THE BIOGENESIS OF MITOCHONDRIA IN MAMMALIAN CELLS

THE BIOGENESIS OF MITOCHONDRIA IN MAMMALIAN CELLS (L CELLS)

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SCOPE AND CONTENTS:

Chloramphenicol has been used to study mitochondrial biogenesis in mammalian cells by examining its effect on: the incorporation of radioactive amino acids into protein by isolated mitochondria, the growth of L cells, the level of representative enzymes and cytochromes in the mitochondria and cytoplasm and the structure of mitochondria and L cells. A reversible inhibition of synthesis of cytochrome c oxidase was obtained by treating cells with D-threo-chloramphenicol for 90 hr. Recovery of cytochrome c oxidase activity was inhibited by cycloheximide, an inhibitor of cytoplasmic protein synthesis. Cycloheximide also reversibly inhibited cytochrome c oxidase formation in cells which were not treated with D-chloramphenicol. It is suggested that the mitochondria and the nucleus have a joint control in the formation of a functionally active cytochrome c oxidase enzyme.

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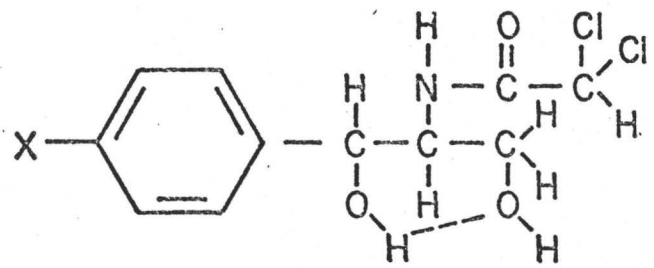
List of Abbreviations

ADP	=	Adenosine diphosphate
ATP	=	Adenosine Triphosphate
DNA	=	Deoxyribonucleic acid
EDTA	=	Ethylenediaminetetra-acetic acid
nm	=	Nano meter (1×10^{-9} meters)
NAD	=	Nicotinamide adenine dinucleotide
NADH	=	Reduced nicotinamide adenine dinucleotide
NADP	=	Nicotinamide adenine dinucleotide phosphate
PBS	=	Phosphate Buffered Saline
RNA	=	Ribonucleic acid
Tris	=	Tris (hydroxymethyl) amino methane

Structural Formulae



X = NO_2 - CHLORAMPHENICOL

= CH_3 S - METHYLTHIO

= CH_3 SO_2 - METHYLSULFONYL

= NH_2 SO_2 - SULFAMOYL (TEVENEL)

Introduction

The presence of DNA in mitochondria (Nass and Nass, 1963) raises the possibility that mitochondria have some degree of autonomy. This idea is supported by the finding of Luck (1963) that mitochondria do not arise de novo but increase by growth and division of pre-existing mitochondria. These findings have led to a great interest in the mechanism of mitochondrial biogenesis and the developments in this area have led to an increased awareness of the problems of synthesis, assembly and integration of mitochondrial components to form a functional unit in the cell. The area has been reviewed by Roodyn and Wilkie (1968), Nass (1969) and Ashwell and Work (1970).

A unique DNA appears to be a characteristic of mitochondria of all species. The presence of DNA in mitochondria has been demonstrated by electron microscopy (Nass and Nass, 1963), by autoradiography (Guttes and Guttes, 1964; Nagata, Shibata and Nawa, 1967) and by the isolation from mitochondria of a unique DNA differing in buoyant density from nuclear DNA of the same species (Borst, Kroon and Ruttenberg, 1967; Luck and Reich, 1964). The mitochondrial DNA from all vertebrates examined appears to be smaller than that found in plants and in lower organisms such as yeasts and Neurospora crassa. Most of the smaller DNA molecules have been characterized as coiled circles whereas the molecular weight and physical form of the larger DNA molecules are uncertain. In vertebrate liver there are on the average 4 or 5 molecules of DNA per mitochondrion (Borst, Ruttenberg and Kroon, 1967) but in some strains of yeast the value may be considerably greater (Mounolou, 1967). It is generally assumed but not proven that all the DNA molecules in one mitochondrion are identical.

Mammalian mitochondrial DNA is homogeneous as judged by centrifugation in caesium chloride and has a molecular weight of 9×10^6. Such a molecular weight could code for about 30 proteins of a molecular weight of 16,500

or one protein of molecular weight about 500,000. There is evidence that the ribosomal RNA in the mitochondrion is coded for by mitochondrial DNA and at least some if not all of the mitochondrial transfer RNA's are as well (Ashwell and Work, 1970). This decreases the amount of DNA available to code for mitochondrial proteins. It is apparent therefore that the amount of DNA associated with a mitochondrion is sufficient to code for only a small fraction of the total mitochondrial protein. For this reason mitochondrial biogenesis must involve integration of both mitochondrial and nuclear coded proteins into the mitochondrion. In order to understand the mechanism of integration it is necessary to first determine the sites of synthesis and genes of the individual mitochondrial proteins.

A simplifying assumption in these studies is that nuclear genes are translated on cytoplasmic ribosomes and mitochondrial genes are translated on mitochondrial ribosomes. Thus by establishing one of either the location of the gene or the site of synthesis of the protein presumptive evidence is obtained for the other. This correspondence has not been completely established in any single system. However, it has been definitively shown that the gene for cytochrome c in yeast is nuclear and the site of synthesis in mammalian cells is on cytoplasmic ribosomes (Ashwell and Work, 1970). Approaches to the problem have included genetic studies in yeast and Neurospora, studies of the recovery of yeast from oxygen and glucose repression, studies on protein synthesis in isolated mitochondria and whole cells and analysis of the effects of two inhibitors of protein synthesis, chloramphenicol and cycloheximide. As will be noted in the discussion that follows, the site of synthesis for several mitochondrial proteins appears to be on cytoplasmic ribosomes. Although there is evidence that some insoluble mitochondrial protein(s) is synthesized in the

mitochondrion, it is still not known which protein(s) is coded for by mitochondrial DNA.

Two types of genetic studies have been used to examine the site of genes which code for mitochondrial proteins. These studies involve either point mutations, which affect one protein or larger mutations, which affect the synthesis of many proteins. With studies of point mutations in yeast it has been demonstrated that the gene for cytochrome c is nuclear. The gene for mitochondrial malate dehydrogenase has also been found to be nuclear in both man (Davidson and Cortner, 1967) and in Neurospora (Munkres, 1968). Genetic studies of larger mutations affecting several mitochondrial proteins have been done mainly in yeast since such mutations are usually lethal in organisms which are not facultative anaerobes.

The "petit" mutants of yeast which have been studied are respiratory deficient and many show cytoplasmic inheritance. The lack of respiratory ability correlates cytologically with degenerate mitochondrial profiles (Yotsuganagi, 1962; Shatz, Tuppy and Klima, 1963) and biochemically by reduced amounts of various dehydrogenases and the loss of cytochromes a, a_3, b and c_1 (Slonimski, 1955; Slonimski and Sherman, 1964; Tewaii, Votsch and Mahler, 1965). The cytoplasmic inheritance and deletions in mitochondrial DNA (Mounolou, Jacob and Slonimski, 1966) which were associated with the "petit" mutations implicated the involvement of mitochondrial DNA in the biogenesis of mitochondria. However these studies on mitochondrial "petits" do not prove that the affected enzymes are coded for by mitochondrial DNA since similar effects have been observed in "petit" mutations in which the mutation is in nuclear genes.

