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BIOGENESIS OF MITOCHONDRIA IN MAMMALIAN CELLS IN CULTURE

By

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Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
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To the great Canadian novel

ABSTRACT

The interrelationship between the mitochondrial synthesis of protein and the synthesis of cellular phospholipids was studied in BHK-21 cells in culture. Cells were cultured over extended periods in D-chloramphenicol and the effects on protein and lipid synthesis determined. Cell division ceased following two generations exposure to the drug while cell size increased. Mitochondria were shown to be enlarged and internal membrane degeneration was visible in the electron microscope. Inner membrane enzymes, cytochrome c oxidase and succinate cytochrome c reductase, and their constituent cytochromes aa_3 and b were depleted by this treatment. Enzymes of the endoplasmic reticulum, mitochondrial matrix and outer membrane were not affected. These effects were reversible unless cell breakdown had occurred.

Mitochondrial protein synthesis constituted less than 5% of the total cellular synthesis as measured by leucine incorporation. This synthesis was sensitive to chloramphenicol and insensitive to cycloheximide, although in the absence of cycloheximide-sensitive cytoplasmic protein synthesis, the mitochondrial protein synthesis products were unstable. Heme synthesis, as measured by δ-aminolevulinic acid incorporation, was sensitive to chloramphenicol if present during both growth and labelling.

The *de novo* synthesis of cellular and in particular mitochondrial phospholipids as measured by phosphate, glycerol or fatty acid incorporation, was sensitive to chloramphenicol treatment. All phospholipids were affected to a similar extent, with the exception of cardiolipin labelling which was reduced to a greater degree. These results were not primarily related to reduced energy levels, since the concentrations of ATP were identical in treated and untreated cells. Removal of chloramphenicol resulted in a rapid synthesis of cardiolipin followed by recovery of the other phospholipids.

Experiments with isolated subcellular fractions revealed an apparent inhibition of the acylation of either *sn*-glycerol-3-phosphate or dihydroxyacetone phosphate in mitochondria from chloramphenicol-treated cells. Glycerol kinase activity may also be affected. Biosynthesis of CDP-glycerides, phosphatidyl inositol, phosphatidyl choline and the polyglycerolphosphatides was stimulated in subcellular fractions from treated cells.

The biosynthesis of polyglycerolphosphatides in BHK-21 cells was found to differ in compartmentalization of reactions compared to other mammalian tissues. The reactions leading to phosphatidyl glycerol phosphate, and conversion of phosphatidyl glycerol to cardiolipin were localized in mitochondria but the hydrolysis of phosphatidyl glycerol phosphate was determined to be a cytoplasmic

rather than mitochondrial function as established for liver, heart or brain.

The implications of the effects of chloramphenicol, an inhibitor of mitochondrial protein synthesis, on cellular phospholipid metabolism are discussed in light of our current knowledge of other mammalian, fungal or bacterial systems.

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NOMENCLATURE

δ-ALA	δ-aminolevulinic acid
CAP	D-chloramphenicol
DHAP	dihydroxyacetone phosphate
DPG	cardiolipin; diphosphatidyl glycerol
EDTA	ethylenediaminetetraacetic acid
FA	fatty acid
F_1-ATPase	mitochondrial adenosine triphosphatase
LPA	lysophosphatidic acid
MES	2-(N-morpholino) ethane sulfonic acid
PA	phosphatidic acid
PC	phosphatidyl choline
PCMB	p-chloromercuribenzoic acid
PE	phosphatidyl ethanolamine
PG	phosphatidyl glycerol
PGP	phosphatidyl glycerol phosphate
PI	phosphatidyl inositol
PS	phosphatidyl serine
SM	sphingomyelin
TCA	trichloroacetic acid
TES	N-tris (hydroxymethyl) methyl-2-aminoethane-sulfonic acid
tris	2-amino 2-hydroxymethyl propane-1,3-diol.

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CHAPTER 1. INTRODUCTION

1.1 MITOCHONDRIAL STRUCTURE

Mitochondria, ubiquitous organelles located in the cytoplasm of virtually all aerobic eukaryotes, are perhaps the best understood cellular organelles with respect to their structural and molecular organization and metabolic functions. The unique feature of the mitochondrion which lends itself to defining the role of the organelle is the mitochondrial membrane system. It serves two functions in terms of compartmentation of cellular functions. In the first case, it defines a boundary which permits the enclosure and regulation of a microenvironment distinct from that of the surrounding cell. Secondly, the membrane permits the functional organization of specific enzymes, proteins and coenzymes into highly efficient assemblages of metabolic machinery (Getz, 1972). Comparative studies of the mitochondria isolated from various sources have confirmed a basic framework common to all mitochondria, yet the presence of features characteristic of the type of cell from which they originate.

The ultrastructure of the mitochondrion is well documented (for recent reviews see Tandler and Hoppel, 1972; Munn, 1974). Pioneered by the work of Palade (1952) and Sjöstrand (1953) using thin section electron microscopy and more recently negative-staining (Horne and Whittaker,

1962) and freeze-etching techniques (Moor and Mühlethaler, 1963), a fairly consistent structural model for the organelle has evolved. The essential components of this model are the two membranes, each showing the classical double track structure. The outer membrane is smooth and free of folds, while the inner membrane is highly convoluted, forming ridges called cristae. These membranes segregate the mitochondrion into two compartments, the matrix which is bounded by the inner membrane, and the intermembrane space, defined by the inner and outer membranes. The inner membrane exhibits a greater particle density in freeze-fracture studies, usually indicative of a higher concentration of integral proteins, than the outer membrane (Malhotra, 1972; Munn, 1974). In addition the elementary particles that are visible in negative staining studies and are now associated with the F_1-ATPase, are found to protrude from the inner membrane into the matrix (Fernandez-Moran, 1962). The density of the matrix and the degree of convolution of the inner membrane can vary with the source of the mitochondria (Tandler and Hoppel, 1972). Furthermore, the internal structure is dramatically dependent on the nutritional or physiological status of the cell.

1.2 MITOCHONDRIAL BIOGENESIS - A GENERAL DISCUSSION

The discovery of mitochondrial DNA and of an associated protein synthesizing system has provided a very powerful

technique for analyzing mitochondrial structure-function relationships. This semi-autonomous synthetic ability of the mitochondrion appears to manifest itself in the production of components of the mitochondrial membranes, particularly the inner membrane, a situation that is appealing analytically in that the majority of components associated with electron transport, oxidative phosphorylation and metabolite transport are localized in this section of the organelle (Harmon et al., 1974). It is with this realization, that the genetics of mitochondria have come to be closely associated with the biogenesis of the proteins of the mitochondrial membrane (Getz, 1972; Sager, 1972). The dynamic state of mitochondrial membrane synthesis involves the biosynthetic systems for mitochondrial nucleic acids, proteins, lipids and heme pigments. The majority of these systems are extramitochondrial and controlled by nuclearly coded structural genes. With this in mind, the remainder of this introduction will deal with a summary of the basic concepts about the origins of these components and how they relate to the problem that is the subject of this thesis.

Mitochondrial membrane organization and function have been discussed in great detail (Racker, 1970), and the biogenesis of the inner membrane has been the subject of many recent symposia and books (Roodyn and Wilkie, 1968; Slater et al., 1968; Boardman et al., 1971; Getz, 1972;

Sager, 1972; Van den Bergh et al., 1972; Kroon and Saccone, 1974; Packer, 1974) and more than twenty review articles. Because of the massive quantity of information available on this subject, one can only make reference to more comprehensive sources of general discussion and attempt to touch on the major points relating to the synthesis of the various cellular components. The main emphasis will be on mammalian mitochondria, although selected references to those from other sources will be made as seems appropriate.

1.3 MITOCHONDRIAL NUCLEIC ACIDS AND TRANSCRIPTION

The existence of mitochondrial DNA in a number of organisms is known and well characterized. As has been determined for most components of mitochondria, there exist many differences in mitochondrial DNA depending on whether its source be animal, plant or fungal. General properties of mitochondrial DNA have been reviewed (Rabodyn and Wilkie, 1968; Borst and Kroon, 1969; Nass, M.M.K., 1969; Nass, S., 1969; Ashwell and Work, 1970; Linnane and Haslam, 1970; Rabinowitz and Swift, 1970; Schatz, 1970; Borst, 1972; Getz, 1972; Linnane et al., 1972; Cohen, 1973; Mahler, 1973; Gillham, 1974; Milner, 1976). Multiple copies (2 to 6) of double-stranded, circular DNA are found in animal mitochondria. These copies appear to be homogeneous small molecules of about

10^7 daltons. They differ in base composition with and do not hybridize to nuclear DNA. Plant, fungal and protozoan mitochondrial DNA's are 3 to 5 times larger and are often isolated as linear molecules, probably as artifacts of the isolation procedure. The small size of mitochondrial DNA's indicates that only a few mitochondrial proteins can be coded for by the organelle DNA. Mitochondrial DNA appears to replicate semi-conservatively, but this finding is by no means a certainty (Borst, 1972).

The subject of mitochondrial transcription has only recently been reviewed in any detail (Kroon and Saccone, 1974; Saccone and Quagliariello, 1975; Avadhani et al., 1975; Milner, 1976). Mitochondrial rRNA and several tRNA's in both animal (up to 12) and fungal cells (up to 20) appear to be coded by the mitochondrial genome (Attardi et al., 1973; Saccone and Quagliariello, 1975), although for most tRNA's, no complementary mitochondrial DNA has been identified. The presence of fMet-tRNA in mitochondria is a striking difference from the cytoplasmic family of tRNA's of eukaryotes (Ashwell and Work, 1970; Rabinowitz and Swift, 1970). Poly(A)-containing species complementary to mitochondrial DNA, have been found in mitochondrial RNA (Perlman et al., 1973) and mitochondrial polysome fractions (Attardi et al., 1974) from HeLa cells, although they have not been demonstrated to act as templates for protein synthesis. However, ten poly(A)-containing RNA molecules

do correspond in number and size to the predicted templates for the *in vivo* products of mitochondrial protein synthesis (Constantino and Attardi, 1975; Saccone and Quagliariello, 1975). It is important to note that mitochondrial DNA from yeast has been transcribed and translated in an *E. coli* cell-free system and the resulting translated products found to correspond to proteins synthesized in isolated mitochondria or in cells in which cytoplasmic protein synthesis has been inhibited (Scragg and Thomas, 1975).

Thus, in summary, mitochondrial DNA appears to code for all mitochondrial rRNA and some of the mitochondrial tRNA's. Although mRNA-like species have been isolated from mitochondria, they are few in number and have not been characterized definitively as the templates for proteins of the mitochondrial membrane in mammalian cells.

1.4 THE BIOSYNTHESIS OF MITOCHONDRIAL MEMBRANE PROTEINS - MITOCHONDRIAL TRANSLATION

The membranes of mitochondria are composed of two groups of proteins - those synthesized on mitochondrial ribosomes and those synthesized on cytoplasmic ribosomes. Several techniques have proved very valuable in determining the translation products of the two systems. They are discussed in a number of reviews (Roodyn and Wilkie, 1968; Slater *et al*., 1968; Borst and Kroon, 1969; Nass, S., 1969; Schatz, 1970; Rabinowitz and Swift, 1970; Linnane

and Haslam, 1970; Ashwell and Work, 1970; Borst and Grivell, 1971; Boardman *et al.*, 1971; Beattie, 1971; Linnane *et al.*, 1972; Borst, 1972; Getz, 1972; Sager, 1972; Van den Bergh *et al.*, 1972; Cohen, 1973; Mahler, 1973; Tzagoloff *et al.*, 1973; Attardi *et al.*, 1973; Kroon and Saccone, 1974; Packer, 1974; González-Cadavid, 1974; Schatz and Mason, 1974; Costantino and Attardi, 1975; Saccone and Quagliariello, 1975; Avadhani *et al.*, 1975; Milner, 1976). The products of protein synthesis have been determined by incubating isolated mitochondria with appropriate precursors, by using selective inhibitors of translation or transcription or alternatively, by the study of mutants in which mitochondrial DNA is defective, such as the petite mutant in *Saccharomyces cerevisiae* (see Gillham, 1974), antibiotic resistant yeast mutants (Mahler, 1973) and the *mi* mutants of *Neurospora crassa* (Roodyn and Wilkie, 1968). Thus by determining which polypeptides are present or absent following these manipulations, an indication of the number and size of the proteins from the inner membrane synthesized on each system can be obtained. More recently, the technique of transcribing and translating mitochondrial DNA with *E. coli* enzymes and ribosomes (Scragg and Thomas, 1975) and the use of somatic cell mutants, temperature sensitive in cytoplasmic protein synthesis (Wallace *et al.*, 1975) have yielded valuable information as to the nature of the mitochondrial translation products.

At this point, it is necessary to discuss specifically the nature of the technique that has been used to determine the extent of mitochondrial protein synthesis and its influence on lipid synthesis in the mammalian cell culture system that is discussed in this thesis. I have made use of the antibacterial antibiotic D(-)-chloramphenicol which has been shown to cause eukaryotic cells to accumulate respiration-deficient mitochondria. Inhibition of mitochondrial protein synthesis by D-chloramphenicol was first demonstrated in rat liver mitochondria in vitro (Rendi, 1959) and has been confirmed in various other eukaryotes (for review, see Schatz and Mason, 1974). Growth of eukaryotic cells in chloramphenicol results in decreased amounts of cytochrome oxidase, F_1-ATPase, cytochrome b and impaired assembly of cytochrome c_1 in mitochondrial membranes. There is no loss of mitochondrial nucleic acids in HeLa cells (Storrie and Attardi, 1972) or in yeast, where a functional protein synthesizing system is retained despite growth for several generations in the drug (Davey et al., 1969).

Chloramphenicol acts to block protein synthesis by preventing the binding of amino acyl tRNA's to the peptidyl site of the ribosome in prokaryotes (Contreras et al., 1974). There are however numerous secondary effects. Mitochondrial electron transport is inhibited at site 1 (Freeman and Haldar, 1967) and oxidative phosphorylation may be uncoupled (Hanson and Hodges, 1963). In these

experiments, the length of exposure of cells to chloramphenicol was sufficiently long to cause a loss of cytochromes, hence reducing or eliminating electron transport and oxidative phosphorylation completely; thus the secondary inhibitory effects were less significant. Other effects that are observed include the suppression of bone marrow ferrochelatase (Jones and Jones, 1969), which is just one aspect of the toxic effects of the use of the drug in the treatment of infections in man (for review, see Franceschinis _et al._, 1974). Inhibition may be incomplete even at high drug concentrations and resistance to the drug may develop. Despite the problems with inhibitor studies, most papers in the field of mitochondrial biogenesis utilize this technique (Schatz and Mason, 1974). Provided that the results are considered in light of those obtained using other techniques, inhibition studies can yield valuable information.

As was discussed earlier, most of the RNA components of the mitochondrial protein synthetic apparatus are synthesized in the mitochondria. However, all the mitoribosomal proteins and the remainder of the enzymes and factors involved in the translation event are cytoplasmically synthesized (Borst and Grivell, 1971; Attardi _et al._, 1973; Saccone and Quagliariello, 1975). The ribosomes and factors from mitochondria are functionally similar and interchangeable with protein-synthesizing components from bacteria.

Two functions have been ascribed to the proteins

synthesized using mitochondrial DNA as a template. The first of these has not been dealt with in great detail and remains essentially speculative. It has been suggested that mitochondrial gene products may act as repressors of nuclear gene expression (Barath and Küntzel, 1972). The evidence for this hypothesis is that the synthesis of certain enzymes coded by nuclear genes, is stimulated by inhibitors of mitochondrial DNA transcription and translation. This may be related to the ability of mitochondria in many systems (Barath and Küntzel, 1972) to divide synchronously and this regulation may be permitted by the hypothetical gene product. Recently, it has been shown that the level of mitochondrial leucyl- and phenylalanyl-tRNA synthetases (nuclearly coded in Neurospora) rise on inhibition of mitochondrial protein synthesis (Beauchamp and Gross, 1976).

Although replication and transcription of mitochondrial DNA in HeLa cells is restricted to or accelerated in S-phase and in G_2-phase, no significant differences in the rate of synthesis or size distribution of mitochondrial proteins have been observed at different phases of the cell cycle (Attardi *et al.*, 1973).

The second function resides in the production of protein components of the inner membrane (Beattie, 1971). As yet, three peptides of cytochrome c oxidase, four of the membrane attachment points of the rutamycin-sensitive

ATPase complex, one or two subunits of cytochrome b and a protein involved in the assembly of cytochrome c_1 but not in the synthesis of the holoenzyme, have been identified as products of mitochondrial protein synthesis in various fungal and animal cells. (For a review of the evidence of these findings and their sources, see Costantino and Attardi, 1975; Saccone and Quagliariello, 1975). The situation is complicated by the fact that many of the low molecular weight peptides identified as mitochondrially synthesized seem to be products of proteolysis of large peptides (Michel et al., 1976).

The unique feature of the polypeptides which are synthesized on mitoribosomes is the fact that they are hydrophobic in nature and in most cases soluble in chloroform-methanol (Tzagoloff and Akai, 1972; Burke and Beattie, 1973; Hadvary and Kadenback, 1973; Costantino and Attardi, 1975). Although no direct evidence exists for their transcription and translation from the mitochondrial genome, they do correspond in size and number to the potential translation products of isolated mitochondrial poly(A)-containing RNA (Ojala and Attardi, 1974; Costantino and Attardi, 1975). Some evidence has been presented for the concept that mitochondria fail to incorporate eight polar amino acids (Costantino and Attardi, 1973), possibly due to the absence of the relevant mitochondrial tRNA's (Saccone and Quagliariello, 1975).

Since the mitochondrially coded polypeptides are all membrane components, their hydrophobicity is not unexpected and may be significant in the association of the enzymes of which they are components, with specific phospholipids. This lipid-protein association will be discussed at a later stage in this thesis.

The remainder of the mitochondrial membrane and matrix proteins are coded for by nuclear genes. Cytochrome c biosynthesis and transfer into the mitochondria is the best studied example (González-Cadavid, 1974). Little is known about the mechanism of transfer of cytoplasmic components, but it may involve selective synthesis on mitochondria-associated cytoribosomes permitting vectorial discharge of the product into the mitochondria (Kellems _et al_., 1974). This hypothesis requires further substantiation as to both the mechanism and the regulation of the synthesis of enzymes with components from both translation systems.

1.5 HEME BIOSYNTHESIS AND INCORPORATION INTO APOCYTOCHROMES

The biosynthesis of heme is a process involving cooperation between enzymes located in different compartments of the cell (Meyer and Schmid, 1973). Condensation of glycine and succinyl coenzyme A by the enzyme δ-aminolevulinic acid synthetase is the initial reaction, occurring in the mitochondria. The product of this reaction, δ-aminolevulinic acid (δ-ALA), is transferred to the cytoplasm, condensed

with a second molecule to yield porphobilinogen which is the immediate precursor of a number of porphyrin derivatives. One of these, coproporphyrinogen, is reintroduced into the mitochondrion where it is oxidized to protoporphyrin and iron (II) is added by ferrochelatase to yield heme. Synthesis of δ-ALA by δ-ALA synthetase is the rate limiting step of the reaction and this reaction is the site of regulation by negative feedback by heme (Meyer and Schmid, 1973; Marks, 1973; Patton and Beattie, 1975).

Attachment of the prosthetic groups to the various apoproteins appears to occur at different sites in the cell (González-Cadavid, 1974). For those cytochromes containing polypeptides coded for on mitoribosomes, the site of attachment is apparently in the mitochondria, while for cytochrome c, the attachment occurs in the cytoplasm. Heme has also been shown to regulate the synthesis of apocytochrome c (González-Cadavid, 1974).

1.6 BIOSYNTHESIS OF MITOCHONDRIAL PHOSPHOLIPIDS

As is the case of mitochondrial membrane proteins, mitochondrial phospholipids consist of species synthesized *in situ* and those synthesized at other sites within the cell, and transported to the mitochondria (Getz, 1972; Morré, 1975). The sites of synthesis of these various phospholipids has been discussed in detail in a number of reviews (McMurray and Magee, 1972; Van den Bosch *et al.*,

1972; McMurray, 1973; Van den Bosch, 1974).

The *de novo* synthesis of the majority of the lipid components is extramitochondrial in animal cells. This includes phosphatidyl choline biosynthesis from CDP-choline and phosphatidyl inositol biosynthesis from CDP-diglyceride, both major constituents of the mitochondrial membrane (McMurray and Dawson, 1969; Van Golde *et al.*, 1974). Both phospholipids are transported to the mitochondria from their site of synthesis in the endoplasmic reticulum by soluble exchange proteins (Dawson, 1973; Wirtz, 1974). The synthesis of phosphatidyl serine and sphingomyelin is also known to occur in the endoplasmic reticulum. However, phosphatidyl serine which is transported to the mitochondria by an exchange protein (Butler and Thompson, 1975) is decarboxylated to yield mitochondrial phosphatidyl ethanolamine (Van Golde *et al.*, 1974). Biosynthesis of the polyglycerophosphatides, phosphatidyl glycerol and cardiolipin involves a series of enzymes that have been shown to be mitochondrial in most cases (Davidson and Stanecev, 1971a). The latter is an interesting finding in that cardiolipin is primarily an inner membrane phospholipid in mitochondria from several sources (White, 1973).

Whereas most of the phospholipids which are constituents of the mitochondrial membranes are eventually synthesized extramitochondrially, many of their precursors can be made in the organelle. The phosphorylation of

glycerol by glycerol kinase is mitochondrial while the phosphorylation of dihydroxyacetone by dihydroxyacetone kinase is cytoplasmic (Jenkins and Hajra, 1976). Acylation of glycerol phosphate or dihydroxyacetone phosphate to yield lysophosphatidic acid or acyldihydroxyacetone phosphate respectively and subsequently phosphatidic acid, can occur in mitochondria as well as in endoplasmic reticulum (Monroy et al., 1973; Hajra, 1968). Phosphatidic acid biosynthesis by phosphorylation of diglyceride occurs only in the endoplasmic reticulum (McMurray, 1975). Finally, the biosynthesis of CDP-diglyceride, the immediate substrate of the polyglycerophosphatide pathway, has been observed in both mitochondria and endoplasmic reticulum (Van Golde et al., 1974).

Despite the intracellular distribution of the enzymes that synthesize mitochondrial phospholipids, it appears that none are determined by the mitochondrial genome (Getz, 1972). Thus the mechanism for regulating the synthesis of mitochondrial phospholipids and their incorporation into the membranes of mitochondria in different stages of development or physiological states, is unknown at this time.

1.7 REGULATION OF LIPID SYNTHESIS BY MITOCHONDRIAL PROTEIN SYNTHESIS

Reconstitution of respiration and oxidative phosphorylation activities from components isolated from mitochondrial

inner membranes is dependent on the addition of the correct phospholipids (Getz, 1970; Kagawa, 1972; Kagawa, 1974). Lipids are implicated in the activity of many mitochondrial enzymes (Lenaz, 1973; Harmon et al., 1974). The physiological state of the mitochondria can result in alterations in phospholipid composition (Getz, 1972). This has been best observed in studies with mitochondria from yeast. In mammalian cells, tight binding between phospholipids and proteins which contain mitochondrially coded polypeptides has been observed. The hydrophobic nature of these polypeptides suggests a possible role in regulation of the synthesis of mitochondrial phospholipids.

With this in mind, I have undertaken a study into the effects of inhibiting mitochondrial protein synthesis on phospholipid synthesis. This has involved characterization of the effects of a specific inhibitor D-chloramphenicol, on various aspects of mitochondrial function and metabolism in cultured animal cells. The results of this investigation will be discussed in subsequent chapters. Each chapter will deal with a particular aspect of the effects of the drug, and will be examined in greater detail in the light of relevant studies reported in the literature.

CHAPTER 2. EFFECT OF CHLORAMPHENICOL ON GROWTH, MORPHOLOGY AND MITOCHONDRIAL RESPIRATORY ENZYMES

2.1 INTRODUCTION

The biogenesis of mitochondria is known to involve two protein synthesizing systems. Experiments with isolated mitochondria have been used to examine the components synthesized on mitoribosomes (for reviews, see Mahler, 1973; Schatz and Mason, 1974), yet this approach fails to account for the interrelationship of the products of the systems for mitochondrial and cytoplasmic protein synthesis (Schatz and Mason, 1974), or the coupling of protein synthesis to mitochondrial DNA and phospholipid biosynthesis. The treatment of whole cells with specific inhibitors provides a method whereby the study of one system in an intact organism is possible in the absence of the second system. Chloramphenicol is an antibacterial antibiotic that was first shown to inhibit mitochondrial protein synthesis selectively in yeast (Clark-Walker and Linnane, 1966; Huang et al., 1966; Clark-Walker and Linnane, 1967). This observation has been verified in other systems (Schatz and Mason, 1974). This study of mitochondrial biogenesis in mammalian cells is of special interest, since the factors regulating this process differ from those in lower eukaryotes in several ways such as disparate sizes

of mitochondrial DNA's (Schatz and Mason, 1974), coupling of electron transport and oxidative phosphorylation (Haslam et al., 1971), mitoribosomal size (Attardi and Ojala, 1971), toxicity of chloramphenicol (Firkin and Linnane, 1968), lack of response to glucose levels (King and King, 1968) and involvement of endocrine controls (Ernster, 1965).

The effects of chloramphenicol on BHK-21 cells in spinner culture have been examined. These are easily manipulated cells with rapid doubling time (12-13 hours) and can be grown to high cell densities (1-2 x 10^6 cells ml^{-1}). BHK-21 cells have been investigated for changes in growth, morphology and mitochondrial enzyme content during chloramphenicol treatment and following its removal from the cultures.

2.2 MATERIALS AND METHODS

2.2.1 Cell Culture

BHK-21 cells were a generous gift from Dr. K.B. Freeman, McMaster University, Hamilton, Canada. They were carried in spinner culture in Modified Alpha MEM (Flow), supplemented with 5% fetal bovine serum (Flow), 100 units ml^{-1} penicillin G (Connaught Laboratories), 100 μg ml^{-1} the anti-PPLO agent, Tylocine® (GIBCO). D-chloramphenicol (Sigma) in ethanol was added to treated cultures at the specified concentrations. The ethanol concentrations used (<0.15%) had no effect upon cell growth. Routine cell counts were determined using a

Coulter Counter, Model B, and cell sizing was done on a Coulter Counter, Model Z_{BI} equipped with recorder.

2.2.2 Harvesting and Preparation of Cells for Enzyme Studies

Cells were harvested during the exponential phase of growth (4-8 x 10^5 cells ml^{-1}) by centrifugation, washed once with phosphate-buffered saline and once with 0.25 M sucrose-0.1 mM EDTA. Cells were suspended in sucrose-EDTA and homogenized for 15 seconds using a Willems Polytron R with P10 Probe (Brinkman) (at a setting of 3.0) (Freeman, 1965; DeVries et al., 1973; McMurray, 1975). This duration and intensity of treatment released more than 90% of cellular cytochrome c oxidase, which was sedimented at 4500 x g for 10 min (McMurray and Dawson, 1969) indicating maximal cell breakage with minimal mitochondrial disreption (Appendix 1).

2.2.3 Enzyme Assays on Cellular Homogenates

Spectrophotometric assays were performed using a Gilford recording spectrophotometer. Cytochrome c oxidase (EC 1.9.3.1), succinate cytochrome c reductase system and NADPH-cytochrome c reductase (EC 3.2.3.5) were assayed as previously described (McMurray and Dawson, 1969), except that Lubrol WX, (0.03% final concentration) was added to the cytochrome c oxidase assay mixture and deoxycholate (0.03% final concentration) to the succinate cytochrome c reductase assay mixture in order to release all latent

enzyme activity. Glutamate dehydrogenase (EC 1.4.1.3) was assayed by the method of Strecker (1955) with potassium cyanide (0.3 mM final concentration) added to block respiration and Lubrol WX (0.03% final concentration) to release latent enzyme. Monoamine oxidase (EC 1.4.3.4) was assayed as previously described (Wurtman and Axelrod, 1963) and modified (Jato-Rodriguez, 1972), using [^{14}C]-tryptamine (New England Nuclear) as substrate.

2.2.4 Cytochrome Spectra

Cells were harvested as before; washed once with phosphate-buffered saline and once with 50% glycerol (w/v). Approximately 10^8 cells were suspended in 50% glycerol and sonicated by immersing the tube in the sonic bath of Branson Sonifier Model S125 at maximum output for 15 seconds. Samples in the reference cuvette were oxidized by adding 0.5 M potassium ferricyanide and those in the second cuvette, reduced with a few grains of sodium hydrosulfite (Williams, 1964). They were then frozen quickly in liquid nitrogen and the difference spectra recorded using an Aminco-Chance spectrophotometer equipped with scatter transmission apparatus (Chance, 1954; Klietmann <u>et al.</u>, 1973).

2.2.5 Preparation of Cells for Electron Microscopy

Cells were fixed in OsO_4 and glutaraldehyde (Polyscience, Inc., Warrington, Pa.) by the method previously

described (Hirsch and Fedorko, 1968), and embedded in Vestopal-W (Martin Jaeger, Geneva) (Ryter and Kellenberger, 1958) using propylene oxide instead of acetone. Sections were examined and photographed using a model 6B AEI electron microscope.

2.2.6 Protein Determinations

Protein samples were analyzed using the method of Lowry et al., (1951) using bovine serum albumin (Sigma) as standard.

2.2.7 Phosphorus Analysis

Phosphorus was assayed by the method of Bartlett (1959) using KH_2PO_4 as standard.

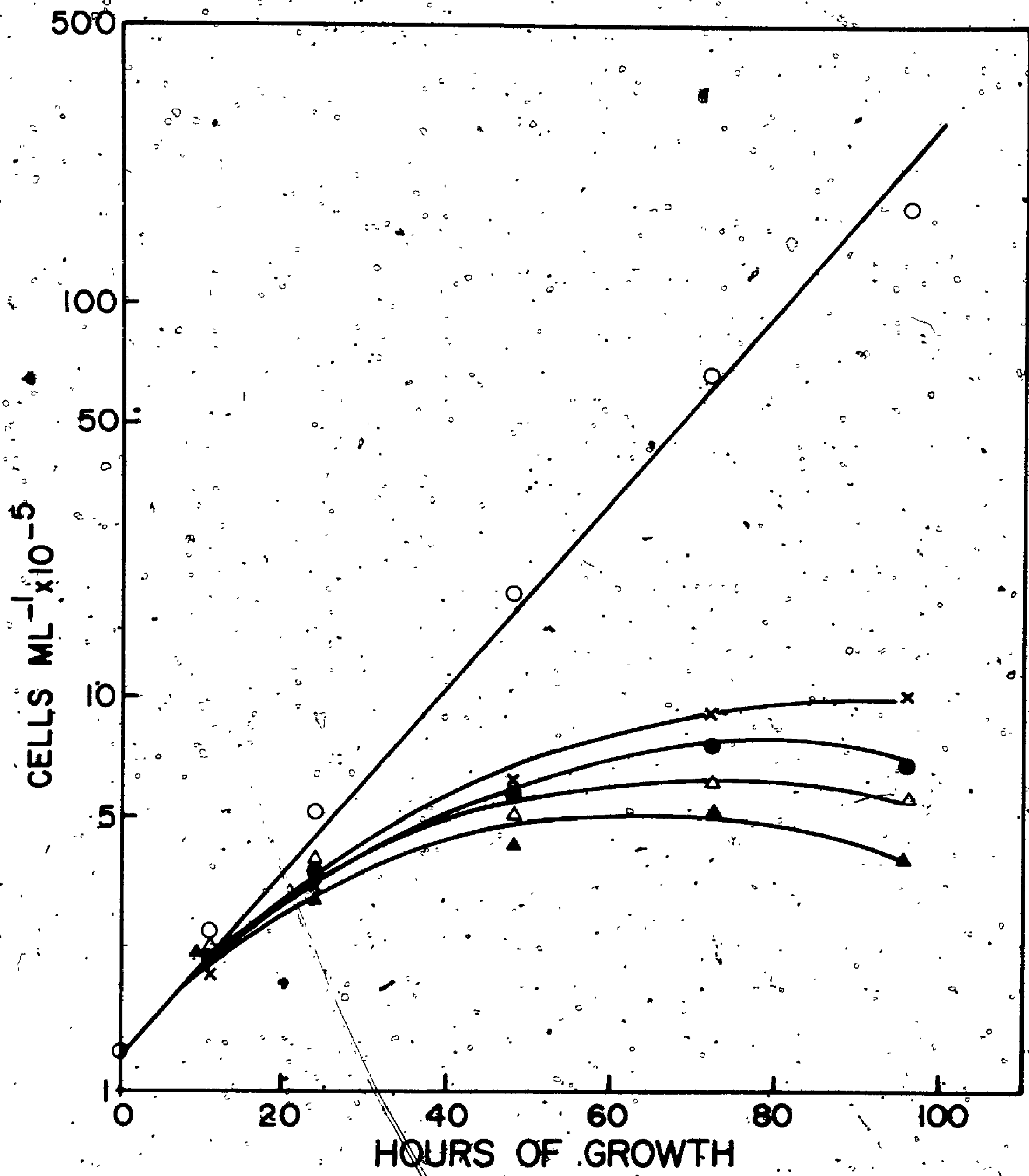
2.3 RESULTS

Figure 1 shows the effects of various concentrations of CAP on the growth of BHK-21 cells in spinner culture. Cells were maintained in culture with the various CAP concentrations, with cell dilution in fresh medium as required to prevent cells from entering stationary growth. It can be seen that CAP at 10, 50 and 100 μg ml^{-1} permits two cell doublings before growth stops, and cell number appears to start to diminish. If cells grown in 100 μg ml^{-1} CAP are resuspended in fresh medium containing 100 μg ml^{-1} CAP at 24 hour intervals, cell growth is enhanced slightly

FIGURE 1

EFFECT OF D-CHLORAMPHENICOL ON THE GROWTH OF BHK-21 CELLS IN SPINNER CULTURE

Cells were grown in the medium described under Materials and Methods. D-chloramphenicol was added to the medium as a concentrated solution in ethanol to the designated final concentration. Cells were diluted to prevent their entry into stationary phase. One culture containing 100 μg ml^{-1} CAP was harvested and resuspended in fresh CAP-containing medium at 24 hour intervals. A 1 ml sample of cells was removed, diluted 1 in 20 with phosphate-buffered saline and counted. o--o no CAP added; ●--● 10 μg ml^{-1} CAP; Δ--Δ 50 μg ml^{-1} CAP; ▲--▲ 100 μg ml^{-1} CAP; ×--× 100 μg ml^{-1} CAP, cells resuspended in fresh medium at 24 hour intervals.


500
100
50
10
5
1
CELLS ML^{-1} x10^{-5}
0
20
40
60
80
100
HOURS OF GROWTH

and three doublings occur before cessation of growth.

Higher concentrations of CAP block all growth by 24 hours (Fig. 2). All cultures were resuspended in fresh medium containing the equivalent concentration of CAP at 24 hours. At 250 and 500 µg ml^{-1} CAP, cell growth is inhibited very quickly. It would appear that resuspension of the cells in fresh medium at 24 hour intervals facilitates the removal of some inhibitor(s) of cellular growth (cf. Fig. 1).

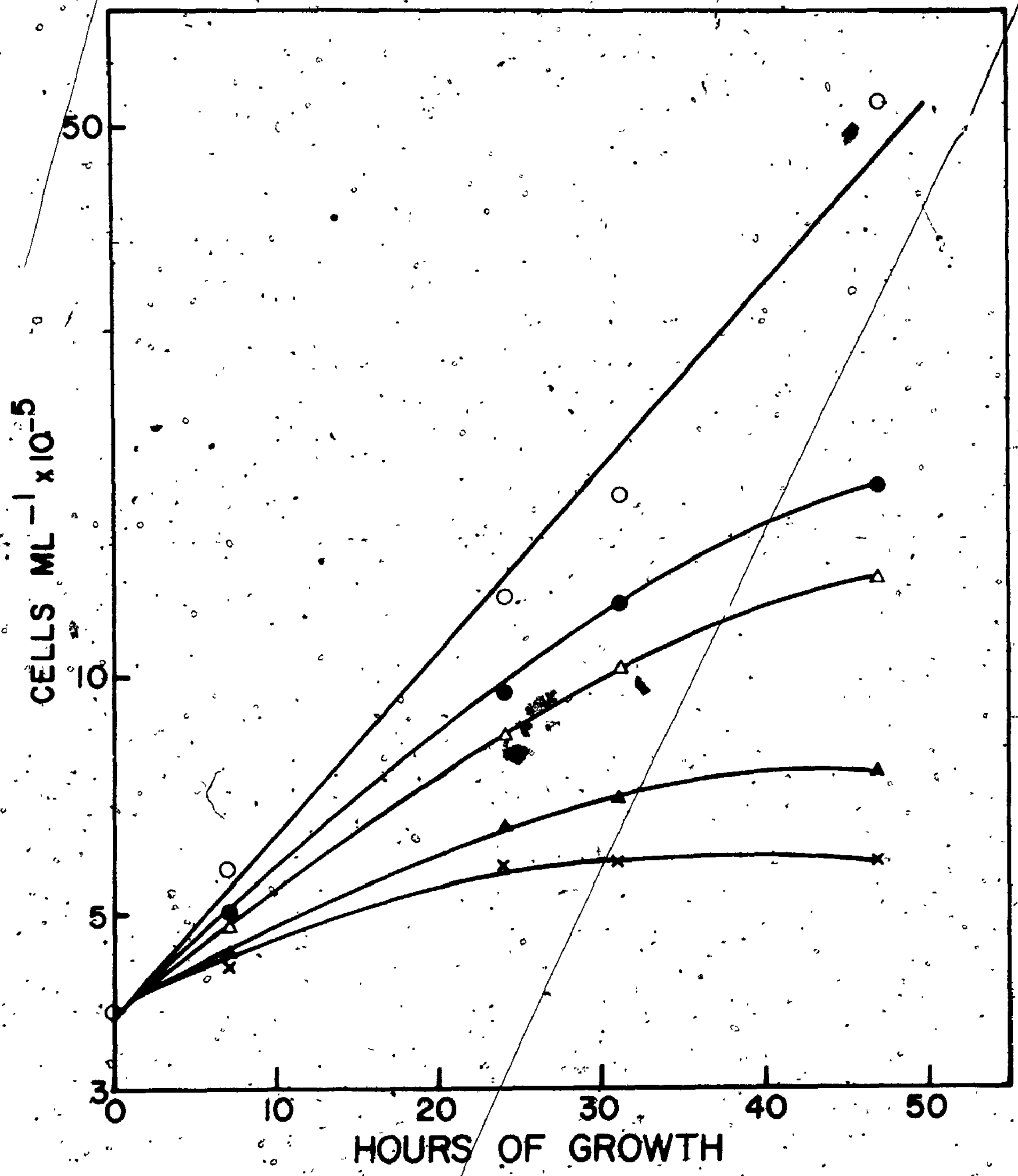
The effect of growth in CAP on cell size was investigated. Figure 3 shows the effect on cell volume as determined by measurement of threshhold values using the Coulter counter. Growth of cells for 48 hours in 100 µg·ml^{-1} CAP results in a mean increase of 29% in the volume of the CAP treated cell population. This increase in size of CAP-treated cells is illustrated further in the increased amounts of cellular protein and lipid phosphorus (Tables 1 and 2). Both cellular protein, indicative of total cellular content, and cellular lipid phosphorus, representative of cellular membranes, were elevated by 20-30% in CAP-treated cells. This probably represents the arrest of cell growth at a pre-mitotic stage because of the absence of the energy or metabolic precursors synthesized by functional mitochondria that are required for cell division.

