 10.4 mM Na_2HPO_4 pH 7.2
Krebs-Henseleit (10X) per 100 ml:	6.9 g NaCl, 0.36 g KCl, 0.13 g KH_2PO_4, 0.144 g $MgSO_4$, 0.322 g $CaCl_2$, 5.96 g HEPES
Buffer A:	10% (v/v) 10X Hanks Balanced Salt Solution without Ca, Mg, phenol red and $NaHCO_3$, 25 mM HEPES, 0.05% (w/v) bovine serum albumin, 0.5 mM EGTA
Buffer B:	10% 10X Hanks solution as above, 25 mM HEPES, 4 mM $CaCl_2$ 0.12% (w/v) collagenase

Buffer C:	10% 10X Krebs-Henseleit, 0.05% bovine serum albumin
Complete media:	L-15 powdered media, 10% fetal bovine serum, 0.3 g/l L-glutamine, 100 U/ml penicillin, 100 ug/ml streptomycin, 0.05 ug/ml insulin, 2.2 ug/ml dextrose
Methionine deficient media:	Identical to complete media without methionine and using 10% dialyzed fetal bovine serum
Sucrose buffer:	0.25 M sucrose, 0.01 M Tris-Cl pH 7.4, 1 mM EDTA
Solubilizing buffer:	5% TX-100, 5 mM EDTA, 1 M KCl, 0.05 M Tris-Cl pH 7.4
Sample dissolving buffer:	4-8 M urea, 4% SDS, 0.01 M phosphate pH 8.3, 2-4% 2-mercaptoethanol

C. Cell isolation.

Hepatocytes were isolated from livers of 180-220 g male rats by an in vivo perfusion method (24). Buffers A, B and C were adjusted to pH 7.4 with 0.2 M Na_2CO_3, sterilized by filtering, and oxygenated with 95% oxygen, 5% carbon dioxide for 30 minutes in a 37°C water bath immediately prior to use. The animals were anesthetized with ether and injected with 100 U heparin after exposure of the abdominal cavity. Sterile gloves were used to complete cannulation of the portal vein and handling of the livers. After the cannula was secured, livers were perfused with Buffer A until free of blood. During this period the vena cava was cut to relieve pressure, and the livers were removed and washed gently in Buffer A. Livers were then transferred to Buffer B and perfused until obvious signs of cell dissociation were observed through the capsule. The capsule was cut open and cells were dispersed into Buffer C. The cells were then filtered through nylon meshes, 900 mesh/cm^2 and 2000 mesh/cm^2, respectively, and collected by

centrifugation at 4.6 g for five minutes. After three washes in complete media, cells were suspended to four times the pellet volume and a 50 µl aliquot was diluted to 1 ml to facilitate counting in a hemocytometer. Cell viability was determined by trypan blue exclusion (24).

D. Cell culture.

Cells were plated in 60 x 15 mm tissue culture dishes to a concentration of 5 x 10^6 cells/plate in complete media and incubated at 37°C in humidified air. The media was replaced after four hours to remove unattached cells. Fifty percent of the cells attach in this period and 80-90% are flattened within 24 hours. The media is then replaced every other day. When freshly isolated hepatocytes were used, they were incubated in sterile tubes in a 37°C shaking water bath to prevent attachment.

E. Cell labeling.

Two procedures were used. The first for monitoring incorporation of radioactivity into TCA precipitable protein required 3-5 µCi of (^{35}S)-methionine per plate (1-2 uCi/ml). Duplicates for each condition were treated each day. When CHI was used, cells were preincubated with this inhibitor for 30-60 minutes before labeling was begun. Cells were washed twice with methionine deficient media. CHI (200 µg/ml), CAP (50 µg/ml), or emetine (100 µg/ml) were then added to each plate at least five minutes before the addition of (^{35}S)-methionine. In some cases a combination of the inhibitors was added. Cells were labelled for two hours (unless otherwise specified), followed by four washes with complete media and allowed to incubate further in complete media (chase) or stopped by two washes with cold PBS. Cells

were collected, centrifuged at 4.6 x g and resuspended to 1 ml. An aliquot was counted and protein was precipitated with 5% TCA in preparation for scintillation counting as described below under Analytical procedures.

When labeling cytochrome c oxidase subunits or mitochondrial gene products, 90-100 μCi/plate (^{35}S)-methionine was used with CHI present to inhibit cytoplasmic protein synthesis. Plates treated with both CAP and CHI were included as a control for nonspecific labeling. Labeling was allowed to proceed for two hours followed by a 30-60 minute chase in complete media. Longer chases were used in pulse-chase experiments. After two washes with cold PBS, the cells were harvested by scraping from the plate.

F. Preparation of cell lysate.

In the determination of mitochondrial gene products and specific subunit labeling, a modification of the Gellerfors and Nelson method (25) for isolating mitochondria was used. All procedures were carried out at 5°C. Cells were harvested from five plates, suspended to 3-6 x 10^6 cells/ml in cold sucrose buffer (4 ml) and sonicated for 30-60 seconds at output setting 2.5 in 15 second bursts on a Heat Systems sonicator (model W-220F). Centrifugation at 430 x g for ten minutes removed cell debris, and centrifugation of the supernatant at 4300 x g for 20 minutes pelleted the crude mitochondrial fraction. The mitochondrial pellet was resuspended to 4 ml and washed by repeating the above two centrifugations. In preparation for immunoprecipitation, this pellet was homogenized with solubilizing buffer (0.4 ml) and subsequently diluted to 2 ml with 0.15 M KCl, 5 mM EDTA, 0.05 M Tris-Cl pH 7.4. Insoluble material was removed by centrifugation at 82,000 x g

for 30-60 minutes. Preparation of the mitochondrial pellet for polyacrylamide gel electrophoresis required a second sonication at output 5 for a total of one minute in a 4 ml volume of sucrose buffer. This was folowed by centrifugations at 17,000 x g for ten minutes to remove debris and 82,000 x g to pellet a membrane fraction.

G. Immunoprecipitation.

Rabbit immunoglobulin against rat liver cytochrome c oxidase and preimmune immunoglobulin were prepared by Dr. James Hare as described (23). Preimmune immunoglobulin (120 µg/ml) was added to the solubilized mitochondrial pellet (2 ml volume) and incubated 5-8 hours at 0°C. After centrifugation for five minutes in an Eppendorf microfuge (Brinkmann), 750-1125 µg/ml of cytochrome c oxidase immunogobulin was added to the supernatant and incubated 20-36 hours at 0°C. The resultant immunoprecipitate was pelleted by microcentrifugation and washed twice in diluted solubilizing buffer.

H. Electrophoresis and fluorography.

Samples were prepared in sample dissolving buffer and incubated for one hour at 37°C prior to application. The SDS-polyacrylamide gel electrophoresis system is that described by Downer (13) as modified (22). Gels were stained with Coomassie Brilliant Blue and destained by the procedure of Fairbanks (26). Two dimensional gels were prepared for the first dimension by the procedure of Cabral and Schatz (27) and as above for the second dimension. A 90 minute equilibration in three volumes of En^3Hance followed by one hour in five volumes of 10% glycerol was used in preparation for fluorography (28). After drying, gels were exposed to Kodak X-Omat R film at -70°C. Length of exposure varied with the radioactivity applied to the gel.