A similar type of mitochondrial mutation to the "petit" mutation in yeast has been found in Neurospora. There is also multiple enzyme and respiratory deficiency in these mutants, which are called poky. Woodward and Munkres (1966) have shown from amino acid analysis of purified

mitochondrial structural protein (defined as insoluble protein with no enzymatic activity) that the poky mutants differ from the wild type strain in only 1 or 2 amino-acids, depending on the mutant. If the isolated structural protein is in fact only one protein, as has been claimed, then it means that a single amino acid replacement in one protein can result in multiple enzyme deficiency. This reflects an interdependance of various mitochondrial proteins. However, interpretation of this work is complicated by the observation that denatured ATPase is often found in structural protein isolated from mitochondria (Shatz and Saltzgaber, 1969) and the finding of so called mitochondrial structural protein in other cellular membranes (Woodward and Munkres, 1967). A final conclusion can only be drawn after further purification and characterization of mitochondrial structural protein is accomplished.

Another approach has used the observation that yeast cells grown under strictly anaerobic conditions or in high concentrations of glucose are free of functional mitochondra, although incomplete mitochondria (containing mitochondrial DNA) are present. The respiratory system can be induced by exposing the aneorobically grown cells to small concentrations of molecular oxygen or by transfering the glucose repressed cells to conditions where there is no glucose. During respiratory adaptation functional mitochondria are formed. This system has been very useful in determining the sites of synthesis of the various mitochondrial proteins which make up a complete mitochondrion. One example of this is discussed below.

In higher organisms, regulation of mitochondrial formation by glucose or oxygen depression has not been extensively studied. Pious (1970) has recently presented some evidence of oxygen induction of cytochromes in human fibroblasts grown in tissue culture, but the spectral studies on which the conclusions are based are not entirely convincing.

The best proof that a protein is synthesized at a particular site is the demonstration of its synthesis by an isolated system containing only one of the sites. It has not yet been possible to demonstrate the synthesis of mitochondrial proteins in an isolated system of cytoplasmic ribosomes. It is however possible to study protein synthesis in isolated mitochondria.

Mitochondria from a wide range of biological organisms have been shown to incorporate radioactive amino acids into protein on incubation in vitro. Many attempts have been made to locate and identify those proteins which had become radioactive and therefore are presumably synthesized in mitochondria in vivo. This approach has demonstrated that the protein synthesized is insoluble (Roodyn, 1962) and is associated only with the inner membrane (Beattie, Basford and Koritz, 1967; Neupert, Brdiczka and Sebald, 1968). Although it has been suggested that this insoluble protein is structural there is no conclusive evidence for this. Due to the difficulties involved in purifying and characterizing this insoluble protein it has not yet been possible to demonstrate with this approach which protein or proteins are coded for by mitochondrial DNA.

Another approach in studying sites of synthesis of mitochondrial proteins is to demonstrate the location of the precursor at its sites of synthesis in vivo. The usual way that this is done is to add a radioactive amino-acid to the system, isolate the protein from mitochondria and microsomes and determine if there is a transfer of radioactivity from microsomes to mitochondria. This technique was used to demonstrate that the site of synthesis of cytochrome c is on the microsomes in rat liver (Gonzalez-Cadavrid and Campbell, 1967).

An alternative approach is studying the sites of synthesis of mitochondrial proteins involves the use of the two inhibitors chloramphenicol and cycloheximide. These inhibitors have been used in both isolated mitochondrial and whole cell systems and have contributed a great deal to an understanding of the

biogenesis of mitochondria.

Chloramphenicol inhibits protein synthesis by isolated rat liver mitochondria (Freeman, 1970), by yeast mitochondria (Wintersberger, 1965) and by bacteria (Brock, 1961) but does not inhibit protein synthesis on cytoplasmic ribosomes of eukaryotic cells. In contrast, cycloheximide inhibits protein synthesis on the cytoplasmic ribosomes (Sisler and Siegel, 1967) but has no effect on protein synthesis by isolated mitochondria (Borst et al, 1967; Ashwell and Work, 1968; Loeb and Hubby, 1968).

The sensitivity of mitochondrial protein synthesis to chloramphenicol and its insensitivity to cycloheximide are two characteristic respects in which it differs from protein synthesis on cytoplasmic ribosomes. Because of this difference, the two inhibitors can be very useful in determining the sites of synthesis of mitochondrial proteins. Those proteins whose appearance (enzyme activity or amount) is inhibited by chloramphenicol are those which are in part at least synthesized in the mitochondria or the synthesis and/or assembly of these proteins are controlled by a product of mitochondrial protein synthesis. Similarly, if the appearance of a protein is inhibited by the presence of cycloheximide, but not chloramphenicol, this would suggest that the protein is synthesized on the cytoplasmic ribosomes. Insensitivity to cycloheximide would strongly indicate that the protein is made on mitochondrial ribosomes, just as insensitivity to chloramphenicol would strongly indicate that the protein is made on cytoplasmic ribosomes. If appearance of the protein were inhibited by both chloramphenicol and cycloheximide then some protein synthesis in both the cytoplasm and mitochondria must be neceassary for formation of the protein. If two sites of synthesis are indicated it would then be necessary to determine whether chloramphenicol and cycloheximide inhibit the incorporation of amino acids into the protein after various periods of treatment. From the specific radioactivity in

the isolated protein it would be possible to determine whether the protein was synthesized partly in the cytoplasm and partly in the mitochondrion or was synthesized completely in the cytoplasm but required some mitochondrial protein synthesis for intergration into the mitochondrion.

The incorporation of radioactive amino acids by isolated mitochondria has been described in several mammalian systems (Roodyn, 1965; Kroon, 1965). In many ways mitochondrial protein synthesis resembles that of bacteria. The antibiotic D-threo-chloramphenicol (structural formula, page ix) inhibits the synthesis of protein in both bacteria and isolated mitochondria. Using D-threo-chloramphenicol, some of its analogues and the L-threo-isomer, Freeman (1970) has shown that the structural requirements for the inhibition of protein synthesis in mitochondria and in bacterial extracts are similar. Fifty percent inhibition was obtained at 10 μM and 15 μM in extracts of Escherichia coli and in rat liver mitochondria respectively with D-threo-chloramphenicol and its p-methylthio, p-methylsulfonyl and p-sulfamoyl analogues. L-threo-chloramphenicol does not inhibit protein synthesis in either bacteria or mitochondria. The similar structural requirements of chloramphenicol for inhibiting protein synthesis indicate that bacteria and mitochondria have a similar binding site on their ribosomes for chloramphenicol and supports the suggestion that mitochondria have evolved from symbiotic bacteria (Roodyn and Wilkie, 1968).

Chloramphenicol or a combination of chloramphenicol and cycloheximide has been used to study mitochondrial biogenesis in whole cells and organisms. Clark-Walker and Linnane (1966 and 1967) observed that yeast grown in the presence of chloramphenicol lacked cytochromes a, a_3, b and c_1. During glucose derepression in the presence of chloramphenicol yeast also failed to develop normal respiration. They suggested that the selective effect of chloramphenicol was on mitochondria and deduced that these cytochromes were

made by the mitochondrion itself. However, as has been previously discussed, inhibition by chloramphenicol alone is insufficient proof for a mitochondrial site of synthesis for the protein. These experiments complemented the earlier genetic studies of the mitochondrial "petit" mutants of yeast. A marked reduction of mitochondrial cristae formation was also observed in yeast grown in the presence of chloramphenicol (Clark-Walker and Linnane, 1967). More recently it has been demonstrated in yeast that the formation of cytochrome oxidase is sensitive to chloramphenicol but the synthesis of the mitochondrial enzymes succinic dehydrogenase, malate dehydrogenase and fumarase are not (Vary, Edwards and Stewart, 1969). These workers found that cycloheximide blocked the induction of all enzymes measured. These results suggests two sites of synthesis (cytoplasmic and mitochondrial) for cytochrome oxidase but only one site of synthesis, which is in this case cytoplasmic, for the mitochondrial enzymes succinic dehydrogenase, malate dehydrogenase and fumarase. They also showed that an inhibition of mitochondrial protein synthesis in yeast resulted in a reduction of mitochondrial cristae.