Figure 4 is a composite of electron micrographs of BHK-21 cells grown for periods up to 120 hours in CAP.

FIGURE 2

EFFECT OF HIGH CONCENTRATIONS OF D-CHLORAMPHENICOL ON GROWTH OF BHK-21 CELLS

Cells were cultured with CAP as described in Fig. 1, except that each culture was harvested and resuspended in fresh medium containing CAP at 24 hours. Cells were counted as in Fig. 1. o--o no CAP added; ●--● 50 μg ml^{-1} CAP; Δ--Δ 100 μg ml^{-1} CAP; ▲--▲ 250 μg ml^{-1} CAP; ×--× 500 μg ml^{-1} CAP.



CELLS ML^{-1} x 10^{-5}
50
10
5
3
0
10
20
30
40
50
HOURS OF GROWTH

FIGURE 3

EFFECT OF D-CHLORAMPHENICOL ON RELATIVE VOLUME OF BHK-21 CELLS GROWN IN SPINNER CULTURE

Cells were grown for 48 hours as in Fig. 2. Samples were taken and cell volume profiles determined by Coulter Counter. ---------- Cells grown 48 hours no CAP; ———— Cells grown 48 hours with 100 µg ml^{-1} CAP.

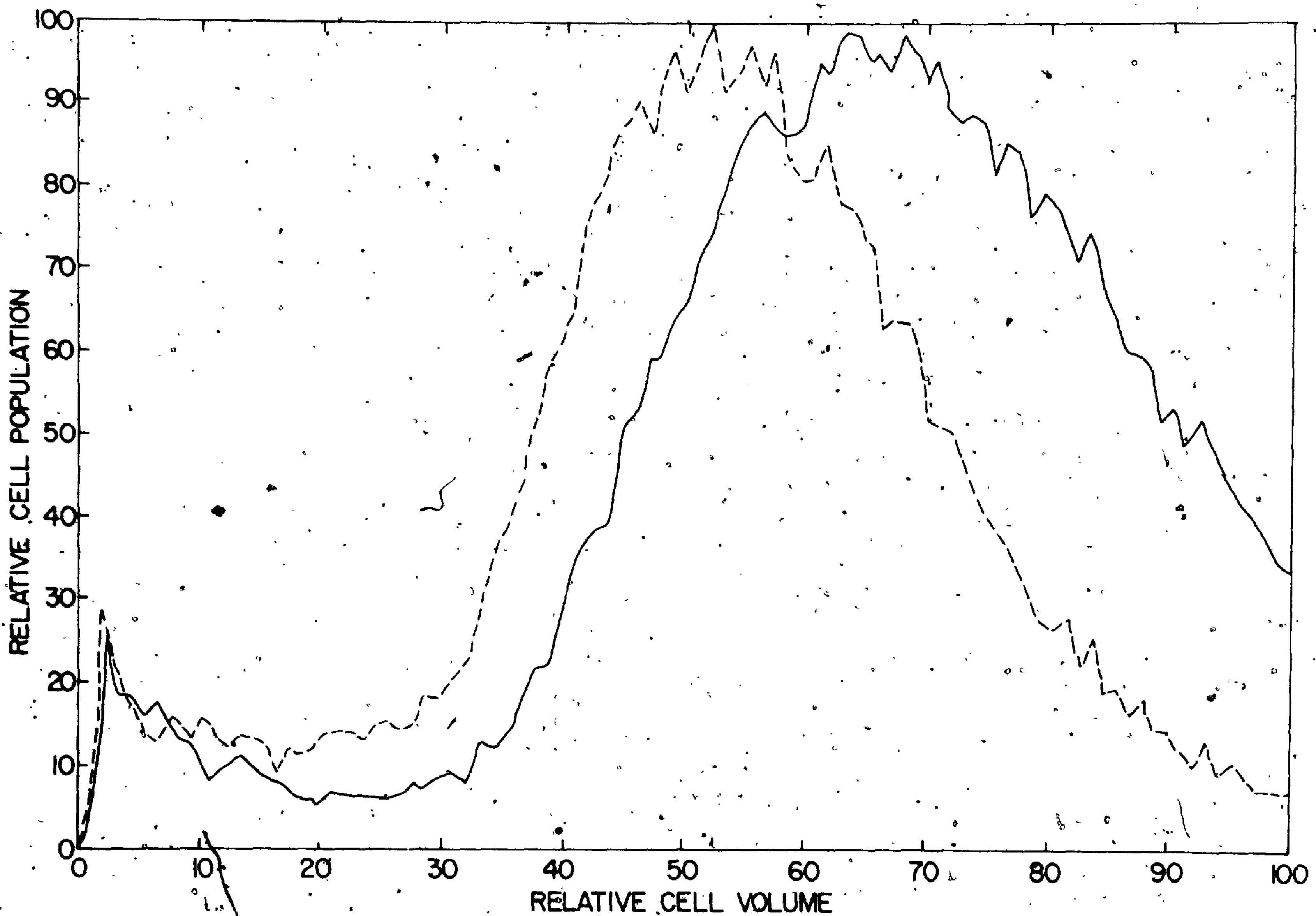

RELATIVE CELL POPULATION
RELATIVE CELL VOLUME
0
10
20
30
40
50
60
70
80
90
100

TABLE 1

EFFECT OF D-CHLORAMPHENICOL ON TOTAL CELLULAR PROTEIN IN BHK-21 CELLS GROWN IN SPINNER CULTURE

BHK-21 cells were grown for 48 hours in spinner culture in medium containing 100 μg ml^{-1} D-chloramphenicol. At 24 hours, 3-5 x 10^5 cells were harvested and resuspended in fresh medium containing D-chloramphenicol. At 48 hours, cells were harvested, washed once with phosphate-buffered saline, once with 0.25 M sucrose-0.1 mM EDTA and suspended in sucrose-EDTA. An equal volume of cold 20% TCA was added to precipitate the cells. The cells were solubilized in 0.4 M NaOH, reprecipitated with cold 20% TCA and solubilized with 0.5 M NaOH-1% sodium dodecyl sulfate.

	mg Cellular Protein / 10^7 Cells (% of Control)	
Experiment	Control	CAP-Treated
I	2.52 (100)	2.98 (118)
II	1.89 (100)	2.57 (136)
III	3.00 (100)	3.28 (109)
Average	2.47 (100)	2.94 (119)

TABLE 2

EFFECT OF D-CHLORAMPHENICOL ON TOTAL CELLULAR LIPID PHOSPHORUS IN SPINNER GROWN BHK-21 CELLS

BHK-21 cells were grown as in Table 1. At 48 hours cells were harvested, washed twice with phosphate-buffered saline. Cells were suspended in phosphate-buffered saline and extracted with chloroform-methanol 1:1 (v/v).

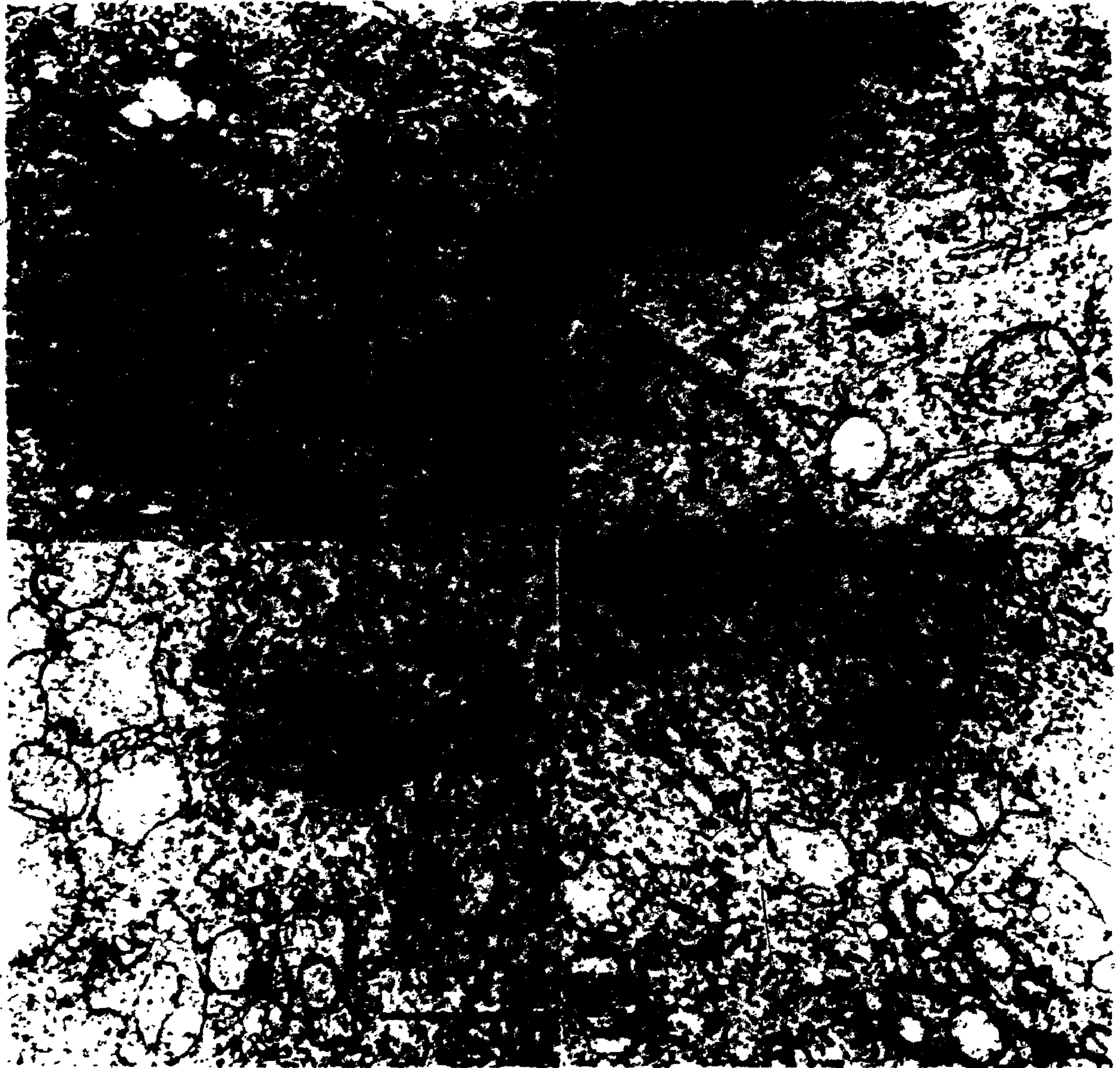
Experiment	μg Cellular Lipid P/10^7 Cells (% of Control)	
	Control	CAP-Treated
I	9.95 (100)	12.75 (128)
II	6.50 (100)	9.26 (142)
III	9.11 (100)	10.89 (120)
Average	8.52 (100)	10.97 (129)

FIGURE 4

EFFECT OF D-CHLORAMPHENICOL ON THE

MORPHOLOGY OF BHK-21 CELLS

Cells were grown in 100 μg ml^{-1} CAP for periods up to 120 hours as described in Fig. 1, for the resuspended culture. At 24 hour intervals, cells were removed, fixed and embedded as described in the Materials and Methods. A - control; B - 24 hours in CAP; C - 48 hours in CAP; D - 120 hours in CAP. Size bar indicates 1 micron.



Growth was characterized by a progressive disruption of mitochondrial cristae with a loss of the plate-like structures. Branched cristae and vesicular profiles appear by 48 hours. The irregularities in membrane structure are accompanied by a decrease in matrix density. Mitochondria appear to be enlarged. Cytoplasmic vacuoles are evident at 48 hours and increase in size and number with length of exposure to CAP. At 120 hours, the cytoplasm is heavily vacuolated.

Enzymes of the mitochondrial inner membrane are specifically affected by CAP. Both succinate cytochrome c reductase (Fig. 5A) and cytochrome c oxidase (Fig. 5B) are affected by growth in 100 $\mu g \cdot ml^{-1}$ CAP. Similar effects on cytochrome c oxidase are observed with 10 and 50 $\mu g\ ml^{-1}$ CAP, but at the lowest concentration, the effect on succinate cytochrome c reductase is marginal (Appendix 2). When the enzyme activities of specific markers of the mitochondrial matrix, outer membrane, and the microsomal fraction, namely glutamate dehydrogenase (Fig. 6A), monoamine oxidase (Fig. 6B), and NADPH-cytochrome c reductase (Fig. 6C) respectively, were assayed, no losses in activity due to growth in chloramphenicol were apparent. In fact, monoamine oxidase activity had almost doubled by 96 hours in CAP.

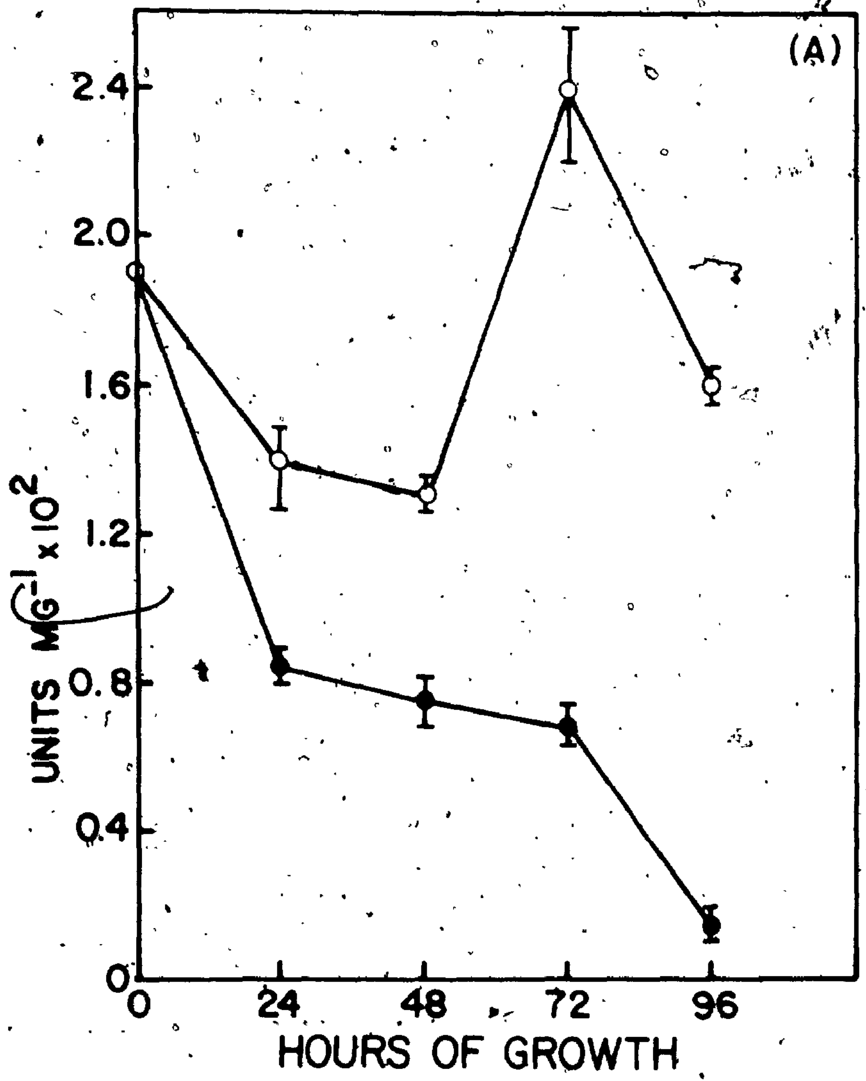
The results of the low temperature difference spectra of the mitochondrial cytochromes were compatible with the

FIGURE 5

EFFECT OF D-CHLORAMPHENICOL ON

MITOCHONDRIAL ENZYMES

Cells were grown for a 96 hour period in 100 μg ml^{-1} CAP, resuspended at 24 hour intervals, cells were harvested, washed once with phosphate-buffered saline and once with 0.25 M sucrose-0.1 mM EDTA. Cells were suspended in sucrose-EDTA and homogenized using a Willems Polytron ®. Enzyme activities and cellular protein were assayed as described in Materials and Methods. A - succinate cytochrome c reductase; B - cytochrome c oxidase; o--o control, no CAP; ●--● 100 μg ml^{-1} CAP. Points expressed as ranges of three cultures.


(A)
UNITS MG^{-1} x 10^2
2.4
2.0
1.6
1.2
0.8
0.4
0
24
48
72
96
HOURS OF GROWTH

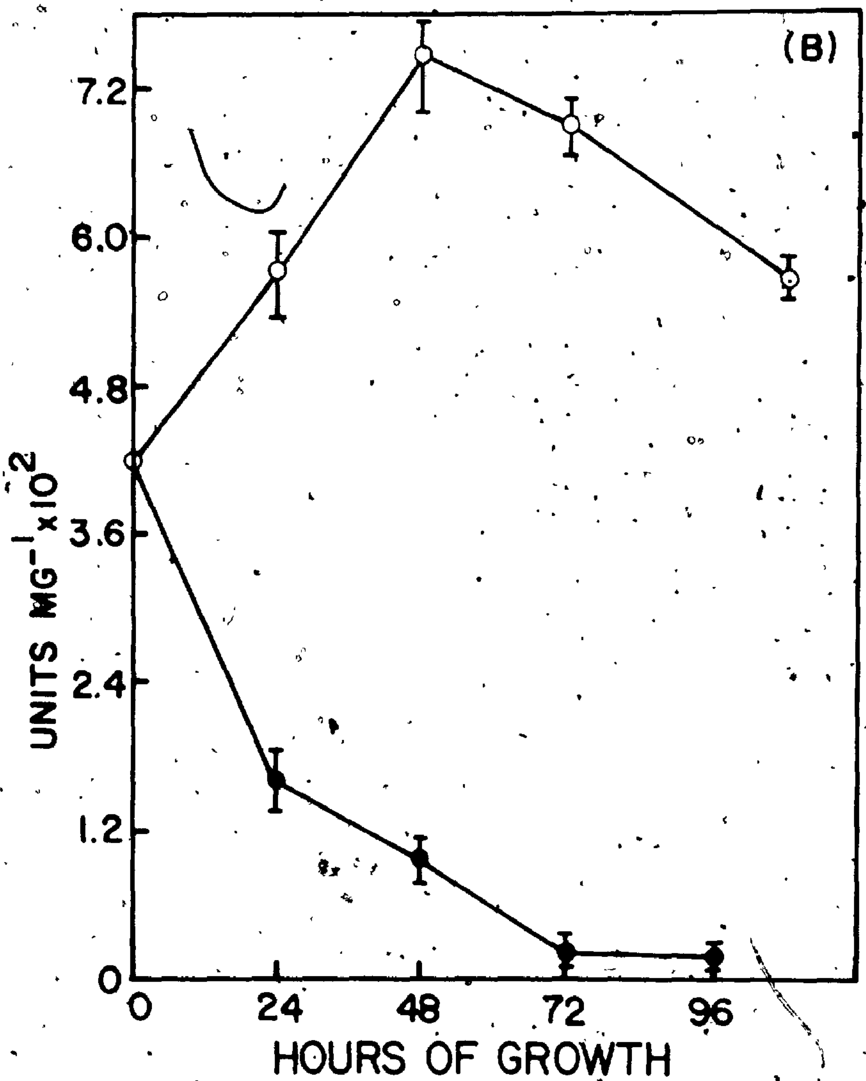
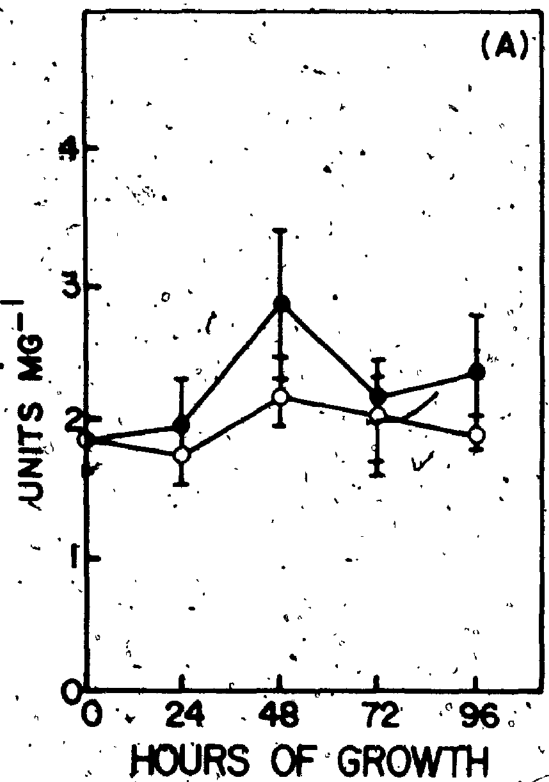
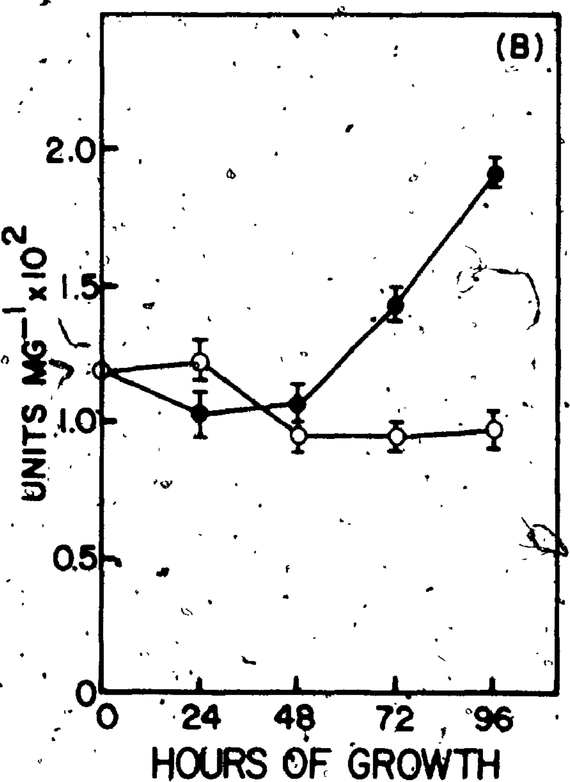

(B)
UNITS MG^{-1} x 10^2
7.2
6.0
4.8
3.6
2.4
1.2
0
24
48
72
96
HOURS OF GROWTH

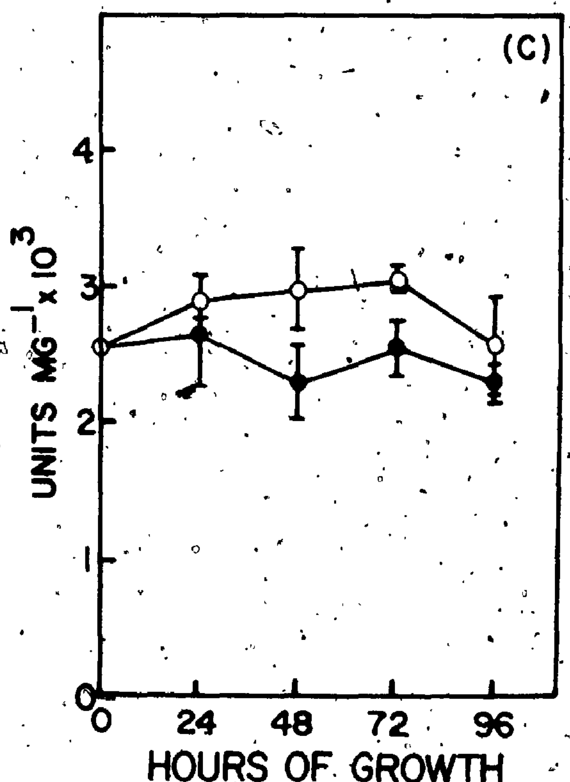
FIGURE 6

EFFECTS OF D-CHLORAMPHENICOL ON BHK-21 CELL ENZYMES

Cells were grown, isolated and homogenized as in Fig. 5. Enzyme activities and protein were assayed as in Materials and Methods. A – Glutamate dehydrogenase; B – Monoamine oxidase; C – NADPH cytochrome c reductase; o--o control, no CAP; ●--● 100 $\mu g\ ml^{-1}$ CAP. Points expressed as ranges of three cultures.


(A)
UNITS MG^{-1}
0
1
2
3
4
0
24
48
72
96
HOURS OF GROWTH


(B)
UNITS MG^{-1} x 10^{2}
0
0.5
1.0
1.5
2.0
0
24
48
72
96
HOURS OF GROWTH


(C)
UNITS MG^{-1} x 10^{3}
0
1
2
3
4
0
24
48
72
96
HOURS OF GROWTH

loss in respiratory enzyme activity. Figure 7 shows the spectra of cells grown over a 72 hour period in 100 μg ml^{-1} CAP. The cytochrome aa_3 peak at 605 nm had disappeared by 48 hours. This compares favourably with the rate of loss of activity of cytochrome c oxidase, of which cytochromes a/a_3 are main components. The cytochrome b peak at 559 nm required 72 hours to disappear entirely, again mirroring the kinetics of activity loss of succinate cytochrome c reductase. Cytochrome c_1 did not separate sufficiently from cytochrome c absorbance at 555 nm under these conditions to determine its response to CAP. There appeared to be no effect on cytochrome c.

If cells are removed from CAP after 48 hours, growth rates return to normal within 12 hours (Fig. 8). This is evident from the growth curve which parallels the growth curve of cultures grown in the absence of CAP over the entire period. A similar recovery profile is seen for cells treated for 24 hours. Extension of the CAP treatment to 72 hours, resulted in a 24 hour lag period before normal growth rates were observed. Extended treatment for 96 hours yielded cultures that showed no recovery following 36 hours post-treatment (Appendix 3).

Cellular morphology appeared to follow a slower recovery time course. Figure 9 shows electron micrographs of cells that had been treated with 100 μg ml^{-1} CAP for 48 hours and then allowed to recover for 24 and 48 hours in

FIGURE 7

LOW TEMPERATURE DIFFERENCE SPECTRA OF BHK-21

CELL CYTOCHROMES

Cells were grown as in Fig. 5. At 24 hour intervals, cells were harvested, washed once with phosphate-buffered saline and once with 50% glycerol (w/v). Cells were suspended in 50% glycerol and sonicated using a Branson Sonifier. Reduced versus oxidized difference spectra were run at 77°K using a Chance-Aminco Spectrophotometer.

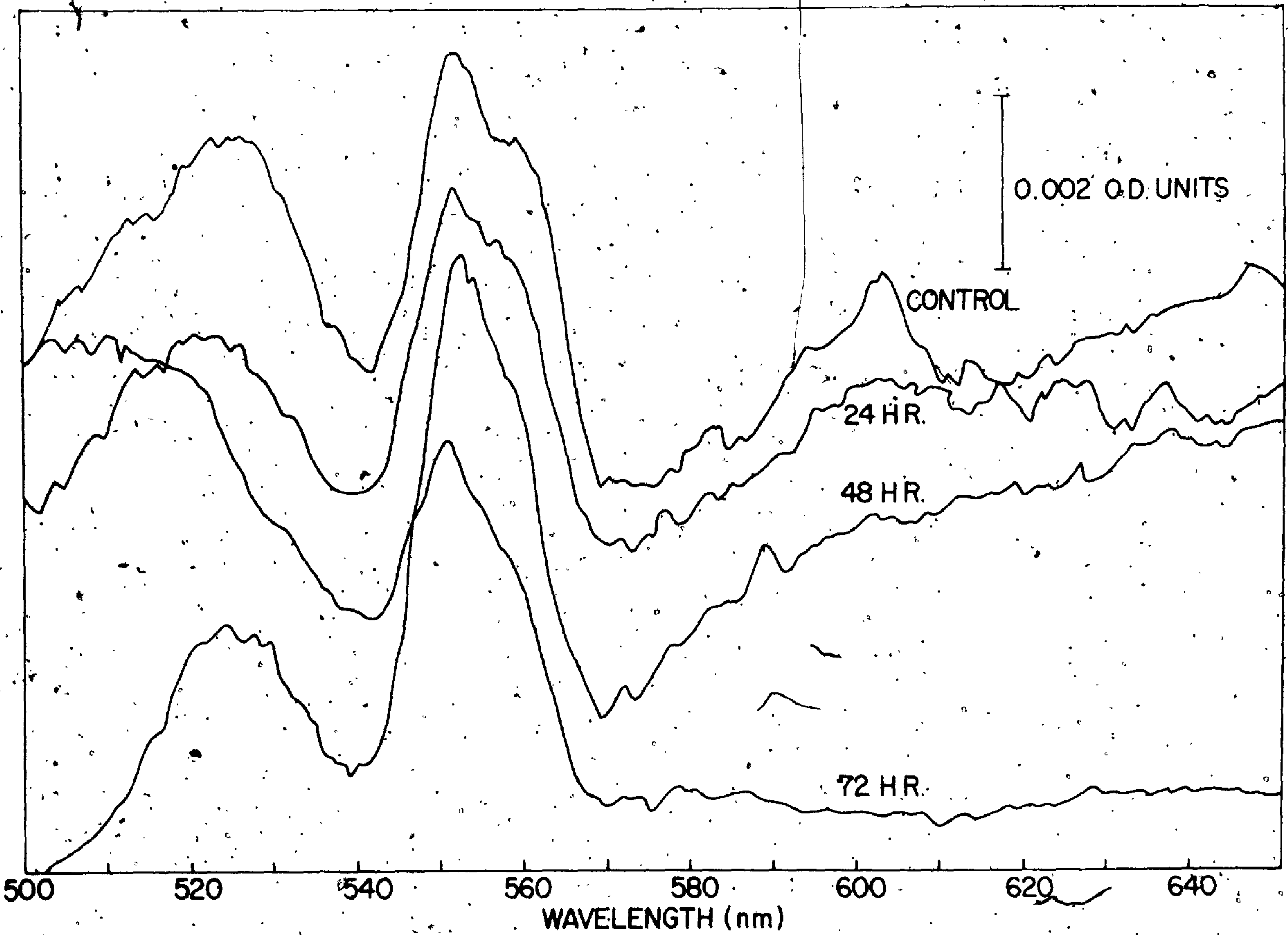

0.002 O.D. UNITS
CONTROL
24 HR.
48 HR.
72 HR.
500
520
540
560
580
600
620
640
WAVELENGTH (nm)

FIGURE 8

RECOVERY OF CELL GROWTH FROM D-CHLORAMPHENICOL TREATMENT

BHK cells were cultured for 48 hours in medium containing 100 μg ml^{-1} CAP. Cells were resuspended in fresh medium with CAP at 24 hours and in fresh medium without CAP at 48 hours. Cell growth was monitored by Coulter Counter over a 96 hour period. o--o control, without CAP; ▲--▲ 96 hours, 100 μg ml^{-1} CAP; Δ--Δ 48 hours, 100 μg ml^{-1} CAP followed by 48 hours without CAP.

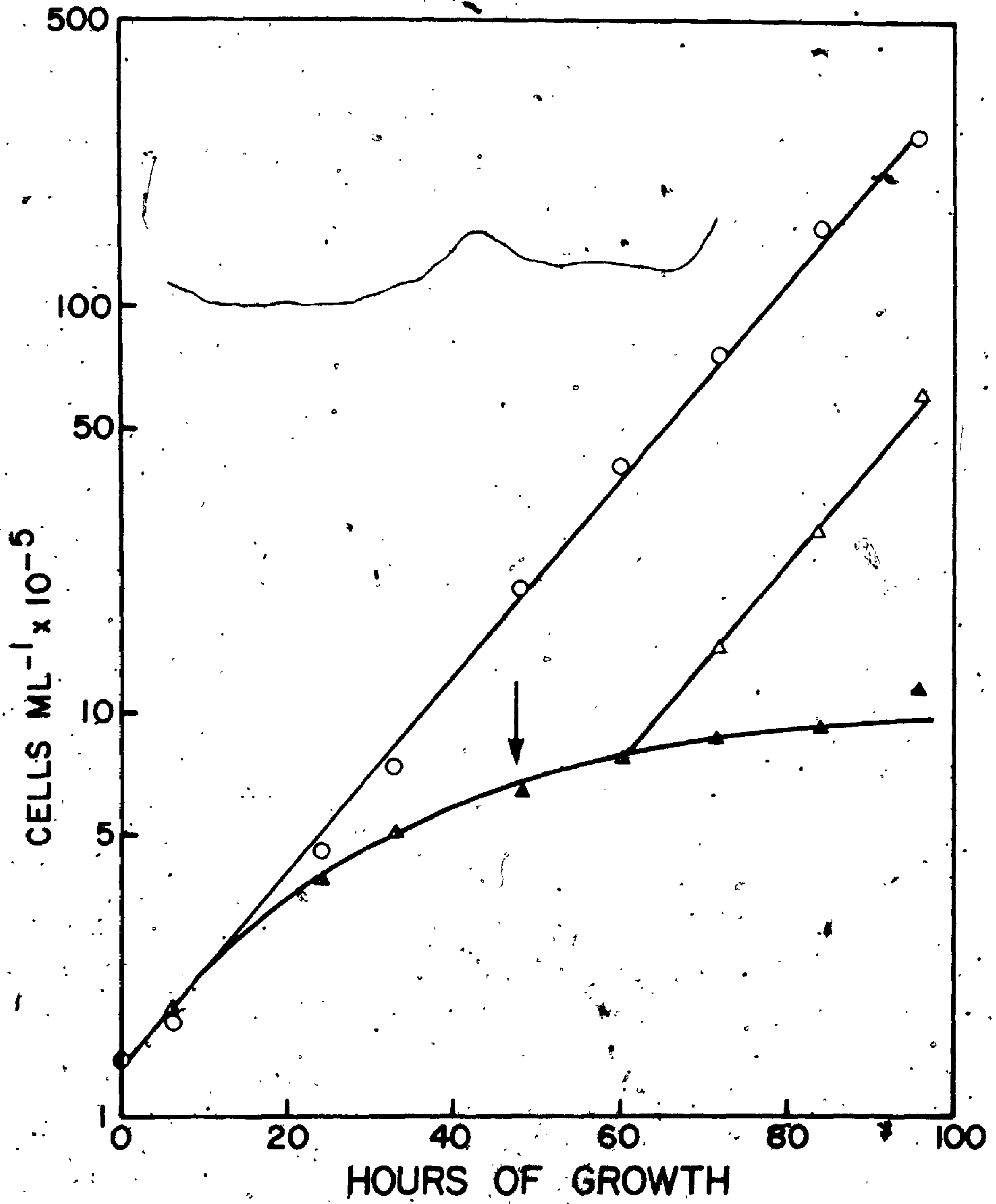
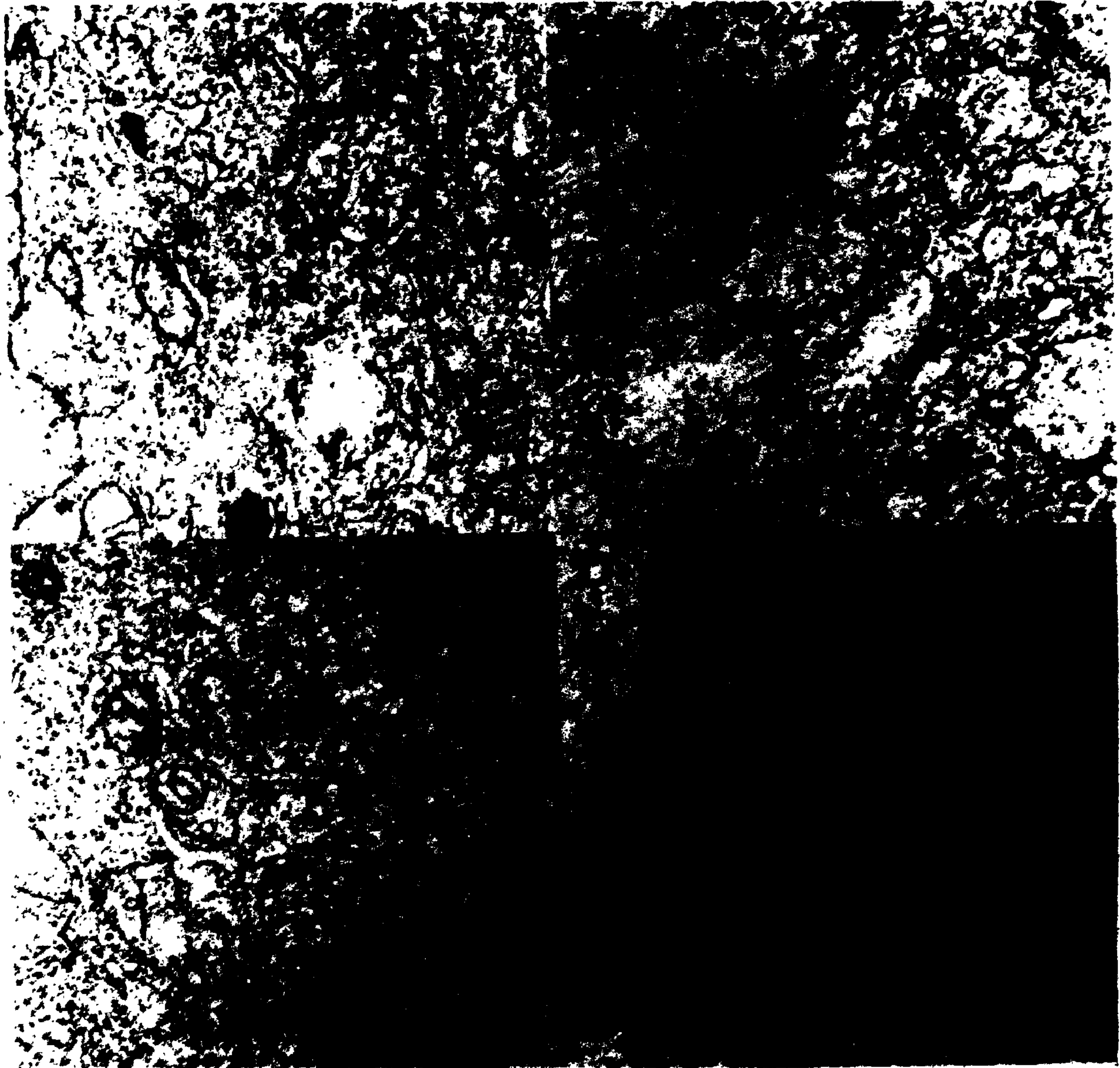

500
100
50
10
5
1
CELLS ML^{-1} x 10^{-5}
0
20
40
60
80
100
HOURS OF GROWTH

FIGURE 9

MORPHOLOGY OF BHK-21 CELLS RECOVERING

FROM TREATMENT WITH CHLORAMPHENICOL

Cells were grown as described in Fig. 8. At 24 hour intervals, cells were removed and prepared as in Fig. 4. A - 96 hours control; B - 96 hours in CAP; C - 48 hours in CAP, 24 hours without CAP; D - 48 hours in CAP, 48 hours without CAP. Size bar indicates 1 micron.