I. Analytical procedures.

Protein concentrations were determined by the method of Lowry (29). Cytochrome c oxidase was assayed by the procedure described in (30). Succinate dehydrogenase activity was measured by the method in (31). DNA concentrations were determined by the method in (32).

For monitoring daily mitochondrial protein synthesis, harvested cells were centrifuged at 4.6 x g for five minutes and resuspended to 1 ml. One ml of 1 M NaOH is added and allowed to incubate at 37°C for ten minutes followed by neutralization with 1 M HCl. Protein was precipitated with 5% TCA and filtered with TCA washing. Filters were allowed to dry overnight They were placed in 5 ml of scintillation fluid and allowed to equilibrate at least 15 minutes before counting. Duplicate samples are counted twice for ten minute periods at settings for ^{14}C.

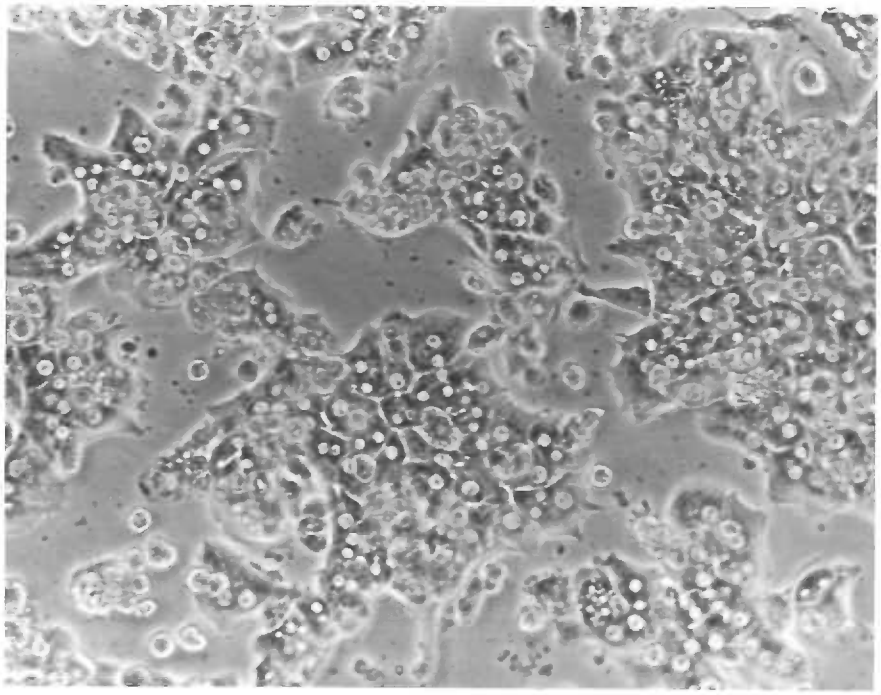

Figure 1. Hepatocytes on day 4 of culture. Magnification 160X.

III. RESULTS

A. Choice of culture conditions.

Several types of media were screened to determine the optimal conditions for attachment, flattening and survival of the hepatocytes over a period of eight days. The media tested were Eagle's Minimum Essential Medium, Dulbecco's Modified Eagle's, Williams E, Ham's F-12 and Leibovitz's L-15. Each of the media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells grown in F-12 and L-15 showed significantly more flattening and the most uniform cell cover over the first three days of culture. Further tests [with the addition of insulin (0.5 µg/ml) to both media, galactose (0.9 mg/ml) to F-12 and dextrose (1.5 mg/ml) to L-15] indicated that L-15 supplemented with both insulin and dextrose was most effective at sustaining cell viability. In this system 50% of the cells attach within four hours and 80-90% of these are flattened with 24 hours. The assumption is made that flattened cells are all viable. The use of collagen-coated plates as described (33,34) did not enhance the response of cells to the culture conditions in the presence of fetal bovine serum. This system is presently under investigation in our laboratory as an alternative to the use of fetal bovine serum for maintenance of cells.

B. Population stability and enzyme levels.

Figure 1 shows that hepatocytes on day 4 of culture are well flattened and compare favorably to other preparations (17). Figure 2 shows that the number of cells remains stable in this system from day 2 through day 6. Table I shows that total protein declines gradually while DNA concentration remains constant. This constant DNA

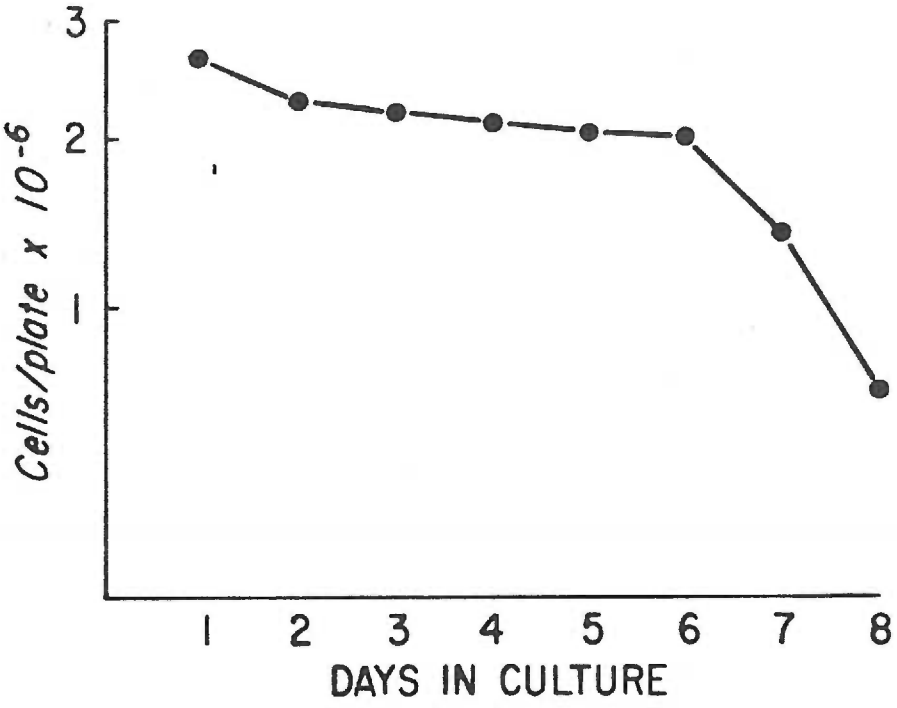

Figure 2. Stability in cell population over eight days of culture. 5×10^6 cells were plated on day 0.

TABLE I. Parameters of cell culture. Results are presented as the average of five preparations ± standard deviation.

Day of culture	Protein mg/ml	DNA µg/ml
1	3.3 ± 1.6	118 ± 47
2	2.5 ± 1.3	109 ± 25
3	2.2 ± 1.5	119 ± 26
4	2.0 ± 1.0	140 ± 61
5	2.1 ± 1.4	127 ± 64
6	2.0 ± 1.4	128 ± 64
7	1.8 ± 1.6	129 ± 78
8	1.8 ± 1.6	102 ± 28

TABLE II. Enzyme activities over the initial period of culture. Results are given as the mean of duplicate assays of three preparations ± standard deviation.