Marchant and Smith (1968) have reported that mycelium of Pythium ultimum grown in the presence of D-chloramphenicol (100 µg/ml for 7 days) is devoid of cytochromes a, a_3 and b, but contain an increased amount of cytochrome c. Although mitochondria from the chloramphenicol treated hyphae appeared normal in thin sections, no knobs could be observed in negatively-stained preparations. The knobs have been shown to contain the mitochondrial enzyme ATPase (Kagawa and Racker, 1966). These results suggest a mitochondrial site of synthesis for the insoluble cytochromes a, a_3 and b and for ATPase and a cytoplasmic site of synthesis for the soluble cytochrome c. However these results must be interpreted with caution since a high concentration of chloramphenicol was used for a long period of treatment. Both of these

factors are likely to increase the number of secondary effects observed with chloramphenicol treatment and so it is uncertain whether they were observing primary (direct) or secondary (indirect) effects. In contrast to yeast the reduced content of the insoluble cytochromes did not result in a decrease in the number of cristae. This may indicate that there is no direct correlation between cytochrome content and the amount of cristae.

Firkin and Linnane (1968) have reported that growth of HeLa cells ceased after two divisions in the presence of D-chloramphenicol at 10-40 µg/ml, was reduced further in the presence of 100 µg/ml and completely inhibited at 150 µg/ml. In HeLa cells grown in the presence of D-chloramphenicol at 20 µg/ml for two cell divisions, the levels of cytochromes a and a_3 were less than 20% of the control and the levels of cytochromes c_1 and b were equally decreased but to a much lesser extent than cytochromes a and a_3. The respiratory activity of these cells was decreased by about 80% (expressed in terms of cyanide sensitive respiration). These results indicate that a reduction in the level of the insoluble cytochromes of the respiratory chain decreases both the respiratory ability and the growth rate of HeLa cells. Firkin and Linnane also suggest that cytochromes a and a_3 are at least partly synthesized in the mitochondria. The small decrease observed in the levels of cytochromes b and c_1 are difficult to interpret. The spectral studies on which their evidence was based are very convincing but the growth studies were certainly incomplete and the observation of cyanide insensitive respiration was not documented.

In contrast to HeLa cells, Kroon and Jansen (1968) observed no inhibition of growth in beating rat heart cells exposed to D- or L-chloramphenicol at 100 µg/ml for 4 days (≃ 2 generations). Although there was no effect on growth, D-chloramphenicol caused 50% inhibition of total cytochrome oxidase activity after 4 days treatment. Kroon has recently reported that cycloheximide

also inhibits formation of cytochrome oxidase in this system (Soc. Exptl. Biol. Symp. 24 in press). The results indicate that an inhibition of cytochrome oxidase can be obtained without a corresponding decrease in cell growth rate. Although sites of synthesis (cytoplasmic and mitochondrial) are indicated for cytochrome oxidase, the activity of cytochrome oxidase was expressed in terms of activity per culture plate which is somewhat vague and the exact results obtained by the use of cycloheximide are not known since the paper is still in press.

A more extensive study of the effect of D-chloramphenicol on representative enzymes in the mitochondria and cytoplasm of mamalian systems was conducted on regenerating rat liver (Firkin and Linnane, 1969). D-Chloramphenicol at 200 mg/kg body wt. was administered to partially hepatectomized rats for 48 hr (≈ 1 generation time for regenerating liver cells). At this time a 25%-decrease was observed in the level of cytochrome oxidase but no change was apparent in the levels of succinic dehydrogenase, malate dehydrogenase or fumarase in isolated mitochondria. Nor was there an effect on fumarase or malate dehydrogenase in the whole homogenate, on glucose-6-phosphatase and cytochrome b_5 in the microsomal fraction or on aldolase and 6-phosphogluconate dehydrogenase in the soluble cytoplasm. The synthesis of lactate dehydrogenase was slightly enhanced. A 40% decrease was observed in the level of cytochrome a, a_3, a 20-25% decrease in cytochromes b and c_1 and a 50% increase in cytochrome c. Some of the mitochondria were swollen and appeared to contain fewer cristae. These results indicate that cytochrome oxidase (cytochrome a, a_3) is at least partly synthesized in the mitochondrion. The site of synthesis for cytochromes b and c_1 is uncertain; since the decrease was small it could be a secondary effect. The decrease in cytochromes observed appeared to be associated with a structural change in the mitochondrion. The mitochondrial enzymes succinic dehydrogenase, malate dehydrogenase and fumarase and cytochrome c appear to be synthesized on cytoplasmic ribosomes. The

effect of chloramphenicol was specific for the mitochondria since the cytoplasmic enzymes were unchanged. The main drawback of this work is that the liver cells went through only 1 generation during the treatment and as is described below, the chloramphenicol treatment appeared to be uneven.

In the regenerating rat liver experiment described, the serum concentration of chloramphenicol was about 30 µg/ml. The liver cells were unevenly affected by this. In 15% of the total population of liver cells there was extensive vacuolation but up to 50% of the cells in the periportal zone showed such vacuolation. A similar vacuolation has been observed in erythroblasts of patients receiving chloramphenicol (Saidi, Wallerstein and Aggeler, 1961) but it is difficult to correlate this finding with that in rat liver due to the uneven effect observed in the liver.

When D-chloramphenicol is used clinically the level found in the serum is 10-20 µg/ml. If the serum chloramphenicol level is kept at this concentration for several days, there is severe bone marrow depression (Saidi et al. 1961; Scott, Finegold, Belkin and Lawrence, 1965). This effect has been correlated with inhibition of mitochondrial protein synthesis by chloramphenicol with subsequent failure of adequate energy production by mitochondria of bone marrow cells (Firkin and Linnane, 1969; Freeman, 1970). It has in fact been shown that therapeutic concentrations of chloramphenicol inhibit protein synthesis in mitochondria isolated from human bone marrow (Martelo, Manyan, Smith and Yunis, 1969).

Inhibition of the primary antibody response by D-chloramphenicol and its p-methylsulfonyl analogue (Weisberger, 1969) could also be due to an inhibition of mitochondrial protein synthesis which would then effect antibody formation.

At the time this investigation was begun the only well documented studies in which chloramphenicol was used to examine the sites of synthesis

of mitochondrial protein were on yeast. It was therefore decided to study a mammalian system. The tissue culture system can be more exactly regulated with respect to growth and concentration of antibiotic. L cells were chosen because of their ready availability. The objectives of the research project to be described were:

(i) to obtain more complete information on the effect of chloramphenicol on the inhibition and recovery of the growth of cells in tissue culture,

(ii) to examine the effect of chloramphenicol on protein synthesis in mitochondria isolated from cells in tissue culture,

(iii) to study the effect of chloramphenicol and cycloheximide on the synthesis of mitochondrial proteins in order to gain a greater insight into the origin of the enzymatic and protein content of the mitochondrion;

to follow recovery of any mitochondrial protein which might be affected,

(iv) to study the ultrastructure of cells and of mitochondria from normal and chloramphenicol-treated cells using a variety of electron microscope techniques.

Chapter 1

Materials and Methods

Cell Line

All studies were performed on a single line of mouse L cells. The subline used is a derivative of Earle's original fibroblast line (Sanford, Earle & Likely, 1948). These cells can be grown very readily either in suspension cultures or attached to glass surfaces.