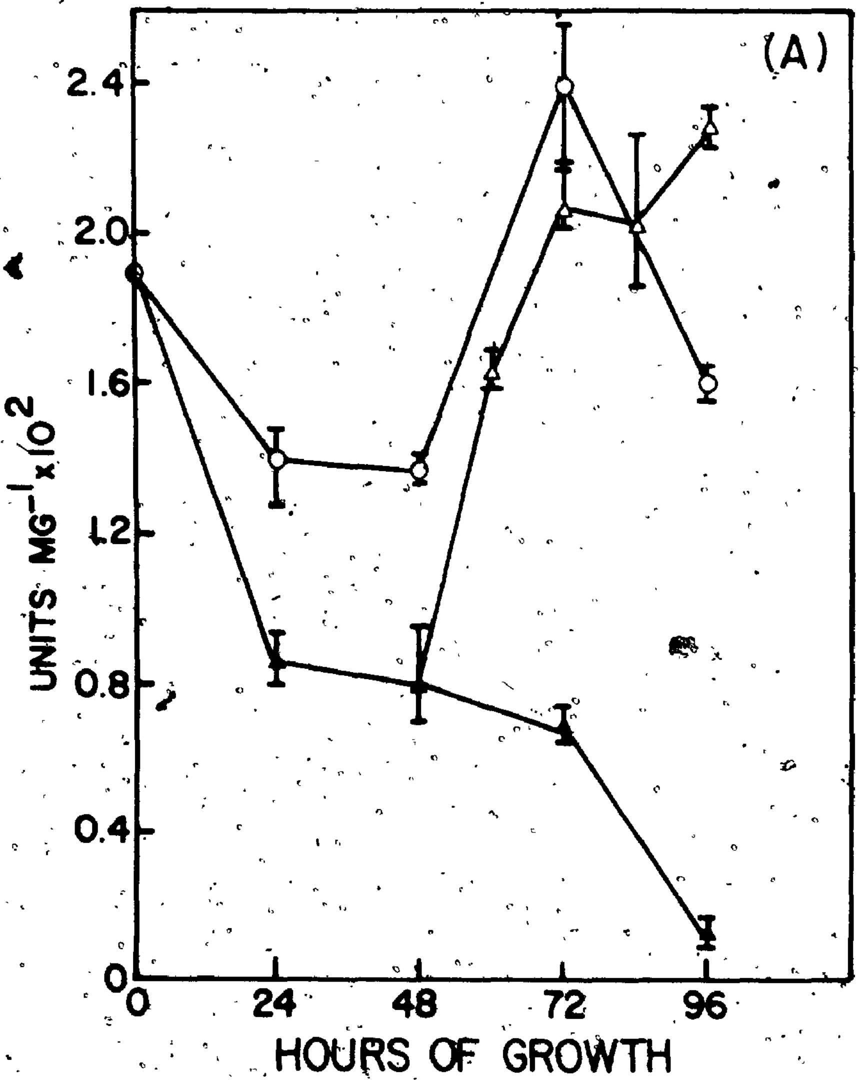
the absence of CAP, in comparison with cells grown for 96 hours in normal medium or 96 hours with CAP. Mitochondrial cristae appear by 24 hours post-treatment and plate-like structure is not evident until 48 hours. Matrix density is increased and cytoplasmic and mitochondrial vacuolation decreased.

Finally, assay of the inner mitochondrial membrane enzymes, cytochrome c oxidase and succinate cytochrome c reductase, revealed different rates of recovery. Succinate cytochrome c reductase (Fig. 10A) appeared to show complete recovery within 12-24 hours. This correlates nicely with the low temperature cytochrome spectra (Fig. 7) which showed some residual cytochrome b activity at 48 hours. Cytochrome c oxidase required 36-48 hours for complete recovery (Fig. 10B). Cytochrome spectra of cells recovered for 24 hours showed nearly normal levels of cytochrome b and some cytochrome aa_3 while the spectra of cells recovered for 48 hours showed both cytochromes aa_3 and b to be present in normal concentrations (Appendix 4). The increased monoamine oxidase activity observed in CAP treated cells (Fig. 6B) was somewhat evident at 12 hours post-treatment; but had returned to normal levels by 48 hours post-treatment with CAP (Fig. 11). No change in either glutamate dehydrogenase or NADPH cytochrome c reductase was observed on removal of the drug.

FIGURE 10

RECOVERY OF MITOCHONDRIAL ENZYMES

Cells were grown as in Fig. 9. At 24 or 12 hour intervals 3-5 x 10^5 cells were harvested, homogenized and assayed for enzyme activity as in Fig. 5. A - succinate cytochrome c reductase; B - cytochrome c oxidase; o--o control, without CAP; ▲--▲ 96 hours 100 μg ml^{-1} CAP, 48 hours followed by 48 hours without CAP. Points expressed as ranges of three cultures.


(A)
UNITS MG^{-1} x 10^2
2.4
2.0
1.6
1.2
0.8
0.4
0
0
24
48
72
96
HOURS OF GROWTH

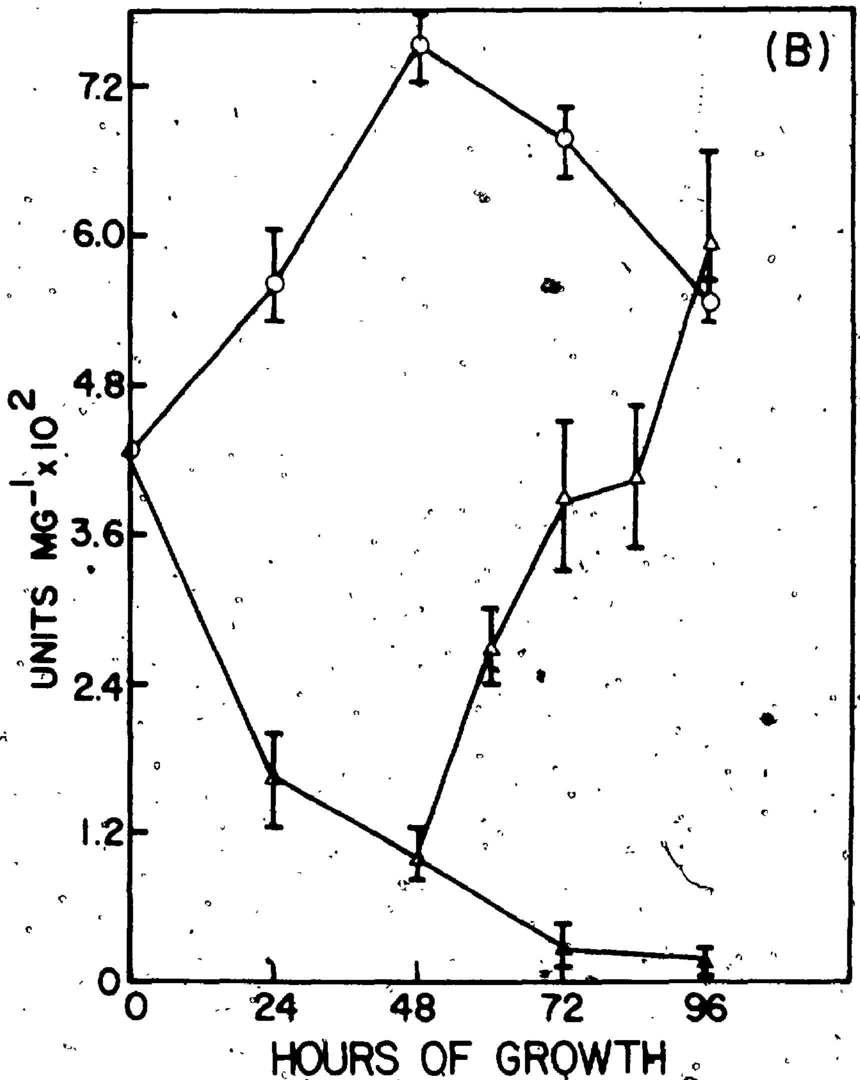
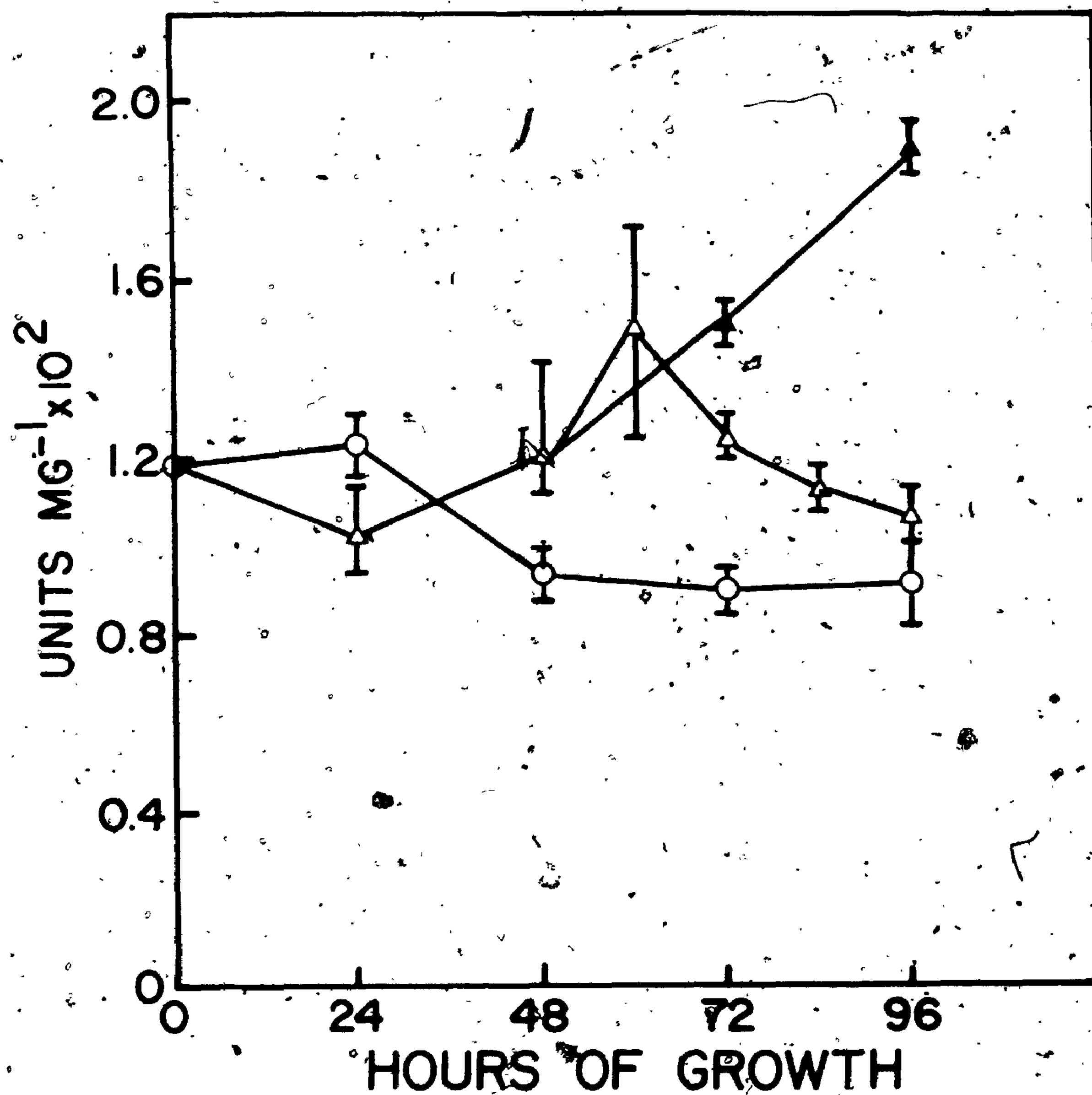

(B)
UNITS MG^{-1} x 10^2
7.2
6.0
4.8
3.6
2.4
1.2
0
0
24
48
72
96
HOURS OF GROWTH

FIGURE 11

RECOVERY OF MONOAMINE OXIDASE ACTIVITY

Cells were grown as in Fig. 10. Cells were harvested, homogenized, and monoamine oxidase assayed as in Fig. 8. o--o control, no CAP; ▲--▲ 96 hours 100 μg ml^{-1} CAP; Δ--Δ 48 hours, 100 μg ml^{-1} CAP, followed by 48 hours no CAP. Points expressed as ranges of three cultures.



2.0
1.6
1.2
0.8
0.4
0
UNITS MG^{-1} x 10^{2}
0
24
48
72
96
HOURS OF GROWTH

2.4 DISCUSSION

Studies of the effects of chloramphenicol on regenerating liver (Firkin and Linnane, 1969; DeVries and Kroon, 1970), primary heart cell cultures (Kroon and Jansen, 1968) or in bone marrow cells (Smith et al., 1970) suffer from the fact that a homogeneous cell population was not used. The use of cultured animal cells such as HeLa (Firkin and Linnane, 1968; Attardi et al., 1973; Lenk and Penman, 1971; King et al., 1972; Fettes et al., 1972), human lung fibroblasts (Packer et al., 1973), lymphoma cells (Bosmann, 1971) or mouse embryo (Piko and Chase, 1973) provides a single cell-type system that can be uniformly treated with chloramphenicol or other drugs, and the effects quantitated.

After two to three cell doublings BHK cells failed to divide in media containing 10-100 μg ml^{-1} CAP, in general agreement with results from similar experiments using L-cells (King et al., 1972; Fettes et al., 1972) and HeLa cells (Firkin and Linnane, 1968; Storrie and Attardi, 1972). Higher CAP concentrations (250 and 500 μg ml^{-1}) inhibited growth almost immediately, again in agreement with results using HeLa cells (Firkin and Linnane, 1968). However, effects of the high CAP concentrations may be related to an immediate inhibition of respiration (Firkin and Linnane, 1968). The fact that differences in cellular growth are noted by 24 hours in BHK-21 cells and L-cells (Fettes et

al., 1972) may indicate faster response to drug treatment, possibly due to faster growth rates (Storrie and Attardi, 1972). In these experiments described above, the cell suspensions were diluted to maintain exponential growth. When cells were resuspended instead in fresh CAP-containing medium, growth rates were less strongly inhibited. Renewing the medium may replace growth components that are rapidly utilized by non-respiring cells, or may remove one or more products of anaerobic metabolism such as lactic acid, or precursors of mitochondria that are not assembled in the presence of CAP (Schatz and Mason, 1974), and which accumulate and secondarily inhibit cell growth.

The increase in cell size as a result of CAP treatment has been noted previously for L-cells (King, et al., 1972) and for HeLa cells (Lenk and Penman, 1971; King et al., 1972). This was not observed by Storrie and Attardi (1972) for HeLa cells. Continued cellular membrane synthesis in the absence of mitochondrial division may result in both enlarged cells and mitochondria. Increased mitochondrial size was not observed in HeLa cells (Lenk and Penman, 1971). If the presence of functioning mitochondria is required for cell division, uncontrolled cell growth without division may continue for some time before it is inhibited by a lack of precursors or energy. The inhibitor itself does not prevent cell division (Mahler, 1973), yet defective mitochondrial function will result in a disruption of cellular anabolic processes.

Within twelve hours of removal of CAP, cells start dividing normally. This period may represent the time needed to regenerate mitochondrial competence, and recover cellular metabolite and/or energy levels. The number of mitochondria per cell does not appear to change in CAP-treated cells, and the increased cellular volume may also be related to the larger volume of cytoplasm occupied by the swollen mitochondria (King et al., 1972). Pious et al. (1972) did not find elevated levels of glycolytic enzymes in CAP-treated cells, suggesting that adaptation to anaerobic energy generating metabolism does not account for the increase in cytoplasmic volume.

Changes in mitochondrial morphology start to become evident by 24-48 hours. The alterations in cristae structure and the decrease in matrix density in BHK cell cultures grown in CAP-containing medium have also been observed in HeLa and L-cells (King et al., 1972), although others have failed to show similar CAP effects in HeLa cells (Attardi et al., 1973) or human fibroblasts (Packer et al., 1973). Despite the degenerative changes in cristae and matrix, both the outer and inner membranes remain intact (Adoutte et al., 1972). The large vacuoles which appear in the cytoplasm, appear to represent lipid droplets containing precursors or unincorporated components of the mitochondrion (Lenk and Penman, 1971; Adoutte et al., 1972).

The reduction in cytochrome c oxidase activity on

CAP treatment, has been well documented (King et al., 1972; Fettes et al., 1972; Pious et al., 1972), as has the effect on the activity of succinate cytochrome c reductase (King et al., 1972). The latter decreases at a slower rate than cytochrome c oxidase in both HeLa and L-cells (King et al., 1972). Since succinate dehydrogenase activity appears to be insensitive to CAP treatment (Fettes et al., 1972), the effect on the coupled system, succinate cytochrome c reductase, is most likely related to the observed effect on cytochrome b. The absence of any reduction in glutamate dehydrogenase activity is in agreement with the conclusion that porteins of the mitochondrial matrix appear to be synthesized entirely on cytoplasmic ribosomes (Schatz and Mason, 1974). Similar results are observed for mitochondrial malate dehydrogenase (Fettes et al., 1972). No changes in endoplasmic reticulum were observed as determined by the constant activity of NADPH cytochrome c reductase. The elevation in monoamine oxidase activity could be attributed to proliferation of the outer membrane, which is not under control of the mitochondrial genome (Adoutte et al., 1972). This observation is consistent with the observed increases in mitochondrial size. The release by CAP treatment of latent activity following mitochondrial damage seems to be a more likely explanation. The results of preliminary studies showed that assay of the enzyme in in the presence of Triton X-100

(Aithal et al., 1976) yields higher values for the specific activity of the control cells, but not for CAP-treated cells (Appendix 5) after 96 hours.

The observed disappearance of the mitochondrial cytochromes on CAP treatment is in agreement with published results. There appears to be little or no change in cytochrome c_1 (King et al., 1972), or cytochrome c (Fettes et al., 1972), both of which are synthesized on cytoplasmic ribosomes (Klietmann et al., 1973; Ross and Schatz, 1976). Cytochromes aa_3 are the most rapidly affected of the mitochondrial cytochromes, while cytochrome b decreases more slowly (King et al., 1972). These results cannot be explained in terms of a faster turnover rate of cytochromes aa_3 than cytochrome b, since all three appear to have similar half-lives of 5.5-6.0 days (Getz, 1972) as measured using ^3H-δ-aminolevulinate or ^{14}C-guanidoarginine (Druyan et al., 1969; Aschenbrenner et al., 1970). The disappearance of both cytochrome c oxidase and succinate cytochrome c reductase activities parallel the disappearance of their respective cytochrome components. On removal of CAP, succinate cytochrome c reductase activity returns to normal within 12-24 hours, followed by cytochrome c oxidase activity. This is in good agreement with results found for yeast cultures either undergoing glucose derepression (Ball and Tustanoff, 1970) or adapting from growth in CAP to growth in cycloheximide (Lin et al., 1974). Thus the

two enzymes may be under different controls, both for synthesis and turnover. This may be related to different rates of synthesis for mitochondrial and cytoplasmic components and their integration into the membrane, both in _de novo_ growth-related enzyme synthesis, and as replacement for enzyme that is turning over in growth restricted cells.

Finally, it appears that the recovery in growth and morphology of cells treated with CAP is related to the recovery of mitochondrial respiratory activity. Once mitochondria regain their potential for electron transport and oxidative phosphorylation, the various processes requiring mitochondrial energy or precursors are revived or accelerated.

Cells exhibit the ability to recover from periods of CAP treatment up to 72 hours. Treatments for longer periods yield cells with little or very slow abilities to recover, possibly due to cell breakdown (Storrie and Attardi, 1972). L-cells on the other hand show the ability to recover rapidly after lengthy treatments (Fettes _et al._, 1972).

The use of CAP to modulate mitochondrial growth in rapidly dividing mammalian cells is a valuable tool for studying mitochondrial biogenesis in a non-microbial system.

CHAPTER 3. MITOCHONDRIAL PROTEIN SYNTHESIS IN CHLORAMPHENICOL-TREATED BHK-21 CELLS

3.1 INTRODUCTION

It has been demonstrated that the antibacterial antibiotic, D-chloramphenicol, selectively inhibits the mitochondrial protein synthesizing system of somatic cells, without affecting the cytoplasmic system (Firkin and Linnane, 1968; Lenk and Penman, 1971; King et al., 1972; Fettes et al., 1972; Storrie and Attardi, 1972; Packer et al., 1972). The block leads to inhibition of hemoprotein formation (Druyan et al., 1973), loss of respiratory enzymes (Firkin and Linnane, 1968; Kroon and Jansen, 1968) and alterations in mitochondrial ultra-structure (Lenk and Penman, 1971; King et al., 1972). However, there is little information concerning the way in which these lesions of the functional inner membrane of the mitochondrion may be related to metabolism of these components. The work described in this chapter represents part of an integrated approach to the study of these parameters of mitochondrial membrane formation in cultured mammalian cells. The growth and morphological alterations as well as changes in respiratory enzyme content of CAP-treated BHK-21 cells have been described in the preceding chapter. The extent of inhibition of mitochondrial and cellular protein

synthesis under these conditions of CAP treatment found to produce optimal inhibitory, but reversible, effects, is examined in this chapter.

3.2 MATERIALS AND METHODS

3.2.1 Cell Culture

Spinner adapted BHK-21 cells were cultured in Modified Alpha MEM supplemented with 5% fetal bovine serum and antibiotics. CAP was added to experimental cultures to a final concentration of 100 $\mu g\ ml^{-1}$ unless otherwise stated. Control cultures were identically maintained except for the absence of CAP. Cells were routinely maintained and labelled under logarithmic growth conditions ($3-6 \times 10^5$ cells ml^{-1}). Cycloheximide (Sigma) was added to cultures to the concentrations described in the text. Cell growth was determined using a Coulter Counter, Model B.

3.2.2 Labelling of Cellular Protein

Rates of protein labelling were measured in cultured cells during the period of linear incorporation by the direct addition of the appropriate isotope to cells freshly suspended in normal growth medium. Unless otherwise noted cells were cultured in CAP-containing medium for a total of 48 hours with resuspension in fresh CAP-containing medium at 24 hours. At 48 hours, cells were resuspended in medium with or without CAP to measure uptake and incorporation of the protein or heme precursor.

L-leucine $[^{14}C(U)]$ (308 mCi $mmole^{-1}$) and L-leucine $[4,5-^{3}H(N)]$ (67.7 Ci $mmole^{-1}$) were purchased from New England Nuclear. δ-Aminolevulinic Acid $[2,3-^{3}H]$ (33 Ci $mmole^{-1}$) was obtained from Schwarz/Mann. Labelled precursor was added to the cultures without any cold carrier, other than the normal medium constituents.

Following the appropriate labelling period, cells were harvested and washed, and fractionated by one of the two procedures described below.

Protein synthesis was also measured by a double labelling technique modified from that described for analyzing specific yeast polypeptides by Groot _et al._ (1972). Control cultures were grown in normal medium containing 0.1 μCi ml^{-1} L-leucine $[^{14}C(U)]$. Additional control cultures and cultures containing 100 μg ml^{-1} CAP were grown in normal medium containing 0.1 μCi ml^{-1} L-leucine $[4,5-^{3}H(N)]$. Following incubation, ^{14}C- and ^{3}H-labelled cells were mixed in a ratio of 1:4 v/v, harvested, washed and fractionated as below. By mixing of the labelled cells before harvesting, the $^{3}H/^{14}C$ ratio introduced should be maintained through all subsequent procedures despite any loss of material. Slight variations in the ratio from one sample to another due to mixing inconsistencies can be eliminated by dividing each ratio by the $^{3}H/^{14}C$ ratio of the TCA-soluble extract of the whole homogenate (see below).

3.2.3 Fractionation of Cells

In experiments where total cellular uptake and incorporation were studied, the washed cellular pellet was suspended in 1 ml of 0.25 M sucrose-0.1 mM EDTA and sonicated for 30 seconds in a Branson Sonifier equipped with bath. An aliquot was removed for measurement of total uptake of precursor and the remainder was precipitated with an equal volume of 20% trichloroacetic acid (w/v).

Where incorporation into various subcellular fractions was analyzed, the washed cells were suspended in 2 ml sucrose-EDTA, homogenized for 15 sec. using a Willems Polytron® (Brinkman), and fractionated by procedures described for rat liver (McMurray and Dawson, 1969). Subcellular fractions were suspended in 1 ml sucrose-EDTA by sonication as above. A sample of the homogenate (0.2 ml) was made up to 1 ml with sucrose-EDTA. A 1 ml sample of the supernatant was also removed. All fractions were precipitated with an equal volume of 20% trichloroacetic acid. The TCA-soluble component of the homogenate was utilized as a measure of the pool size of the precursor used in the labelling procedure.

The TCA-precipitated fractions were subsequently processed by a modification of the method of McMurray and Rogers (1973) as modified from Beattie _et al_. (1966). The pellet was washed once with 5% TCA, and then dissolved in 0.4 M NaOH containing 1 mg ml^{-1} L-leucine or 1 mg ml^{-1}

δ-aminolevulinic acid (prepared immediately before use) and incubated for 60 minutes at 37°. The proteins were reprecipitated with an equal volume of 20% TCA and the slightly moist precipitates dissolved by standing overnight in 0.2 ml of 0.5 M NaOH-1% sodium dodecyl sulfate at room temperature. Samples were then diluted to 1 ml and warmed slightly to clarify.

Aliquots were removed immediately for determination of protein by the method of Lowry et al. (1951). Samples were prepared for liquid scintillation counting by neutralization with excess ethyl formate and addition of Aquasol® (New England Nuclear) (McMurray and Rogers, 1973). The samples were then counted in a Packard Tri-Carb® scintillation spectrophotometer optimized for double label determination. Results were corrected for spill-over of one channel into the other channel. TCA-soluble pools were counted by neutralizing the extract with 0.5 M NaOH-1% sodium dodecyl sulfate and counting as above. Specific activities were reported as cpm per mg protein or as the ratio of ^{3}H-counts to ^{14}C-counts. These latter values were normalized by dividing by the TCA-soluble $^{3}H/^{14}C$ ratio, a measure of the intracellular pool of labelled precursor at the end of the incubation. Subcellular fractions were normalized relative to incorporation values for whole homogenates in order to detect selective effects on particular organelles.

3.3 RESULTS

Uptake of ^{3}H-leucine with time in the presence or absence of CAP, was examined in cells that had been cultured for 48 hours in the presence or absence of 100 μg ml^{-1} CAP (Fig. 12A). It is evident from these data, that the rate of uptake of leucine is slightly decreased by culturing the cells in the presence of CAP. The presence of CAP during the uptake period does not appear to affect the amount of leucine taken up by the cells. In all cases the maximum uptake appears to be the same.

When the amount of leucine incorporated into cellular protein was examined with time, the results indicated in Figure 12B were obtained. The rate of incorporation into total cellular protein is slightly reduced in CAP-treated cells but appears to reach the same maximum as the control cultures by 10 hours. Again it would appear that the pretreatment with CAP is important in determining the incorporation rate, while the presence of CAP during the incorporation period has little effect.

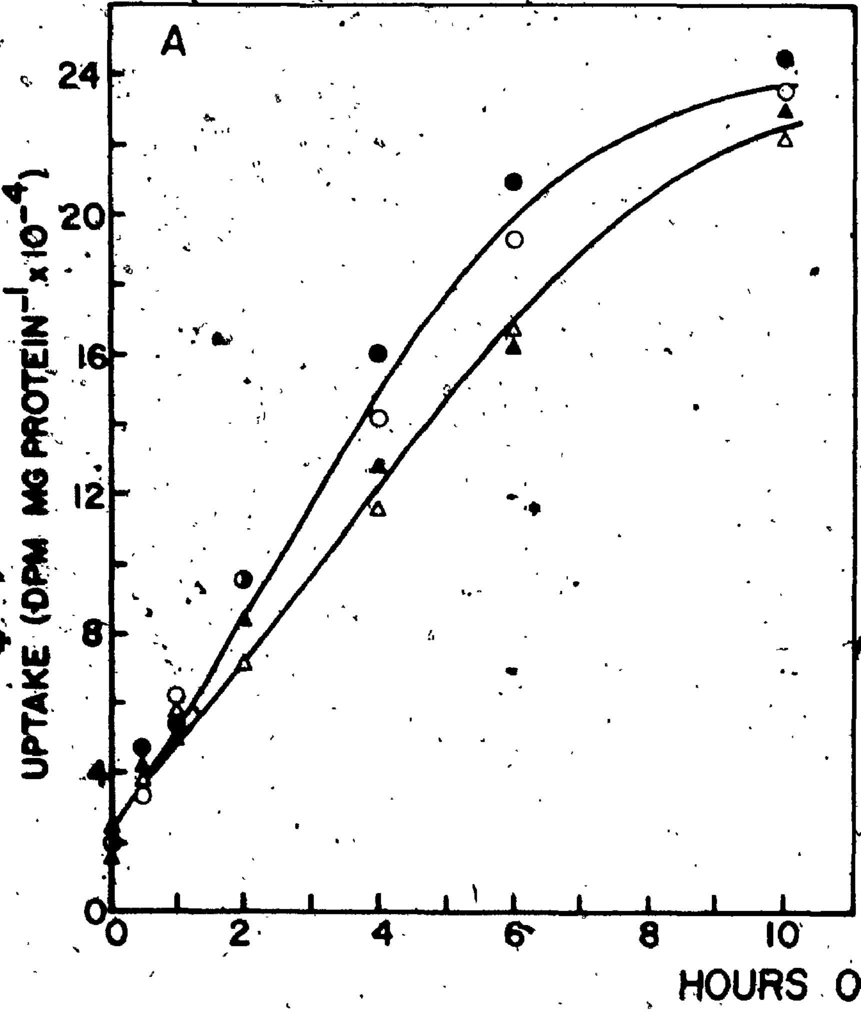
The effects on the labelling of subcellular fractions were determined in cells grown for 48 hours in 100 μg ml^{-1} CAP and then labelled for 4 hours with leucine. The double label results are given in Table 3. The results indicate a slight though probably unimportant increase in the precursor pool of CAP-treated cells. When the ratio of counts incorporated into the various subcellular fractions is

FIGURE 12

TOTAL UPTAKE AND INCORPORATION OF ^{3}H-LEUCINE

BY D-CHLORAMPHENICOL-TREATED BHK-21 CELLS

BHK-21 cells were grown for 48 hours in the presence and absence of 100 μg ml^{-1} CAP. Cells were then resuspended in fresh medium (3 x 10^{5} cells ml^{-1}) with and without CAP and cultured for an additional 10 hours in the presence of 1 μCi ml^{-1} ^{3}H-leucine. At various time intervals, 3-5 x 10^{7} cells were removed, washed and total uptake of leucine was determined as described in the Materials and Methods (A). Samples were precipitated with 20% TCA and fractionated as described in the Materials and Methods (B), o--o 48 hours growth in Alpha + 10 hours uptake in Alpha; ●--● 48 hours growth in Alpha + 10 hours uptake in Alpha + 100 μg ml^{-1} CAP; Δ--Δ 48 hours growth in Alpha + 100 μg ml^{-1} CAP + 10 hours uptake in Alpha; ▲--▲ 48 hours growth in Alpha + 100 μg ml^{-1} CAP + 10 hours uptake in Alpha + 100 μg ml^{-1} CAP.


A
UPTAKE (DPM MG PROTEIN^{-1} x 10^{-4})
0
4
8
12
16
20
24
0
2
4
6
8
10

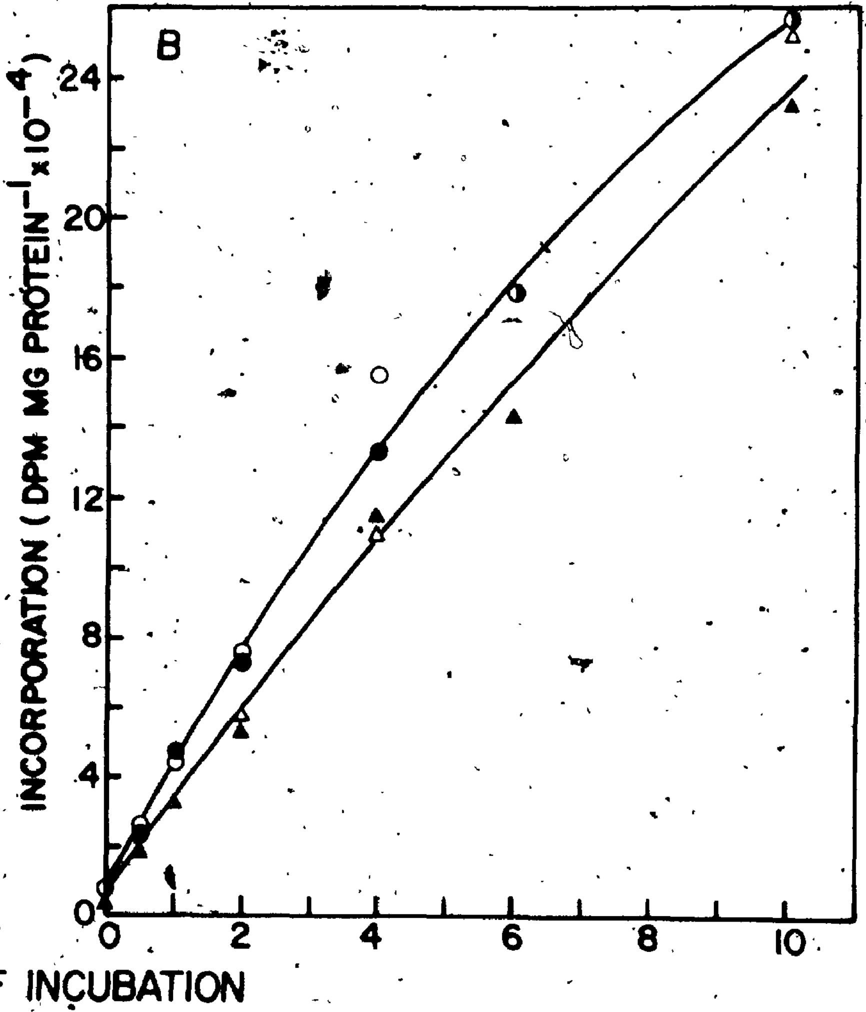

B
INCORPORATION (DPM MG PROTEIN^{-1} x 10^{-4})
0
4
8
12
16
20
24
0
2
4
6
8
10
HOURS OF INCUBATION

TABLE 3

THE EFFECTS OF D-CHLORAMPHENICOL ON THE INCORPORATION OF LEUCINE INTO THE PROTEINS OF SUBCELLULAR FRACTIONS ISOLATED FROM BHK-21 CELLS

BHK-21 cells were grown for 48 hours in 100 μg ml^{-1} CAP, resuspended (5.5×10^5 cells ml^{-1}) and then labelled for 4 hours with ^3H-leucine (0.1 μCi ml^{-1}) in the presence or absence of CAP. A control culture was labelled for the same period with 0.1 μCi ml^{-1} ^{14}C-leucine. Following incubation, the cultures were mixed (10^8 cells total), precipitated with 20% TCA and fractionated as described in the Methods.