Day of culture	Cytochrome c oxidase [1]	10^4 x Succinate dehydrogenase [2]
0	.67 ± .24	4.1 ± 2.3
1	1.89 ± .81	12.3 ± 6.6
2	1.26 ± .45	5.1 ± 1.5
3	.57 ± .31	6.0 ± 5.2
4	.59 ± .33	5.1 ± 1.4
5	.21 ± .13	3.8 ± 0.9

[1]Activity expressed as first order rate constant per mg protein (15).

[2]Specific activity: µmoles/min/mg protein.

concentration is interesting because the total number of cells is decreased to one-third of the original number by day 8 and might indicate heteroploidy of these non-dividing cells. Since there appeared to be a loss of viability after six days, further studies were focused on days 0-5 of culture. Table II shows the activity of cytochrome c oxidase and succinate dehydrogenase over this period. Both enzymes show a significant increase in activity on day 1 followed by declining activity with continued culture.

C. (^{35}S)-methionine incorporation.

On day 2 of culture, cells were labeled for time periods of 15 minutes to four hours with (^{35}S)-methionine in the presence of CHI or CHI + CAP. CHI is reported to inhibit cytoplasmic protein synthesis by interfering with both initiation and elongation in 80s ribosomes (35). CAP inhibits mitochondrial protein synthesis by binding to 70s ribosomes, possibly near the catalytic center of the peptidyltransferase (36). The use of the two inhibitors together as a control is indicative of non-specific labeling. Figure 3 shows that non-specific labeling increases with the labeling period but at a rate less than labeling of CHI inhibited cells. The difference between the two conditions reflects the actual incorporation of counts into mitochondrial proteins. These findings agree with Gellerfors et al. (17) who report linear labeling of mitochondrial proteins for the first two hours. Two hours were chosen as the optimal labeling period for two reasons. First, a two-to-one ratio of label between the CHI labeling condition and the control was obtained. Secondly, it has been reported (14) that CHI inhibition is decreased after two hours of incubation, indicating a possible inactivation of the inhibitor by the cells. The two-hour labeling

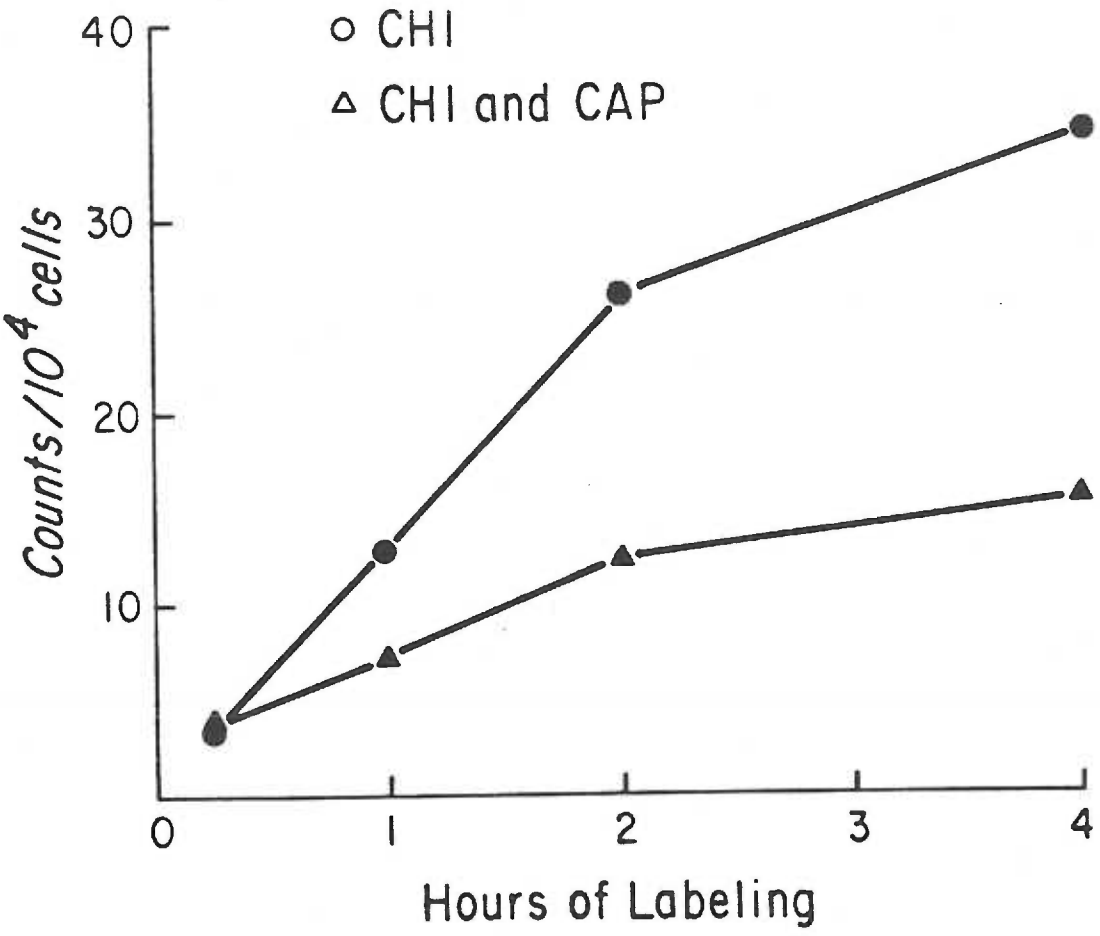

Figure 3. TCA precipitable counts/10^4cells on labeling for various time periods.

TABLE III. Effect of inhibitors on incorporation of (^{35}S)-methionine at high label concentration.

Fraction	Inhibitor	DPM[1]	Percent of cell fraction[2]	Percent of control[2]
Cells (2.5×10^6)	None	1.5×10^7		100
Cells	CHI & CAP	2.9×10^6		
Cells	CAP	8.1×10^6		43
Cells	CHI	4.1×10^6	100	10
Mitochondria	CHI	3.7×10^5	14	
Membrane	CHI	3.4×10^4	2	
Immunoprecipitate	CHI	1.2×10^4	1	

[1]Counting efficiency 70%.
[2]See Results, section C.

TABLE IV. Daily incorporation of (^{35}S)-methionine into TCA precipitable protein in uninhibited cells. Average of duplicate samples.

Day of culture	DPM/10^4 cells	DPM/mg protein
0	100	7,000
1	1000	58,000
2	600	36,000
3	1100	84,000
4	2700	93,000
5	2600	83,000

period also minimizes any deleterious effects of the inhibitors that might interfere with the incorporation of radioactivity into the cells.