Growth Conditions

All experiments discussed in this thesis involved cultures of cells grown in suspension in spinner flasks. The cells were grown in a Fullview Tissue Culture Incubator (Precision Scientific) at 37°. They were maintained in the logarithmic phase of growth through daily dilution with fresh medium. In the concentration range used ($1\text{-}5\times10^5$ cells/ml) the cells were able to maintain a suitable pH for growth. The growth of cells was followed by taking periodic counts with an electric cell counter (Coulter Counter). Cell viability was tested with 1% Eosin-Y (Goldstein & Okada, 1969). Doubling times were claculated from the growth equation $N=N_o e^{0.693t/t_2}$ on an Olivetti Underwood Programma 101 desk computer.

The growth medium used was Joklik's modification of Eagle's minimal essential medium (Eagle, 1959). This modification has 10 times the phosphate concentration but lacks the $CaCl_2$ component of Eagle's medium. The medium was supplemented with the following antibiotics: 75 units of penicillin/ml, 50 µg of streptomycin/ml, and anti-PPLO agent (tylocine) (1.0 ml/100 ml medium). Fresh medium was prepared every two weeks. Fetal calf serum was added to 5% prior to sterilization by millipore filtration. This level of serum

was adequate to support growth and doubling times of 16-20 hr were obtained.

Chemical Modifications of Growth Conditions

D-chloramphenicol, L-chloramphenicol or Tevenel (the sulfamoyl analogue of D-chloramphenicol) were the usual additions to the growth medium. Stock solutions of chloramphenicol were made up to 2 mg/ml in medium and re-sterilized by millipore filtration. Solutions of the concentration desired for the experiment (10 µg/ml or 100 µg/ml) were prepared every two or three days during the course of the experiment. Similarly a stock solution of $1x10^{-4}$ M cycloheximide was prepared and diluted to the appropriate concentration with medium just before use.

Experiments in which cells were grown in the presence of chloramphenicol were initiated with cells grown in ordinary medium and recovered by centrifugation. This was done by spinning at 70xg for 6 min in an IEC-PR6 centrifuge at room temperature. The cells were then resuspended in medium containing chloramphenicol, cycloheximide or in ordinary medium. Other changes in the growth medium of cells were done in the same way.

Measurement of Respiratory Ability

The respiratory ability of L cells was examined polarographically with the use of a Clark fixed-voltage probe and a Biological Oxygen Monitor (Yellowspring Instrument Co.). For this purpose samples were harvested at 350xg for 10 minutes in an IEC-PR6 centrifuge at 0°C. The cells were washed twice with 40 ml of phosphate buffered saline (PBS) (Parker, 1961) lacking $CaCl_2$ and $MgCl_2$, and resuspended in the same medium. The oxygen consumption by $1-2x10^7$ cells was measured polarographically at 37° in 3.0 ml of the isolation medium.

ATP Levels

The ATP level of L cells was determined spectrophotometrically by following the extent of NADP reduction at 340 nm in the presence of glucose, hexokinase and glucose-6-phosphate dehydrogenase (Lamprecht & Trautschold, 1956). For every mole of ATP present, a mole of NADP is reduced. Samples of $2\text{-}5x10^7$ cells were harvested at 650xg for 10 min, suspended in PBS ($-CaCl_2$, $-MgCl_2$) and $HClO_4$ was added to 0.1N. After centrifugation an aliquot of the supernatant was recovered, neutralized with 1N KOH and stored at 0° for 1 hr for precipation of $KClO_4$. The reaction mixture contained 33mM glucose, 20mM potassium phosphate buffer, pH 7.4, 5mM $MgSO_4$, hexokinase (33 µg/ml), glucose-6-phosphate dehydrogenase (100 µg/ml) and NADP (67 µg/ml) in a final volume of 3.0 ml. The reaction was started by the addition of 0.5 ml of supernatant and NADP (67 µg/ml) was added at 5 and 10 min to ensure completion of the reaction (Haldar and Freeman, 1968).

Preparation of Mitochondria

Rat and mouse liver mitochondria were isolated by a modification of the technique of Roodyn, Reis & Work (1961). After sacrificing the rat or mouse, the liver was removed rapidly and minced in 225mM sucrose, 75mM mannitol and 1mM EDTA at pH 7.4 for electron microscopy. For amino acid incorporation studies 0.3 M sucrose - 2mM EDTA, pH 7.2 was used. The mince was homogenized in 3 volumes of medium with 2-3 strokes of a Potter-Elvejhem homogenizer. The homogenate was centrifuged at 500xg for 10 minutes to remove nuclei and cell debris. The supernatant was decanted and centrifuged at 750xg for 10 min. Lipid was skimmed off before decanting any supernatant. The resulting supernatant was centrifuged at 8500xg for 10 min to yield a crude mitochondrial pellet. The mitochondria were

washed once for electron microscopy and three times for amino acid incorporation experiments.

Mouse L cell and human KB cell mitochondria were prepared by the following procedure. The cells ($5x10^7$-$5x10^8$ for enzyme assays and $5x10^8$-$1x10^9$ for protein synthesis) were harvested by sedimentation at 650xg for 10 min. After one wash in 0.3M sucrose—2mM EDTA—2mM Tris-HCl at pH 7.4, the cells were suspended in 10 ml of this medium and disrupted with an Ultra-Turrax homogenizer (Freeman, 1964). Disruption required homogenization for at least 30 sec at 75v and 15 sec at 80v followed by 30 sec at 75v to obtain 100% free nuclei. KCl (1M) was added to 0.02 or 0.05 M to cause nuclear contraction. The homogenate was centrifuged at 480 g for 10 min. The resulting supernatant was decanted and the nuclear pellet washed. Both the nuclear suspension and supernatant were then centrifuged at 480 g for 10 min. The supernatants obtained were combined and centrifuged at 5090 g for 15 min to yield a crude mitochondrial pellet. The mitochondria were washed once for enzyme assays and electron microscopy and twice for amino acid incorporation. The final suspension was usually in the sucrose medium described above.

Inner and outer mitochondrial membranes were prepared by the swelling, shrinking and sonication method described by Sottocasa, Kuylenstierna, Ernster and Bergstrand (1967). These fractions were used for freeze-etching and cleaving.

Protein Determination

Protein concentration in cytoplasmic-supernatants and isolated mitochondria was determined colorimetrically by a modification of the method of Lowry et al. (1951). The protein was dissolved in 0.1N NaOH at 50° for 2 min prior to the reaction. To a 1.0 ml sample, 5 ml of a freshly combined solution of 2% Na_2CO_3 and 0.02% NaK tartrate in 0.1N

NaOH: 0.5% $CuSO_4 \cdot 5H_2O$ (50:1) were added and rapidly mixed on a Vortex mixer. After 30 min at room temperature, 0.5 ml of a solution of the Folin-Ciocalteau reagent diluted 1:1 (u/v) was added rapidly and mixed immediately. The $\underline{E}_{660}$ is read after one hour at room temperature. Bovine serum albumin to 100 µg was used as a standard.