	$^3H/^{14}C$ Ratio/Pool $^3H/^{14}C$[a] (Relative to Homogenate[b])			
	48 Hour Control Growth		48 Hour CAP Growth	
Fraction	Uptake − CAP	Uptake + CAP	Uptake − CAP	Uptake + CAP
Pool[c]	1.49	1.55	1.58	1.60
Homogenate	1.08 (1.00)	1.04 (1.00)	1.03 (1.00)	1.01 (1.00)
Mitochondrial	1.09 (1.01)	1.05 (1.00)	1.02 (0.99)	1.02 (1.01)
Microsomal	1.05 (0.97)	1.01 (0.97)	1.00 (0.97)	0.98 (0.97)
Supernatant	1.02 (0.94)	1.00 (0.96)	0.97 (0.94)	0.96 (0.95)

[a] $^3H/^{14}C$ TCA-precipitated fraction / $^3H/^{14}C$ TCA-soluble homogenase, i.e. pool.

[b] $^3H/^{14}C$ TCA-precipitated fraction / $^3H/^{14}C$ TCA-precipitated homogenase.

[c] $^3H/^{14}C$ TCA-soluble homogenate.

examined, there is a very slight decrease in fractions from cells grown for 48 hours in CAP. This decrease in relative specific activity is probably related to the slightly larger pool in these cells. However, when the effect on the various subcellular fractions is compared to the gross effect on the TCA-precipitable homogenate, it is evident that there is basically no decrease specific to the mitochondria.

The effect of different concentrations of CAP on the incorporation of leucine was examined (Table 4). Since higher concentrations of CAP were shown to inhibit cell growth severely, the effect on protein synthesis might be more pronounced. It was observed that as the concentration of CAP was increased from 0 to 250 μg ml^{-1}, there was an apparent stimulation of leucine incorporation into mitochondrial protein as well as microsomal protein. At 500 μg ml^{-1}, there was a sharp decrease in labelling of protein. Soluble protein was labelled to a decreasing degree relative to other fractions with increased CAP, but again there was no selective decrease of mitochondrial protein labelling.

Since it was apparent that mitochondrial protein synthesis constituted a very small percentage of total cellular protein synthesis, cells were treated with cycloheximide, a specific inhibitor of cytoplasmic protein synthesis, in order to eliminate the bulk of the cellular

TABLE 4

THE EFFECTS OF D-CHLORAMPHENICOL CONCENTRATION ON LEUCINE INCORPORATION INTO BHK-21 CELL SUBCELLULAR FRACTIONS

BHK cells were cultured for 48 hours in various concentrations of CAP. At 48 hours, they were resuspended in fresh medium containing CAP (4×10^5 cells ml^{-1}) and incubated for 4 hours with 0.1 μCi ml^{-1} ^3H-leucine. A control culture containing 0.1 μCi ml^{-1} ^{14}C-leucine was incubated simultaneously. Cells were then mixed ($^3H/^{14}C$ 5:1 v/v) (5×10^8 cells total) and fractionated as in Table 3.

Fraction	$^3H/^{14}C$ Ratio/Pool $^3H/^{14}C$[a] (Relative to Homogenate[b])				
	Control	50 μg ml^{-1} CAP	100 μg ml^{-1} CAP	250 μg ml^{-1} CAP	500 μg ml^{-1} CAP
Pool[c]	2.75	2.56	2.43	2.23	2.20
Homogenate	0.110(1.00)	0.096(1.00)	0.107(1.00)	0.129(1.00)	0.109(1.00)
Mitochondrial	0.100(0.91)	0.100(0.96)	0.106(0.99)	0.152(1.18)	0.098(0.90)
Microsomal	0.102(0.93)	0.107(1.11)	0.099(0.92)	0.171(1.32)	0.089(0.82)
Supernatant	0.118(1.07)	0.107(1.11)	0.101(0.94)	0.116(0.90)	0.076(0.70)

[a] $^3H/^{14}C$ TCA-precipitated fraction / $^3H/^{14}C$ TCA soluble homogenate.

[b] $^3H/^{14}C$ TCA-precipitated fraction / $^3H/^{14}C$ TCA-precipitated homogenate.

[c] $^3H/^{14}C$ TCA-soluble homogenate.

incorporation of leucine. It was first necessary to determine the optimal concentration of cycloheximide for inhibition of cellular growth (Fig. 13). Cycloheximide at a concentration of 10^{-5} M was shown to maintain a steady number of cells while concentrations below resulted in net cell growth and those above net cell death. This concentration of cycloheximide (10^{-5}M) was used to examine the extent of CAP-sensitive protein synthesis.

The inhibition of total cellular protein synthesis by CAP was shown to be minimal (Fig. 12B). Since protein synthesis by CAP was measured at a period when cells had already been exposed to CAP for 48 hours, it was conceivable that any dramatic reduction may have occurred during the initial period of exposure. Cells were therefore treated with CAP, CAP plus cycloheximide or cycloheximide during the initial 32 hours of growth, and labelled with ^{14}C-leucine. The results are shown in Figure 14. Initially, the ^{14}C/^{3}H leucine ratio in the TCA-soluble fraction (a measure of the intracellular pool) in cycloheximide-treated cells is larger than the pool in cells grown in the absence of cycloheximide, regardless of whether CAP is present or not. With time however, this situation is reversed so that by 30 hours, the free pool of leucine is two- to three-fold greater in the absence of cycloheximide. In addition, although the relative labelling of the TCA-precipitable material is initially about the same for all

FIGURE 13

EFFECTS OF CYCLOHEXIMIDE ON BHK CELL GROWTH

BHK-21 cells were grown for 48 hours in the cycloheximide concentrations indicated.

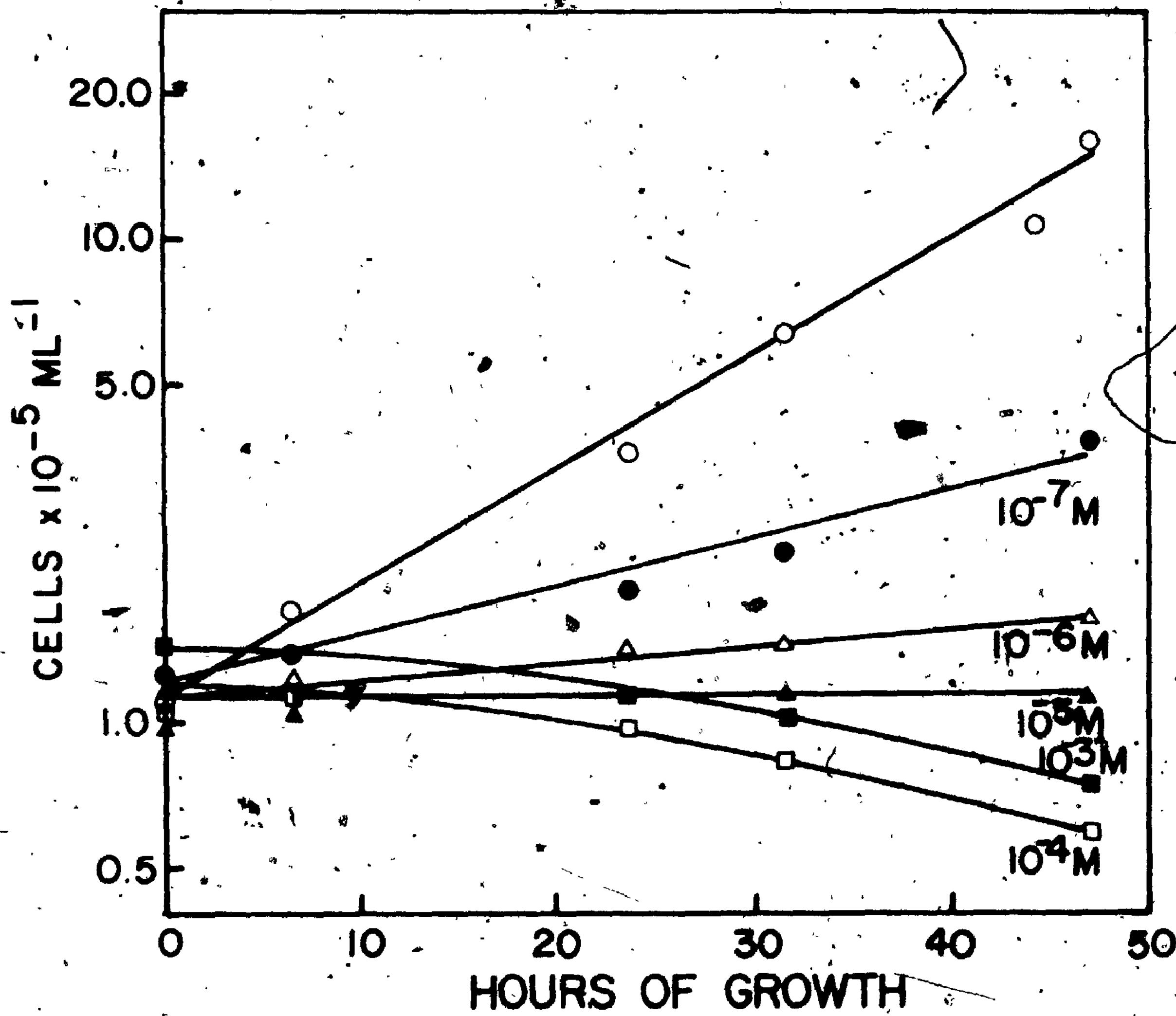
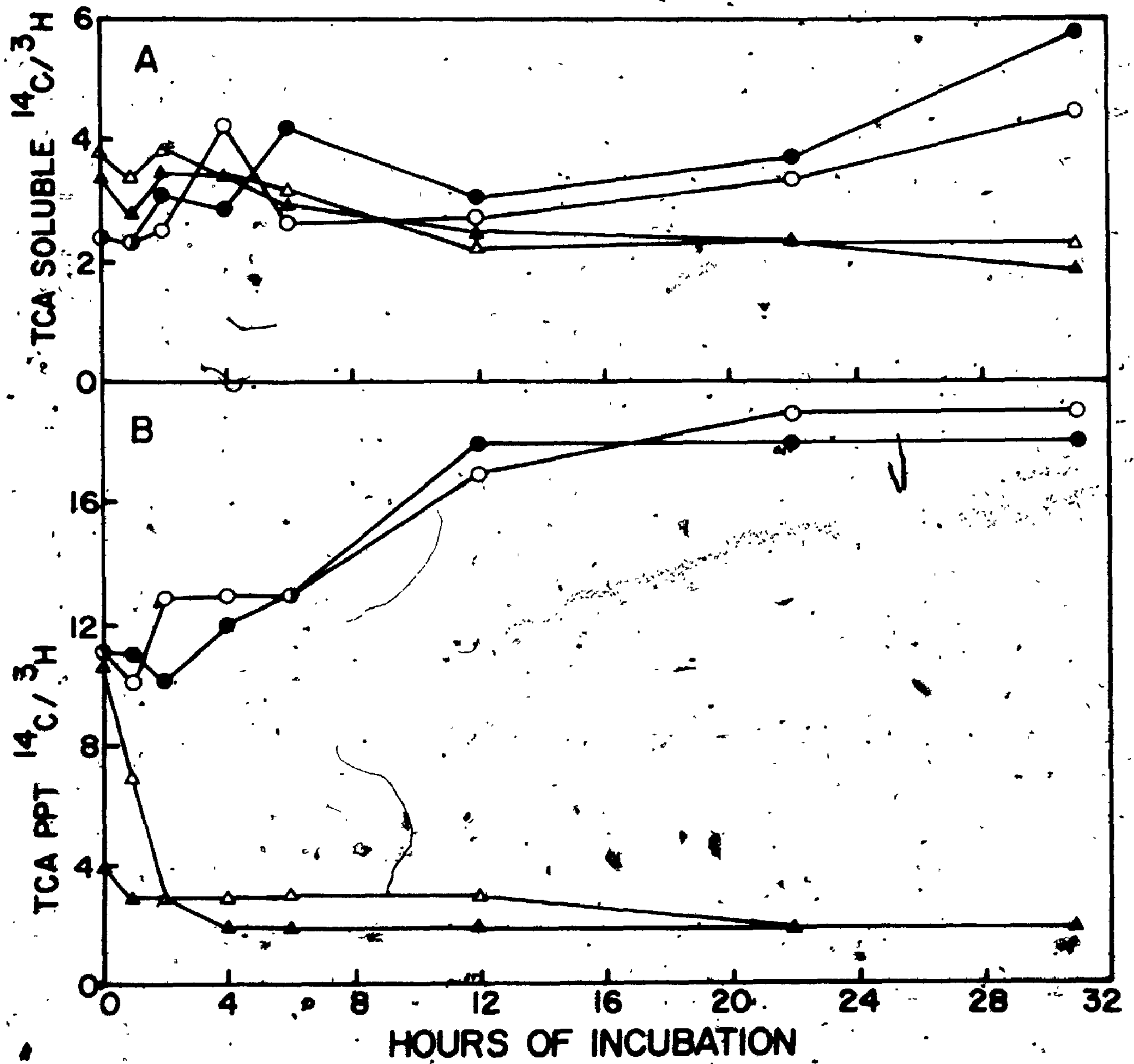

CELLS x 10⁻⁵ ML⁻¹
20.0
10.0
5.0
1.0
0.5
0
10
20
30
40
50
HOURS OF GROWTH
10⁻⁷M
10⁻⁶M
10⁻⁵M
10⁻³M
10⁻⁴M

FIGURE 14

EFFECTS OF D-CHLORAMPHENICOL AND CYCLOHEXIMIDE ON BHK CELL PROTEIN SYNTHESIS AND LEUCINE POOL SIZE

Cells were grown at various times up to 32 hours in the presence of 0.1 μCi ml^{-1} ^{14}C-leucine and either 100 μg ml^{-1} CAP, 10^{-5} M cycloheximide or both. A second control culture was grown simultaneously with 0.1 μCi ml^{-1} ^{3}H-leucine. Cells were sampled, mixed in a ratio of 1:4 v/v $^{14}C/^{3}H$, (7 x 10^{6} cells total) washed, and precipitated with 20% TCA. The TCA-soluble fraction was counted as described in the Methods (A). The precipitable material was fractionated and counted (B). o--o control; ●--● 100 μg ml^{-1} CAP; Δ--Δ 10^{-5}M cycloheximide; ▲--▲ 100 μg ml^{-1} CAP + 10^{-5} M cycloheximide.



A
B
TCA SOLUBLE $^{14}C/^{3}H$
TCA PPT $^{14}C/^{3}H$
0
2
4
6
0
4
8
12
16
0
4
8
12
16
20
24
28
32
HOURS OF INCUBATION

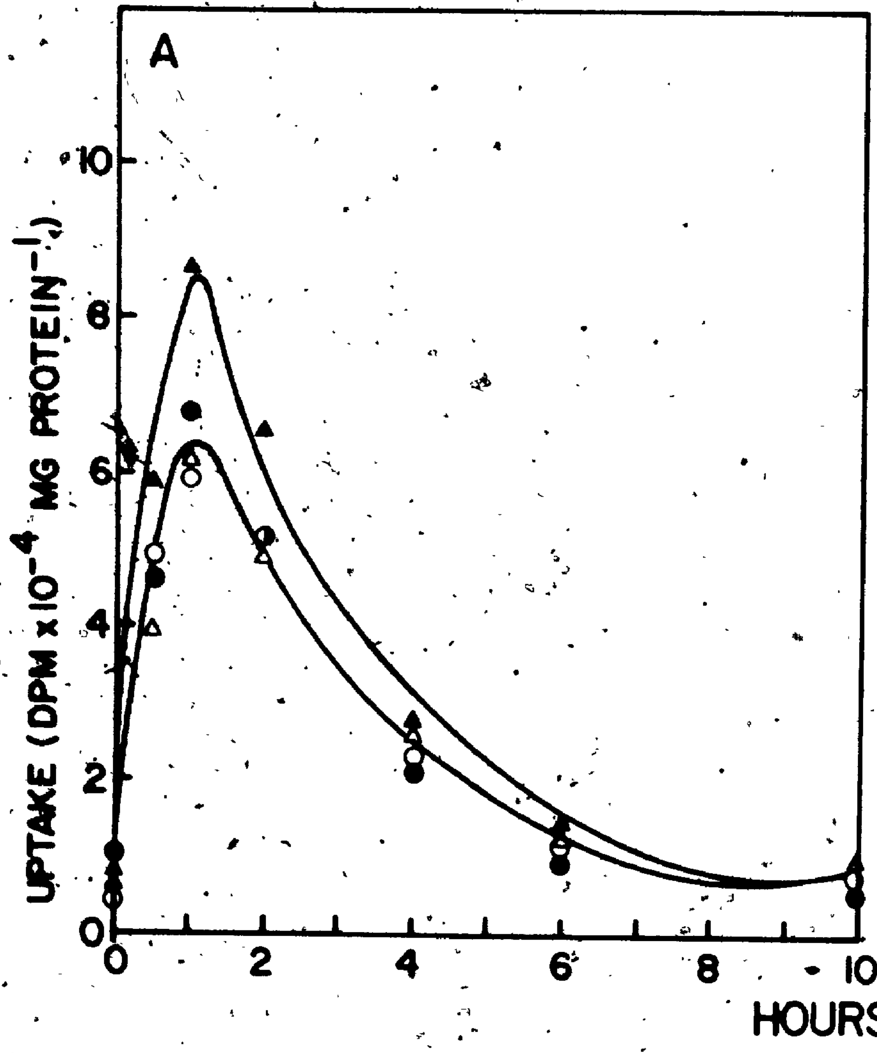
cultures, by the end of the incubation period, the control cultures have an 8- to 10-fold greater incorporation than those treated with cycloheximide. Chloramphenicol seems to have little or no effect on the leucine pool, whether or not cycloheximide is present. However, when the initial period (0-60 min.) of labelling of the TCA-precipitable material is examined, if CAP is added in the presence of cycloheximide, there is a further decrease in the ratio. At later times (from 2 hr. on) there is no additional effect when CAP is added to the cycloheximide inhibited cells. It is also evident that the results observed in earlier experiments (Table 4) do not arise from a preferential metabolism of the ^{3}H- versus ^{14}C-labelled leucine by control or CAP-treated cells, as the isotopes were reversed in this experiment.

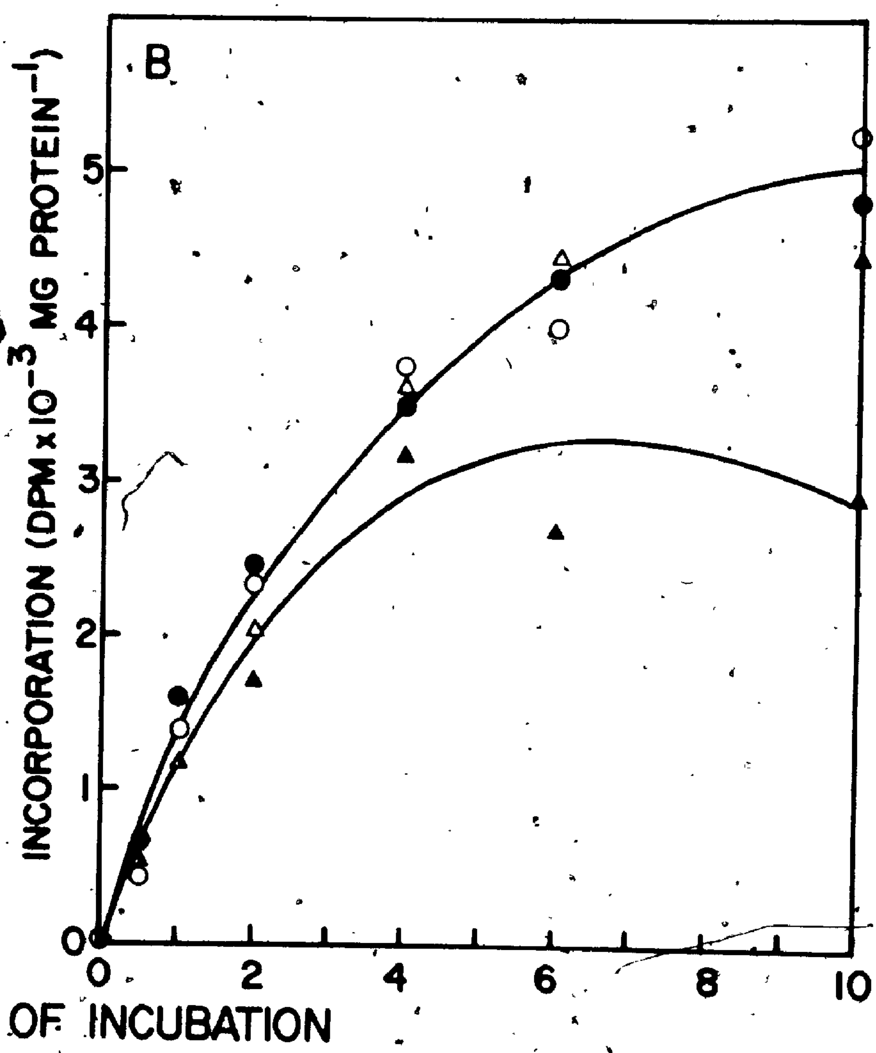
Because some of the products of mitochondrial protein synthesis are integrated into hemoproteins, the effects of CAP on the incorporation of δ-aminolevulinic acid in BHK cells were examined. The effects of CAP on uptake and on incorporation into TCA-precipitable material are shown in Figure 15. CAP does not appear to inhibit the uptake of δ-aminolevulinic acid into the cells. In fact, if cells were cultured and labelled in the presence of CAP, the initial rate of uptake as well as the maximum uptake, may be slightly enhanced (Fig. 15A). However when cells are cultured and labelled in the presence of CAP, the incorporation into TCA-precipitable material at 10 hours is reduced by about one third (Fig. 15B).

FIGURE 15

EFFECTS OF D-CHLORAMPHENICOL ON THE UPTAKE AND INCORPORATION OF δ-AMINOLEVULINIC ACID

Cells were grown for 48 hours in the presence and absence of 100 μg ml^{-1} CAP as before. At 48 hours cells were suspended in fresh medium with and without CAP (3 x 10^5 cells ml^{-1}) plus [^{3}H]-δ-aminolevulinate (1 μCi ml^{-1}) and incubated. 3 x 10^6 cells were removed at various times, washed and suspended by sonication. Part was retained for total uptake (A) and the remainder was precipitated with an equal volume of 20% TCA and fractionated (B). o--o 48 hour growth in Alpha + 10 hour uptake in Alpha; ●--● 48 hour growth in Alpha + 10 hour uptake in Alpha + CAP; Δ--Δ 48 hour growth in Alpha + CAP + 10 hour uptake in Alpha; ▲--▲ 48 hour growth in Alpha + CAP + 10 hour uptake in Alpha + CAP.


A
UPTAKE (DPM x 10^{-4} MG PROTEIN^{-1})
0
2
4
6
8
10
0
2
4
6
8
10


B
INCORPORATION (DPM x 10^{-3} MG PROTEIN^{-1})
0
1
2
3
4
5
0
2
4
6
8
10
HOURS OF INCUBATION

This reduction is not related to uptake, since control cultures, grown without CAP and labelled in the presence of CAP, do not show this inhibition. Cells cultured in CAP and labelled in the absence of the antibiotic also fail to show the inhibition.

If this reduction is related to the absence of incorporation of prosthetic groups into mitochondrially coded protein, the effect should be evident specifically in the mitochondrial fraction. These results are indicated in Table 5. The observed reduction in total cellular precipitable material after 4 hours labelling does not seem to be related entirely to an effect specific to the mitochondria, as all subcellular fractions seem to be affected to some extent, despite the fact that the specific activity of the mitochondria was three-fold higher than the microsomal fraction. This latter point is a reasonable finding in view of the concentration of hemoproteins in mitochondrial membranes, and the importance of mitochondrial reactions in the terminal assembly of heme (Meyer and Schmid, 1973).

3.4. DISCUSSION

The incorporation of leucine into whole cells or into mitochondrial proteins is not dramatically affected by prior growth or labelling in the presence of CAP. This is consistent with the fact that the bulk of cellular protein, and of mitochondrial protein is synthesized on cytoplasmic

TABLE 5

THE EFFECTS OF D-CHLORAMPHENICOL ON THE INCORPORATION OF δ-AMINOLEVULINIC ACID INTO THE PROTEINS OF SUBCELLULAR FRACTIONS ISOLATED FROM BHK-21 CELLS

BHK-21 cells were grown for 48 hours in 100 μg ml^{-1} CAP resuspended (6×10^5 cells ml^{-1}) and labelled for 4 hours with ^3H-δ-aminolevulinic acid (1 μCi ml^{-1}). 5×10^7 cells were harvested, washed and fractionated and the isolated fractions were precipitated with an equal volume of 20% TCA as described in Methods.

	Incorporation[a] (Relative to Homogenate[b])			
	48 Hour Control Growth		48 Hour Growth in CAP	
Fraction	Uptake - CAP	Uptake + CAP	Uptake - CAP	Uptake + CAP
Homogenate	1.08 (1.00)	1.06 (1.00)	1.15 (1.00)	0.93 (1.00)
Mitochondrial	3.73 (3.45)	3.60 (3.40)	4.23 (3.68)	3.10 (3.33)
Microsomal	1.18 (1.09)	1.06 (1.05)	1.15 (1.00)	0.93 (1.00)
Supernatant	0.62 (0.57)	0.68 (0.64)	0.51 (0.44)	0.49 (0.53)

[a] DPM Incorporated $\times 10^{-4}$ per mg protein

[b] Incorporation fraction / Incorporation Homogenate

ribosomes rather than the CAP-sensitive mitoribosomes (Beattie, 1971). The total amount of protein synthesized intramitochondrially is minimal in comparison to the vast numbers of mitochondrial proteins synthesized in the cytoplasm (Schatz and Mason, 1974). The slight reduction in labelling of cellular protein observed in Figure 12 may be related to the reduced uptake, but the amount incorporated relative to the amount taken up is constant. It is only at concentrations of CAP where cell growth is essentially non-existent that inhibition is evident, yet this is not restricted to the mitochondria.

Cycloheximide has a dramatic effect on the incorporation of leucine into BHK-21 cells. Following an initial rate of labelling comparable to that of cultures grown in the absence of inhibitor, the synthesis drops off quickly and reaches a low basal level by two hours. If CAP is added to the cycloheximide containing cultures, the initial synthesis is reduced. Thus, the initial protein synthesis is probably of mitochondrial origin. Mitochondrially coded proteins appear to be synthesized in significant quantities only if they can be combined with the appropriate cytoplasmically coded peptides that are needed to generate the complete component (Schatz and Mason, 1974; Tzagoloff et al., 1973). Thus a pool of mitochondrially synthesized precursors is present initially, but disappears within two hours in the absence of cytoplasmic

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synthesis. Again, it is evident that the level of mitochondrial protein synthesis is very low in comparison to cytoplasmic protein synthesis.

By contrast with the quite general distribution of leucine labelling among cell fractions, incorporation of δ-aminolevulinate was several-fold greater in the mitochondrial proteins. This is consistent with the fact that many hemoproteins are localized in the mitochondrial inner membrane, and that the final step in the production of heme which involves the insertion of iron(II) by ferrochelatase is mitochondrial (Meyer and Schmid, 1973; Kadenbach, 1971). Since heme is not being incorporated into cytochromes aa_3 or b in the presence of CAP, it may accumulate in the cells and eventually be removed by turnover after several hours. The only main regulatory step in the biosynthesis of heme involves δ-aminolevulinate synthetase (Meyer and Schmid, 1973; Strand et al., 1972) and this step is bypassed by the addition of δ-aminolevulinate to the cultures and hence no feedback inhibition occurs. Partial inhibition of ferrochelatase by chloramphenicol has been reported (Druyan et al., 1973) and this may account for some of the reduced labelling of cells cultured and labelled in the presence of CAP. The absence of some cellular proteins that bind heme as a prosthetic group, would probably account for the eventual decrease in protein bound heme. This is concluded from the fact that the reduction is observed in all subcellular fractions and is therefore most

likely related to a stage of the synthetic process prior to attachment of prosthetic group to either mitochondrially or microsomally located apoproteins.

It seems evident that mitochondrial protein synthesis in BHK-21 cells is regulated by events similar to those of other eukaryotes. The fact that CAP produces a fairly selective effect on mitochondrial protein synthesis without disrupting cytoplasmic protein synthesis even at concentrations which have been shown to inhibit respiration (Firkin and Linnane, 1968; Fettes et al., 1972), makes this cell system valuable for the examination of the subtle effects of the inhibition of mitochondrial protein synthesis on mitochondrial lipid metabolism.

CHAPTER 4. MITOCHONDRIAL PHOSPHOLIPID BIOSYNTHESIS IN CHLORAMPHENICOL-TREATED BHK-21 CELLS

4.1 INTRODUCTION

Studies on the biosynthesis of the mitochondrial membrane have involved two approaches - identification of the genetic systems responsible for the coding and synthesis of mitochondrial polypeptide components (Schatz and Mason, 1974; Tzagoloff et al., 1973; Mahler, 1973) and studies on the processes involved in the biosynthesis of mitochondrial phospholipids (McMurray and Magee, 1972; Van Den Bosch et al., 1974; Van Golde et al., 1974). However, very few workers have endeavoured to relate the interdependence of mitochondrial protein and phospholipid synthesis in mammalian systems. Two studies have indicated the concurrent synthesis of cardiolipin and proteins of the mitochondrial inner membrane (Hallman and Kankare, 1971; Liskova et al., 1974). Other studies have shown simultaneous labelling of phospholipid and protein of the endoplasmic reticulum and their eventual incorporation into the mitochondrion (Schiefer, H.-G., 1969; Beattie, 1969).

In mammalian mitochondria, a strong physical association has been demonstrated between phospholipids, particularly cardiolipin, and inner membrane proteins which have polypeptide components that are synthesized on

mitoribosomes (Chuang *et al*., 1970; Awasthi *et al*., 1970; Awasthi *et al*., 1971; Chuang and Crane, 1973; Chuang *et al*., 1973; Yu *et al*., 1973; Swanjung *et al*., 1973; Lopez-Maratalla *et al*., 1973; Wilschut and Scherphof, 1974; Yu *et al*., 1975). Therefore, it was decided to investigate the effects of chloramphenicol, an inhibitor of mitochondrial protein synthesis, on the synthesis of mitochondrial phospholipids in BHK-21 cells in culture under conditions where growth and respiratory enzyme content have been established to be impaired.

4.2 MATERIALS AND METHODS

BHK-21 cells were maintained in spinner culture as previously described. CAP was added to cultures at 100 μg ml^{-1} and its effects were routinely checked by cell counting in a Coulter counter.

4.2.1 Labelling of Cells in Culture

All labelling was done by adding isotope to cells suspended in fresh medium with or without CAP. $[^{32}P]$-P_i (Atomic Energy Canada) was added to cultures at 1 μCi ml^{-1} and cells were harvested following incubation during the exponential phase of growth (4-8 x 10^5 cells ml^{-1}), washed once with phosphate-buffered saline and once with 0.25 M sucrose-0.1 mM EDTA. Cells were suspended in sucrose-EDTA, and sonicated if whole cell homogenates were required.

Alternatively, the cells were homogenized with a Willems Polytron® (Brinkman) and subcellular fractionation was carried out by procedures described for mouse liver (McMurray and Rogers, 1973). Lipids were extracted as previously described (McMurray and Dawson, 1969) and separated by either two-dimensional (Rouser *et al.*, 1970) or one-dimensional thin layer chromatography (Possmayer *et al.*, 1969). Spots were identified by autoradiography, by spraying with 1% iodine in methanol or charring with 40% sulphuric acid. The areas were scraped off and digested in 70% perchloric acid. After the silica gel was removed by centrifugation, the Ĉerenkov radiation was counted (Clausen, 1968) and then phosphorus was determined (Bartlett, 1959; Rouser *et al.*, 1970). Protein was determined by the method of Lowry *et al.* (1951).

Incorporation of glycerol into lipids was normalized using a $^{3}H/^{14}C$ ratio technique analogous to the $^{3}H/^{14}C$ leucine labelling described in Chapter 3. A control culture was grown containing 0.1 μCi ml^{-1} [2-^{14}C]-glycerol (133.4 mCi/mole) (New England Nuclear). A second control culture and the various CAP treated cultures were grown simultaneously containing 0.1 μCi ml^{-1} [2-^{3}H]-glycerol (6.48 Ci $mmole^{-1}$) (New England Nuclear). ^{14}C- and ^{3}H-labelled cells were mixed in a ratio of 1:4 v/v, harvested and fractionated as above. Cells were extracted as before, except the lower phase was washed three times with

theoretical upper phase containing 10 mM glycerol. Following TLC of the washed extract (Rouser et al., 1970; Possmayer et al., 1969), spots were visualized by spraying with 1% iodine in methanol, scraped off the plates and counted in 0.5 ml water and 10 ml Aquasol ® (New England Nuclear) (Webb and Mettrick, 1972), using a Packard Tri-Carb ® Scintillation Spectrophotometer to obtain a $^{3}H/^{14}C$ ratio. Use of this ratio technique for determining the labelling of phospholipids has several inherent advantages. First of all, normalization is not dependent on the recovery of low amounts of P in minor lipids such as PG and PA. Secondly, the mixing of two differently labelled cultures before harvesting means that the ratio should be maintained through all subsequent procedures despite any loss of material. Slight variations in cell populations from one culture to another may be corrected by dividing each ratio by the $^{3}H/^{14}C$ ratio of the TCA-soluble extract of whole homogenate.

De novo fatty acid synthesis and incorporation into phospholipid was determined by labelling cells with 1 μCi ml^{-1} [1-^{14}C]-sodium acetate (24.1 mCi mg^{-1}) (Amersham). Following incubation, cells were harvested, washed, fractionated and extracted as above with the exception that theoretical upper phase containing 10 mM sodium acetate was used to wash the chloroform-methanol extract. The extracts were subjected to TLC (Possmayer

et al., 1969), and the spots were analysed for radioactivity as described above. In the case where mitochondrial extracts of cells, labelled with any of the various isotopes utilized, were chromatographed, cold carrier cardiolipin, phosphatidyl glycerol, phosphatidic acid and lysophosphatidic acid (Serdary Research, London, Canada) were added to the chloroform-methanol extract in order to facilitate identification of the low amounts of lipid with iodine spray.

The ability of cells to incorporate saturated versus unsaturated fatty acids was also examined. [1-^{14}C]-linoleic acid (52.8 mCi mmole^{-1}) or [1-^{14}C]-palmitic acid (52.0 mCi mmole^{-1}) (New England Nuclear) were added to cultures at 0.1 μCi ml^{-1} as the albumin complexes. The fatty acid albumin complexes were prepared as follows. The fatty acid solution (hexane) was dried under nitrogen and the fatty acid titrated with 10 mM KOH and sonicated in the sonic bath until uniformly suspended. Fatty acid-free albumin (Sigma) was added in solution until a fatty acid to albumin molar concentration ratio of approximately 6 was achieved. The complex was sonicated again until the solutions appeared homogeneous and the complexes were added to the cultures. Cells were harvested following incubation, washed and fractionated as above. The lipid extract was washed with theoretical upper phase and chromatographed as for acetate-labelled cells.

4.2.2 Fatty Acid Content of Cardiolipin from CAP-Treated Cells

Cultures containing 100 μg ml^{-1} CAP were allowed to grow for 48 hours as before and then allowed to recover in medium without CAP for an additional 24 hours. Samples were taken at 48 hours treatment and after recovery the cells were harvested and washed, and the lipids extracted. The extracts were chromatographed (Possmayer et al., 1969) and the cardiolipin identified by spraying the edges of the thin layer plates with iodine. The cardiolipin band was scraped off and the lipid was extracted from the gel with chloroform-methanol 1:1 (v/v). The extracted material was transmethylated, and the methyl esters extracted (Carroll and Khor, 1971) and analyzed by gas liquid chromatography in a Hewlett Packard, Model 402, with hydrogen flame detector, using a column of 15% EGSS-X on Chromosorb P (Carroll and Khor, 1971). The methyl ester positions were checked using a standard mixture of fatty acid methyl esters (Serdary, London, Canada).

4.2.3 ATP Determination by the Luciferin-Luciferase System

Cells were grown for 48 hours in 100 μg ml^{-1} CAP. At this point, cultures were split and half allowed to continue growth in CAP and the other half transferred to medium without CAP for an additional 24 hours. At 12 hour time intervals, samples (approx. 10^7 cells) were removed, harvested, washed once with phosphate-buffered saline and

once with 0.25 M sucrose. Pellets were suspended in 1 ml of 0.1 M Tris-HCl buffer pH 7.5, sonicated in the sonic bath and stored at -20°C. ATP contents were determined using the method of Ebadi _et al._ (1971) as modified from the method of Stanley and Williams (1969) in a Packard Tri-Carb Model 3375 scintillation spectrophotometer with the coincidence gate switched off. Results are expressed as 10^{-11} moles ATP per mg protein determined by the method of Lowry _et al._ (1951).

4.3 RESULTS

The effects of growth in 100 μg ml^{-1} CAP on uptake and incorporation of $[^{32}P]-P_i$ are shown in Figure 16. Both the uptake of $[^{32}P]-P_i$ as a function of the total cellular P content (Figure 16A) and the incorporation into lipid P (Figure 16B) are inhibited in cells in which CAP is present during the 48 hour growth period and the 6 hour labelling period. If CAP is removed from the medium during labelling, cells recover the ability to take up P_i and incorporate it into phospholipid. All cultures showed an initial 1-2 hour lag in incorporation. This probably represents the period needed for synthesis of the necessary P-containing lipid precursors.