Table III shows the effect of inhibitors on the incorporation of (^{35}S)-methionine into cell fractions. This was monitored during the preparation of cytochrome c oxidase immunoprecipitates involving high concentrations of label. Identical results were obtained for the degree of inhibition of protein synthesis when low concentrations of label were used and only TCA precipitable counts were measured. Using a high concentration of label, a significant amount of non-specific labeling (see above) occurs as is represented by the CHI + CAP condition. The percent columns are obtained by subtracting these non-specific counts from the other conditions and dividing by the control condition. CHI-resistant methionine incorporation was 10% of that seen in the absence of drug. Treatment with CAP reduced incorporation of radioactivity to 43% of control, indicating some inhibition of cytoplasmic protein synthesis. Proteins from mitochondria isolated from CHI blocked cells account for 14% of the detected label and 1% is traced to the immunoprecipitable fraction. The rest represents non-specific labeling or pools of methionine found in cytoplasm or otherwise removed on mitochondrial purification. Half of the label incorporated in the membrane fraction by mitochondrial protein synthesis is recovered in the immunoprecipitate.

The daily incorporation of (^{35}S)-methionine into uninhibited cells is shown in Table IV. On isolation the cells appear to have impaired ability to incorporate methionine into precipitable protein. They seem to recover this capacity on culture.

Mitochondrial protein synthesis was monitored each day under

inhibitor treatment conditions with emetine in addition to CHI and the control, CHI + CAP. Emetine has been shown to irreversibly inhibit cytoplasmic protein synthesis in mammalian cells (20). The cells were harvested immediately in cold PBS, counted and TCA precipitated. Figure 4 shows the daily mitochondrial protein synthesis measured and represents the CHI + emetine treated precipitable counts minus the control values. This indicates depressed mitochondrial protein synthesis on isolation with apparent recovery of this system on culture.

D. Labeling of subunits.

Mitochondria were prepared from cells labeled with (^{35}S)-methionine in the presence or absence of CHI and CAP. The membrane fraction was solubilized and exposed to cytochrome c oxidase specific immunoglobulin which precipitated the relevant subunits in each case. Coomassie blue stained patterns of the SDS-urea polyacrylamide gel electrophoresis are shown on the left side of Figure 5 which reveal the seven subunits of cytochrome c oxidase. It is evident that there is no significant difference in the migration of immunoprecipitates of purified mitochondria and the mitochondria prepared from hepatocytes cultures in our system. The stained pattern shown in the IPH track is representative of the cytochrome c oxidase specific immunoprecipitates formed under the labeling conditions shown in the fluorograph. This demonstrates that all of the subunits are present in any given inhibitor condition, but as explained below, not all of the subunits are labeled.

Gels were prepared for fluorography and the results for each inhibitor condition are shown on the right side of Figure 5. With CHI alone, three bands are visible which are sensitive to CAP and co-migrate with the three large subunits of cytochrome c oxidase. This indicates

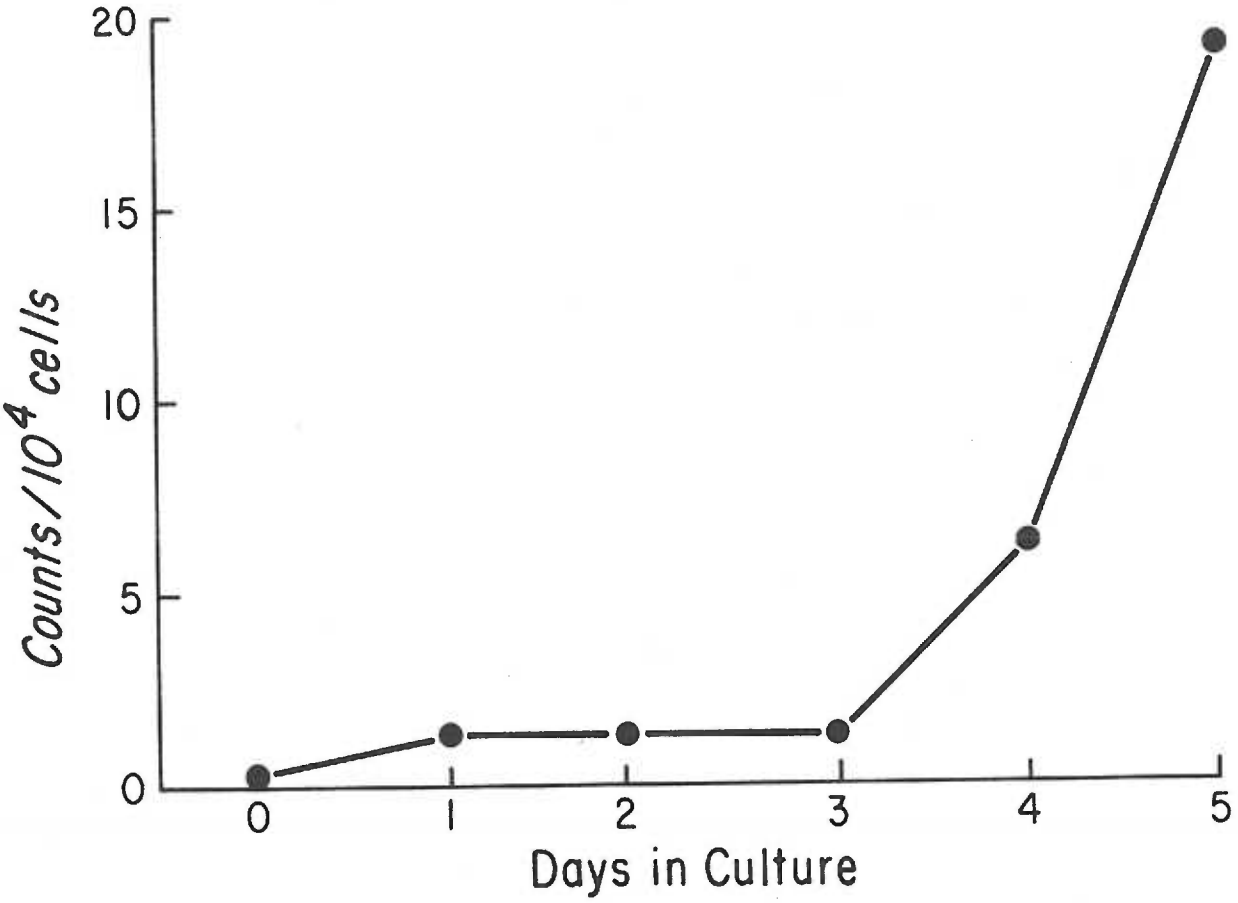

Figure 4 Daily mitochondrial protein synthesis. Average of duplicate (emetine + CHI) - (emetine + CHI + CAP) labeling conditions. See Results, section C.