Amino Acid Incorporation by Isolated Mitochondria

The effect of chloramphenicol on protein synthesis by isolated mitochondria was studied using either the incubation medium of Haldar and Freeman (1969) or of T.S. Work (personal communication). The former medium contains 50mM KCl, 10mM succinate, 20mM potassium phosphate (pH 7.2), 1mM ADP, 5mM MgCl, C^{14}-leucine (2.5 µCi/ml) and other amino acids as a mixture (50 µg/ml), whereas Work's medium contains 90mM KCl, 10mM succinate, 16mM potassium phosphate (pH 7.2), 10mM ADP, 8mM $MgSO_4$, 1.3mM EDTA, 0.5mM NAD, 20mM nicotinamide, 35mM $(NH_4)_2$ SO_4, cytochrome $\underline{c}$ (0.2 mg/ml) bovine serum albumin (1 mg/ml), C^{14}-leucine (0.1 µCi/ml) and a mixture of other amino acids at 50 µg/ml. The mitochondria (2-3 mg protein/ml) were incubated for 20 min at 37° in a water bath shaker set at 80 cycles/min. The reaction was stopped by the addition of 3 ml of 5% trichloracetic acid containing non-radioactive leucine (1.6 mg/ml). In the case of mitochondria from tissue culture, less variation was obtained in zero time controls by adding 0.5 ml of 0.1% bovine serum albumin to each sample before precipitation. The samples were heated for 15 min at 90°, cooled and filtered in a Millipore unit, using 25 mm nitrocellulose filters with a 1.2 µ pore size. After two washes with 5 ml 5% trichloracetic acid and one with 1 ml water, the filters were glued to planchets and allowed to dry. They were counted on a Nuclear Chicago low background counter.

Enzyme Assays

All assays were performed at room temperature and activities were determined from the initial slope. Aldolase and phosphofructokinase were measured in the Gilford recording spectrophotometer in 1.0 ml cuvettes 1.0 cm path length. All other assays involving continuous measurement of an optical density change were run in a Cary model 14 spectrophotometer in 3.0 ml cuvettes. Assays were conducted on the first mitochondrial-free supernatant or on the once-washed mitochondrial fraction. Cytochrome c oxidase and ATPase activities were measured with intact mitochondria. Malate dehydrogenase, succinic dehydrogenase and NADH-cytochrome c reductase activities were measured with mitochondria disrupted with 0.2% triton X-100. Cytosol enzymes measured were malate dehydrogenase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, aldolase and phosphofructokinase.

Cytochrome c oxidase was assayed by following the oxidation of reduced cytochrome c at 550 nm. Horse heart cytochrome c either from Sigma Chemical Co. (type III, $550_{red}/280_{ox}$=1.36) or from Boehringer Mannheim further purified on Amberlite IRC-50 (Margoliash, 1954, $550_{red}/280_{ox}$=1.3) were used. The cytochrome c was dissolved in 0.03M ammonium acetate (pH 7.4) to 0.38 mg/ml and reduced with sodium dithionite (1.0 mg/ml). Nitrogen was bubbled through the cytochrome c solution to maintain it in the reduced state. Before each assay, 3.0 ml of the reduced cytochrome c was inverted in a cuvette until there was a drop in E_{550}. The time required for this was 30 sec to 1 min. Mitochondria at 1-2 mg protein/ml were then added to give a final concentration of approximately 32 µg protein/ml in the reaction mixture.

Dinitrophenol-stimulated ATPase activity was assayed by estimating colorimetrically the inorganic phosphate liberated from ATP (Myers and Slater, 1957). The incubation medium contained 75mM KCl, 1mM $MgCl_2$, 0.1mM

dinitrophenol, 0.05M sucrose, 0.05M Tris-HCl, pH 7.2 and 0.6mM EDTA and 0.2-0.3 mg mitochondrial protein per ml. The reaction was started by the addition of ATP to 2mM, in a final volume of 1.0 ml. After 15 min the reaction was stopped by the rapid addition of 0.2 ml of 30% trichloractic acid. The sample was centrifuged and inorganic phosphate was measured on 1.0 ml of the supernatant of the reaction and appropriate control by a modification of the method of Fiske and Subba Row (Sumner, 1944).

NADH cytochrome c reductase was assayed by following the reduction of cytochrome c at 550nm (Sottocasa, Kuylenstierna, Ernster and Bergstrand, 1967). Each reaction contained cytochrome c (1.3 mg/ml), 0.3mM KCN, 0.04M KCl, 0.03M phosphate buffer, pH 7.4 and mitochondrial protein (50 µg/ml). After a 2 min equilibration the reaction was started by the addition of NADH to 76 µg/ml in a final volume of 3.0 ml.

Succinic dehydrogenase was assayed by the method of Singer, as modified by Hauber (1967). The reaction mixture contained 1mM KCN, 13mM sodium succinate, 0.04M phosphate buffer, pH 7.4 and mitochondrial protein (0.1 mg/ml) in a final volume of 3.0 ml. After a 7 min equilibration the reaction was started by the rapid addition of 20 µl of 0.1M KCN, 0.1 ml of 0.05% (w/v) dichlorophenol indolephenol and 0.1 ml of 0.33% (w/v) phenazine methosulphate. The E_{600} was followed for 1 min.

Malate dehydrogenase activity was measured by following the reduction of NAD in the presence of malate at 340nm (Roodyn, Suttie & Work, 1962). Each reaction mixture contained 18mM sodium malate, NAD (0.45 mg/ml), 75mM glycine-NaOH buffer, pH 9.9 and the mitochondrial or mitochondrial-free supernatant fraction (approximately 33 µg protein/ml) in a final volume of 3.0 ml.

Lactate dehydrogenase activity was measured by following the oxidation

of NADH at 340nm in the presence of pyruvate, as described by Kubowitz and Ott (1943). Each cuvette contained 0.33mM sodium pyruvate, NADH (38.4 µg/ml), 33mM phosphate buffer, pH 7.4 and mitochondrial free supernatant (approximately 8 µg protein/ml) in a final volume of 3.0 ml.

Glucose-6-phosphate dehydrogenase activity was measured by following the reduction of NADP in the presence of glucose-6-phosphate at 340nm. (Warburg and Christian, 1936). Each reaction mixture contained 0.075mM NADP, 0.01M $MgCl_2$, 5mM N-Tris-HCl acid, pH 7.5 and mitochondrial-free supernatant (100 µg protein/ml). After a 6 min equilibration the reaction was started by adding glucose-6-phosphate to 4mM in a final volume of 2.0 ml.

Aldolase activity was assayed by the method of Racker (1947) in which the oxidation of NADH is followed at 340nm. In this method the products of aldolase action were coupled to the reduction of NAD with the enzyme trisephosphate isomerase and glycerophosphate dehydrogenase. Each cuvette contained in 1.0 ml, 1mM fructose diphosphate, glycero= phosphatedehydrogenase/triosphosphate isomerase (0.02 mg/ml), NADH (0.1 mg/ml), 0.04M triethanolamine, pH 7.5 and 0.01M EDTA. The reaction was started by the addition of 0.05ml of mitochondrial-free supernatant (1-2 mg protein/ml) and was followed for 10 min.

Phosphofructokinase activity was determined by coupling with aldolase, triose phosphate isomerase and α-glycerophosphate dehydrogenase as described by Racker (1947). Each reaction mixture contained 0.16mM NADH, 2mM ATP, pH 7, 5mM $MgSO_4$, 0.05M KCl, 33mM Tris-HCl, pH 8.0, 2mM fructose 6-phosphate, aldolase (0.03 mg/ml), and glycerophosphate dehydrogenase/ triosphosphate isomerase (0.007 mg/ml) in 1.0 ml. The reaction was started by the addition of 0.1 ml of mitochondrial-free supernatant (1-2 mg protein/ml) and was followed for 10 min.

Mitochondrial Cytochromes

Mitochondria were isolated from normal cells ($5x10^8$-$1x10^9$) and cells grown in D-chloramphenicol for 90 hr. The mitochondria were suspended to 4-5 mg of protein/ml with a small Potter Teflon homogenizer in 0.05M sucrose, 0.04M KCl, 0.01M $MgCl_2$ and 0.02M phosphate buffer, pH 7.5. The oxidized reference sample contained 0.01M dinitrophenol and the reduced sample 0.01M succinate or a few grains of sodium dithionite. The difference spectrum was examined with the Cary model 14 spectrophotometer using a high intensity light source.