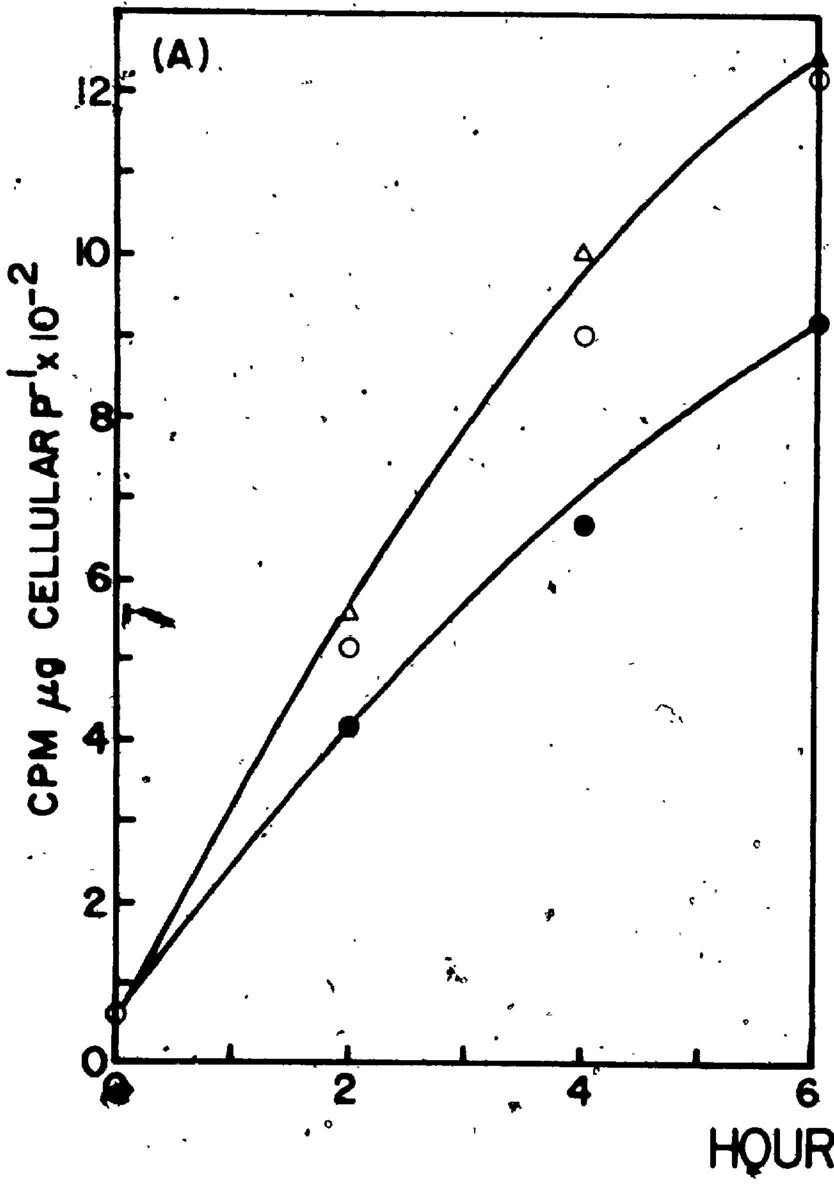
$[^{32}P]$-labelling of phospholipids in cells grown for 48 hours in CAP and then labelled for 4 hours in the presence or absence of CAP was examined in more detail.

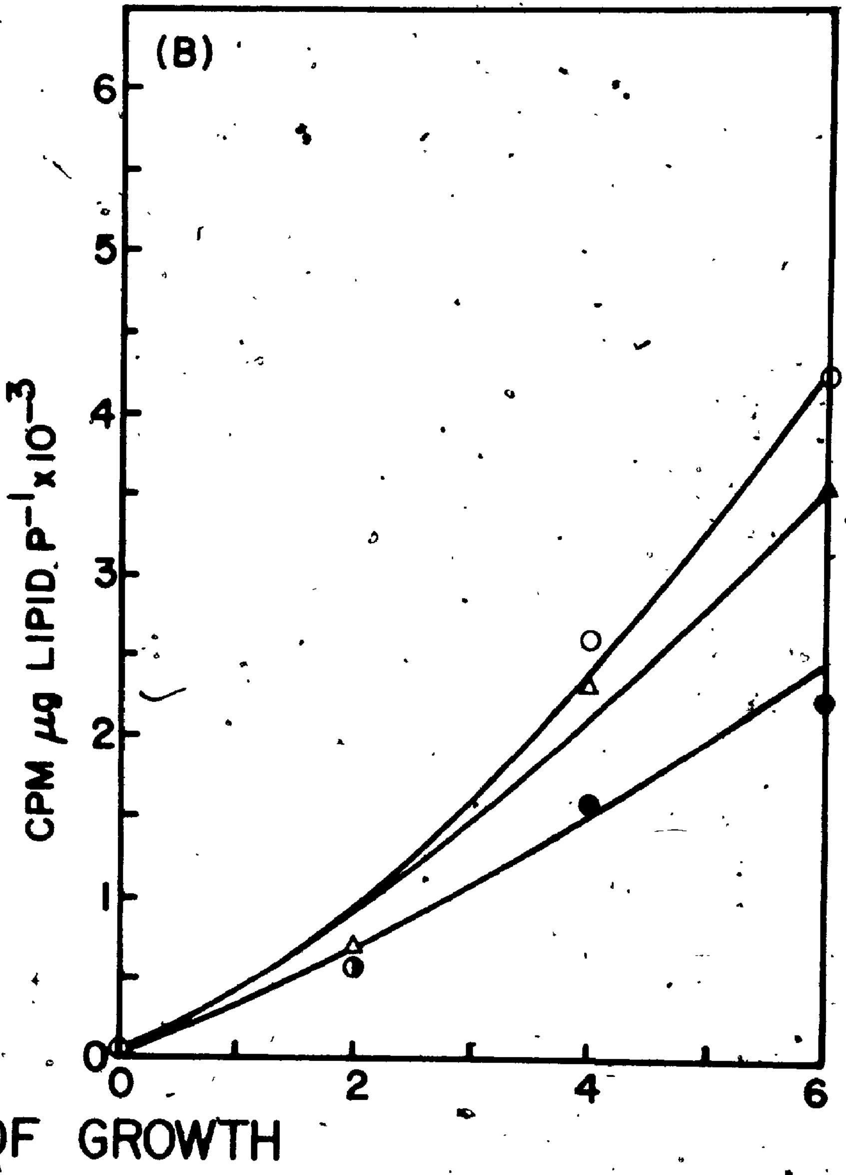
FIGURE 16

UPTAKE AND INCORPORATION OF $[^{32}P]$-P_i BY BHK-21

CELLS IN CULTURE

Cells were grown in the presence and absence of 100 $\mu g\ ml^{-1}$ CAP for 48 hours. At this time they were resuspended ($2 \times 10^5\ ml^{-1}$) in fresh medium containing 1 $\mu Ci\ ml^{-1}$ $[^{32}P]$-P_i with or without CAP and samples (5×10^6 cells) were taken over a 6 hour period. Cells were harvested, washed and suspended in 0.25 M sucrose-0.1 mM EDTA and sonicated. An aliquot was removed for determination of total uptake (A) and the remainder was extracted with chloroform-methanol 1:1 and the incorporation into lipid determined (B). o--o cells grown 48 hours in Alpha, uptake in Alpha, ●--● cells grown 48 hours in Alpha + CAP, uptake in Alpha + CAP; Δ--Δ cells grown 48 hours in Alpha + CAP, uptake in Alpha.


(A)
CPM μg CELLULAR P⁻¹ x 10⁻²
0
2
4
6
8
10
12
0
2
4
6


(B)
CPM μg LIPID P⁻¹ x 10⁻³
0
1
2
3
4
5
6
0
2
4
6
HOURS OF GROWTH

The results are indicated in Table 6. Although considerable fluctuation of the total counts incorporated was observed from experiment to experiment, it was found that growth for 48 hours in CAP before labelling resulted in a reduction of 30-50% in the amount of [^{32}P]-P_i incorporated into cellular phospholipid relative to control cultures. The presence of CAP during the labelling period of cells grown in the presence or absence of CAP did not have a consistent effect on the incorporation of [^{32}P]-P_i and is probably not important.

Total cellular phospholipids were isolated in order to determine if there was a selective inhibition (Table 7). Several features are evident. PC, PS and SM all showed reduced labelling (approximately 15-20%) if CAP was present during the uptake period, regardless of the growth conditions. PE showed reduced labelling if CAP was present during the initial growth period, while DPG labelling was reduced during any period of exposure to CAP. The incorporation into PG and PA showed slight increases if cells were cultured for 48 hours in CAP. There were difficulties however in quantitating these data. Since the specific activities are based on the amount of P present in each lipid spot, the low and inconsistent recoveries of P from PS, PA, PI and PG make interpretation of these results difficult. Others have shown the turnover of PI and PS to be slow as compared to other BHK cell lipids, and they

TABLE 6

^{32}P LABELLING OF BHK-21 CELL PHOSPHOLIPIDS

Cells were grown in the presence and absence of 100 μg ml^{-1} CAP for 48 hours as in Figure 16. They were then labelled with 1 μCi ml^{-1} [^{32}P]-P_i in the presence or absence of CAP, 5 x 10^7 cells were harvested after 4 hours of incubation and the lipids extracted as in Figure 16.

Culture Conditions		CPM ^{32}P μg P^{-1} (Percent of controls without CAP)				
48 Hours Growth	4 Hour Label	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
-CAP	-CAP	938(100)	4960(100)	7060(100)	6690(100)	7620(100)
-CAP	+CAP	632(67.4)	3850(77.6)	7580(107)	5090(76.1)	8770(115)
+CAP	-CAP	463(49.4)	3470(69.9)	3850(54.5)	4420(66.1)	4760(62.5)
+CAP	+CAP	311(33.2)	3330(67.2)	4990(70.6)	3550(53.1)	4930(64.8)

TABLE 7

EFFECT OF CAP ON LABELLING OF BHK-21 PHOSPHOLIPIDS WITH $[^{32}P]-P_i$

The lipid extract from Figure 16 was chromatographed (Rouser *et al.*, 1970) and the ^{32}P counts in each lipid spot determined as described in Materials and Methods.

	CPM μg lipid P^{-1} [a] (Relative to total[b])			
	48 hour growth -CAP		48 hour growth +CAP	
Phospholipid	uptake -CAP	uptake +CAP	uptake -CAP	uptake +CAP
total	7120(1.00)	7150(1.00)	4340(1.00)	4490(1.00)
PC	6860(0.96)	5800(0.81)	4570(1.05)	3950(0.88)
PE	7180(1.01)	7590(1.06)	3250(0.75)	3470(0.77)
PI	11200(1.58)	9190(1.29)	7080(1.63)	6160(1.37)
DPG	7440(1.05)	5190(0.73)	3350(0.77)	3070(0.68)
PA	3840(0.54)	4570(0.64)	2960(0.68)	4880(1.09)
SM	1880(0.26)	1340(0.19)	980(0.23)	844(0.19)
PG	6050(0.85)	5530(0.77)	4460(1.03)	4080(0.91)
PS	7180(1.01)	4040(0.57)	4740(1.09)	3570(0.79)

[a] average of three experiments

[b] specific activity phospholipid relative to specific activity total lipid

may not be equilibrated, under these conditions (Gallaher and Blough, 1975). The turnover of PA is rapid but not easily quantitated (Gallaher and Blough, 1975). To what extent the observed decreases are a result of a generalized decrease in labelling due to a block in precursor formation or a result of specific inhibition of a particular pathway is uncertain.

Since the site of CAP inhibition of protein synthesis is mitochondrial, the effect in subcellular fractions was examined in order to determine if the reduced labelling of phospholipids occurs to a greater extent in that organelle. The results are reported in Table 8. A small reduction in the ^{32}P labelling of both mitochondrial and microsomal phospholipids in cells grown 48 hours in CAP was observed, but this decrease was consistent with a generalized reduction in the labelling of the whole homogenate. When phospholipid extracts of mitochondria were chromatographed in one dimension (Possmayer _et al_., 1969), the results in Table 9 were observed. In this experiment better separation of PG and DPG was obtained by use of a one dimensional system, and total recovery of the ^{32}P was obtained. A significant decrease in the amount of label incorporated into cardiolipin was evident, with smaller decreases in PA and PG. A concomitant increase in the labelling of PC was also observed. Although the incorporation into the total mitochondrial lipid was reduced by 60%, the only phospho-

TABLE 8

SUBCELLULAR EFFECTS OF CAP ON [^{32}P]-P_i LABELLING OF LIPIDS

Cells were grown and labelled with [^{32}P]-P_i as described in Figure 16. 5×10^7 cells were harvested after 4 hours incubation, washed, homogenized and fractionated as described in the Materials and Methods. The lipids were extracted from each subcellular fraction and the lipid ^{32}P counts determined.

	Relative Specific Activity[a] (Relative to Homogenate[b])			
	48 Hour Growth -CAP		48 Hour Growth + CAP	
Fraction	Uptake - CAP	Uptake + CAP	Uptake - CAP	Uptake + CAP
Homogenate	1.09 (1.00)	1.25 (1.00)	0.97 (1.00)	0.88 (1.00)
Mitochondrial	0.89 (0.82)	0.78 (0.63)	0.83 (0.85)	0.71 (0.82)
Microsomal	1.35 (1.24)	1.44 (1.55)	1.02 (1.05)	1.12 (1.28)

[a] CPM μg P^{-1} chloroform-methanol extract/CPM μg P^{-1} TCA-soluble pool of homogenate

[b] relative specific activity fraction/relative specific activity homogenate

TABLE 9

CAP EFFECTS ON [^{32}P]-P_i LABELLING OF MITOCHONDRIAL PHOSPHOLIPIDS

The lipid extract of mitochondria labelled as in Table 8 was chromatographed in one dimension (Possmayer et al., 1969) and the radioactivity determined as a percent of the total label recovered from the TLC plate.

Phospholipid	Labelling[a] (percent of total label recovered from chromatogram[b])	
	48 hours growth -CAP (uptake -CAP)	48 hours growth +CAP (uptake +CAP)
DPG	19.2	9.9
PA	1.4	1.1
PG	8.3	5.8
PE + PI	28.9	29.9
PC	36.9	49.5

[a] total counts incorporated - control = 1760 CPM μg P^{-1}, CAP-treated = 580 CPM μg P^{-1}

[b] average of two experiments

lipid to show a specific decrease greater than the general decrease in labelling was cardiolipin. Thus there may be a generalized effect on one or more universal phospholipid precursors, superimposed on which is a specific effect on cardiolipin involving the polyglycerolphosphatide biosynthetic pathway.

Due to the fluctuations that resulted from variable P recovery, these experiments were repeated utilizing the double label technique with glycerol as the precursor. With this method, sample recovery from subcellular fractionation or chromatography is not important in determining the ratio of ^{3}H to ^{14}C. Table 10 gives the results of the labelling of cells for a four hour period following 48 hours of growth in the presence or absence of CAP. In this case, the decrease in incorporation due to the presence of CAP is evident in both mitochondria and microsomes. There is a specific decrease in mitochondrial labelling of 15% relative to the homogenate in CAP-treated cells, not seen in the microsomal fraction. The mitochondrial fraction was chromatographed to examine the effect upon individual phospholipids (Table 11). All lipids were reduced in the extent of their labelling. The cardiolipin labelling was reduced to the greatest extent relative to the total lipid extract. PG appeared to be increased in labelling, although only to a small extent. The presence of CAP during the labelling period seemed to have little effect. Thus there appears

TABLE 10

EFFECT OF CAP ON THE INCORPORATION OF GLYCEROL INTO BHK-21 CELL LIPIDS

Cells were grown for 48 hours in 100 μg ml^{-1} CAP. They were then resuspended in fresh medium with or without CAP and labelled for 4 hours with 0.1 μCi ml^{-1} [2-^{3}H]-glycerol or 0.1 μCi ml^{-1} [2-^{14}C]-glycerol. Cells were mixed (6×10^{7} cells total) as indicated for the $^{3}H/^{14}C$ labelling technique described in Materials and Methods and fractionated as before (Table 8).

		Relative Specific Activity[a] (Relative to Homogenate[b])			
		48 hours growth -CAP		48 hours growth +CAP	
		uptake -CAP	uptake +CAP	uptake -CAP	uptake +CAP
Expt. 1	Total Homogenate	2.20	1.93	1.42	1.48
Expt. 2	Total Homogenate	2.37(1.00)	2.10(1.00)	1.90(1.00)	1.64(1.00)
	Mitochondrial	2.43(1.03)	2.12(1.01)	1.62(0.85)	1.40(0.85)
	Microsomal	2.40(1.01)	2.16(1.03)	1.90(1.00)	1.62(0.97)

[a] $^{3}H/^{14}C$ chloroform-methanol soluble/$^{3}H/^{14}C$ TCA-soluble pool of homogenate

[b] Relative specific activity fraction/Relative specific activity homogenate

TABLE 11

THE EFFECT OF CAP ON MITOCHONDRIAL LIPID SYNTHESIS

The chloroform-methanol extract from a mitochondrial fraction labelled and isolated as in Table 10 was chromatographed (Rouser *et al.*, 1970) and the $^{3}H/^{14}C$ ratio in each lipid spot determined.

	Relative Specific Activity[a] (relative to total mitochondrial $^{3}H/^{14}C$[b])			
	48 hour growth -CAP		48 hour growth +CAP	
Phospholipid	uptake -CAP	uptake +CAP	uptake -CAP	uptake +CAP
Total	2.43(1.00)	2.12(1.00)	1.62(1.00)	1.40(1.00)
DPG	2.36(0.97)	2.07(0.98)	1.31(0.81)	1.10(0.79)
PG	2.45(1.01)	2.30(1.08)	2.11(1.30)	1.70(1.21)
PE	2.47(1.02)	2.39(1.13)	1.81(1.18)	1.59(1.14)
PC	2.37(0.98)	1.96(0.92)	1.72(1.06)	1.39(0.99)
PI	2.28(0.94)	2.00(0.94)	1.41(0.87)	1.53(1.09)

[a] $^{3}H/^{14}C$ phospholipid/$^{3}H/^{14}C$ TCA-soluble pool of homogenate

[b] $^{3}H/^{14}C$ phospholipid/$^{3}H/^{14}C$ chloroform-methanol soluble mitochondrial fraction

to be a general decrease in the labelling of cellular phospholipids in cells grown in CAP. In addition, the pathway from PG to DPG may be inhibited as seen by the 20% decrease in cardiolipin labelling relative to the total, and the slight elevation in labelled PG in CAP-treated cells. The amount of mitochondrial phospholipid per mg mitochondrial protein remains unchanged on treatment with CAP and cardiolipin represents the same proportion of total mitochondrial lipid P in treated and normal mitochondria (Appendix 6).

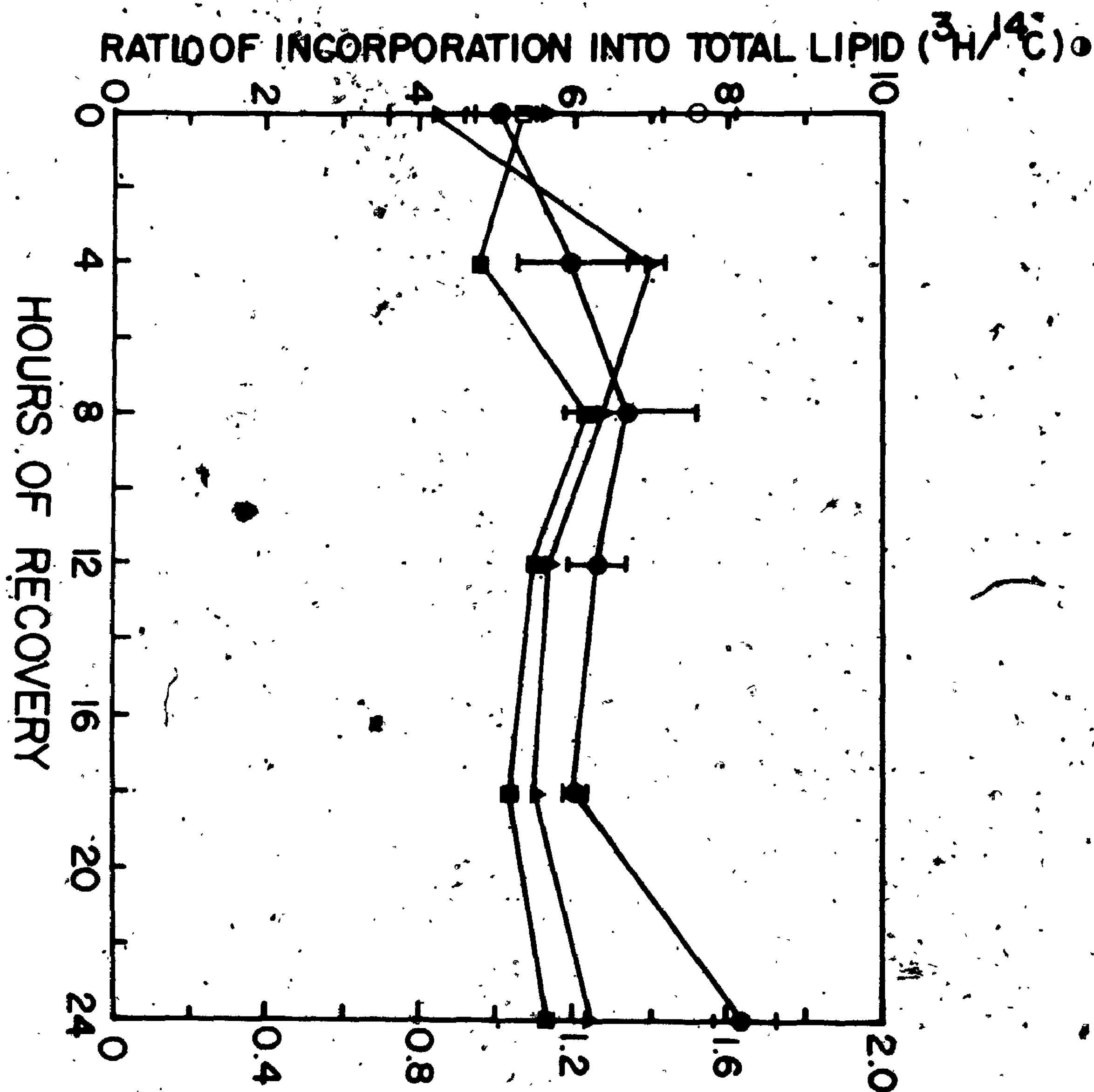
The recovery of cellular lipid synthesis on removal of CAP was examined with the double label technique. These results are shown in Figure 17. The rate of synthesis of cardiolipin increased by 75% within 4 hours of removal of CAP from the cultures and dropped off thereafter. On the other hand, the rate of total lipid synthesis and that of microsomally synthesized lipid (indicated by the rate of PE synthesis) did not reach maximal rates until 8 hours following removal of CAP. Thus the specific effect on DPG is rapidly overcome on removal of the CAP. The general effect that results in reduced labelling of all the phospholipids requires additional time before the rate of lipid synthesis recovers to control values.

The incorporation of [^{14}C]-acetate into BHK-21 lipid was used to ascertain whether fatty acid synthesis was impaired in CAP-treated cells as has been observed in yeast

FIGURE 17

RECOVERY OF LIPID SYNTHESIS FROM TREATMENT WITH CAP

Cells were grown for 48 hours in 100 μg ml^{-1} CAP. They were then resuspended in fresh medium without CAP and 10^7 cells were removed at various time intervals and pulsed for 2 hours with [2-^{3}H]-glycerol (1 μCi ml^{-1}). Simultaneously, a control culture was pulsed for 2 hours with [2-^{14}C]-glycerol (0.5 μCi ml^{-1}). Ten volumes of the ^{3}H-labelled cells were mixed with one volume of ^{14}C-labelled cells, and then washed and sonicated. The lipids were extracted and chromatographed (Possmayer et al., 1969). The rate of incorporation into total lipids and individual lipids was determined. Results are plotted as average ± maximum error of individual cultures. -o- 48 hour growth no CAP - control synthesis rate of total lipid synthesis; -●- 48 hour growth in CAP, recovered 24 hours no CAP - recovery rate of lipid synthesis; -Δ- control rate of DPG synthesis; -▲- recovered rate of DPG synthesis; -□- control rate of PE synthesis; -■- recovered rate of PE synthesis.



RATE OF INCORPORATION INTO CARDIOLIPIN RELATIVE TO RATE OF INCORPORATION INTO TOTAL LIPID ($^3H/^{14}C$ DPG/$^3H/^{14}C$ TOTAL) ▲

RATE OF INCORPORATION INTO PE ($^3H/^{14}C$ PE/$^3H/^{14}C$ TOTAL) ■

(Kovac et al., 1967; Gordon and Stewart, 1971; Gordon and Stewart, 1972; Janki et al., 1974a; Rogers et al., 1974 Janki et al., 1975). Results of the subcellular distribution of label at 4 hours where incorporation is still linear are shown in Table 12. Incorporation into homogenates was reduced by 50% in CAP-treated cells. This is similar to the generalized decrease in de nous labelling of lipid observed earlier. In the microsomal fraction where the bulk of acylation occurs, the relative specific activity is reduced indicating that the endoplasmic reticulum may be the site of a block in the incorporation into phospholipid. No relative decrease in the mitochondrial labelling was observed. Thin layer chromatography of the mitochondrial lipid extracts yielded the results shown in Table 13. No selective effect on any phospholipid, including cardiolipin, was observed. Thus the DPG specific effect observed with labelled glycerol or P_i as precursors is not related to fatty acid synthesis, but rather de novo phospholipid synthesis.

Phospholipid acylation in CAP-treated cells was the next biosynthetic event examined. The fatty acid composition of cardiolipin from BHK-21 cells is given in Table 14. The significant feature is that the amounts of saturated fatty acids (16:0 and 18:0) are reduced on treatment with CAP while the levels of unsaturated fatty acids (18:1 and 18:2) are elevated and retained even after 24 hours recovery.

Palmitate and oleate make up the greatest percentage

TABLE 12

EFFECT OF CAP ON [^{14}C]-ACETATE INCORPORATION INTO BHK-21 CELL PHOSPHOLIPID

BHK-21 cells were grown for 48 hours in 100 μg ml^{-1} CAP. They were resuspended in fresh medium (7.5 x 10^5 cells ml^{-1}) containing 1 μCi ml^{-1} [1-^{14}C]-sodium acetate for 4 hours. 8 x 10^7 cells were harvested, washed and extracted as before (Fig. 16).

Fraction	CPM μg lipid P^{-1} [a] (relative to homogenate[b])	
	48 hours growth + 4 hours label -CAP	48 hours growth + 4 hours label +CAP
Homogenate	4260 (1.00)	2190 (1.00)
Mitochondria	3270 (0.77)	1710 (0.78)
Microsomal	3520 (0.83)	1490 (0.68)

[a] average of two experiments

[b] CPM μg lipid P^{-1} fraction/CPM μg lipid P^{-1} homogenate

TABLE 13

CAP EFFECTS ON [^{14}C]-ACETATE INCORPORATION INTO MITOCHONDRIAL PHOSPHOLIPIDS

The lipid extracts of the mitochondria from cells described in Table 12 were chromatographed (Possmayer *et al.*, 1970) and the ^{14}C radioactivity was determined on each area of the chromatogram.

Phospholipid	Percent of Total Mitochondrial Lipid Label[a]	
	Control - no CAP during growth or labelling	CAP - present during growth and labelling
DPG	23.1	24.1
PA	6.8	6.3
PG	4.3	4.8
PE + PI	15.6	15.7
PC	48.8	47.5

[a] average of two experiments

TABLE 14

FATTY ACID CONTENT OF CARDIOLIPIN FROM CAP-TREATED BHK-21 CELLS

Cells were grown in 100 μg ml^{-1} CAP for 48 hours and allowed to recover for 24 hours in the absence of CAP. Cardiolipin was isolated from 108 cell samples taken and prepared for gas liquid chromatography as described in Methods.

Fatty Acid	48 hours growth -CAP		48 hours growth +CAP		48 hours +CAP -24 hours recovery	
	expt. 1	expt. 2	expt. 1	expt. 2	expt. 1	expt. 2
12:0	0.2	N.D.	0.5	0.4	0.2	0.3
14:0	6.8	6.1	6.5	6.2	3.8	3.4
14:1	1.2	1.1	2.1	1.8	2.6	4.2
16:0	30.3	31.0	24.4	19.0	19.9	28.0
16:1	11.2	9.0	12.0	11.6	11.9	8.7
18:0	12.4	14.9	7.0	6.1	8.1	9.8
18:1	35.5	34.7	44.6	46.9	46.7	42.5
18:2	2.6	3.2	4.4	5.0	6.7	3.1
18:3	trace	trace	0.3	0.2	trace	trace

N.D. not detected

trace < 0.2%

of the cardiolipin fatty acid content. This is contrasted with the high levels of linoleate usually found in cardiolipin from animal cells (White, 1973), and reflects the fatty acid composition of the serum added to the medium. Calf serum has low levels of polyunsaturated fatty acids and the composition of cell membranes reflects this (Harary et al., 1967).

Cells were labelled with ^{14}C-palmitate and ^{14}C-linoleate to examine any differences in the effects of CAP on the incorporation of saturated versus unsaturated fatty acids. A two hour pulse was used as the incorporation is linear for only 3 hours (Appendix 7). The effects of CAP on the labelling of cells and subcellular fractions are shown in Table 15. Incorporation of palmitate is decreased in microsomes of CAP-treated cells. This would indicate that de novo synthesis which occurs primarily in microsomes is probably inhibited. Total incorporation of palmitate into mitochondrial lipid, does not seem to be inhibited. In the case of linoleate, the labelling of both microsomal and mitochondrial lipid is reduced. This may represent inhibition of reacylation of the bulk lipid of the two subcellular fractions, or selective inhibition of the de novo biosynthetic routes which may incorporate linoleate. Saturated fatty acids are usually introduced in the de novo pathways and polyunsaturated fatty acids via deacylation-reacylation mechanisms (McMurray and Magee, 1972).

TABLE 15

CAP EFFECTS ON THE INCORPORATION OF FATTY ACID INTO BHK-21 CELLS

Cells grown for 48 hours on 100 $\mu g\ ml^{-1}$ CAP were labelled with [1-^{14}C]-palmitate or [1-^{14}C]-linoleate for 2 hours. 6 X 10^7 cells were then harvested, washed and the lipids extracted from subcellular fractions as before (Fig. 16).

	CPM μg lipid P^{-1} [a] (relative to homogenate[b])			
	[^{14}C]-palmitate		[^{14}C]-linoleate	
Fraction	cells grown -CAP	cells grown +CAP	cells grown -CAP	cells grown +CAP
Homogenate	1.53×10^{5} (1.00)	1.10×10^{5} (1.00)	1.03×10^{5} (1.00)	0.70×10^{5} (1.00)
Mitochondrial	1.14×10^{5} (0.74)	1.12×10^{5} (1.03)	0.84×10^{5} (0.89)	0.39x10[illegible] (0.55)
Microsomal	1.23×10^{5} (0.80)	0.73×10^{5} (0.66)	1.29×10^{5} (1.26)	0.80×10^{5} (1.16)

[a] average of two experiments

[b] CPM μg lipid P^{-1} fraction/CPM μg lipid P^{-1} homogenate

The fatty acid labelling profiles of the phospholipids isolated from BHK cell mitochondria are given in Table 16. It is evident that the incorporation of palmitate into mitochondria follows a different profile than the incorporation of linoleate. The large amount of lable in the PA + FFA spot was found to be due to the presence of unincorporated FFA. This was ascertained by chromatography of the extract, using a system for separation of neutral lipids (Appendix 8). Thus the ability of mitochondria to incorporate palmitate *de novo* into PA is impaired in CAP-treated cells. DPG is labelled with palmitate to a greater degree in CAP-treated mitochondria, either because of rapid turnover of existing DPG or due to reacylation with palmitate. In the case of cells labelled with linoleate, there is a slight increase in the labelling of DPG in mitochondria relative to other phospholipids from CAP-treated cells. No pool of free linoleate was observed indicating that it may be incorporated into mitochondrial phospholipids *via* the reacylation pathway (Bard *et al*., 1972), and that this pathway is not impaired in CAP-treated cells.

A generalized decrease of *de novo* phospholipid synthesis has been observed in cells grown in CAP. Since several mitochondrial cytochromes are absent or deficient in CAP-grown cells, it seemed likely that this inhibition was a result of decreased ATP levels resulting from impaired respiration and oxidative phosphorylation. CAP has also

TABLE 16

FATTY ACID LABELLING PROFILES OF MITOCHONDRIA FROM CAP-TREATED BHK-21 CELLS

Mitochondrial extracts from Table 15 were chromatographed (Possmayer *et al.*, 1970) and the lipids identified and counted.

Phospholipid	Percent of Total Counts[a]			
	[^{14}C]-palmitate		[^{14}C]-linoleate	
	cells grown -CAP	cells grown +CAP	cells grown -CAP	cells grown +CAP
DPG	13.1	21.1	14.6	18.1
PG	3.7	2.2	1.7	1.1
PGP	1.4	0.7	0.9	0.6
PE + PI	10.8	5.5	11.8	9.4
PC	50.2	27.5	66.7	66.8
PA + FFA	20.5	42.9	4.1	3.8

[a]average of 2 experiments

been shown to inhibit respiration in cultured animal cells (Firkin and Linnane, 1968; Fettes et al., 1972). However, there appears to be no decrease in the ATP concentration of CAP-treated cells compared with cells grown in the absence of CAP (Figure 18). Since no ATP can be produced from oxidative phosphorylation in mitochondria lacking cytochrome c oxidase and cytochrome b or in mitochondria inhibited at Site 1 (Firkin and Linnane, 1968; Fettes et al., 1972), any ATP produced must be at the level of substrate phosphorylation.

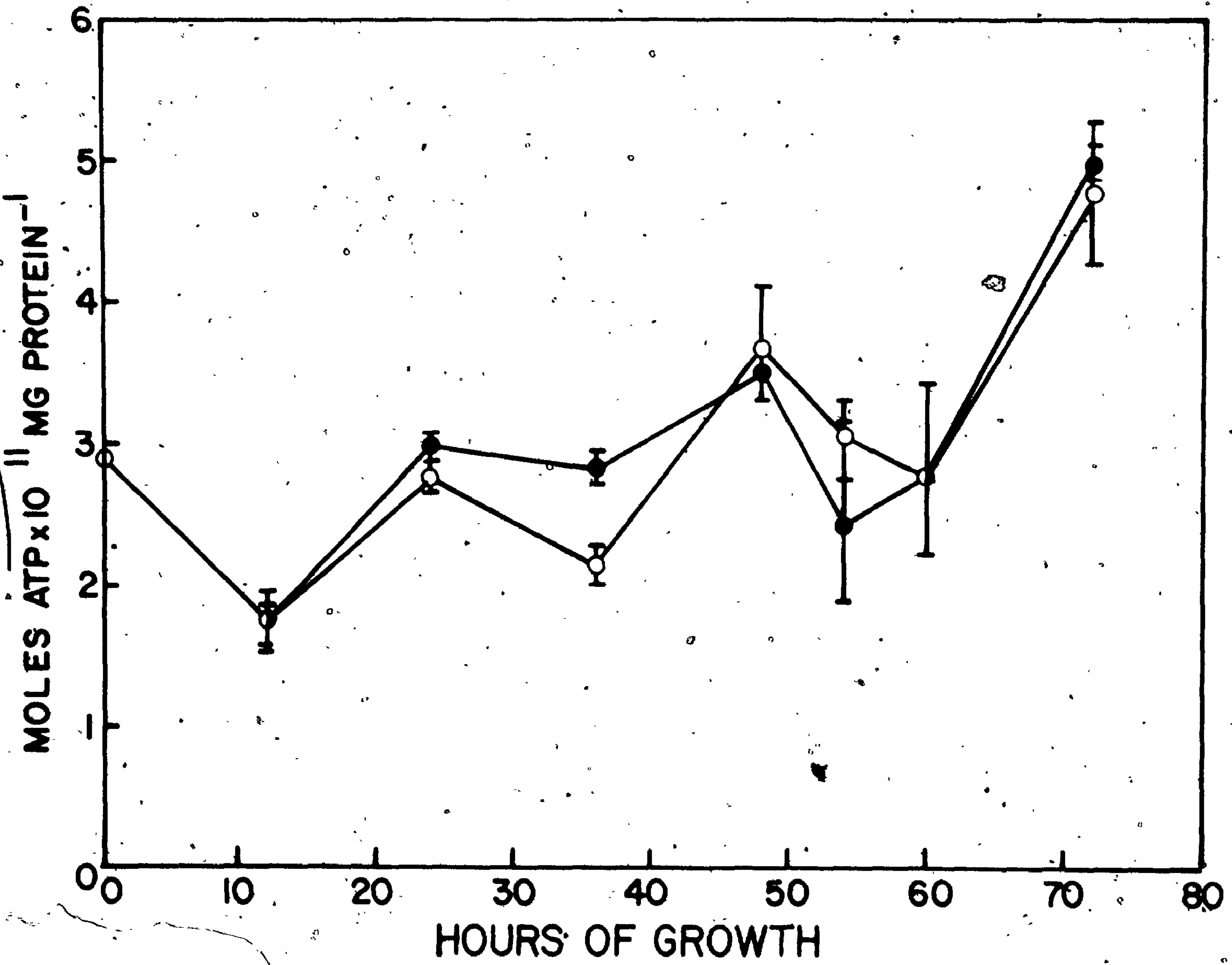
4.4 DISCUSSION

Growth of cultured animal cells in the presence of 100 μg ml^{-1} CAP results in the decreased labelling of cellular lipids, although the greatest reduction in labelling occurs in lipids of the mitochondria. The results of labelling cells with either $[^{32}P]$-P_i or $[^3H]$-glycerol would indicate that the reduction in counts incorporated occurs at a step very early in the biosynthetic routes to phospholipid. $[^{32}P]$-P_i is first incorporated into ATP. Glycerol can be incorporated into lipid via at least two pathways - acylation of either sn glycerol-3-phosphate or dihydroxyacetone phosphate (Pollock et al., 1975a). It has been shown that the latter pathway is utilized to a significant extent in glycerolipid synthesis in BHK-21 cells (Pollock et al., 1975b).