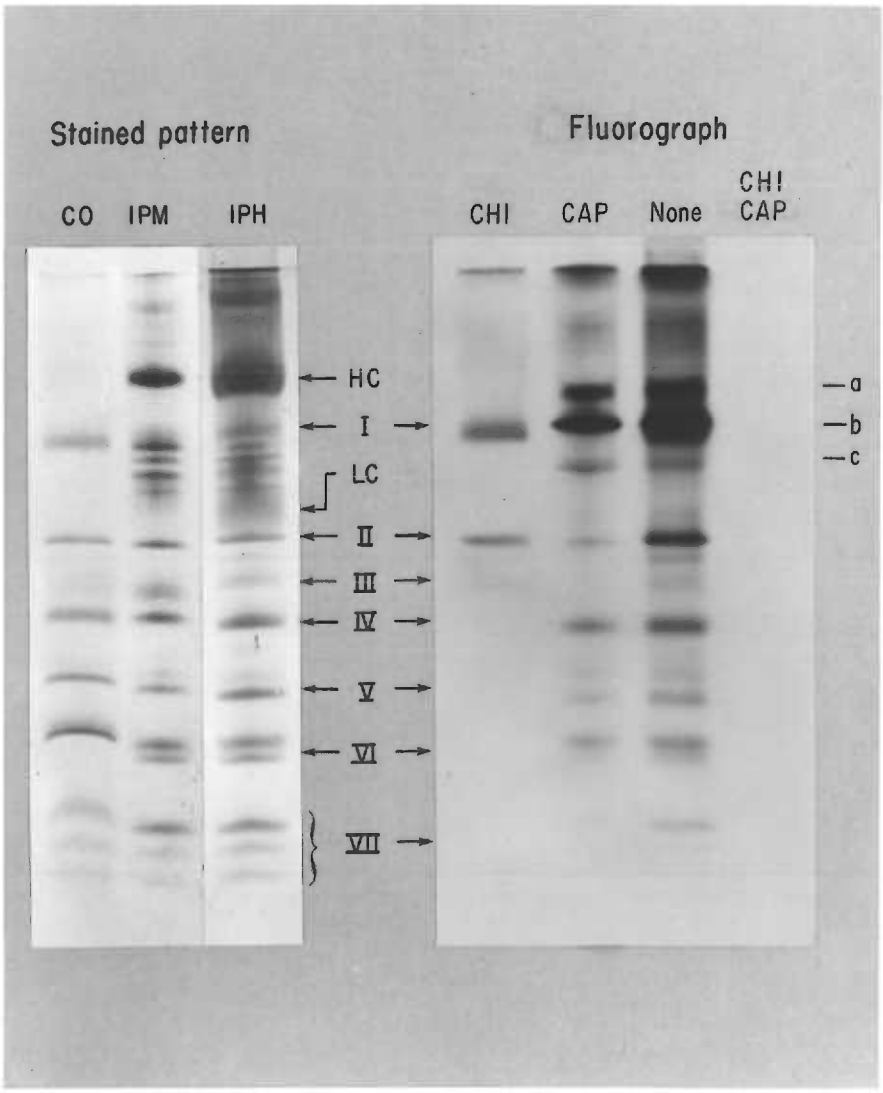

Figure 5. Electrophoretic separation of cytochrome c oxidase immunoprecipitates of purified rat liver mitochondria (IPM), hepatocytes (IPH), and hepatocytes labeled in the presence of inhibitors. Co-purified rat liver cytochrome c oxidase, HC-heavy chain immunoglobulin, LC-light chain of immunoglobulin, Roman numerals denote the subunits of cytochrome c oxidase by the numbering system of Capaldi (13). a, b and c indicate co-precipitated CHI-sensitive polypeptides.

that they are mitochondrially synthesized polypeptide subunits selectively precipitated by cytochrome c oxidase specific immunoglobulin. The CAP condition reveals the presence of four small molecular weight polypeptides which are sensitive to CHI and co-migrate with the four small subunits of cytochrome c oxidase. Because the three larger polypeptides (a, b and c) are also CHI sensitive, they appear to be cytoplasmically synthesized, but they do not correspond to the larger subunits of the enzyme complex. They may represent precursor forms of the lower molecular weight subunits. The sample prepared in the absence of inhibitors shows all seven immunoprecipitated cytochrome c oxidase subunits as well as the high molecular weight polypeptides. Finally, the CHI + CAP condition is included to demonstrate that there was no non-specific incorporation of label detectable in the immunoprecipitates.

E. Pulse-chase precursor study.

Uninhibited cells were labeled for two hours with (^{35}S)-methionine (50 μCi/plate) and chased in complete media for periods ranging from 0 - 22 hours. After harvesting and preparation of mitochondria, samples were stored on ice until all cells were collected. The supernatants from the mitochondrial preparations were also saved. The mitochondria were then solubilized and prepared for immunoprecipitation. After SDS-urea polyacrylamide gel electrophoresis of the immunoprecipitates, Coomassie blue staining revealed all seven subunits for each chase period. The fluorograph is shown in Figure 6. The lower molecular weight subunits (V-VII) as well as subunit III are not visible due to the lower concentration of label in this preparation (see also Discussion). No incorporation of radioactivity into subunit

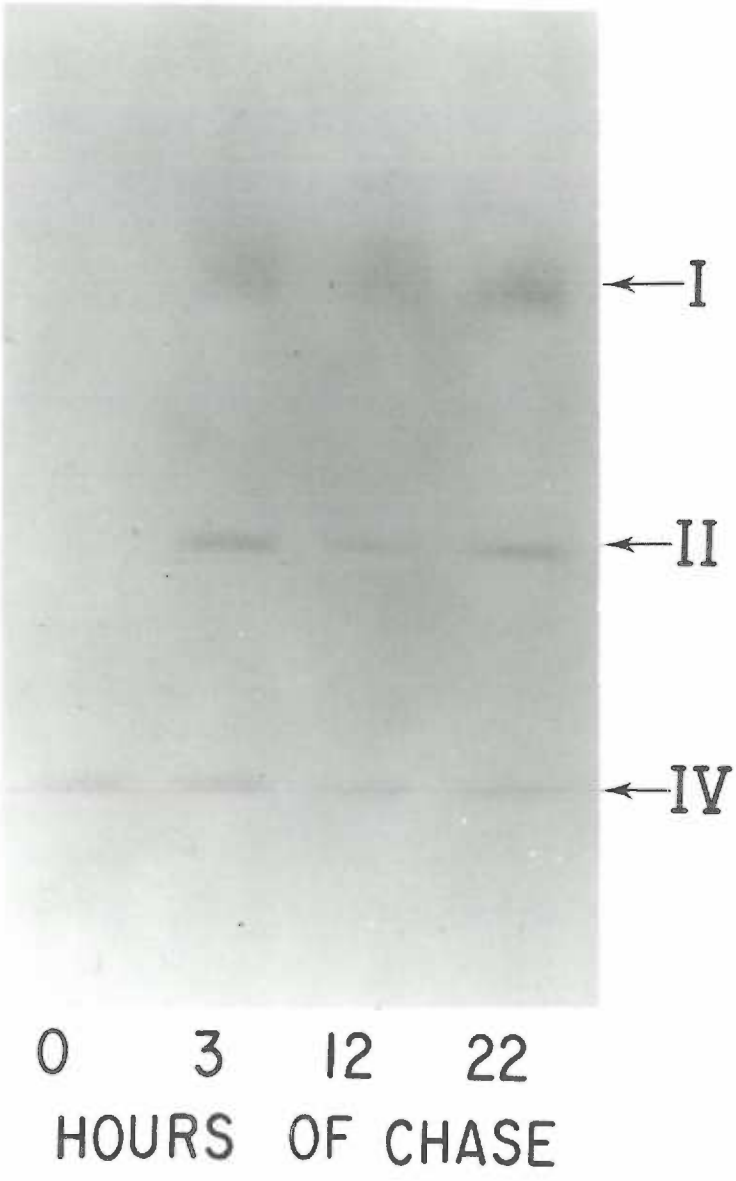

Figure 6. Fluorograph of cytochrome c oxidase specific immunoprecipitates from uninhibited cells following labeling with (^{35}S)-methionine and variable chase periods. Roman numerals indicate subunits of cytochrome c oxidase.