Electron Microscopy

In preparation for electron microscopy the cells were harvested at 650 g for 10 min at 0°. The cells were washed once with 40 ml of PBS. Mitochondria were prepared as described earlier. All material was examined in a Phillips 300 electron microscope.

Negative Staining

At neutral pH, electron opaque stains such as ammonium molybdate and phosphotungstate have little affinity for proteins or lipids, so cellular organelles are unstained and remain transparent to electrons. This was employed to study the appearance of isolated mitochondria from control cells and cells grown in D-chloramphenicol.

A copper grid (300 mesh) coated with both formvar and carbon, was placed on a mixed drop of mitochondrial suspension and freshly made 2 1/2% ammonium molybdate (pH 7.4). After 30 sec the grid was lifted and excess suspension removed with filter paper. The grids were stored in grid boxes until observation.

Freeze-Etching and Cleaving

The method of freeze-etching was introduced by Steere (1957). It is fundamentally a physical preparation which involves six steps:

pretreatment of the specimen with a cryoprotective agent, rapid freezing, fracturing of the frozen specimen with a cooled knife, etching (freeze-drying) of the fractured surface, replication of this surface with a heavy metal and carbon, and cleaning of the replica. Unlike the usual sectioning techniques, the specimen can be treated in such a way that it remains viable and consequently the problem of artifacts is probably greatly reduced. In addition to the cross-sectional views obtained with the usual techniques, one also observes surface views of membranes and organelles.

L cells, isolated mitochondria from L cells and isolated inner and outer membranes of rat liver and L cells mitochondria were examined with the use of this technique. The cryoprotective agent employed was glycerol, added to 16-20%. The specimens were rapidly frozen in liquid freon 22 and stored in liquid nitrogen until cleaved, etched and replicated in a Balzers high vaccum unit. Platinum and carbon were used for replication. The replicas were cleaned with 70% H_2SO_4 and subsequently 5% hypochlorous acid (Javex), and washed with distilled water after each. The washed replicas were recovered with formvar-coated support grids and examined in the electron microscope.

Thin Sectioning

With this method the demonstration of cell structures depends on their chemical reaction with heavy metal compounds. After chemical fixation the specimens are dehydrated, embedded, thin-sectioned and stained for examination.

Cells which had been washed once in PBS were fixed with 2% osmic acid in PBS. After dehydration with alcohol and propylene oxide the specimens were embedded in epon. Thin-sectioning was carried out with a LKB microtome. The embedded tissue was recovered on copper mesh grids and stained with

uranyl acetate and lead citrate.

Chemicals

Amino acids were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Nucleotides were from C.F. Boehringer G.M.b.H., Mannheim Germany. NAD, NADH and NADP were obtained from Mann Research Laboratories Inc., New York, N.Y. All enzymes were from Sigma Chemical Co., St. Louis, Mo. C^{14} leucine (51 mCi:mmole) was obtained from The Radiochemical Centre, Amersham, Bucks. D-threo-chloramphenicol, its methylthio and methylsulfonyl analogues and the L-threo-isomer.were a gift from Dr. H.E. Machamer, Parke, Davis and Co., Ltd., Detroit, Michigan. The Tevenel was a gift from Dr. C.E. Hoffman, E.I. du Pont de Nemours and Co., Newark, Del. Cycloheximide (Actidione) was obtained from the Upjohn Company, Kalamazoo, Michigan. All other chemicals were reagent grade where possible. Joklik's modification of minimal essential medium (powder), fetal calf serum and anti-PPLO agent were obtained from Grand Island Biological Co., Grand Island, New York.

Chapter 2

Results

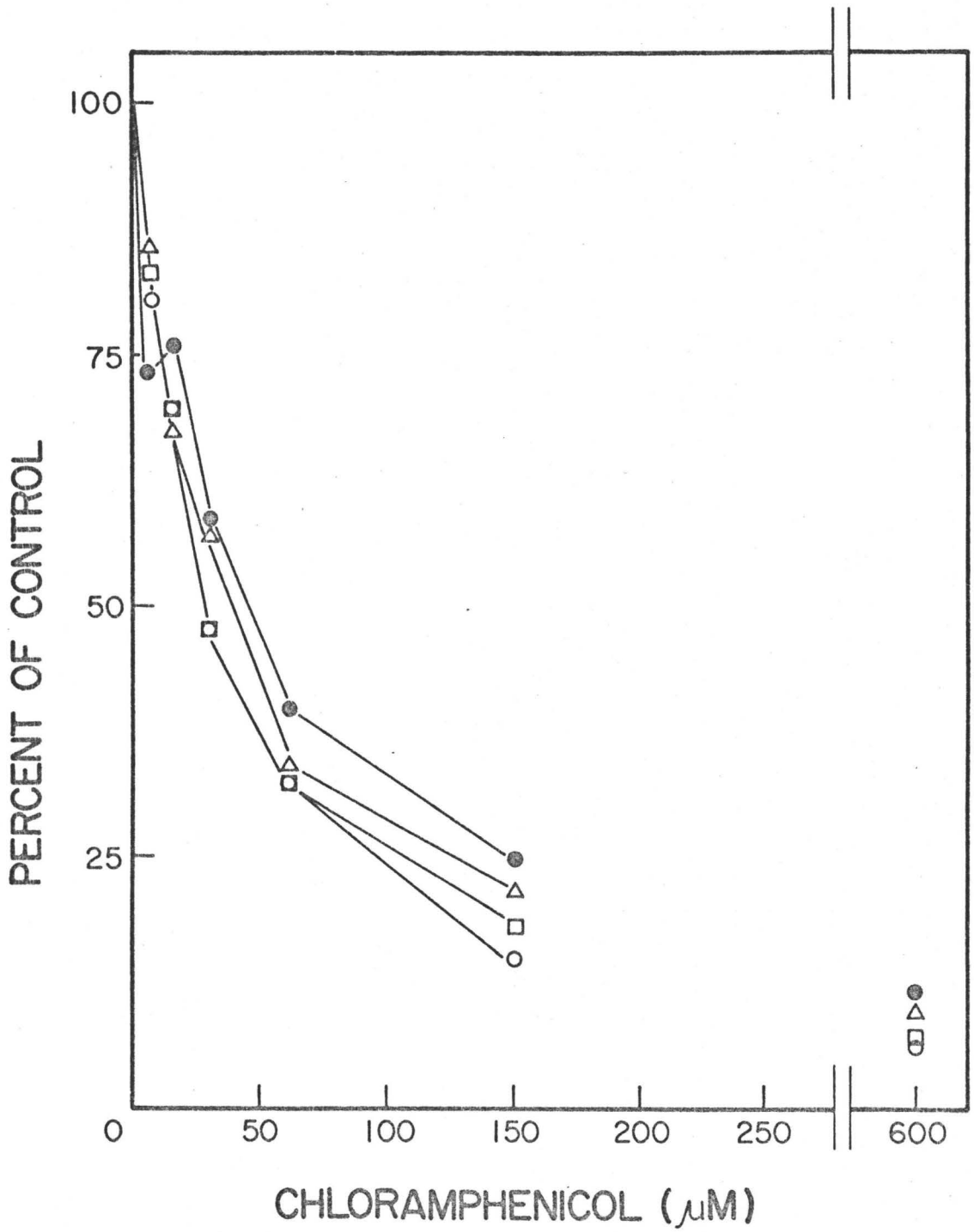
I Protein Synthesis in Isolated Mitochondria