FIGURE 18

ATP CONTENT OF CAP-TREATED BHK CELLS

BHK-21 cells were grown in medium containing 100 μg ml^{-1} CAP. Cells were resuspended in fresh medium containing CAP at 24 hour intervals. At 12 hour intervals 10^7 cells were removed, harvested, washed once with phosphate-buffered saline, once with 0.25 M sucrose and suspended in 1 ml 0.1 M Tris-HCl pH 7.5 and sonicated. ATP was determined by the luciferin-luciferase system (32). o--o 72 hours - no CAP; ●--● 72 hours - 100 μg ml^{-1} CAP.


MOLES ATP x 10^{11} MG PROTEIN^{-1}
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
80
HOURS OF GROWTH

Since the level of ATP in CAP-treated BHK-21 cells does not appear to be reduced, it is possible that the block lies at the level of glycerol kinase, dihydroxyacetone kinase (Jenkins and Hajra, 1976) or other enzymes involved in the interconversion of the two precursors. One area which may bear examination lies in the interconversion of glycerol phosphate and dihydroxyacetone phosphate. The levels of ATP in CAP-treated cells must be maintained at normal concentrations through substrate level phosphorylation. If this process involves glycolysis, one end product would be NADH. If the level of NADH is raised greatly, it is possible that the equilibrium involving cytoplasmic glycerol phosphate dehydrogenase is such that dihydroxyacetone phosphate would be produced at a very reduced rate. Similarly, since the mitochondrial electron transport chain would be reduced, there would be no oxidized flavoprotein to act as receptor for the reducing equivalents produced from glycerol phosphate by the mitochondrial glycerol phosphate dehydrogenase. Thus, the lipid biosynthetic pathways which proceed via dihydroxyacetone phosphate may be slowed down by the reduced levels of substrate. It is interesting to note that mitochondrial $NADH/NAD^+$ levels have been shown to regulate the rate of β-oxidation in isolated rat liver mitochondria (Lumeng et al., 1976).

The general decrease in lipid synthesis is also observed when cells are grown on $[^{14}C]$-acetate, $[^{14}C]$-palmitate

and [^{14}C]-linoleate. This would confirm that the block is in the *de novo* pathway in the case of the first two precursors. Reduction in labelling with linoleate may reflect reduced *de novo* synthesis, but also may be indicative of reduced reacylation of *de novo* synthesized phospholipid. The fact that all cellular lipids seem to be synthesized to a lower extent in CAP-treated cells would indicate that the block affects a pathway common to all lipid classes. The magnitude of reduction is fairly consistent for all phospholipids with the exception of cardiolipin, which is reduced to a greater extent. Incorporation of fatty acid, either added or synthesized from acetate, would be expected to be reduced in cells in which the acylation rate of dihydroxyacetone phosphate is reduced due to the shortage of precursor. The reduction in the relative amounts of saturated fatty acid determined in cardiolipin may reflect the effects of long term growth under essentially anaerobic type conditions. Treated cells may reduce the amount of saturated fatty acid synthesized *de novo* and even attempt to utilize fatty acid as an energy source. The result may be that the relative amount of unsaturated fatty acid is elevated by preferential retention in an effort to conserve membrane integrity. Mammalian cells in culture have been shown to retain acyl groups from membrane phospholipids (Lynch *et al.*, 1976).

Besides the general reduction in phospholipid

biosynthesis, a further decrease in synthesis of cardiolipin is observed. Furthermore, when the acylation of other lipids appears reduced, cardiolipin labelling by both palmitate and linoleate is elevated, possibly indicating an increased turnover of the mitochondria—specific lipid or rapid reacylation. Two pools of cardiolipin, one involving cardiolipin reacylated with linoleate, have been shown to exist in rat (Bard _et al._, 1972). The pool containing linoleyl cardiolipin may be turning over more rapidly (Landriscina _et al._, 1976) in mitochondria from CAP-treated cells. Glycerol or $[^{32}P]-P_i$ labelling of cardiolipin indicates that _de novo_ synthesis is blocked to an even greater degree than for the other cellular lipids. Since cardiolipin is so intimately associated with membrane proteins containing mitochondrially-coded peptides (Chuang _et al._, 1970; Lopez-Moratalla _et al._, 1973), it is possible that inhibition of the synthesis of polypeptide components of these proteins by CAP may reduce the amount of associated phospholipid that can be incorporated into the membrane. In yeast, synthesis and incorporation of inner membrane components has been shown to involve formation of lipoprotein complexes from _de novo_ synthesized polypeptides and lipids while outer membrane proteins, not coded for on mitoribosomes are inserted into preexisting lipid (Janki _et al._, 1974b). Studies with yeast have indicated a relationship between restoration of respiratory activity and phospholipid content

on glucose derepression (Castelli _et al._, 1972). Aeration of anaerobically grown yeast in the presence of chloramphenicol, permitted phospholipid levels to rise normally in the absence of respiratory adaptation (Kovac _et al._, 1967) but only if the unsaturated fatty acids or sterol were present in the growth medium (Gordon and Stewart, 1972; Janki _et al_, 1974a; Janki _et al._, 1975; Marzuki _et al._, 1975). Yeast with little or no respiratory activity as a result of glucose repression or anaerobic growth, have reduced cardiolipin levels when compared to respiratory-competent cells (Jakovcic _et al._, 1971). On derepression of glucose repressed yeast, cardiolipin content was shown to increase concurrently with respiratory development (Gailey and Lester, 1968). The appearance of cytochrome oxidase in rat liver has been shown to be related to the synthesis of cardiolipin (Hallman and Kankare, 1971; Liskova _et al._, 1974). It is not inconceivable therefore, that the _de novo_ synthesis of cardiolipin and its incorporation into BHK cell mitochondria would be related to the synthesis of mitochondrially-coded polypeptides. The synthesis of cardiolipin on removal of CAP peaks at 4 hours (Figure 17), ahead of the other phospholipids and prior to the recovery of the respiratory enzymes containing mitochondrially-coded components. Cardiolipin biosynthesis has also been shown to be inhibited by CAP in regenerating liver (Getz, 1975). In bacterial systems, inhibition of

protein synthesis by amino acid starvation or CAP treatment causes inhibition of the synthesis of cardiolipin and other lipids (Sokawa et al., 1968; Mindich, 1970; Glenn and Gould, 1973; Arbogast and Henderson, 1975). On the other hand, CAP does not inhibit lipid synthesis under conditions of impaired protein synthesis in either Mycoplasma laidlawii (Kahane and Razin, 1969) or in the protozoan, Tetrahymena pyriformis (Gleason and Ooka, 1974).

The reduced synthesis of BHK cell phospholipids by CAP is not related to any gross inhibition of protein synthesis or to reductions in the energy content of the cells. The reduction may be related to cellular levels of reduced NAD or to control by a mitochondrial gene product of an enzymatic step in the synthesis of sn-glycerol-3-phosphate or dihydroxyacetone phosphate. The synthesis of cardiolipin is additionally inhibited, either as a result of mandatory mitochondrial protein synthesis for its incorporation or due to the fact that glycerol phosphate is involved in several steps of its biosynthesis (Stanacev et al., 1973). Studies of the biosynthesis of mitochondrial phospholipids in isolated sub-cellular fractions will be reported in the next chapter.

CHAPTER 5. SYNTHESIS OF MITOCHONDRIAL PHOSPHOLIPIDS BY SUBCELLULAR FRACTIONS ISOLATED FROM NORMAL AND CHLORAMPHENICOL-TREATED BHK-21 CELLS

5.1 INTRODUCTION

Experiments described in the previous chapter have indicated that BHK-21 cells and in particular the mitochondria of these cells, show reduced labelling of phospholipids in culture, when the cells are grown in chloramphenicol. The reduction in labelling with $^{32}P-[P_i]$, ^{3}H-glycerol, ^{14}C-acetate, ^{14}C-linoleate or ^{14}C-palmitate is not restricted to any particular class of phospholipid, but is observed in all phospholipids of all subcellular fractions isolated from the cells. In addition, there appears to be a secondary reduction in the labelling of cardiolipin, a specific mitochondrial phospholipid, when the former two isotopes are utilized.

The inhibition appears to be at the level of *de novo* phospholipid synthesis. Since it has been shown that cellular ATP levels do not differ significantly from control values in chloramphenicol-treated cells, the site of inhibition probably lies in the biosynthesis of one or more universal phospholipid intermediates. In addition, the biosynthesis of polyglycerolphosphatides may be secondarily inhibited.

It was, therefore, decided to examine the synthesis of a number of the key intermediates of phospholipid

biosynthesis in subcellular fractions isolated from chloramphenicol-treated cells, in order to determine whether or not the inhibition resulted from alterations in enzymes involved in these pathways. The subcellular localization of the phospholipid-biosynthetic steps has been extensively reviewed (Van den Bosch *et al.*, 1972; Thompson, 1973; Van den Bosch, 1974). The following reactions were examined:

1. (a) Acyl-CoA + *sn*-Glycerol-3-Phosphate → Lysophosphatidic Acid
   (b) Acyl-CoA + Lysophosphatidic Acid → Phosphatidic Acid
2. (a) Acyl-CoA + Dihydroxyacetone Phosphate Acyldihydroxyacetone Phosphate
   (b) Acyldihydroxyacetone Phosphate + NADPH → Lysophosphatidic Acid + $NADP^+$
   (c) Acyl-CoA + Lysophosphatidic Acid → Phosphatidic Acid
3. Phosphatidic Acid + CTP → CDP-Diglyceride + PP_i
4. CDP-Diglyceride + Inositol → Phosphatidyl Inositol + CMP
5. Diglyceride + CDP-Choline → Phosphatidyl Choline + CMP
6. Glycerol + ATP → *sn*-Glycerol-3-Phosphate
7. (a) CDP-Diglyceride + *sn*-Glycerol-3-Phosphate → Phosphatidyl Glycerol Phosphate + CMP

7. (b) Phosphatidyl Glycerol Phosphate →
Phosphatidyl Glycerol + P_i

(c) Phosphatidyl Glycerol + CDP-Diglyceride →
Cardiolipin + CMP

5.2 MATERIALS AND METHODS

BHK-21 cells were cultured for 48 hours in 100 μg ml^{-1} CAP as previously described in Chapter 2. They were harvested (3-7 x 10^{-7} cells per culture), washed once with phosphate-buffered saline, once with 0.25 M sucrose-0.1 mM EDTA, suspended in 1 ml of sucrose-EDTA, and homogenized using a Polytron® cell disrupter. In experiments in which a post-microsomal supernatant fraction was needed, the homogenate was centrifuged at 50,000 x g for 150 min. to bring down nuclei, mitochondria, and microsomes at one time. This provided a concentrated supernatant, undiluted by washings from other subcellular fractions. The pellet was resuspended in sucrose-EDTA and subcellular fractionation was conducted by differential centrifugation (McMurray and Dawson, 1969). Fractions were resuspended if necessary in sucrose-EDTA and assayed for protein (Lowry et al., 1951) or lipid phosphorus (Bartlett, 1959). Sprague-Dawley rats (200-300 gm) were used as a source of liver mitochondria (McMurray and Dawson, 1969).

The acylation of sn-glycerol-3-phosphate to yield lysophosphatidic acid and phosphatidic acid was examined

using the *in vitro* assay of Monroy *et al.* (1972) for both mitochondria and microsomes. *sn*-Glycerol-3-phosphate, disodium salt [^{14}C(U)] (128 mCi mmole^{-1}) was purchased from New England Nuclear. Palmityl-CoA, MES, TES, and glycylglycine were purchased from Sigma (St. Louis, Mo.). All other chemicals, if not specifically described, were reagent grade. The reaction was stopped with an equal volume of 6N HCl and the lipids were extracted by the method of Daae and Bremer (1970) using *n*-butanol. Aliquots were removed and dried for scintillation counting in Aquasol® using a Packard Scintillation Spectrophotometer®. The remainder of the extract was taken to dryness, mixed with standard phosphatidic acid and lysophosphatidic acid (Serdary, London, Canada) and chromatographed in one-dimension on oxalate-impregnated silica gel plates (Possmayer *et al.*, 1969). Spots were identified by spraying with 1% iodine in methanol, scraped off and counted (Webb and Mettrick, 1972).

The acylation of dihydroxyacetone phosphate to yield acyldihydroxyacetone phosphate and its subsequent reduction to lysophosphatidic acid and acylation to phosphatidic acid was examined by the method of LaBelle and Hajra (1972) in both mitochondria and microsomes. Dihydroxyacetone [^{14}C(U)] (60 mCi mmole^{-1}) was purchased from I.C.N. Pharmaceuticals. Dihydroxyacetone phosphate [^{14}C(U)] was prepared using glycerol kinase (Sigma) (Possmayer, 1976). The reaction

mixture contained 0.2 mmole glycine buffer (pH 8.5), 7.5 μmoles ATP, 15 μmoles $MgCl_2$, 50 μCi dihydroxyacetone [$^{14}C(U)$] and 8 units glycerol kinase in 2.5 ml total volume. After incubation for 4 hours at 23°C, the reaction was stopped with 2.5 ml 5% TCA. Following treatment with 375 mg Norit A, the solution was brought to pH 4.5 with 0.1 N KOH. An ion exchange column of Bio-Rad AG1-X2, 200-400 mesh (Calbiochem) was prepared by washing 20g of the resin with 2N NaOH, followed by water, 5N formic acid and water. After loading the sample, the column was washed with water and the dihydroxyacetone phosphate eluted with a linear gradient of 0-5N formic acid. The peak was identified by counting aliquots from each fraction. The tubes containing labelled DHAP were pooled and lyophilized and the sample solubilized with water and brought to pH 4.5 with 0.1N KOH. It is stable at this pH if kept frozen. The purity of the DHAP could be checked by paper chromatography in butanone: glacial acetic acid:borate saturated water 36:4:4 (v/v/v). Carrier DHAP was prepared from the cyclohexylamine salt, dimethyl ketal (Sigma) as described in the literature accompanying the reagent.

The acylation reaction was stopped using 2.1 ml chloroform-methanol 1:2 (v/v) and the lipid extracted (Hajra, 1974). Aliquots were removed for counting and the remainder chromatographed as above with carrier phosphatidic acid (Serdary) and palmityl dihydroxyacetone

phosphate (a generous gift from Dr. B. W. Agranoff, Ann Arbor, Michigan). Spots were scraped off and counted as above.

CDP-diglyceride formation was assayed in both mitochondria and microsomes as described by Van Golde *et al.* (1974) using dioleyl phosphatidic acid (Serdary Research, London) and cytidine 5'-triphosphate, tetrasodium salt [5-^{3}H] (26.2 Ci mmole^{-1}) (New England Nuclear). G3634A cationic detergent (Atlas Chemical Industries, Brantford, Ontario) was added to a final concentration of 65 mg ml^{-1} to activate the enzyme (Bishop and Stickland, 1976). Samples were removed from the incubation mixture at 0, 5, 10 and 20 minutes and the reaction stopped with chloroform-methanol 1:1 (v/v). Following the addition of 0.05 μmole cold CDP-diolein (Serdary), samples were cooled to 0°C and washed with theoretical upper phase (McMurray and Dawson, 1969) containing 0.1 mM CTP and 0.1N HCl (Bishop and Stickland, 1976). Aliquots were taken to dryness and counted in Aquasol ®.

Phosphatidyl inositol biosynthesis in microsomes was assayed according to Van Golde *et al.* (1974) using CDP-diolein (Serdary) and *myo*-inositol-2-^{3}H(N) (2.84 Ci mmole^{-1}) (New England Nuclear). Samples were removed from the incubation mixture at 0, 2.5, 5, 10 and 20 minutes, extracted with chloroform-methanol 1:1 (v/v) and washed with theoretical upper phase (McMurray and Dawson, 1969)

containing 10 mM inositol (Sigma), taken to dryness and counted in Aquasol®.

The biosynthesis of phosphatidyl choline by isolated microsomes was assayed according to McMurray (1975) except that the 1,2-diolein (Serdary) concentration used was either 0.8 or 4.0 mM (Sribney and Lyman, 1973). Following incubation, samples were extracted, washed and counted (McMurray, 1975). Cytidine diphosphocholine [methyl-^{14}C] (40 mCi mmole^{-1}) was purchased from New England Nuclear.

Polyglycerolphosphatide biosynthesis was examined using a system modified from that described by Domazet et al. (1973). Labelling with mitochondria, mitochondria plus supernatant or whole homogenate was determined using CDP-diolein (Serdary) and either sn-glycerol-3-phosphate, disodium salt [^{14}C(U)] (130 mCi mmole^{-1}) (New England Nuclear) or glycerol [^{14}C(U)] (133.4 mCi mmole^{-1}) (New England Nuclear) plus ATP (Sigma). The reactions were stopped by extracting the samples with chloroform-methanol 1:1 (v/v) and washing the extracts with theoretical upper phase (McMurray and Dawson, 1969) containing 10 mM glycerol. Lipids were separated on TLC (Possmayer et al., 1969), the spots identified by spraying with 1% iodine in methanol, scraped off and counted (Webb and Mettrick, 1972).

Phosphatidyl glycerol phosphate hydrolysis was examined using the first stage of the assay system for polyglycerolphosphatide biosynthesis (Domazet et al., 1973) containing

[^{14}C]-phosphatidyl glycerol phosphate minus the exogenous CDP-diolein, sn-glycerol-3-phosphate or glycerol. [^{14}C]-Phosphatidyl glycerol phosphate was prepared using mitochondria isolated from rat liver (McMurray and Dawson, 1969) and the first stage of the polyglycerolphosphatide synthetic system (Domazet et al., 1973) to which was added p-chloromercuribenzoic acid (Sigma) to a final concentration of 2.42 mM. The phosphatase which converts phosphatidyl glycerol phosphate to phosphatidyl glycerol has been shown to be inhibited by sulfhydryl reagents (Kiyasu et al., 1963). Chloroform-methanol extracts of this reaction mixture were chromatographed (Possmayer et al., 1969) and side strips of each TLC plate sprayed with 1% iodine in methanol. Phosphatidyl glycerol phosphate was found to chromatograph slightly behind lysophosphatidic acid. This area was scraped off and the [^{14}C]-phosphatidyl glycerolphosphate (5 μCi mmole^{-1}) eluted with 50 ml chloroform-methanol-water, 2:1:0.1 (v/v/v). The phosphatase reaction was stopped with chloroform-methanol 1:1 (v/v) and the extracts washed and chromatographed (Possmayer et al., 1969) and the spot corresponding to phosphatidyl glycerol was counted (Webb and Mettrick, 1972).

5.3 RESULTS

The results of the acylation of glycerol-3-phosphate by CAP-treated mitochondria and microsomes are shown in

Table 17. In mitochondria from CAP-treated cells, there is a 40% decrease in the amount of sn-glycerol-3-phosphate acylated to lysophosphatidic acid and a 60% reduction in that converted to phosphatidic acid. This decrease is not observed in the microsomal fraction where a slight stimulation in synthesis may exist. The method of preparation of mitochondria used results in very low microsomal contamination, usually less than 5% (McMurray and Dawson, 1969).

Acylation of dihydroxyacetone phosphate has been described as the preferred biosynthetic route to phosphatidic acid in BHK-21 cells (Pollock et al., 1975b; Pollock et al., 1976). This alternate pathway was examined in order to determine whether the observed decrease in sn-glycerol-3-phosphate acylation in mitochondria or the reduced labelling with glycerol in cultured cells was related to an effect of the less preferred substrate (Table 18).. A reduction of 60% in acyldihydroxyacetone phosphate and 50% in phosphatidic acid labelling was observed in CAP-treated mitochondria. This reduction is similar to that observed for sn-glycerol-3-phosphate acylation. A slight reduction in acylation of DHAP by CAP-treated microsomes is evident, but of a much smaller magnitude than the decrease observed in mitochondria. No decrease in acyl DHAP formation by microsomes is evident. Phosphatidic acid formation from either sn-glycerol-3-phosphate or acyl DHAP proceeds at approximately equivalent

TABLE 17

ACYLATION OF sn-GLYCEROL-3-PHOSPHATE BY MITOCHONDRIA AND MICROSOMES ISOLATED FROM CAP-TREATED BHK-21 CELLS

The incubation mixture contained 30 μmoles MES-TES-glycylglycine buffer pH 7.5, 1 μmole $MgCl_2$, 10 nmoles palmityl CoA, 37.5 μmoles sucrose, 0.75 μmole sn-glycerol-3-phosphate [$^{14}C(U)$] (1.5×10^6 DPM $\mu mole^{-1}$) and either 0.1-0.2 mg mitochondrial or 0.3-0.4 mg microsomal protein in 0.5 ml final volume. Following a 5 minute incubation period at 37°C (Monroy *et al.*, 1972), the reaction was stopped with 0.5 ml 6N HCl and the lipids extracted by the method of Daae and Bremer (1970). The extracts were chromatographed as described (Possmayer *et al.*, 1969).

	Specific Activity (pmoles min^{-1} mg $protein^{-1}$)[a]			
	Lysophosphatidic Acid		Phosphatidic Acid	
Subcellular Fraction	Control	CAP-Treated	Control	CAP-Treated
mitochondria	1070	409	589	371
microsomes	847	896	684	735

[a] average of four samples

TABLE 18

ACYLATION OF DIHYDROXYACETONE PHOSPHATE BY MITOCHONDRIA AND MICROSOMES FROM CAP-TREATED BHK-21 CELLS

The incubation mixture contained 37.5 μmoles tris-HCl pH 7.4, 2 μmoles KF, 2 μmoles $MgCl_2$, 10 nmoles palmityl CoA, 12.5 μmoles sucrose, 1 μmole NADPH, 1 μmole dihydroxyacetone phosphate [$^{14}C(U)$] (3.5×10^5 DPM $\mu mole^{-1}$) and either 0.1-0.2 mg mitochondrial or 0.3-0.5 mg microsomal protein in 0.5 ml final volume. Following a 30 minute incubation at 37°C (LaBelle and Hajra, 1972), the reaction was stopped with 2.1 ml chloroform-methanol 1:2 (v/v) (Hajra, 1974) and the lipids chromatographed (Possmayer <u>et al</u>., 1969).

	Specific Activity (pmoles min^{-1} mg $protein^{-1}$)[a]			
	Acyldihydroxyacetone phosphate		Phosphatidic Acid	
Subcellular Fraction	Control	CAP-treated	Control	CAP-treated
mitochondria	79.6	30.3	429	214
microsomes	13.2	14.8	555	438

[a]average of four samples.

orders of magnitude in mitochondria or microsomes *in vitro*. This contrasts markedly with the difference in the amount of lysophosphatidic acid accumulated relative to the amount of acyl DHAP accumulated; a much greater amount of the latter is converted to phosphatidic acid. On the other hand, much more lysophosphatidic acid is present in *sn*-glycerol-3-phosphate-labelled fractions than phosphatidic acid (Table 17). No lysophosphatidic acid could be detected in DHAP-labelled fractions. Although mitochondria synthesized more acyl DHAP or lysophosphatidic acid than microsomes, the conversion to phosphatidic acid was greater in the latter fraction (Table 18).

CDP-diglyceride biosynthesis by isolated mitochondria and microsomes was examined (Table 19). No inhibition of the enzyme CTP:*sn*-3-phosphatidic acid cytidylyltransferase (EC 2.7.7.41) was observed in CAP-treated mitochondria, while a 3-fold stimulation was evident in the microsomal fraction. The latter may be due to some release of latent activity, although the addition of detergent makes this unlikely. Proliferation of microsomal membranes does not seem to occur as the level of NADPH-cytochrome c reductase (EC 3.2.3.4), a microsomal marker, does not rise in CAP-treated cells (Chapter 2). It is possible that a selective increase in CTP-*sn*-3-phosphatidic acid cytidylyltransferase occurs in an effort to offset the reduced production of phosphatidic acid, in order to maintain necessary levels of the phospholipids which are derived from the CDP-diglyceride

TABLE 19

BIOSYNTHESIS OF CDP-DIGLYCERIDE BY MITOCHONDRIA

AND MICROSOMES FROM CAP-TREATED BHK-21 CELLS

The incubation mixture consisted of 250 μg dioleylphosphatidic acid, 30 μmoles tris-HCl, pH 7.4, 0.37 μmole CTP [5-^{3}H] (15.1 x 10^6 DPM $\mu mole^{-1}$), 22.5 μg Atlas G-3634A, 12 μmoles $MgCl_2$, 12.5 μmoles sucrose, and 0.15 mg mitochondrial or 0.4 mg microsomal protein in 0.35 ml final volume (Van Golde *et al.*, 1974). Samples were removed at 0, 5, 10 and 20 minutes, and the lipids were extracted as described in Methods.

Subcellular Fraction	Specific Activity (pmoles min^{-1} mg $protein^{-1}$)[a] Control	CAP-Treated
mitochondria	107	112
microsomes	232	666

[a] average of two experiments

pathway.

The biosynthesis of two phospholipids known to be synthesized extramitochondrially was examined. Phosphatidyl inositol biosynthesis is reported in Table 20. A stimulation in phosphatidyl inositol biosynthesis was observed in microsomes from CAP-treated cells. Although not of the same magnitude as the increase in CDP-diglyceride synthesis, the trend is the same and does not account for the decrease in labelling of phosphatidyl inositol that was observed in cultured cells labelled with glycerol, P_i or fatty acid (Chapter 4).

CDP-choline:1,2 diglyceride choline phosphotransferase (EC 2.7.8.2), a second microsomal enzyme involved in phospholipid synthesis, was assayed in isolated microsomes in the presence of exogenous diolein (Fig. 19). Although no difference is observed at the lower diolein concentration, the activity of the enzyme is stimulated in microsomes from CAP-treated cells at the higher concentration, indicative of the trend observed for CDP-diglyceride and phosphatidyl inositol biosynthesis. Enzyme activity is not linearly related to protein concentration, a result that has been noted previously (McMurray, 1975).

Polyglycerolphosphatide biosynthesis from sn-glycerol-3-phosphate was examined in isolated mitochondria. The first stage of the incubation measures the incorporation into PGP and PG; addition of divalent cations (Mg^{++}) in the second stage promotes the conversion of PG to DPG

TABLE 20

PHOSPHATIDYL INOSITOL BIOSYNTHESIS IN

MICROSOMES FROM CAP-TREATED BHK-21 CELLS

The incubation mixture consisted of 100 μmoles tris-HCl pH 8.4, 0.2 μmole CDP-diolein, 2 μmoles *myo*-[2-^{3}H] inositol (11.0 x 10^6 DPM $\mu mole^{-1}$), 2 μmoles $MnCl_2$, 50 μmoles sucrose and 2.5 mg microsomal protein (Van Golde *et al.*, 1974) in 1 ml final volume. Samples (200 μl) were removed at 0, 2.5, 5, 10 and 20 minutes, extracted with 3.2 ml chloroform-methanol 1:1 (v/v) and washed as described in the Methods.

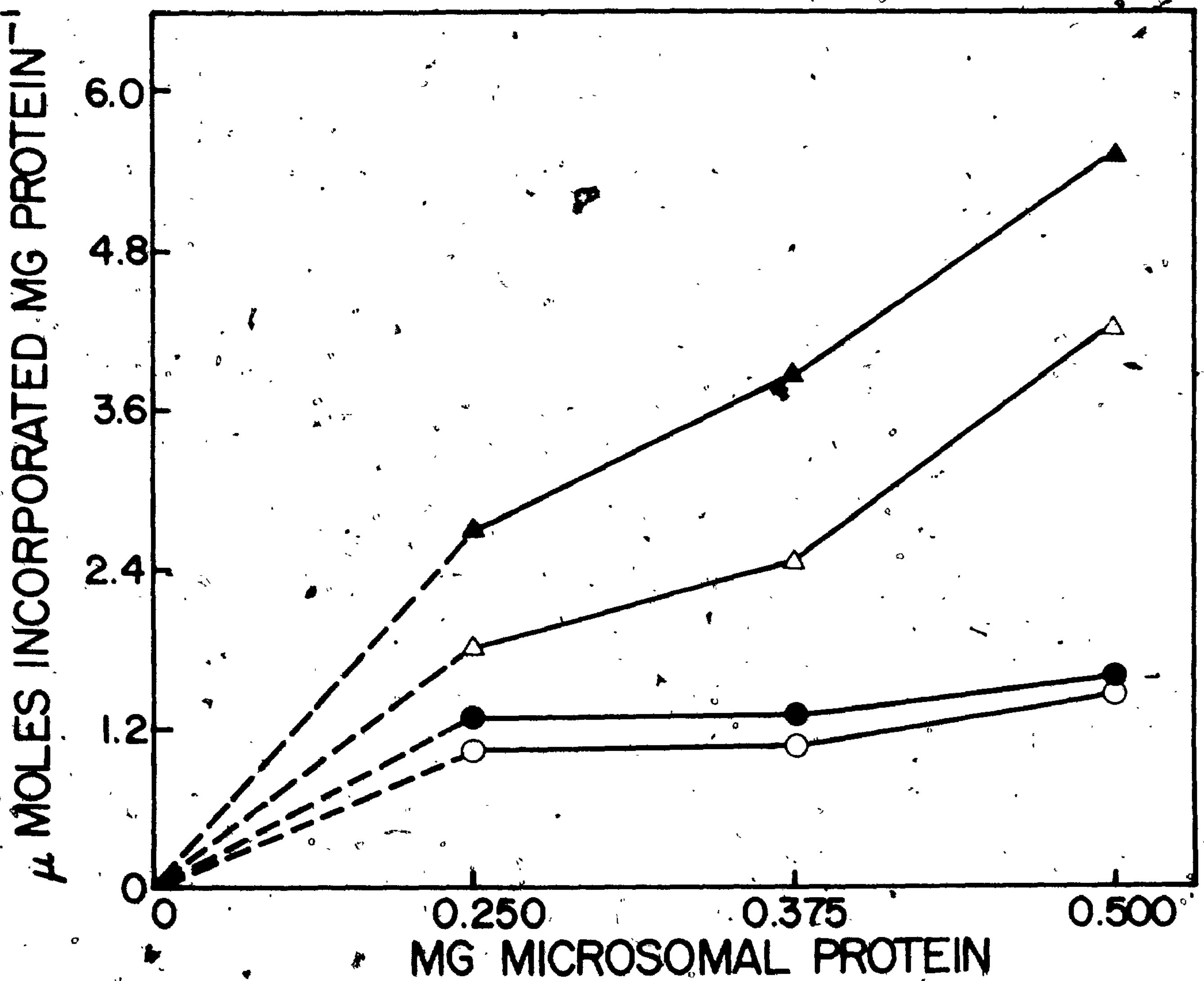
	Specific Activity (pmoles min^{-1} mg $protein^{-1}$)[a]	
	Control	CAP-treated
microsomes	5.82	7.96

[a] average of duplicate experiments

FIGURE 19

PHOSPHATIDYL CHOLINE BIOSYNTHESIS IN CAP-TREATED BHK-21 CELLS

The incubation mixture consisted of 18.75 µmoles tris-HCl, pH 7.4, 10 µmoles $MgCl_2$, 5 µmoles cysteine-HCl pH 7.4 (fresh), 0.2 µmole CDP-choline [methyl-^{14}C] (3.33×10^5 DPM $\mu mole^{-1}$), 50 µmoles sucrose, 20 nmoles EDTA, 0.5 mg Tween 20, either 0.4 µmole or 2 µmoles, 1,2 diolein and 0.25-0.50 mg microsomal protein in 0.5 ml final volume (McMurray, 1975). Following a 30 minute incubation at 37°C, the samples were extracted and washed as described in the methods section. 0.4 µmole 1,2 diolein: o--o, control, ●--●, CAP-treated; 2 µmoles 1,2 diolein: Δ--Δ, control, ▲--▲, CAP-treated.


µ MOLES INCORPORATED MG PROTEIN⁻¹
6.0
4.8
3.6
2.4
1.2
0
0
0.250
0.375
0.500
MG MICROSOMAL PROTEIN

(Domazet *et al.*, 1973). The incorporation of *sn*-glycerol-3-phosphate (Table 21) into CAP-treated mitochondria under these conditions with added CDP-diglyceride was not reduced when compared to controls. Addition of ATP to the second stage of the incubation (Domazet *et al.*, 1973) had no effect on incorporation or distribution of the label and was omitted from further experiments. Unlike the results for mitochondria isolated from rat liver or guinea pig liver (Davidson and Stanacev, 1974), the conversion of PGP to PG seems to be rate limiting in BHK-21 mitochondria. This step is slightly stimulated in mitochondria from CAP-treated cells (i.e. there is less accumulation of radioactivity in PGP and more in PG and DPG). The experiments were repeated to determine whether the presence of CAP in either stage of the incubation *in vitro* would affect the reaction (Table 22). If CAP was present during the first stage of the incubation, no change or a slight decrease in total incorporation and conversion of PGP to PG was observed in mitochondria from both control and CAP-treated cells. During later experiments using rat liver mitochondria or BHK-21 cell mitochondria and 5 mM $CoCl_2$ instead of 100 mM $MgCl_2$ (Hostetler *et al.*, 1975), no effect of CAP *in vitro* could be observed on the conversion of PG to DPG (McMurray, 1976). Thus the effect of CAP on DPG formation in cells in culture is probably not related to direct inhibition of enzymes of the polyglycerolphosphatide pathway in mitochondria by CAP itself.

TABLE 21

POLYGLYCEROLPHOSPHATE BIOSYNTHESIS IN ISOLATED MITOCHONDRIA FROM CAP-TREATED BHK-21 CELLS

The incubation mixture consisted of 50 μmoles tris-HCl pH 7.4, 5 μmoles, 2-mercaptoethanol (fresh), 0.5 μmole CDP-diolein, 0.1 μmole sn-glycerol-3-phosphate [$^{14}C(U)$] (11.1×10^6 DPM $\mu mole^{-1}$), 25 μmoles sucrose, 10 nmoles EDTA and 0.2 mg mitochondrial protein in 0.35 ml final volume (Domazet *et al.*, 1973). After 1 hour incubation at 37°C, 50 μl of 50 mM ATP or 50 mM Na_2HPO_4 pH 7.4 and 100 μl 1M $MgCl_2$ were added and the incubation continued for an additional 2 hours. The samples were then extracted with chloroform-methanol 1:1 (v/v), washed and counted as described in the Materials.

	Percent of Label Incorporated[a]			
	Control		CAP-Treated	
Phospholipid	+ATP	-ATP	+ ATP	-ATP
DPG	0.9	1.2	6.7	8.2
PG	2.1	1.3	8.5	8.2
PGP	95.5	94.6	80.2	78.3
Total Incorporation[b]	5.05	5.90	6.10	6.40

[a] average of four samples

[b] nmoles mg $protein^{-1}$

TABLE 22

EFFECT OF D-CHLORAMPHENICOL ON THE BIOSYNTHESIS
OF POLYGLYCEROLPHOSPHATIDES IN BHK-21 MITOCHONDRIA
FROM CONTROL OR CAP-TREATED CELLS

The assay conditions were identical to those described in Table 21 except that the ATP was omitted in the second stage of the incubation. 100 μg CAP was added to the incubation during the first or second stage, to a final volume of 0.45 ml. Extraction of lipid was done as in Table 21.

	Percent of Label Incorporated[a]					
	Control			CAP-Treated		
Phospholipid	no CAP	CAP Stage 1	CAP Stage 2	no CAP	CAP Stage 1	CAP Stage 2
DPG	1.1	1.0	1.1	2.4	2.0	2.4
PG	2.2	2.0	2.3	3.2	2.7	3.3
PGP	95.4	95.9	95.3	92.4	93.9	92.6
Total Incorporation[b]	6.56	6.18	6.42	7.82	6.10	6.90

[a] average of four samples
[b] nmoles mg $protein^{-1}$

The effects of detergents on the incorporation of sn-glycerol-3-phosphate by isolated mitochondria were examined (Table 23), as the elevated incorporation observed in CAP-treated mitochondria might have been due to a release of latent activity resulting from membrane alteration. Treatment of mitochondria with either Triton X-100 or deoxycholate did not increase incorporation of sn-glycerol-3-phosphate and, in fact, slightly inhibited incorporation by mitochondria from control cells. Addition of potassium oleate stimulated the total incorporation of label into CAP-treated mitochondria and stimulated by three- to four-fold, the conversion of PGP to PG and then to DPG, in both control and CAP-treated mitochondria. The mechanism of this stimulation is not known and was not simply due to acylation of sn-glycerol-3-phosphate to produce lysophosphatidic and phosphatidic acid. In no case was the conversion of PGP to PG in mitochondria from control cells elevated to that of mitochondria from CAP-treated cells. Furthermore, when labelled PGP was added to mitochondria from CAP-treated or control cells (Table 24) no difference could be observed between the phosphatase activity of the treated or untreated cells. It was not possible to account for the increased hydrolysis of PGP in mitochondria from CAP-treated cells when the coupled assay described in Table 21 was used.

It is possible that components localized in some other subcellular fraction are involved in polyglycerol-phosphatide biosynthesis in the cells in culture, although

TABLE 23

DETERGENT EFFECTS ON sn-GLYCEROL-3-PHOSPHATE INCORPORATION

IN MITOCHONDRIA FROM CAP-TREATED BHK-21 CELLS

The incubation mixture was the same as described in Table 22 except that CAP was omitted. Detergent (40 μg) was added to the samples. Following 1 hour incubation at 37°C, 5 μmoles $CoCl_2$ was added to a final volume of 0.4 ml and the incubation continued for 2 hours. Lipids were extracted as in Table 21.