II occurs without a chase period. Subunit I is difficult to distinguish from a possible high molecular weight precursor, but a chase is still required to incorporate label into high molecular weight immunoprecipitable protein(s). Subunit IV is labeled in the absence of any chase period. At this concentration of label no chasing of larger molecular weight polypeptides into smaller subunits is observed. Immunoprecipitates from the supernatants were also run on the gel system. Material that reacts with cytochrome c oxidase immunoglobulin is present, but no diffinitive results concerning possible precursors were obtained. Investigations with short pulse labeling are required to clarify the issue.

F. Mitochondrial gene product labeling.

In order to block cytoplasmic synthesis, cells were labeled in the presence of CHI. Again the CAP + CHI labeling condition was used as a control. Some of the CHI inhibited cells were prepared for immunoprecipitation, and the resulting immunoprecipitates were applied to SDS-urea polyacrylamide gel electrophoresis with the other solubilized membrane samples. Results for freshly isolated hepatocytes and the same cells after days 2 and 4 of culture are shown in Figure 7. On day 0 there is no apparent incorporation of label into protein. Labeling on day 2 shows a minimum of seven mitochondrially synthesized proteins, three of which are immunoprecipitated with immunoglobulins to cytochrome c oxidase, including the two most strongly labeled bands. The results for day 4 are similar to those for day 2. Subunits I and II are apparent on immunoprecipitation, and there is no qualitative change in mitochondrial gene products between the two days.

Attempts were made to separate mitochondrial membrane

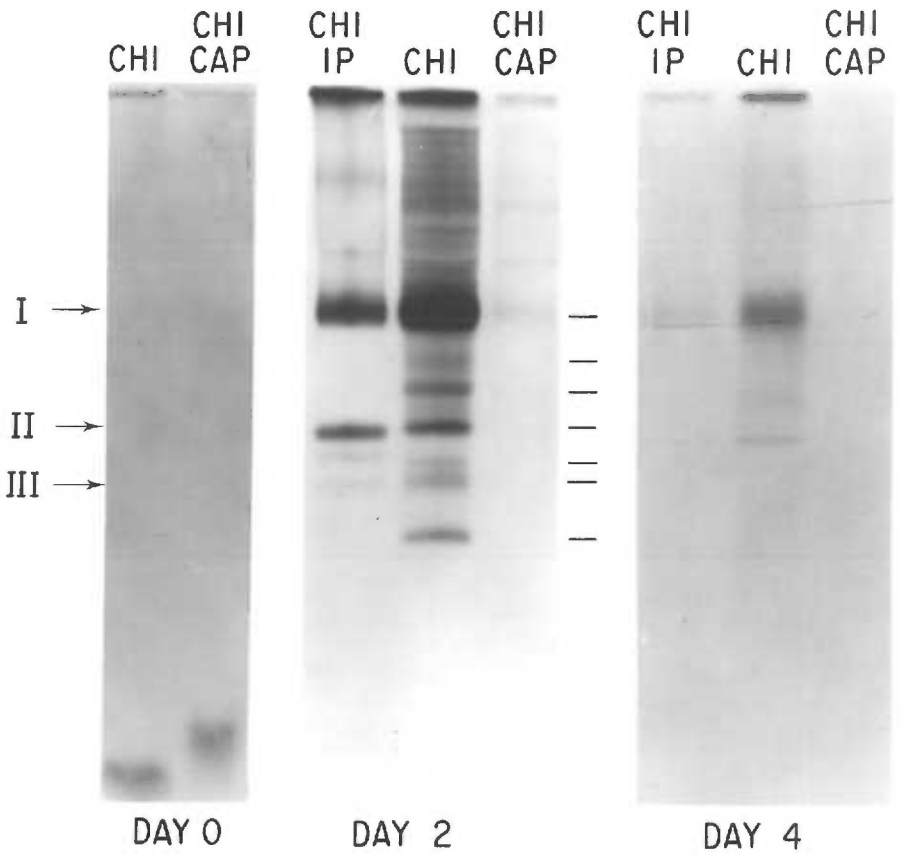

Figure 7. Mitochondrial gene products synthesized on days 0, 2 and 4 of culture. Roman numerals denote cytochrome c oxidase subunits. Headings indicate inhibitor conditions, IP - immunoprecipitate. Bars indicate seven mitochondrial gene products. All are 18 day exposures.

components which had been labeled in the presence of CHI over two dimensions using isoelectric focusing followed by SDS-urea polyacrylamide gel electrophoresis with samples from day 0. Coomassie blue stained patterns were obtained, but no labeling was observed on fluorography. This is consistent with Figure 7 which shows no labeling of mitochondrial gene products on day 0.

G. CAP pretreatment.

Preliminary results with low concentrations of (^{35}S)-methionine had indicated a two-fold increase in the incorporation of label into TCA precipitable protein following a 24 hour pre-incubation of cells with CAP. This could be explained by a build-up of cytoplasmic subunits of the cytochrome c oxidase complex and other jointly made enzymes during the pre-incubation and a resultant increase in the assembly of the labeled mitochondrial subunits into membrane during the labeling and chase period. Using a high concentration of label (60 μCi/plate), CAP pre-incubated cells (day 2) were labeled for two hours in the presence of CHI or CHI + CAP. Mitochondria were isolated and prepared for SDS-urea polyacrylamide gel electrophoresis along with an immunoprecipitate for the CHI inhibited condition. Staining with Coomassie blue revealed all seven subunits of cytochrome c oxidase in the immunoprecipitate track and various mitochondrial proteins in the other tracks, but fluorography after a 20-day exposure showed no labeling of subunits or peptides.

IV. DISCUSSION

Tanaka et al. (19) have shown that freshly isolated hepatocytes have impaired induction of tyrosine transaminase and hormone stimulated protein synthesis. This is supported by the depressed activity of cytochrome c oxidase and succinate dehydrogenase (Table II) on isolation and the low incorporation of radioactivity into TCA precipitable protein in uninhibited cells (Table IV). The failure to label mitochondrial gene products in hepatocytes on day 0 may be explained by impairment of metabolic function immediately following the trauma of isolation. However, evidence presented on cell population, protein and DNA concentrations shows good population stability under culture conditions (Figure 2 and Table I), and enzyme activities appear to rebound on adaptation of the cells to culture (Table II). All of these effects argue in favor of the cultured cells as a valid and easily manipulated system for the study of mitochondrial biosynthesis. For example, oxygen concentrations can be controlled in this system, allowing any oxygen induction of respiratory enzymes or mitochondrial protein synthesis to be monitored. Based on the increased incorporation of (^{35}S)-methionine into TCA precipitable protein in uninhibited cells (Table IV) and increased mitochondrial protein synthesis (Figure 4) over the period of culture, the plateau and decline in enzyme activities measured (Table II) might indicate increased turnover of cellular and particularly mitochondrial components. This could contribute to the limited viability of these cells.