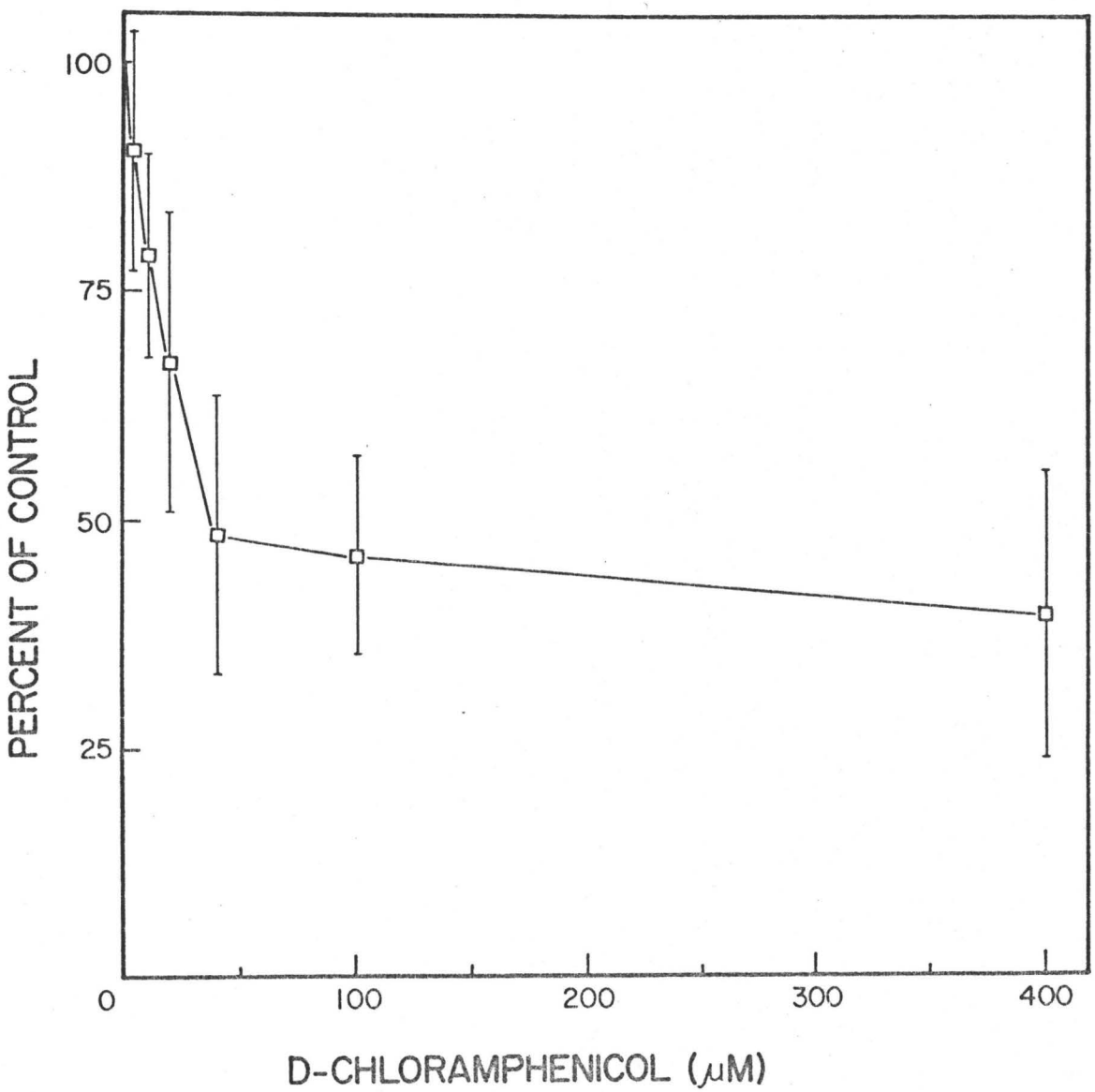
In order to understand the biogenesis of mitochondria it is necessary to know the cellular sites of synthesis of individual mitochondrial proteins. One approach is to selectively inhibit the synthesis of protein by mitochondria. If the appearance of a protein is inhibited by such treatment this indicates that it is in part at least synthesized in the mitochondrion or its synthesis or assembly is controlled by a product of mitochondrial protein synthesis. D-threo chloramphenicol is known to inhibit the incorporation of amino acids into protein by isolated mitochondria from yeast, mammalian liver and bone marrow cells. Using mouse liver as a standard the incorporation of C^{14}-leucine into protein in the presence of chloramphenicol was examined in isolated mitochondria from mouse L cells and human KB cells, both grown in tissue culture (Fig. 1, 2 and 3). The corresponding specific activities were 459, 401 and 218 counts/min/mg protein. Only the medium of Haldar and Freeman (1969) was used for incubating mitochondria from mouse liver and KB cells whereas both media were used for L cell mitochondria and the results averaged. In mouse liver mitochondria there was 50% inhibition at 28 μM D-chloramphenicol or the methylsulfonyl analogue, 39 μM Tevenel and 44 μM of the methylthio analogue. In isolated mitochondria from L cells and KB cells there was 50% inhibition at 38 and 90 μM D-chloramphenicol, respectively (38 μM = 12.3 μg/ml). The standard deviations indicate a large variation in the results obtained with mitochondria from the cells grown in tissue culture. Unlike mouse liver mitochondria (N=2) the

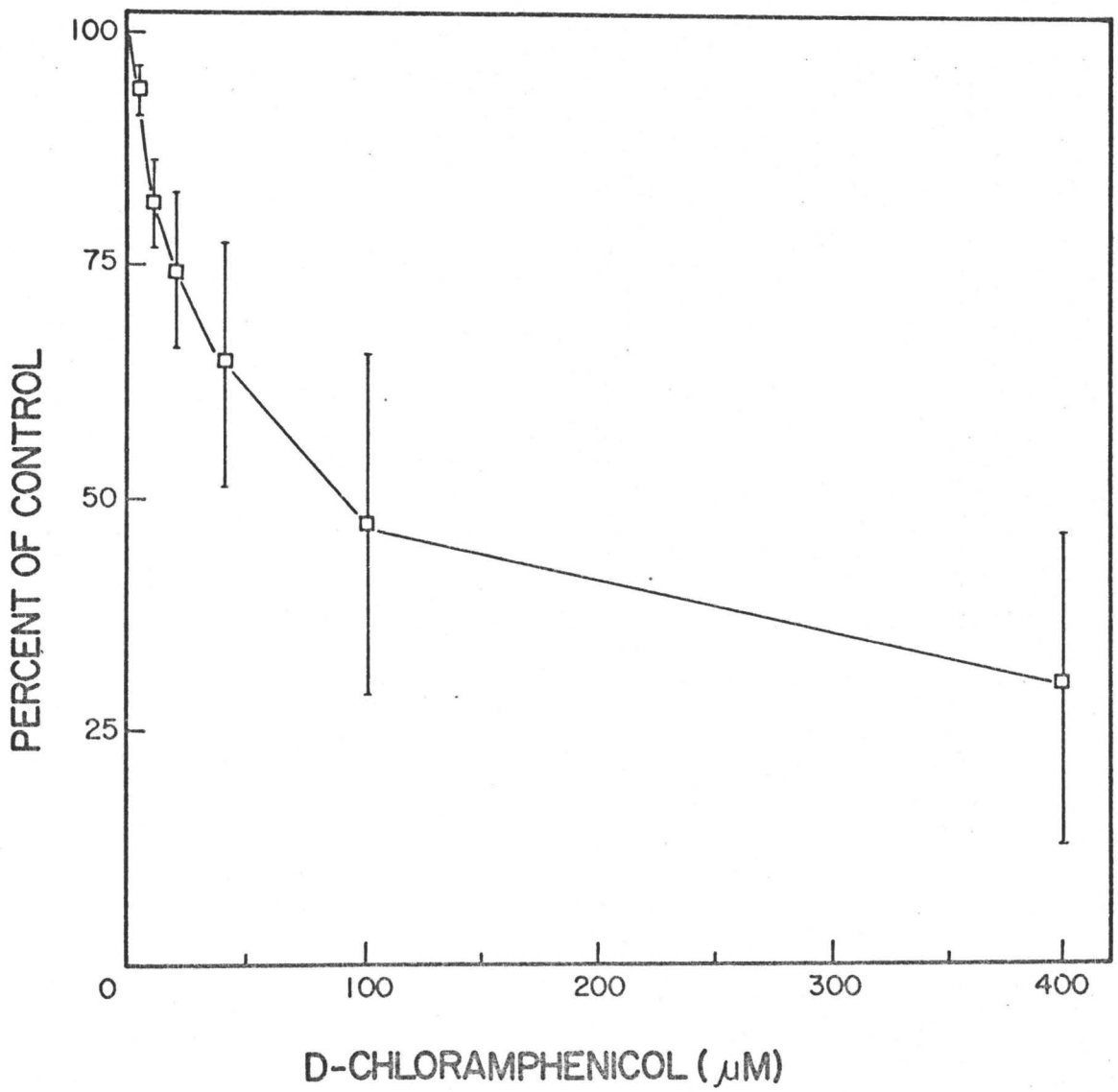
Figure 1. Effect of Chloramphenicol on Protein Synthesis by Isolated Mouse Liver Mitochondria. □, D-threo-Chloramphenicol; △, p-sulfamoyl analogue; ○, p-methylsulfonyl analogue; ●, p-methylthio analogue.