	Percent of Label Incorporated[a]							
	Mitochondria		+ Triton X-100		+ Deoxycholate		+ K Oleate	
Phospholipid	-CAP	+ CAP	-CAP	+CAP	-CAP	+CAP	-CAP	+CAP
DPG	1.9	3.4	1.8	2.1	1.8	2.2	4.2	8.4
PG	5.8	10.4	5.9	10.1	5.9	11.2	14.4	27.6
P[illegible]	90.8	82.6	92.0	83.1	91.6	84.0	79.7	60.7
Total Incorporation[b]	4.48	4.58	3.16	4.61	3.11	4.19	3.95	6.27

[a] average of two samples

[b] nmoles mg protein^{-1}

TABLE 24

EFFECT OF DETERGENT ON THE HYDROLYSIS OF PHOSPHATIDYL GLYCEROL PHOSPHATES BY MITOCHONDRIA ISOLATED FROM CAP-TREATED BHK-21 CELLS

The incubation mixture consisted of 50 µmoles tris-HCl, pH 7.4, 5 µmoles 2-mercaptoethanol, 25 µmoles sucrose, 10 nmoles EDTA, and 8.5 nmoles [^{14}C]-phosphatidyl glycerol phosphate (11.1 x 10^6 DPM $\mu mole^{-1}$) sonicated 3 x 1 min. Detergent was added to various tubes (0.5 mg) and 0.1 mg mitochondrial protein to 0.50 ml final volume. Control cultures were either left or sonicated in a Branson Sonifier with bath at maximum intensity. Samples were incubated 1 hour at 37°C and extracted as in Table 21.

	Percent of Label Incorporated[a]							
	Mitochondria		Sonicated Mitochondria		Mitochondria +Deoxycholate		Mitochondria +Triton X-100	
Phospholipid	-CAP	+CAP	-CAP	+CAP	-CAP	+CAP	-CAP	+CAP
PG	7.3	8.2	6.8	6.1	5.8	7.3	7.0	8.3
PGP	90.1	88.4	91.4	90.3	91.3	90.0	89.8	90.1

[a] average of two samples

this is not the case with other systems (Kiyasu *et al*., 1963; Davidson and Stanacev, 1974; Hostetler and Van den Bosch, 1972). This pathway was reexamined using the same assay system, except that whole homogenates were substituted for mitochondria (Table 25). Several points are evident. First, the greater effectiveness of Co^{2+} than Mg^{2+} in the conversion of PG to DPG (Hostetler *et al*., 1975) is confirmed. Secondly, the total incorporation into polyglycerolphosphatides is stimulated over that observed with isolated mitochondria (cf. Table 21). Thirdly, the conversion of PGP to PG is greatly enhanced. Thus the pathway from CDP-diglyceride to cardiolipin is not entirely mitochondrial in BHK-21 cells.

The possibility that a soluble factor was involved in the hydrolysis of PGP to PG was investigated. A system was reconstituted with mitochondria plus the post-microsomal supernatant fraction from control or CAP-treated cells or mixture of both (Table 26). As was the case with the homogenate, the bulk of the PGP was converted to PG and then to DPG. In addition, the amount of *sn*-glycerol-3-phosphate incorporation is elevated two- to three-fold. The supernatant has a dual effect. First, it enhances the amount of label incorporated, possibly through the removal of an inhibitory end-product, PGP, and, secondly, it activates or contains the phosphatase that converts PGP to PG. It is also evident that the source of the supernatant, either from control or CAP-treated cells, has no effect on

TABLE 25

POLYGLYCEROLPHOSPHATIDE BIOSYNTHESIS IN HOMOGENATES

OF CAP-TREATED BHK-21 CELLS

The assay system was identical to that described in Table 22. Homogenate, 1.4-1.6 mg, was added to each tube. Following 1 hour incubation at 37°C, 2.5 μmoles $CoCl_2$ or 100 μmoles $MgCl_2$ was added to a final volume of 0.4 ml and further incubated for 2 hours. Lipids were extracted as in Table 21.

Phospholipid	Percent of Label[a]			
	Control		CAP-Treated	
	$+Co^{2+}$	$+Mg^{2+}$	$+Co^{2+}$	Mg^{2+}
DPG	15.2	8.6	27.8	11.5
PG	65.7	60.8	56.9	60.7
PGP	15.1	28.7	11.5	25.0
Total Incorporation[b]	11.7	9.32	11.0	8.80

[a] average of four samples

[b] nmoles mg $protein^{-1}$

TABLE 26

EFFECT OF SUPERNATANT ON MITOCHONDRIAL SYNTHESIS OF POLYGLYCEROLPHOSPHATIDES

The incubation mixture was as described in Table 22. To each sample was added 0.15 mg mitochondrial protein and 0.5 mg post-microsomal supernatant protein and incubated for 1 hour at 37°C. $CoCl_2$ (5 μmoles) was added and the incubation continued for 2 hours. Lipids were extracted as in Table 21.

	Percent of Label[a]			
	Control Mitochondria		CAP-Treated Mitochondria	
Phospholipid	+Control Supernatant	+CAP Supernatant	+Control Supernatant	+ CAP Supernatant
DPG	12.2	13.1	19.7	19.9
PG	55.2	56.8	46.7	46.9
PGP	31.3	28.9	30.9	31.9
Total Incorporation[b]	16.8	18.1	31.7	31.5

[a] average of four samples

[b] nmoles mg $protein^{-1}$

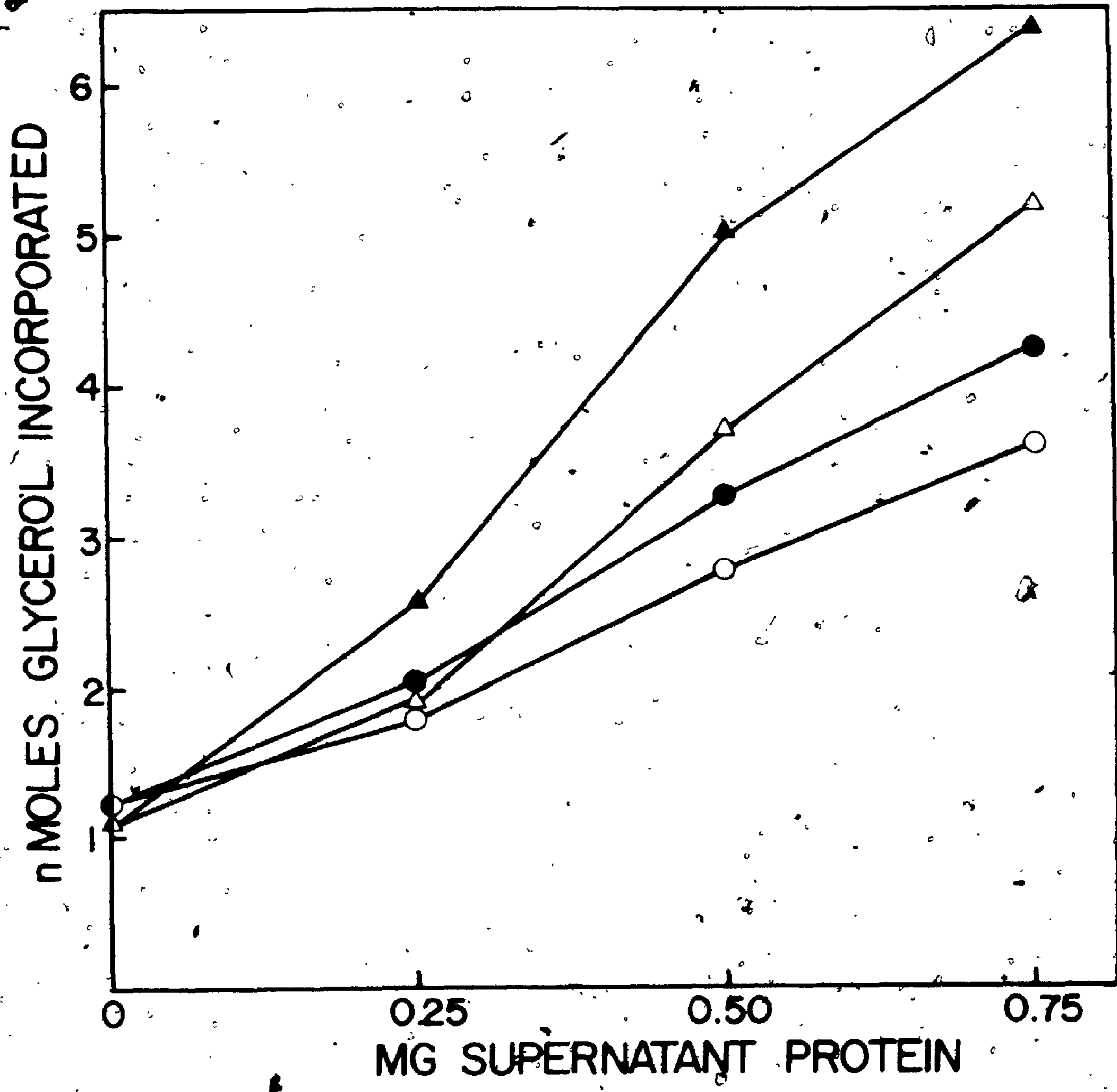
the incorporation. It is apparent that the synthesis of cardiolipin from sn-glycerol-3-phosphate and CDP-diglyceride is not impaired in CAP-treated cells.

Since sn-glycerol-3-phosphate is an important precursor for cell phospholipids, either through its incorporation into PA and subsequently CDP-diglyceride, or directly into polyglycerolphosphatides, the synthesis of sn-glycerol-3-phosphate was examined using a coupled assay system. Glycerol and ATP were added to an *in vitro* system reconstituted from BHK-21 mitochondrial and post-microsomal supernatant which contained the polyglycerolphosphatide precursors described earlier except for sn-glycerol-3-phosphate. The results are shown in Figure 20. In the presence of supernatant from CAP-treated cells, mitochondria from either control or CAP-treated cells showed a greater ability to incorporate glycerol than if the supernatant was derived from control cells. Mitochondria from control cells were able to incorporate 50% more glycerol into lipid in the presence of either supernatant. Supernatant was essential for maximal incorporation and no differences between control and CAP-treated mitochondria were observed in its absence. When the distribution of label among the polyglycerolphosphatides was examined as a function of the amount of supernatant (Fig. 21), mitochondria from CAP-treated cells showed the typically enhanced ability to convert PG to DPG that was observed in *in vitro* experiments utilizing sn-glycerol-3-phosphate as precursor.

FIGURE 20

INCORPORATION OF GLYCEROL INTO POLYGLYCEROLPHOSPHATIDES IN MITOCHONDRIA FROM CAP-TREATED BHK-21 CELLS IN THE PRESENCE OF POST-MICROSOMAL SUPERNATANT

The incubation mixture contained 50 µmoles tris-HCl, pH 7.4, 5 µmoles 2-mercaptoethanol (fresh), 0.5 µmole CDP-diolein, 0.3 µmole glycerol [^{14}C(U)] (11.1 x 10^6 DPM $\mu mole^{-1}$), 50 moles sucrose, 20 nmoles EDTA, 2 µmoles ATP, 0.1 mg mitochondrial protein and 0.25-0.75 mg supernatant protein. Following 1 hour incubation, 5 µmoles $CoCl_2$ was added to a final volume of 0.5 ml and the incubation was continued for a further 2 hour period. Lipids were extracted as in Table 21. Δ--Δ, mitochondria and supernatant from control cells; ▲--▲, mitochondria from control cells, supernatant from CAP-treated cells; o--o, mitochondria from CAP-treated cells, supernatant from control cells; ●--●, mitochondria and supernatant from CAP-treated cells.

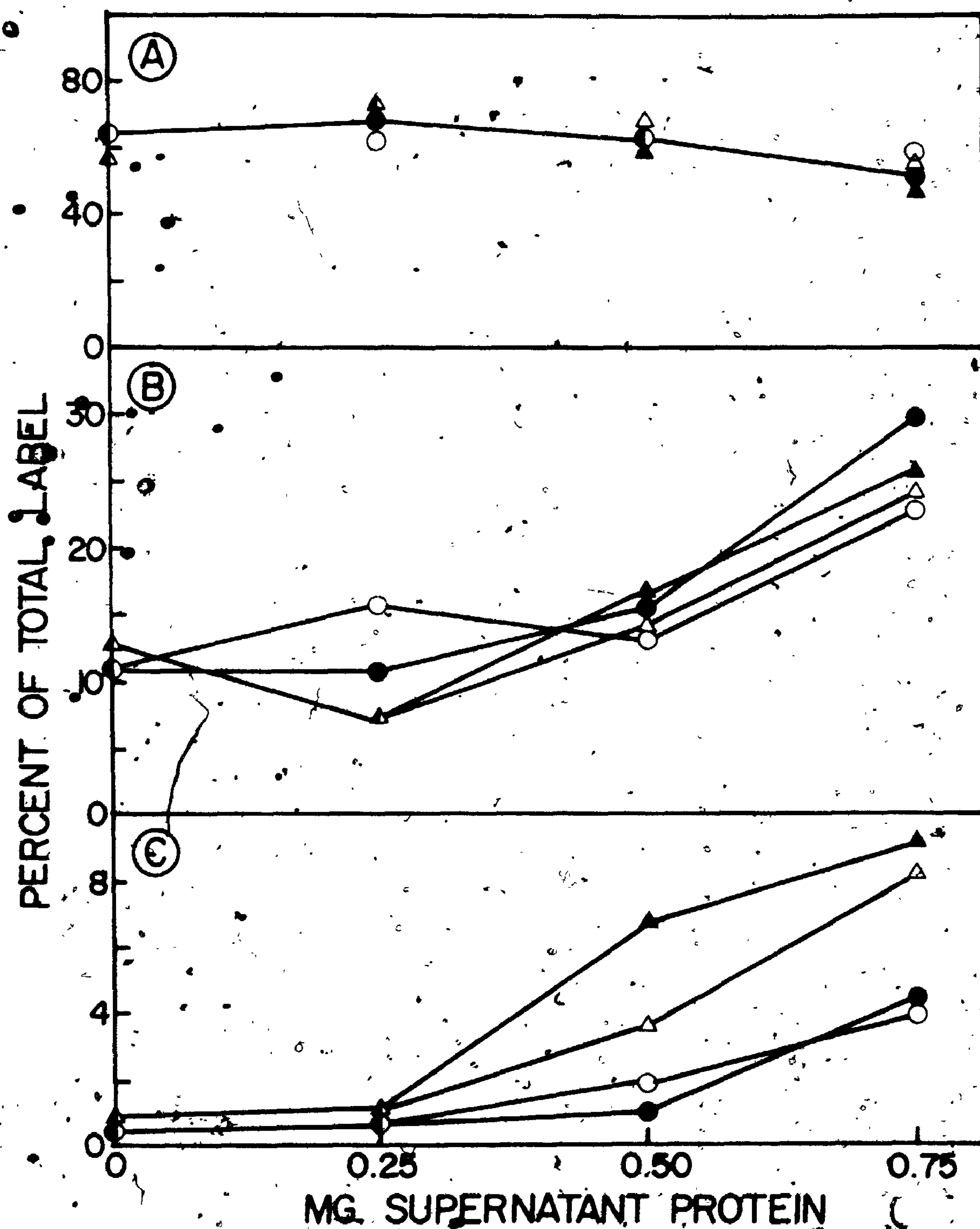


n MOLES GLYCEROL INCORPORATED
1
2
3
4
5
6
0
0.25
0.50
0.75
MG SUPERNATANT PROTEIN

FIGURE 21

DISTRIBUTION OF GLYCEROL [^{14}C(U)] AMONG THE POLYGLYCEROLPHOSPHATIDES IN BHK-21 MITOCHONDRIA

The lipid extract from Figure 20 was chromatographed (Possmayer et al., 1975) and the spots detected with 1% iodine and counted (Webb and Mettrick, 1972). A - PGP; B - PG; C - DPG; o--o, control mitochondria and supernatant; ●--●, control mitochondria and CAP-treated supernatant; Δ--Δ, CAP-treated mitochondria and control supernatant; ▲--▲, CAP-treated mitochondria and supernatant.


A
B
C
80
40
0
30
20
10
0
8
4
0
PERCENT OF TOTAL LABEL
0
0.25
0.50
0.75
MG. SUPERNATANT PROTEIN

5.4 DISCUSSION

Studies with mitochondria isolated from rat liver show that they possess limited autonomy with respect to the synthesis of their own lipid components: the *de novo* generation of the majority of phosphoglycerides by the cytidine pathway is exclusively extramitochondrial (McMurray and Dawson, 1969). The biosynthesis of phosphatidyl choline from CDP-choline in isolated rat liver or BHK-21 mitochondria is strictly a function of the degree of contamination by endoplasmic reticulum (McMurray, 1974; McMurray, 1975). Similar conclusions can be drawn for the biosynthesis of phosphatidyl inositol (McMurray and Dawson, 1969; Van Golde *et al.*, 1974; Williamson and Morre, 1976) and phosphatidyl ethanolamine (McMurray and Dawson, 1969; Williams and Bygrave, 1970) from $[^{32}P]-P_i$. Mitochondria from rat liver show no detectable synthesis of phosphatidyl inositol, sphingomyelin or phosphatidyl serine (Van Golde *et al.*, 1974). These results differ markedly from those obtained with isolated yeast mitochondria (Ostrow, 1971; Mangnall and Getz, 1971; Cobon *et al.*, 1974) which were able to synthesize phosphatidyl serine, phosphatidyl ethanolamine, phosphatidyl inositol, and some phosphatidic acid and neutral lipid as well as phosphatidyl glycerol, cardiolipin. In mammalian cells, a mechanism exists for the transfer of these phospholipids from their site of synthesis in the endoplasmic reticulum to the newly formed membranes of mitochondria Wirtz and Zilyersmit, 1970; Butler and Thompson, 1975; Stewart-Dehaan and McMurray, 1976). This transfer parallels

the labelling kinetics of mitochondrial soluble proteins (Beattie, 1969). The intracellular exchange of phospholipids has been summarized in a number of reviews (Dawson et al., 1972; Dawson, 1973; McMurray, 1973; Wirtz, 1974). Phosphatidyl serine that has been transferred to the mitochondria (Butler and Thompson, 1975) is immediately decarboxylated to yield mitochondrial phosphatidyl ethanolamine (Dennis and Kennedy, 1972; Van Golde et al., 1974).

Mitochondria have been shown to possess the machinery for the synthesis of phosphatidic acid (Shephard and Hubscher, 1969; McMurray and Dawson, 1969; Monroy et al., 1972) or at least lysophosphatidic acid (Daae, 1972; Davidson and Stanacev, 1974) from sn-glycerol-3-phosphate. The alternate pathway involving the phosphorylation of diglyceride by diglyceride kinase is not functional in isolated mitochondria (McMurray, 1975). A third pathway involving the acylation of dihydroxyacetone phosphate is very active in cultured cells (Pollock et al., 1975a); BHK-21 cells have been shown to utilize this pathway preferentially (Pollock et al., 1975b; Pollock et al., 1976). However, in the in vitro experiments reported here where variability of flux into precursor pools was eliminated, no such preference was observed. The enzymes in this pathway have been demonstrated in both mitochondria and microsomes (Labelle and Hajra, 1972). Once produced

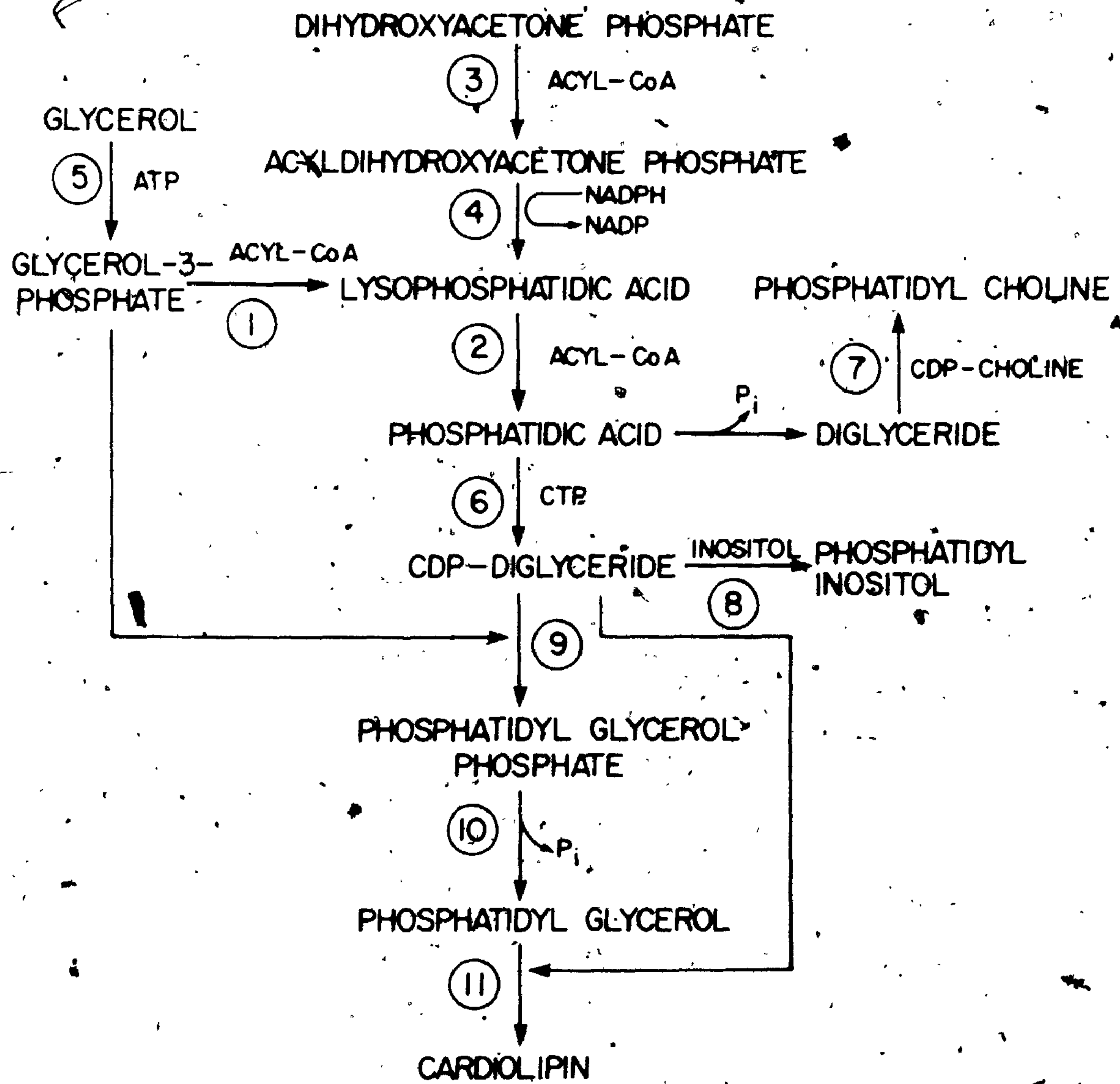
phosphatidic acid can be utilized by both mitochondria and microsomes in the synthesis of CDP-diglyceride (Vorbeck and Martin, 1970; Van Golde et al., 1974; Bishop and Strickland, 1976). Mitochondrial CDP-diglyceride can be utilized in the biosynthesis of polyglycerolphosphatides in situ (Davidson and Stanacev, 1971; Hostetler et al., 1971; Stanacev et al., 1972).

The biosynthetic pathways of a number of the phospholipids and precursors of BHK-21 mitochondria have been examined in the presence of chloramphenicol (Fig. 22). Decreased synthesis of both lysophosphatidic acid and phosphatidic acid was observed in both pathways (Reactions ① - ② and ③, ④, ②) in isolated mitochondria and, to a lesser extent, in microsomes. Since phosphatidic acid represents the central intermediate in the biosynthesis of virtually all the cellular phospholipids, any defect in its synthesis should be reflected in reduced synthesis of all cellular phospholipids. The observed inhibition of phosphatidic acid synthesis in vitro by CAP-pretreatment of BHK cells would account for the generalized decrease in the synthesis of cellular phospholipids by cultured cells, as measured using fatty acid, glycerol or P_i as the radioactive precursors (Chapter 4). In addition, the reduced ability of mitochondria from CAP-treated cells to utilize glycerol in the presence of post-microsomal supernatant as a precursor for polyglycerolphosphatides (Reaction ⑤) and probably other phospholipids would also contribute to the

FIGURE 22

BIOSYNTHETIC PATHWAYS FOR

MITOCHONDRIAL PHOSPHOLIPIDS



DIHYDROXYACETONE PHOSPHATE
3
ACYL-CoA
GLYCEROL
5
ATP
ACYLDIHYDROXYACETONE PHOSPHATE
4
NADPH
NADP
GLYCEROL-3-PHOSPHATE
ACYL-CoA
1
LYSOPHOSPHATIDIC ACID
PHOSPHATIDYL CHOLINE
2
ACYL-CoA
7
CDP-CHOLINE
PHOSPHATIDIC ACID
P_i
DIGLYCERIDE
6
CTP
CDP-DIGLYCERIDE
INOSITOL
PHOSPHATIDYL INOSITOL
8
9
PHOSPHATIDYL GLYCEROL PHOSPHATE
10
P_i
PHOSPHATIDYL GLYCEROL
11
CARDIOLIPIN

observed effects of chloramphenicol in culture. The cellular concentrations of ATP are not reduced in CAP-treated cells and hence the energy state of the cell should not be a factor.

Several enzyme activities were shown to be elevated in subcellular fractions from CAP-treated cells. These included CTP-phosphatidate cytidylyltransferase (Reaction (6)) of both mitochondria and microsomes, phosphatidyl inositol biosynthesis (Reaction (8)) and choline phosphotransferase (Reaction (7)) of microsomes, as well as the three steps in polyglycerolphosphatide biosynthesis in mitochondria (Reactions (9), (10) and (11)). It is possible that in compensatory response to reduced levels of precursor, phosphatidate, these enzymes are increased in concentration in the attempt to maintain the necessary content of cellular membrane components.

The enzymes that were demonstrated to have reduced activity *in vitro*, namely glycerol kinase, *sn*-glycerol-3-phosphate acyl transferase, dihydroxyacetone phosphate acyl transferase and possibly lysophosphatidate acyl transferase are all entirely or partially located in the mitochondria (Monroy *et al.*, 1972; LaBelle and Hajra, 1972; Jenkins and Hajra, 1976). There is no evidence for the translation of any components of these enzymes on mitoribosomes. Since the primary effect of CAP is the inhibition of mitochondrial protein synthesis, it is possible that CAP may be inhibiting the translation of one or more components or activators of

lipid biosynthetic enzymes. A seond possibility is that a mitochondrial gene product is involved in activation of the nuclear genome that is responsible for coding these enzymes. Thirdly, it is possible that these enzymes are turning over rapidly in mitochondria that have stopped dividing and thus have reduced needs for phospholipid biosynthesis.

The activation of the mitochondrial glycerol incorporation by post-microsomal supernatant from CAP-treated cells may either reflect a release of glycerol kinase from the mitochondria due to membrane breakdown or turnover, or activation of the mitochondrial enzyme by a soluble factor which is elevated in CAP-treated cells. Without direct analysis of the levels of the enzyme, it is impossible to distinguish between these possibilities. The ability of supernatant from CAP-treated cells to activate the incorporation of glycerol to a greater extent than that of control cell supernatant, may simply be due to enhanced conversion of PGP to PG and removal of product inhibition of the second enzyme in the coupled assay.

During culture in chloramphenicol-containing media, cardiolipin labelling was reduced to greater extent than for the other cellular or mitochondrial phospholipids. No reduction in the biosynthesis of any polyglcerolphosphatides (Reactions (4), (10) and (11)) was detected in mitocondria from CAP-treated cells. Two possibilities for this difference may be considered. First, the *in vitro*

assay utilized sn-glycerol-3-phosphate and CDP-diglyceride that were added exogenously, while the effect on cardiolipin was observed when cells were labelled with glycerol or P_i. Thus, the cells in culture must synthesize sn-glycerol-3-phosphate (or dihydroxyacetone phosphate) and CDP-diglyceride before attaining the immediate precursors added *in vitro*. Since both sn-glycerol-3-phosphate and CDP-diglyceride (due to reduced phosphatidate) levels are probably reduced by CAP, precursors of the *in vitro* polyglycerolphosphatide assay that enter the reaction series in at least four enzymatic steps (Reactions (6), (9), (10) and (11)) would be present in lower amounts. No other phospholipid synthetic reaction uses as many of these precursors and hence the effect may simply be cumulative. The second possibility which could not be assessed from these experiments is that the assembly of cardiolipin into mitochondrial inner membranes is dependent on its association with polypeptides that are translated on mitoribosomes and hence would not be synthesized in the presence of CAP.

Finally, the dependence upon the supernatant fraction for phosphatidylglycerol phosphate phosphatase activity is in contrast to the results found with other systems (Kiyasu *et al.*, 1963; Davidson and Stanacev, 1970; Hostetler and Van den Bosch, 1972; Stanacev *et al.*, 1973; Davidson and Stanacev, 1974). This finding poses a number of questions concerning the reason for the difference with other cell

types in which mitochondrial PGP phosphatase is autonomous, and whether this difference is inherent in BHK-21 cells or in all cultured cells. It is possible that this enzyme is involved in the regulation of mitochondrial development and function, in relation to the cell cycle and/or cell density.

CHAPTER 6. PHOSPHATIDYL GLYCEROL PHOSPHATE PHOSPHATASE IN BHK-21 CELLS: A COMPARISON WITH RAT LIVER MITOCHONDRIA

6.1 INTRODUCTION

Mitochondria have been shown to possess the capacity to catalyze the synthesis of several phospholipids, such as phosphatidic acid, CDP-diglyceride, phosphatidyl glycerol and cardiolipin (for review, see Van den Bosch, 1974). The biosynthesis of the polyglycerolphosphatides, i.e. phosphatidyl glycerol and cardiolipin, from _sn_-glycerol-3-phosphate and CDP-diglyceride is a process intrinsic to the inner membrane of mitochondria (Kiyasu _et al._, 1963; Davidson and Stanacev, 1970; Hostetler and Van den Bosch, 1972; Stanacev _et al._, 1973; Davidson and Stanacev, 1974). This pathway involves the conversion of an obligatory intermediate, phosphatidyl glycerol phosphate, to phosphatidyl glycerol, a process that has been demonstrated in mitochondria from a number of tissues, such as chicken liver (Kiyasu _et al._, 1963), sheep brain (Davidson and Stanacev, 1970), rat liver (Hostetler _et al._, 1971; Hostetler and Van den Bosch, 1972; Stanacev _et al._, 1973; Domazet _et al._, 1973), guinea pig heart (Domazet _et al._, 1973), rat brain (Possmayer _et al._, 1968), rat heart (Stanacev _et al._, 1969), and guinea pig liver (Davidson and Stanacev, 1971b). Phosphatidyl glycerol is subsequently converted to

cardiolipin (Hostetler *et al.*, 1971; Davidson and Stanacev, 1971a; Davidson and Stanacev, 1971b; Hostetler and Van den Bosch, 1972; Hostetler *et al.*, 1972; Domazet *et al.*, 1973; Hostetler *et al.*, 1975).

While investigating the biosynthesis of polyglycerol-phosphatides in mitochondria isolated from chloramphenicol-treated BHK-21 cells, it was observed that the dephosphorylation of phosphatidyl glycerol phosphate was rate limiting and that the activity of the phosphatase was very low. Partial characterization of the phosphatase and its probably soluble nature are described in this Chapter.

6.2 MATERIALS AND METHODS

Spinner adapted BHK-21 cells were maintained in culture in Alpha-MEM (Flow), containing 5% fetal bovine serum (Flow). Following centrifugation of exponentially growing cells (5-10 x 10^8 cells), they were washed and homogenized in 1 ml 0.25M sucrose-0.1mM EDTA using a Polytron® (McMurray, 1975). Male Sprague-Dawley rats (100-250g) were used in the liver experiments. Liver homogenates were prepared in 0.25M sucrose-0.1mM EDTA, and subcellular fractions were isolated as described previously (McMurray and Dawson, 1969). Subcellular fractions from BHK-21 cells were similarly prepared except that the supernatant fraction was prepared on the first centrifugation in order to maintain its concentration. The pellet was resuspended and the remaining subcellular

fractions isolated as before (McMurray and Dawson, 1969).

The assay of polyglycerolphosphatide synthesis and/or phosphatidyl glycerol phosphate hydrolysis was based on the *in vitro* system described by Stanacev *et al*. (1972) and Domazet *et al*. (1973), using *sn*-glycerol-3-phosphate [^{14}C(U)] (130.5 mCi mmole^{-1}) (New England Nuclear) and CDP-diolein (Serdary Research, London, Canada). Modifications of this assay are described in the legends of each figure or table. Following incubation, the samples were extracted with 8 volumes chloroform-methanol 1:1 (v/v) and washed with theoretical upper phase as described by McMurray and Dawson (1969). Lipids were chromatographed (Possmayer *et al*., 1969), localized by spraying with 1% iodine in methanol, scraped off and counted (Webb and Mettrick, 1972). Protein was determined by the method of Lowry *et al*. (1951).

6.3 RESULTS

Table 27 indicates the relative distribution of label in mitochondria from BHK-21 cells, and rat liver mitochondria with or without PCMB. In rat brain or liver, the conversion of phosphatidyl glycerol phosphate to phosphatidyl glycerol has been shown to be sensitive to sulfhydryl inhibitors (Kiyasu *et al*., 1963; Possmayer *et al*., 1968). The labelling profile of BHK-21 mitochondria is identical with that of PCMB-treated rat liver mitochondria. This would seem to indicate that the activity of

TABLE 27

DISTRIBUTION OF LABELLED GLYCEROL-3-PHOSPHATE

INCORPORATED BY MITOCHONDRIA FROM BHK-21 CELLS AND RAT LIVER

The incubation mixture consisted of 50 μmoles tris-HCl, pH 7.4, 25 μmoles sucrose, 10 nmoles EDTA, 0.5 μmole CDP-diolein, and 0.1 μmole *sn*-glycerol-3-phosphate [^{14}C(U)] (11.1 x 10^6 DPM $\mu mole^{-1}$). Samples containing BHK-21 mitochondria (0.1 mg) and normal rat liver mitochondria (0.25 mg) were added to 5 μmoles 2-mercaptoethanol and for treated rat liver mitochondria to 0.4 μmole *p*-chloromercuribenzoic acid (PCMB), potassium salt pH 8.5 to final volumes of 0.35 ml. Following 1 hour incubation at 37°C, the lipids were extracted as described in the Methods.

Phospholipid	Percent of Label Incorporated[a] BHK-21 cells	Normal rat liver	PCMB-treated rat liver
PG	4.5	90.6	1.8
PGP	92.2	6.7	94.7
Total Incorporation[b]	16.5	14.0	6.2

[a] average of two experiments

[b] nmoles mg $protein^{-1}$

the PGP phosphatase in the BHK-21 mitochondria is very low, a feature not observed in rat liver mitochondria under normal conditions. Addition of rat liver mitochondria to labelled BHK-21 mitochondria resulted in conversion of PGP to PG (Appendix 9).

Since the PGP to PG conversion occurs in homogenates of BHK-21 cells (Chapter 5), the effect of the addition of post-microsomal supernatant from BHK-21 cells or the supernatant from sonicated rat liver mitochondria on the labelling profile in BHK-21 mitochondria was examined (Fig. 23). Examination of the individual polyglycerol-phosphatides revealed that although total incorporation was stimulated to a greater extent by the sonicated rat liver mitochondrial supernatant (Appendix 10), at optimal concentrations of both types of supernatant the production of PG and PGP occur at the same relative rates. In both cases, conversion of PGP to PG is stimulated many-fold.

A partial characterization of the nature of this stimulation was undertaken (Table 28). The total incorporation of labelled sn-glycerol-3-phosphate was stimulated by both BHK-21 post-microsomal supernatant and sonicated rat liver mitochondrial supernatant, as is the percent of the label converted to PG and DPG. Dialysis has no effect on either stimulation. When an equivalent original volume of either heat-treated supernatant is utilized, total incorporation is reduced to close to the control values. It appears that the PGP to PG conversion

FIGURE 23

THE EFFECTS OF BHK-21 POST-MICROSOMAL SUPERNATANT AND SUPERNATANT FROM SONICATED RAT LIVER MITOCHONDRIA ON THE INCORPORATION OF [^{14}C]-GLYCEROL-3-PHOSPHATE BY BHK-21 CELL MITOCHONDRIA

The assay mixture was the same as the untreated system described in Table 27. To each tube was added 0.1 mg BHK-21 mitochondrial protein. Post-microsomal supernatant (7.5 mg protein ml^{-1}) was added to one set of tubes at the protein concentrations described. Frozen rat liver mitochondria were sonicated for 3 minutes with cooling using a microprobe equipped Branson Sonifier. The sonicate was centrifuged as described for the post-microsomal supernatant for BHK-21 cells and this supernatant (10.0 mg ml^{-1}) added to a second set of tubes. Following 1 hour incubation at 37°C, 5 μmoles $CoCl_2$ was added to a final volume of 0.5 ml and incubated for a further 2 hours. Lipids were extracted as previously described (Table 21). The lipid extracts were chromatographed and the spots identified by spraying with 1% iodine in methanol, scraped off and counted. o—o, DPG; Δ —— Δ, PG and □——□, PGP, with BHK-21 post-microsomal supernatant added. ●——●, DPG; ▲——▲, PG and ■——■ PGP with sonicated rat liver mitochondrial supernatant added.