The nature of the non-specific labeling represented by the CHI + CAP inhibitor condition is not clear. This label does not show up on fluorography after SDS-urea polyacrylamide gel electrophoresis, but its

presence is consistently detected by scintillation counting. Jeffreys and Craig (20) report that it cannot be attributed to (^{35}S)-methionyl tRNAs since these would be destroyed in the alkali wash used prior to scintillation counting. The possibility of cytoplasmic protein synthesis occurring in spite of the CHI-block was previously mentioned (see Results, section C); however non-specific label is still detected in the presence of emetine + CHI + CAP. Since this non-specific label is most apparent in whole cell TCA precipitates on scintillation counting, it could be representative of membrane- or protein-associated cellular pools of (^{35}S)-methionine. The importance of controls in this system cannot be ignored though the cause of the non-specific labeling is unknown.

In this system the incorporation of (^{35}S)-methionine into CHI-inhibited cells indicates that 10% of the total labeling takes place in the absence of cytoplasmic protein synthesis (Table III). After correcting for non-specific labeling, this value represents mitochondrial synthesis. In other systems CAP has been shown to have little effect on cytoplasmic protein synthesis (36), reducing the incorporation of radioactivity by 1-5% (20). In this system it reduces the incorporation of radioactivity to 43% of control, indicating that it affects more than mitochondrial protein synthesis. Also, the fact that CAP pre-treatment of cells prohibits labeling of subunits, as described in Results, section G, indicates that there are further complications with prolonged use of the drug. In this study CAP was used mostly in conjunction with CHI as a control for non-specific labeling, and no other difficulties were noted.

Cytochrome c oxidase specific immunoglobulin precipitated 50%

of the radioactivity from the mitochondrial membrane fraction in agreement with Bernstein et al. (37). This indicates that a large percentage of membrane incorporated mitochondrial protein synthesis results in cytochrome c oxidase related polypeptides. This is further borne out by the mitochondrial gene product labeling shown in Figure 7. The immunoprecipitate for day 2 shows clearly the three mitochondrial subunits (I-III). It is obvious that subunits I and II are the most strongly labeled products of mitochondrial protein synthesis and could account for a large fraction of the incorporated label.

The non-discrete radioactivity above subunit I of cytochrome c oxidase seen in the fluorograph of day 2 of mitochondrial gene product labeling (Figure 7, CHI band and control) could be explained by the aggregation and slow migration of mitochondrial products. Such aggregation is not unknown (20), and I conclude there to be a minimum of seven mitochondrial gene products based on the clearly visible bands in Figure 7. The possibility of proteolytic degradation of mitochondrially made proteins exists since it was necessary to dissociate the samples at 37°C to prevent aggregation of cytochrome c oxidase subunit I. The presence of urea and SDS would, however, tend to exclude this possibility. The high degree of hydrophobicity of the products of mitochondrial protein synthesis also makes it difficult to assume that all polypeptides produced are present in the polyacrylamide gel. Interest in the number of mitochondrial gene products in mammalian systems is high due to the large range in the number of reported products (3-19) (20,37,39). Results presented here agree with those of Gellerfors (17) that the rat liver mitochondrial genome with seven to

subunits IV-VII contain even less. In addition, subunit III is a smaller peptide with a molecular weight of 19,000 as opposed to 36,000 and 28,000 for subunits I and II, respectively. Subunit III is also hydrophobic and difficult to introduce into polyacrylamide gels. With this in mind, the pulse chase precursor study (Figure 6) might be interpreted to indicate that mitochondrially synthesized peptides require a chase period to be incorporated into the membrane. The fact that subunit IV is present even without a chase many indicate that it can incorporate into the mitochondrial membrane more easily or perhaps is the first subunit to associate with the membrane.

The immunoglobulins generated against purified rat liver cytochrome c oxidase have been demonstrated to be somewhat non-specific, as shown by the immunoprecipitation of components a, b and c, in addition to cytochrome c oxidase subunits in Figure 5. These components are seen in the fluorograph as labeled bands but are not readily identifiable on Coomassie blue staining. Poyton (10, 11) has reported the presence of three polypeptides which are cytoplasmically translated and antigenically related to cytochrome c oxidase in yeast. In view of the CHI-sensitivity of bands a, b and c, the nature of these immunoprecipitated polypeptides requires further investigation. They fall in the same molecular weight range (37,000-55,000) as polypeptides seen by Poyton, one of which is reported to be a precursor to the four smallest subunits of the enzyme complex. Bands a, b and c are immunologically related to cytochrome c oxidase, but the pulse chase study described here does not provide any conclusive evidence either for or against the possibility of a precursor relationship. In order to establish a definite precursor role for any of these components, several

areas of investigation should be pursued. Their rate of synthesis and possible degradation into known subunits should be measured with short pulse-chase studies. Localization of such potential precursors in the cytoplasm should be attempted. Immunoprecipitation of polypeptides from hepatocyte mRNA programmed cell-free synthesis systems that might co-migrate with components a, b and c would confirm any precursor role. Another important source of evidence would result from their isolation and tryptic peptide mapping. This would allow the direct determination of a possible precursor relationship of any of these large molecular weight components with the smaller subunits.

V. SUMMARY

The localization of cytochrome c oxidase subunit synthesis has been determined in yeast and Neurospora, as well as Xenopus oocytes, but various conflicts have arisen in the investigations involving mammalian systems. Using a gel system that gives adequate resolution of the cytochrome c oxidase subunits and immunoglobulins generated toward the purified rat liver enzyme complex, subunits are labeled with (^{35}S)-methionine in the presence of inhibitors specific for cytoplasmic (CHI) or mitochondrial (CAP) protein synthesis. On the basis of resistance to one inhibitor combined with sensitivity to the other inhibitor, it is determined that the three largest subunits are synthesized on mitochondrial ribosomes, while the four smaller subunits are cytoplasmically synthesized. The immunoglobulin used in this investigation was able to immunoprecipitate half of the radioactivity in the membrane fraction of CHI-inhibited cells, indicating that cytochrome c oxidase subunits I-III are major products of mitochondrial protein synthesis. Labeling of a minimum of seven mitochondrial gene products confirms the incorporation of a high percentage of label into cytochrome c oxidase subunits I and II. An attempt was also made to determine whether large molecular weight co-immunoprecipitates were precursor forms of the subunits. In addition, this study has compared freshly isolated hepatocytes with those that have been cultured over a period of days. Results indicate that isolated cells have impaired ability to incorporate (^{35}S)-methionine into TCA precipitable cytoplasmic and mitochondrially translated protein, and decreased activity of cytochrome c oxidase and succinate dehydrogenase.

VI. REFERENCES

1. Schatz, G. and Mason, T. The biosynthesis of mitochondrial proteins. Ann. Rev. Biochem. 1974. 43, 51-87.

2. Tzagoloff, A., Macino, G. and Sebald, W. Mitochondrial genes and translation products. Ann. Rev. Biochem. 1979. 48, 419-441.

3. Mason, T. and Schatz, G. Cytochrome c oxidase from bakers yeast. II. Site of translation of the protein components. J. Biol. Chem. 1973. 248, 1355-1360.

4. Rubin, M. and Tzagoloff, A. Assembly of the mitochondrial membrane systems. X. Mitochondrial synthesis of three of the subunit proteins of yeast cytochrome oxidase. J. Biol. Chem. 1973. 248, 4275-4279.

5. Koch, G. Synthesis of the mitochondrial inner membrane in cultured Xenopus laevis oocytes. J. Biol. Chem. 1976. 251, 6097-6107.

6. Borst, P. and Grivell, L. The mitochondrial genome of yeast. Cell 1978. 15, 705-723.

7. Ludwig, B., Downer, N. and Capaldi, R. Labeling of cytochrome c oxidse with (^{35}S)diazobenzene sulfonate. Orientation of this electron transfer complex in the inner mitochondrial membrane. Biochemistry 1979. 18, 1401-1407.

8. Sebald, W., Schwab, A. and Bucher, T. Cycloheximide resistant amino acid incorporation into mitochondrial protein from Neurospora crassa in vivo. FEBS Lett. 1969. 4, 243-246.

9. Sebald, W., Weiss, H. and Jackl, G. Inhibition of the assembly of cytochrome oxidase in Neurospora crassa by chloramphenicol. Eur. J. Biochem. 1972. 30, 413-417.

10. Poyton, R. and McKemmie, E. A polyprotein precursor to all four cytoplasmically translated subunits of cytochrome c oxidase from Saccharomyces cerevisiae. J. Biol. Chem. 1979. 254, 6763-6771.

11. Poyton, R. and McKemmie, E. Post-translational processing and transport of the polyprotein precursor to subunits IV to VII of yeast cytochrome c oxidase. J. Biol. Chem. 1979. 254, 6772-6780.

12. Eytan, G., Carrol, R., Schatz, G. and Racker, E. Arrangement of the subunits in solubilized and membrane bound cytochrome c oxidase from bovine heart. J. Biol. Chem. 1975. 250, 8598-8603.

13. Downer, N., Robinson, N. and Capaldi, R. Characterization of a seventh different subunit of beef heart cytochrome c oxidase. Similarities between the beef heart enzyme and that from other species. Biochemistry 1976. 15, 2930-2936.

14. Ries, G., Hundt, E. and Kadenbach, B. Immunoprecipitation of a cytoplasmic precursor of rat-liver cytochrome oxidase. Eur. J. Biochem. 1978. 91, 179-191.

15. Rascati, R. and Parsons, P. Purification and characterization of cytochrome c oxidase from rat liver mitochondria. J. Biol. Chem. 1979. 254, 1586-1593.

16. Beattie, D. The synthesis of mitochondrial proteins. Sub-Cell. Biochem. 1971. 1, 1-23.

17. Gellerfors, P., Wielburski, A. and Nelson, B. D. Synthesis of mitochondrial proteins in isolated rat hepatocytes. FEBS Lett. 1979. 108, 1, 167-170.

18. Ricca, G., Liu, D., Coniglio, J. and Richardson, A. Rates of protein synthesis by hepatocytes isolated from rats of various ages. J. Cell Physiol. 1978. 97, 137-146.

19. Tanaka, K., Sato, M., Tomita, Y. and Ichihara, A. Biochemical studies on liver functions in primary cultured hepatocytes of adult rats. I. Hormonal effects on cell viability and protein synthesis. J. Biochem. 1978. 84, 937-946.

20. Jeffreys, A. and Craig, I. Analysis of proteins synthesized in mitochondria of cultured mammalian cells. Eur. J. Biochem. 1976. 68, 301-311.

21. Constantino, P. and Attardi, G. Metabolic properties of the products of mitochondrial protein synthesis in HeLa cells. J. Biol. Chem. 1977. 252, 1702-1711.

22. Hare, J. F., Ching, E. and Attardi, G. Human cytochrome c oxidase. Biochemistry 1980. in press.

23. Hare, J. F. Rat liver cytochrome c oxidase: Subunit composition of the purified and immunoisolated enzyme. In press.

24. Moldeus, P., Hogberg, J. and Orrenius, S. Isolation and use of liver cells. Meth. Enzymol. 1978. 52, 60-64.

25. Gellerfors, P. and Nelson, B. D. A rapid method for the isolation of intact mitochondria from isolated rat liver cells. Anal. Biochem. 1979. 93, 200-203.

26. Fairbanks, G., Steck, T. and Wallach, D. F. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 1971. 10, 2931-2936.

27. Cabral, F. and Schatz, G. High resolution one- and two-dimensional electrophoretic analysis of mitochondrial membrane polypeptides. Meth. Enzymol. 1979. 56, 602-613.

28. Bonner, W. and Laskey, R. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 1974. 46, 83-88.

29. Lowry, O. H., Rosebrough, N., Farr, A., and Randall, R. Protein measurement with the folin phenol reagent. J. Biol. Chem. 1951. 193, 265-275.

30. Smith, L. Spectrophotometric assay of cytochrome c oxidase. Methods of Biochem. Analysis. 1955. 2, 427-434.

31. King, T. Preparation of succinate dehydrogenase and reconstitution of succinate oxidase. Meth. Enzymol. 1967. 10, 322-331.

32. Dische, Z. Color reactions of nucleic acids and their constituents based on reactions of their sugars. in: Chargaff and Davidson, The Nucleic Acids, 1955, Vol. I, 286.

33. Michalopoulos, G. and Pitot, H. C. Primary culture of parenchymal liver cells on collagen membrane. Exp. Cell. Res. 1975. 94, 70-78.

34. Rubin, K., Oldberg, A., Hook, M. and Obrink, B. Adhesion of rat hepatocytes to collagen. Exp. Cell. Res. 1978. 117, 165-177.

35. Traub, P. Localization of streptomycin-resistance in the 30s ribosomes of E. coli. Mode of action of cycloheximide. in: Inhibitors Tools in Cell Research. Bucher and Sies, eds., Springer-Verlag, New York, 1969, 79-99.

36. Vasquez, D., Staehelin, T., Celma, M., Battaner, E., Fernandez-Munoz, R. and Munro, R. Inhbitors as tools in elucidating ribosomal function. in: Inhibitors Tools in Cell Research. Bucher and Sies, eds., Springer-Verlag, New York, 1969, 100-123.

37. Bernstein, J., Bucher, J. and Benniall, R. Origin of mitochondrial enzymes. V. The polypeptide character and the biosynthesis of rat liver cytochrome c oxidase polypeptides by mitochondria. J. Bioen. and Biomemb. 1978. 10, 59-74.

38. Steffens, G. and Buse, G. Studien an Cytochrom-c-oxidase. I. Hoppe-Seyler's Z. Physiol. Chem. 1976. 357, 1125-1137.

39. Attardi, G. and Ching, E. Biogenesis of mitochondrial proteins in HeLa cells. Meth. Enzymol. 1979. 56, 66-78.