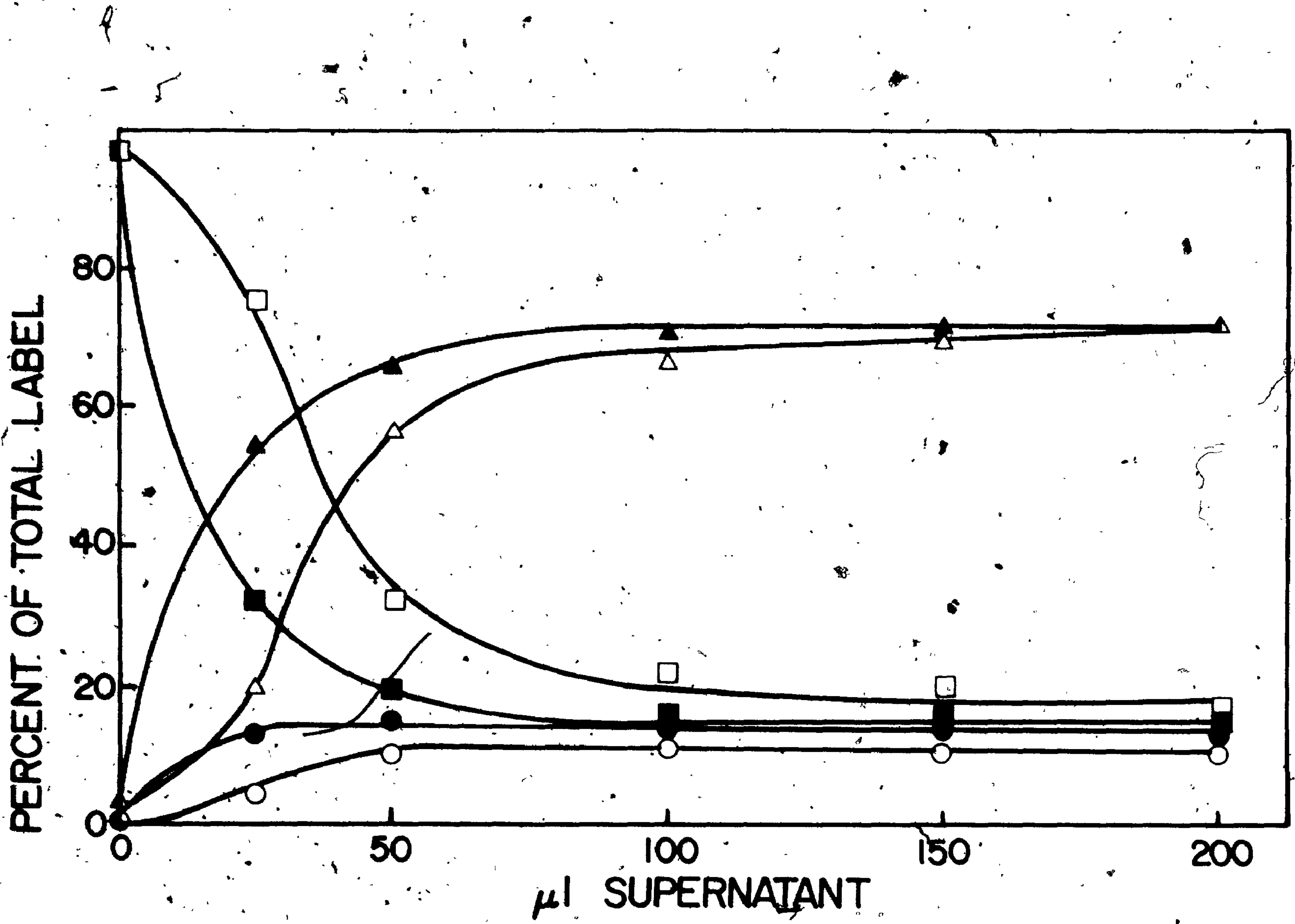

PERCENT OF TOTAL LABEL
0
20
40
60
80
0
50
100
150
200
μl SUPERNATANT

TABLE 28

EFFECTS OF VARIOUS TREATMENTS ON THE STIMULATION BY SUPERNATANT OF PHOSPHATIDYL GLYCEROL PHOSPHATE CONVERSION TO PHOSPHATIDYL GLYCEROL

The assay mixture was identical to that described in Figure 23. Each tube contained 150 μg BHK-21 mitochondrial protein and the various supernatant fractions described below. Following 1 hour at 37°C, 5 μmoles $CoCl_2$ was added to a final volume of 0.5 ml and the samples incubated for an additional 2 hours. Lipids were extracted as in Table 21.

Sample	Lipid Synthesized[a] Total	DPG	(Percent of Total) PG	PGP
Control - no supernatant	1.51	0.056(3.7)	0.087(5.8)	1.37(90.5)
+ 750 μg BHK-21 post-microsomal supernatant	3.49	1.39(39.8)	1.42(40.7)	0.68(19.5)
+ 750 μg dialyzed[b] BHK-21 post-microsomal supernatant	3.42	1.44(42.1)	1.44(42.1)	0.54(15.8)
+ 250 μg heat treated[c] BHK-21 post-microsomal supernatant	2.18	0.98(45.0)	0.79(36.2)	0.41(18.8)
+ 1.0 mg sonicated[d] rat liver mitochondria supernatant	4.65	2.06(44.3)	1.99(42.8)	0.60(12.9)
+ 1.0 mg dialyzed[b] sonicated rat liver mitochondrial supernatant	4.76	2.03(42.6)	1.97(41.4)	0.76(16.0)
+ 0.5 mg heat treated[c] sonicated rat liver mitochondrial supernatant	1.80	0.23(12.8)	0.34(18.9)	1.23(68.3)
+ 2.0 mg rat liver post-microsomal supernatant	1.55	0.64(41.3)	0.57(36.8)	0.34(21.9)

[a] nmoles per sample - average of two experiments
[b] dialyzed against 1000 volumes 0.25M sucrose-0.1mM EDTA-0.2% 2-mercaptoethanol for 20 hours at 4°C
[c] 10 min. at 100°C, centrifuged at 10,000g x 10 min.
[d] prepared as described in Figure 23.

by BHK-21 post-microsomal supernatant is not as heat labile as for the rat liver extract, and that the enhancement of total incorporation of label is an effect not related to conversion of PGP to PG and DPG. In addition, a factor in the rat liver post-microsomal supernatant will stimulate conversion of PGP to PG, with no enhancement of total incorporation.

The hydrolysis of PGP by post-microsomal supernatant from BHK-21 cells was examined using [^{14}C]-PGP isolated from rat liver mitochondria treated with PCMB as in Table 27. These results (Table 29) indicate that the hydrolysis of PGP to PG by supernatant is probably due to a sulfhydryl-containing phosphatase rather than a factor which activates a mitochondrial phosphatase.

6.4 DISCUSSION

Unlike rat liver mitochondria, mitochondria from BHK-21 cells are not autonomous for the synthesis of polyglycerolphosphatides. Instead, it appears that the phosphatase involved in the conversion of PGP to PG is a cytoplasmic enzyme that has free access to the mitochondrially-synthesized PGP and rapidly stimulates PG formation. The rate of DPG synthesis is dependent on the synthesis of PG from PGP. In addition, a cytoplasmic factor stimulates the total synthesis of polyglycerol-phosphatides in BHK-21 cells. This latter factor is heat-labile, while the phosphatase seems to be relatively stable.

TABLE 29

CONVERSION OF PGP TO PG BY BHK-21 POST-MICROSOMAL SUPERNATANT

The assay mixture consisted of 1.21 nmoles [^{14}C]-PGP (6.6 x 10^3 DPM nmole^{-1}), 25 μmoles tris-HCl, pH 7.4 ± 2.5 μmoles 2-mercaptoethanol sonicated together for 30 seconds in a bath-equipped Branson Sonifier. BHK-21 post-microsomal supernatant (250 μg protein, 12.5 μmoles sucrose, 5 nmoles EDTA) was added to a final volume of 0.25 ml. Following up to 90 minutes incubation at 37°C, the lipids were extracted as in Table 21.

Sample	Total PG Produced (pmoles)
Control - 0 min.	12.3
- 30 min.	66.4
- 60 min.	121
- 90 min.	182
- 90 min.-2-mercaptoethanol	105
- 90 min.-2-mercaptoethanol + PCMB	30.6

Both these activities can be replaced by adding a heat-labile soluble factor(s) isolated from rat liver mitochondria.

The reason for this interesting difference in compartmentalization of this biosynthetic pathway is uncertain. Whether it is a function of kidney or kidney-derived cells in general or whether it is an inherent property of cultured cells is unknown. The latter possibility may provide a mechanism for the regulation of mitochondrial function under varying conditions of cell culture.

CHAPTER 7. SUMMARY

1. Treatment of BHK-21 cells with D-chloramphenicol results in cessation of cell growth following two doublings of cell number. Cell size is increased and mitochondria are enlarged and show progressive degeneration of cristae with time. This is accompanied by a loss in mitochondrial respiratory enzyme activity. The loss of cytochrome c oxidase activity is rapid, while the decline in succinate cytochrome c reductase activity lags by 24 hours. These decreases parallel the disappearances of cytochromes aa_3 present in cytochrome oxidase and cytochrome b present in succinate cytochrome c reductase. No effects on the mitochondrial matrix enzyme, glutamate dehydrogenase, or on the endoplasmic reticulum marker, NADPH-cytochrome c reductase, are observed. The outer mitochondrial membrane marker, monoamine oxidase, is activated, probably due to release of latent activity.

2. The effects of chloramphenicol are reversible within the experimental period examined. Growth and mitochondrial structure return to normal within 12 to 24 hours of removal of the drug. Succinate cytochrome c reductase activity is rapidly regained (within 12 hours) while cytochrome c oxidase recovery is slower (within 48 hours). The recovery of mitochondrial respiratory activity seems to be a prerequisite to normal cellular growth.

3. Protein synthesis by mitochondrial ribosomes, as measured by chloramphenicol-sensitive leucine incorporation, constitutes a very small percentage of either total cellular or even total mitochondrial protein synthesis. Inhibition of bulk cellular protein synthesis by the cytoplasmic inhibitor cycloheximide, reveals a small and highly labile cycloheximide-insensitive component, whose synthesis is chloramphenicol-sensitive.

4. The synthesis of hemoproteins, as measured by δ-aminolevulinic acid incorporation, is sensitive to prolonged and continuous exposure to chloramphenicol. As anticipated, localization of hemoprotein synthesis is primarily mitochondrial.

5. The _de novo_ synthesis of cellular phospholipid, measured by inorganic phosphate, glycerol or fatty acid incorporation, is inhibited by cellular growth in chloramphenicol. This effect is not localized solely in the mitochondria and is observed in phospholipids synthesized in all membrane fractions. Precursor pools of these various components (phosphate, glycerol and fatty acid) are elevated in chloramphenicol-treated cells. The effect does not appear to be related to the cellular energy state, as ATP levels are normal in treated cells.

6. Cardiolipin biosynthesis is inhibited to a greater extent than other cellular phospholipids as measured by glycerol and inorganic phosphate incorporation. Labelling of cardiolipin with fatty acid reveals that the acyl groups of cardiolipin may be turning over faster than the other mitochondrial phospholipids in treated cells. Long term experiments reveal a selective retention of unsaturated fatty acids in cardiolipin from treated cells. Cardiolipin synthesis recovers quickly on removal of the drug. This is followed by a recovery in the bulk lipid synthesis.

7. The inhibition of phospholipid synthesis appears to occur at two levels. First is the phosphorylation of glycerol to yield sn-glycerol-3-phosphate. Second is the formation of phosphatidic acid from either sn-glycerol-3-phosphate or dihydroxyacetone phosphate by mitochondria from treated cells.

8. The biosynthesis of phosphatidyl choline, phosphatidyl inositol, CDP-diglyceride, and the polyglycerolphosphatides are all stimulated in subcellular fractions from treated cells.

9. The compartmentalization of polyglycerolphosphatide biosynthesis in BHK-21 cells is different from that described in animal tissues in that the phosphatase

responsible for the conversion of phosphatidyl glycerol phosphate to phosphatidyl glycerol is located in the cytoplasm rather than the mitochondria.

CHAPTER 8. CONCLUSIONS

BHK-21 cells and isolated mitochondria have been shown to behave in the same manner as other somatic cells when treated with chloramphenicol, and a relationship between the synthesis of mitochondrial polypeptides and cellular phospholipids has been established. Several possibilities seem likely and bear future scrutiny. The first is that a selective inhibition of the synthesis of a small number of polypeptides parallels the inhibition and stimulation of a number of lipid-synthesizing enzyme activities, until now not implicated in any relationship with products of the mitochondrial genome. Elucidation of this relationship requires examination of the specific polypeptides whose synthesis is inhibited by chloramphenicol in BHK-21 cells in order to ascertain whether or not they correspond to components, inhibitors, or activators of the affected phospholipid synthetic machinery. Alternatively, biosynthesis of mitochondrial phospholipids may be subject to mitochondrial genetic regulation of nuclear gene expression. It is possible, for example, that the lipid synthesizing enzymes which are affected by chloramphenical, although nuclearly-coded, are genetically regulated by mitochondrial gene expression.

A second avenue of approach has been alluded to earlier. In view of the apparently normal levels of ATP

in chloramphenicol-treated cells, it may prove fruitful to ascertain the nature of this energy production. Although Pious *et al.* (1972) have shown no elevation in glycolytic enzyme levels on chloramphenicol treatment of human diploid fibroblasts, this may not be the case for BHK-21 cells. If not produced by oxidative phosphorylation, substrate level phosphorylation is an obvious alternative as the source of ATP production. The latter event may involve allosteric regulation by nucleotide pools, a feature that has been demonstrated as being responsible for maintaining bacterial energy flux (Sanwal, 1970). Substrate level phosphorylation and metabolite flow through glycolytic or hexose monophosphate pathways must produce large amounts of reduced nucleotides. The question then arises as to how they are removed; and if they are not, what is the effect of their accumulation on cellular metabolism? Extension of this hypothesis further raises the possibility that the affected lipid synthesizing enzymes, both those that are inhibited or those that are stimulated, may also be allosterically controlled by nucleotide levels (Possmayer *et al.*, 1973; Possmayer, 1974; Sribney *et al.*, 1976).

In Chapter 4 the coupling between bacterial protein and lipid synthesis was mentioned briefly. This coupling has been shown to involve the expression of the relA gene and subsequent to it the cellular levels of the "magic spots", guanosine 5'-diphosphate, 3'-diphosphate (ppGpp)

and guanosine 5'-triphosphate, 3'-diphosphate (pppGpp) and other highly phosphorylated nucleotides (for review see Cashel, 1975). Recently, a number of these nucleotides have been isolated from several cultured cell lines, including BHK-21 cells (Rhaese, 1975) and have been synthesized in isolated rat liver mitochondria (Horvath et al., 1975). These nucleotides have been implicated by analogy to bacterial nucleotides, as regulators of differentiation and development. However, they remain as regulators without a known function. In bacteria, antibiotics such as chloramphenicol and erythromycin have been shown to cause accumulation of large amounts of ppGpp and pppGpp due to their action on the ribosome (Rhaese et al., 1975), and to inhibit the synthesis of a number of phospholipids including cardiolipin and phosphatidyl glycerol (Tropp et al., 1970; Arbogast and Henderson, 1975). It is interesting to speculate that the observed effects of chloramphenicol on BHK-21 cellular lipid metabolism and particularly cardiolipin, are being mediated via one or more of these nucleotides. Some evidence supports this contention. Fatty acid synthesis in *E. coli* is controlled by the relA gene (Nunn and Cronan, 1976). It is also significant to note, in view of the observed inhibition of *sn*-glycerol-3-phosphate and dihydroxyacetone phosphate acylation in chloramphenicol-treated BHK-21 mitochondria, that with palmityl-CoA (but not palmityl acyl carrier protein) as donor, the acylation of *sn*-glycerol-3-

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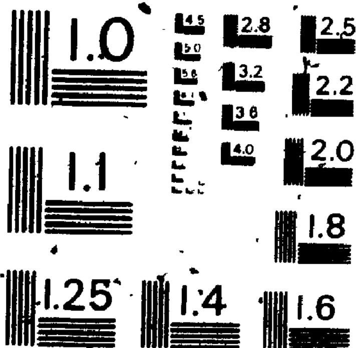

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phosphate in E. coli is inhibited by ppGpp (Merlie and Pizer, 1973; Lueking and Goldfine, 1975). Still more interesting is the finding that ppGpp inhibits the enzyme sn-glycerol-3-phosphate CMP:phosphatidyl transferase which produces phosphatidyl glycerol phosphate in E. coli (Merlie and Pizer, 1973). Should this regulation also be determined in mitochondria, it may be sufficient evidence to choose the endosymbiont model of mitochondrial evolution over the plasmid model (Mahler and Raff, 1975). Purification of the enzymes that are inhibited in CAP-treated cells and examination of their response to various regulators, as well as determination of their absolute cellular concentrations, seems a likely investigative approach in respect to the above hypothesis.

Finally, the different compartmentalization of the phosphatase involved in phosphatidyl glycerol phosphate hydrolysis in BHK-21 cells, is worth examining in greater detail. Before any definitive conclusions about its possible involvement in the regulation of mitochondrial development can be made, a number of experiments are required. A specific assay for this enzyme is necessary. This is needed in order to study its subcellular distribution in the presence of non-specific phosphatase activity. Detailed marker enzyme studies are needed to correlate with its distribution. The apparent heat stability of this enzyme may prove valuable in its purification. If similar distributions are evident for other cultured cells, a study

of the enzyme's activity in response to cell cycle, density, nutritional status and metabolite build-up may prove fruitful in elucidating a possible method of relating mitochondrial development to cellular development.

The potential to expand this study is endless. So little is known about the interplay and regulation of various subcellular components in mitochondrial biogenesis in somatic cells. This system may provide a useful tool in expanding this knowledge.

APPENDIX 1

MITOCHONDRIAL MARKER ENZYME ACTIVITIES IN POLYTRON-DISRUPTED BHK-21 CELLS

BHK-21 cells, harvested during exponential growth phase, were washed once with phosphate-buffered saline and once with 0.25M sucrose-0.1 mM EDTA. 10^8 cells were suspended in sucrose-EDTA and disrupted using a 15 second burst at a setting of 3.0 on a Willems Polytron R. Cells were fractionated by the standard method of differential centrifugation (McMurray and Dawson, 1969). Cytochrome c oxidase and succinate cytochrome c reductase were assayed as described in the methods section of Chapter 2, except that no detergent treatment was utilized. Addition of detergent stimulated the activity 20-fold.

	percent of total activity[a]	
Fraction	cytochrome c oxidase	succinate cytochrome c reductase
Homogenate	100	100
Nuclear	5.2	4.6
Mitochondrial	89.6	96.6
Microsomal	9.3	6.3
Supernatant	0	0
Total activity recovered in fractions	104.1	107.5

[a] average of two different cultures

APPENDIX 2

EFFECTS OF VARIOUS CONCENTRATIONS OF CHLORAMPHENICOL ON MITOCHONDRIAL RESPIRATORY ENZYMES IN BHK-21 CELLS

BHK-21 cells were grown in various concentrations of CAP as described in Figure 1 of Chapter 2. 10^7 Cells were harvested at 24 hour intervals, washed in phosphate-buffered saline and 0.25 M sucrose-0.1 mM EDTA, suspended in sucrose-EDTA and sonicated for 30 seconds in a Branson Sonifier equipped with bath. Cytochrome c oxidase and succinate cytochrome c reductase were assayed as described in Appendix 1.

Specific Activity (units per mg protein)

Time	Cytochrome c Oxidase				Succinate cytochrome c reductase			
	Control	10 μg ml^{-1} CAP	50 μg ml^{-1} CAP	100 μg ml^{-1} CAP	Control	10 μg ml^{-1} CAP	50 μg ml^{-1} CAP	100 μg ml^{-1} CAP
0	0.018	0.018	0.018	0.018	0.0037	0.0037	0.0037	0.0037
24 hr	0.012	0.004	0.005	0.005	0.0041	0.0041	0.0036	0.0028
48 hr	0.011	0.003	0.003	0.002	0.0027	0.0032	0.0025	0.0030
72 hr	0.014	0.003	0.002	0.002	0.0050	0.0053	0.0016	0.0016
96 hr	0.012	0.002	0.002	0.002	0.0041	0.0043	0.0011	0.0010

APPENDIX 3

RECOVERY OF BHK-21 CELLS FOLLOWING DIFFERENT PERIODS OF EXPOSURE TO CHLORAMPHENICOL

BHK-21 cells were exposed for different periods of time to 100 $\mu g\ ml^{-1}$ CAP. CAP was removed at 24, 48, 72 or 96 hours of exposure to the drug and cell number determined as in Figure 8 of Chapter 2.

Cells x $10^{-5}\ ml^{-1}$ [a]

Time (hours)	Control (no CAP)	CAP	CAP removed at 24 hours	CAP removed at 48 hours	CAP removed at 72 hours	CAP removed at 96 hours
0	1.09	1.08	1.08	1.11	1.10	1.14
24	4.85	2.95	2.90	2.80	2.93	2.95
36	8.94	3.67	3.70	3.54	3.70	3.70
48	20.8	4.94	6.90	4.89	4.91	4.43
60	37.3	6.06	12.1	6.12	6.53	6.08
72	67.9	6.31	24.2	11.1	6.82	6.12
84	153	6.42		21.8	7.11	5.98
96	309	7.04		41.2	7.28	6.21
108	584	6.92			11.3	6.20
120	1121	6.74			20.2	6.04
132	2038	6.12				5.98

[a] cell count assuming no dilution to maintain exponential growth

APPENDIX 4

CYTOCHROME CONCENTRATIONS OF BHK-21 CELLS RECOVERING FROM TREATMENT WITH CHLORAMPHENICOL

Cells were grown in 100 μg ml^{-1} chloramphenicol as described in Figure 8, Chapter 2. At 48 hours, the CAP was removed and cells were sampled at 72 hours and 96 hours and the low temperature cytochrome spectra measured as described in Figure 7, Chapter 2. The difference in absorbance for each cytochrome was determined as described by Klietmann et al., (1973).

Time	Cytochrome aa_3 $OD_{600\ nm} - OD_{630\ nm}$	Cytochrome b $OD_{558\ nm} - OD_{575\ nm}$
48 hours CAP	-0.0003	0.0026
48 hours CAP + 24 hours recovered	0.0004	0.0037
48 hours CAP + 48 hours recovered	0.0011	0.0042
Control (no CAP)	0.0013	0.0040

APPENDIX 5

EFFECTS OF TRITON X-100 ON ACTIVITY OF MONOAMINE OXIDASE FROM CHLORAMPHENICOL-TREATED BHK-21 CELLS

Cells were grown for 96 hours in 100 $\mu g\ ml^{-1}$ CAP as described in Figure 5, Chapter 2. At 24 hour intervals, samples were removed and assayed for enzyme activity and protein as described in Figure 6, Chapter 2 except that cells were incubated for 5 minutes with Triton X-100 (0.02% final concentration) before addition of substrate.

Time	Specific activity (units per mg protein)			
	Expt. 1 (no Triton X-100)		Expt. 2 (+ Triton X-100)	
	Control	CAP-Treated	Control	CAP-Treated
0 hr	0.012	0.012	0.029	0.029
24 hr	0.013	0.011	0.024	0.026
48 hr	0.010	0.012	0.026	0.031
72 hr	0.010	0.015	0.020	0.023
96 hr	0.010	0.020	0.023	0.024

APPENDIX 6

EFFECTS OF D-CHLORAMPHENICOL ON THE LIPID PHOSPHORUS CONTENT OF BHK-21 MITOCHONDRIA

Cells were grown for 48 hours in 100 $\mu g\ ml^{-1}$ CAP as described in Figure 5, Chapter 2. At 48 hours, 5×10^7 cells were harvested, washed once with phosphate-buffered saline, once with 0.25 M sucrose-0.1 mM EDTA and the mitochondria isolated (McMurray and Dawson, 1969). The mitochondrial pellets were suspended in sucrose-EDTA by sonication as described in the Methods section of Chapter 3. Protein was determined by the method of Lowry *et al.*, (1951) and phosphorus by the method of Bartlett (1959). Extracts were chromatographed (Rouser *et al.*, 1970), spots identified with iodine, scraped and phosphorus determined (Bartlett, 1959).

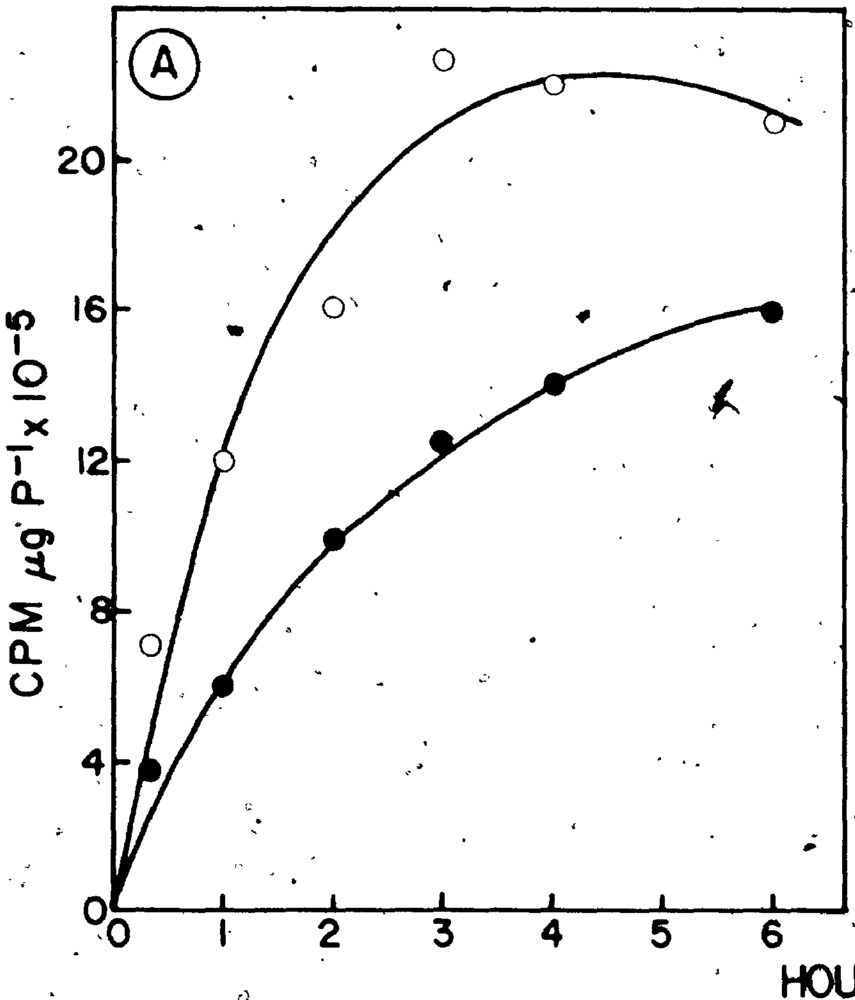
Phospholipid	Lipid P (percent of total[a]) Control	CAP-Treated
PC	50.7	45.5
PE	22.3	25.9
PI	10.1	9.0
PS	7.1	7.3
DPG	5.3	5.4
PG	1.4	1.0
SM	3.1	5.5
PA	1.0	0.6
Total	12.7 μg P/mg protein	12.8 μg P/mg protein

[a]average of three samples

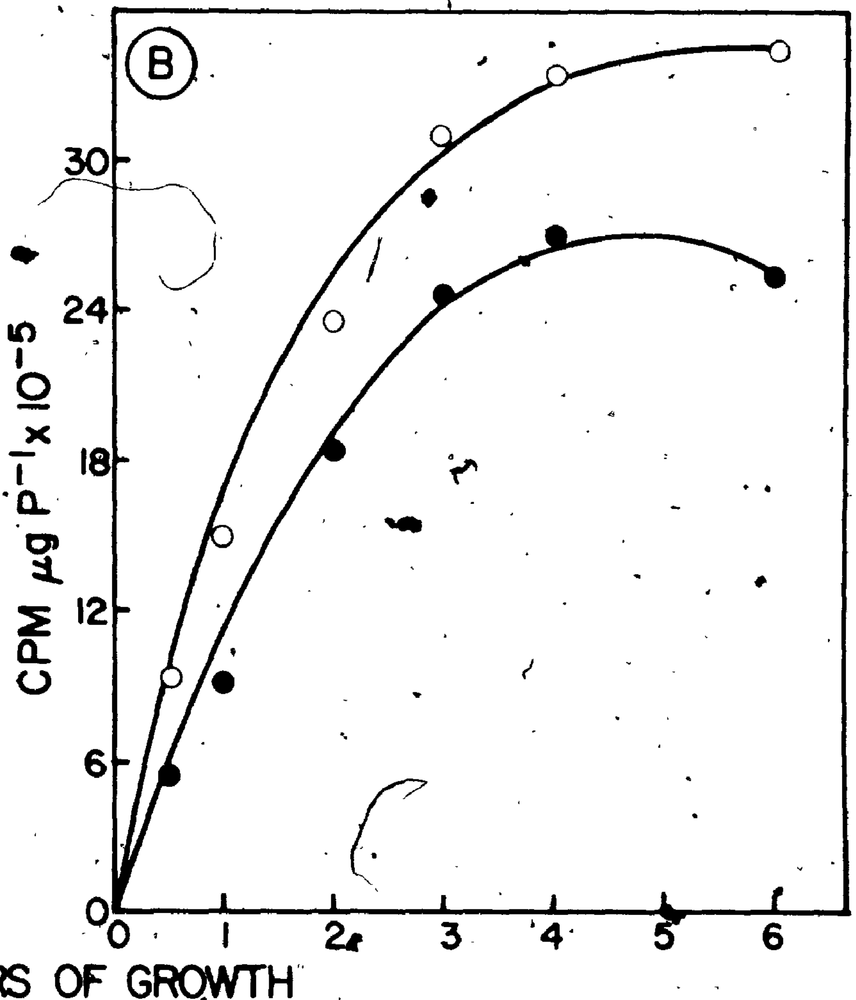
APPENDIX 7

INCORPORATION OF LINOLEIC AND PALMITIC ACIDS INTO CHLORAMPHENICOL TREATED BHK-21 CELLS

BHK-21 cells were grown in 100 μg ml^{-1} CAP for 48 hours as described in Table 15, Chapter 4 and labelled for 6 hours with either [^{14}C]-linoleic acid or [^{14}C]-palmitic acid as described in the Methods section of Chapter 4. At various times, cells were sampled, washed as described in Appendix 6 and then extracted with chloroform-methanol 1:1 (v/v) and the total CPM determined. Phosphorus was determined by the method of Bartlett (1959). A - linoleic acid; B - palmitic acid; o-o control; ●-● CAP-treated.



A
CPM μg P^{-1} x 10^{-5}
0
4
8
12
16
20
0
1
2
3
4
5
6



B
CPM μg P^{-1} x 10^{-5}
0
6
12
18
24
30
0
1
2
3
4
5
6
HOURS OF GROWTH

APPENDIX 8

DETERMINATION OF EXTENT OF LABELLING OF PHOSPHATIDIC ACID WITH [^{14}C]-PALMITATE IN CAP-TREATED BHK-21 CELL MITOCHONDRIA

Cells were grown for 48 hours in 100 μg ml^{-1} CAP and labelled with [1-^{14}C]-palmitate as described in Table 15, Chapter 4. They were then harvested, washed and the lipids extracted from isolated mitochondria. Aliquots were chromatographed in two dimensions (Rouser et al., 1970), one dimension (Possmayer et al., 1969) or in one dimension on thin layer plates made with silica gel H and developed with petroleum ether (30°-60°C): diethyl ether: acetic acid, 85:15:2 (v/v/v) (P. Shum, Personal Communication).

CPM per 1 ml Aliquot[a]

	Rouser		Possmayer		Shum	
Spot	Control	CAP	Control	CAP	Control	CAP
PA	1,220	685	-	-	-	-
FFA	6,730	11,100	-	-	6,100	12,000
PA + FFA	-	-	6,340	10,300	-	-

[a] average of two samples

APPENDIX 9

EFFECT OF ADDING RAT LIVER MITOCHONDRIA TO BHK-21 CELL MITOCHONDRIA PRE-LABELLED WITH [^{14}C]-GLYCEROL-3-PHOSPHATE

A batch mixture consisting of 500 μmoles Tris-HCl pH 7.4, 250 μmoles sucrose, 0.1 μmole EDTA, 5 μmoles CDP-diolein, 50 μmoles 2-mercaptoethanol, 1 μmole sn-glycerol-3-phosphate [$^{14}C(U)$] (6.66 x 10^6 DPM $\mu mole^{-1}$) and 1 mg BHK-21 cell mitochondria was incubated for 90 min. at 37°C. The mitochondria were pelleted at 10,000 x g x 10 min., washed once with 0.25M sucrose-0.1mM EDTA and suspended in 1 ml sucrose-EDTA. Labelled mitochondria (0.1mg) were incubated for 1 hour at 37°C with 50 μmoles Tris-HCl, pH 7.4, 75 μmoles sucrose, 30 μmoles EDTA, 5 μmoles 2-mercaptoethanol, 0.1 μmole sn-glycerol-3-phosphate and 0.125 mg rat liver mitochondria. $CoCl_2$ (5 μmoles) was added to a final volume of 0.45 ml and the mixture incubated for an additional hour at 37°C. The lipids were extracted as described in the methods.

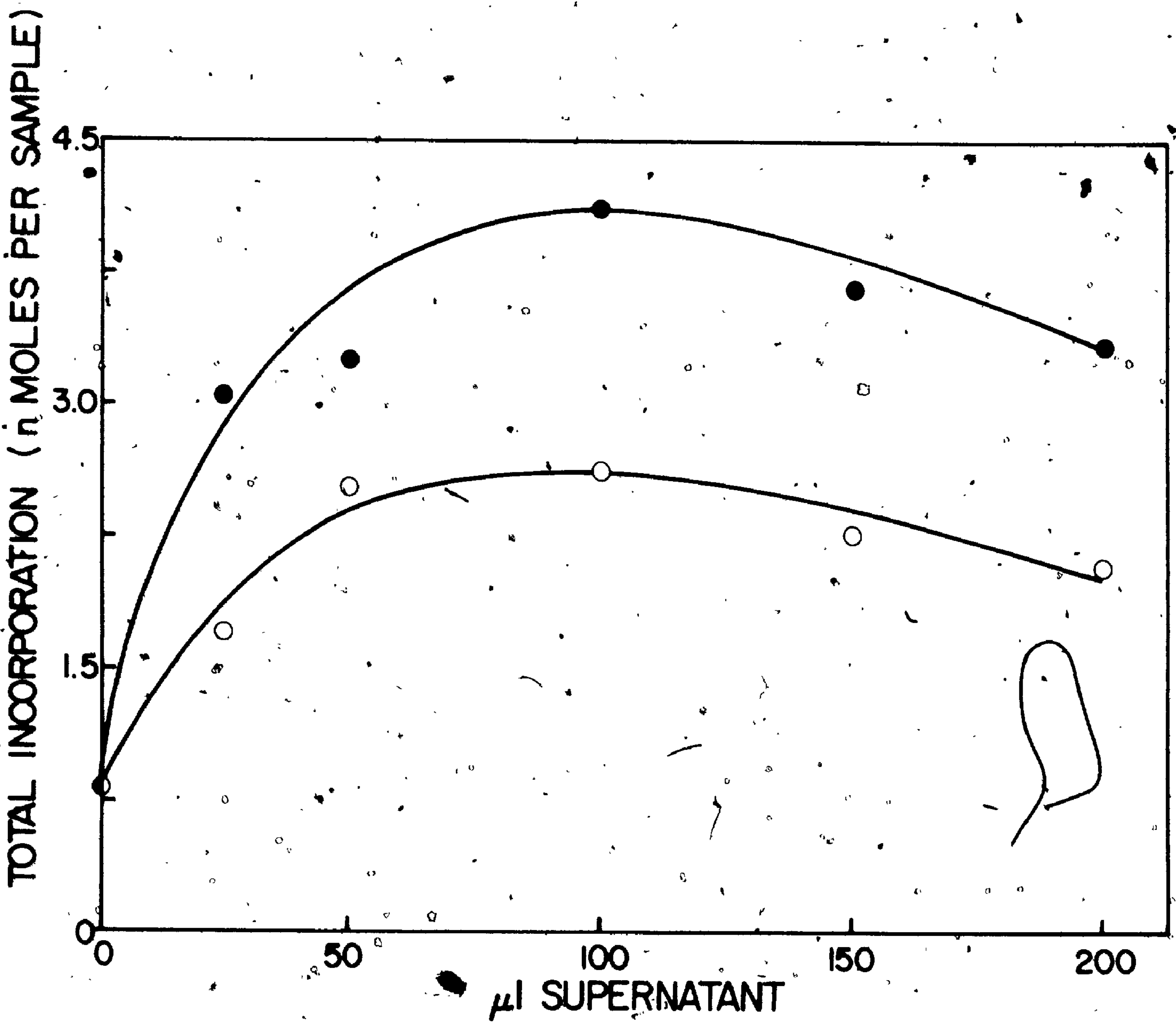
Addition	Percent of Label Incorporated		
	PGP	PG	DPG
1) BHK-21 mitochondria only	47.2	26.3	16.5
2) BHK-21 and rat liver mitochondria	16.3	59.7	17.2
- rat liver mitochondria sonicated[a]	15.7	62.3	19.7
- BHK-21 mitochondria sonicated[a]	24.0	59.4	10.0
- both sonicated together[a]	18.7	58.1	15.5
- +0.5 mg deoxycholate	9.6	79.6	6.6
- +0.5 mg Triton x-100	5.6	83.8	5.5

[a] sonicated for 30 sec. at maximum output in Branson Sonifier with Bath.

APPENDIX 10

THE EFFECTS OF BHK-21 POST-MICROSOMAL SUPERNATANT AND SONICATED RAT LIVER MITOCHONDRIAL SUPERNATANT ON TOTAL SN-GLYCEROL-3-PHOSPHATE INCORPORATION BY BHK-21 MITOCHONDRIA

The incubation was done as in Figure 23, Chapter 6. Total lipid extracts were counted: o--o BHK-21 Post-Microsomal Supernatant; ●--● Sonicated Rat Liver Post-Microsomal Supernatant.


TOTAL INCORPORATION (n MOLES PER SAMPLE)
4.5
3.0
1.5
0
0
50
100
150
200
µl SUPERNATANT

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