Figure 2. Effect of Chloramphenicol on Protein Synthesis by Mitochondria Isolated from L Cells.

Figure 3. Effect of Chloramphenicol on Protein Synthesis by Mitochondria Isolated from KB Cells.


100
75
50
25
0
50
100
150
200
250
600
PERCENT OF CONTROL
CHLORAMPHENICOL (μM)


100
75
50
25
0
100
200
300
400
PERCENT OF CONTROL
D-CHLORAMPHENICOL (μM)


100
75
50
25
0
PERCENT OF CONTROL
100
200
300
400
D-CHLORAMPHENICOL (μM)

inhibition of incorporation in mitochondria from KB cells (N=4) and L cells (N=8) tended to start leveling off after about 50% inhibition. Since the addition of cycloheximide to 1 x 10^{-6}M did not further decrease incorporation, the chloramphenicol-resistant incorporation cannot be accounted for by cytoplasmic contamination.

II Cells

In studying the biogenesis of mitochondria by specifically inhibiting mitochondrial protein synthesis in cells, it is important to know the overall effects of the inhibitors on cell growth.

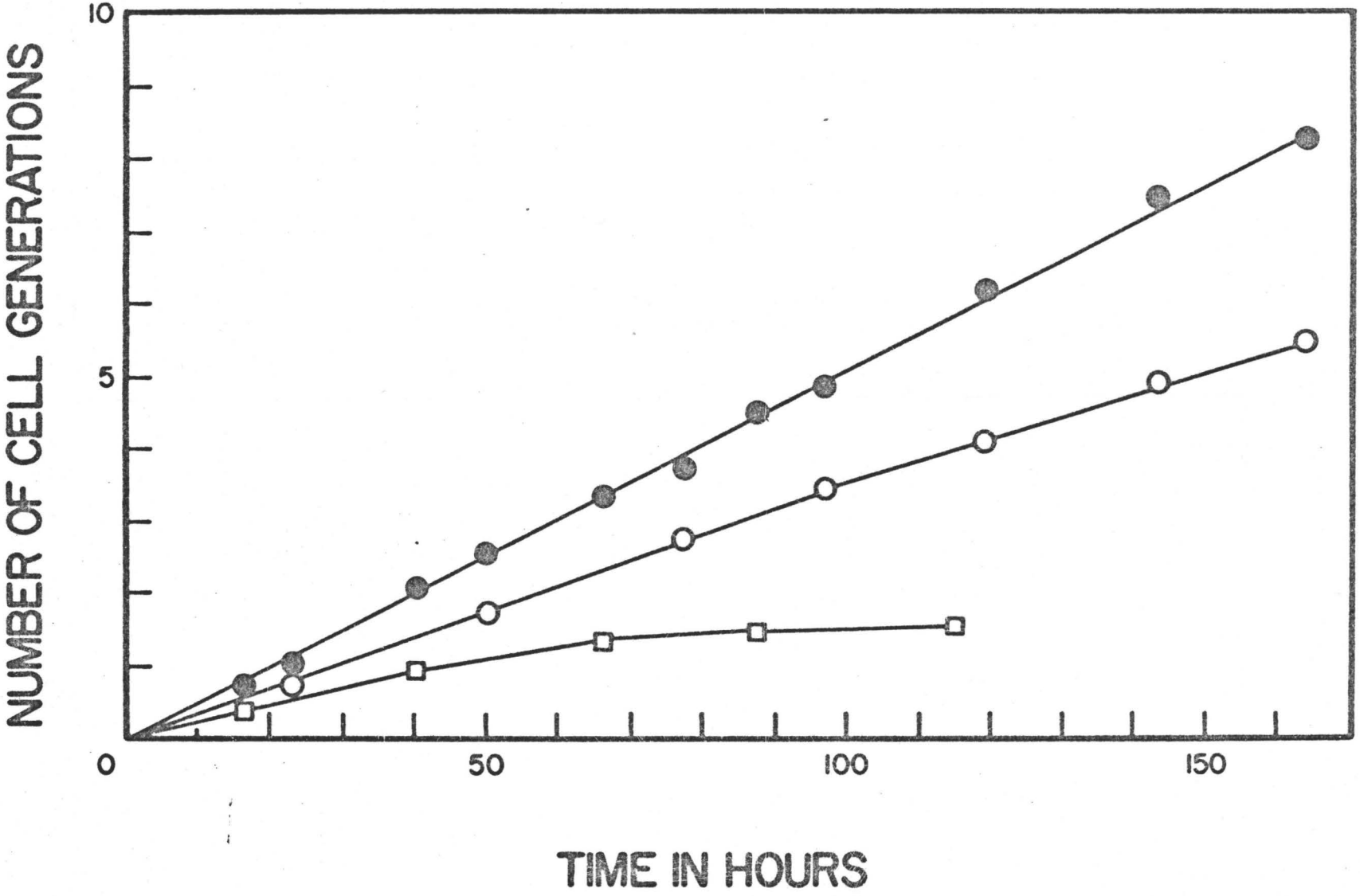
The experiments of Freeman (1970) on protein synthesis in mitochondria isolated from rat liver showed that 15 µM (4.8 µg/ml) D-chloramphenicol or Tevenel was sufficient to cause 50% inhibition of amino acid incorporation. L-chloramphenicol was found to have no effect on protein synthesis in rat liver mitochondria. In the experiments reported in the previous section 38 µM (12.3 µg/ml) D-chloramphenicol was required for 50% inhibition of amino acid incorporation into mitochondria isolated from L cells. It has also been shown (Freeman and Haldar, 1968; Freeman, 1970) that 1 mM (0.32 mg/ml) D- or L-chloramphenicol causes 50% inhibition of NADH oxidase activity in beef heart mitochondria whereas 10.6 mM (3.8 mg/ml) Tevenel was required to cause the same inhibition. It was thought then that D- or L-chloramphenicol at 0.1 mg/ml might cause some inhibition of NADH oxidase, but the same concentration of Tevenel would not. There should also be a complete inhibition of mitochondrial protein synthesis by D-chloramphenicol or Tevenel (but not by L-chloramphenicol) at this concentration. Thus by using all three inhibitors, it would be possible to distinguish between the two inhibitory effects of D-chloramphenicol. Another

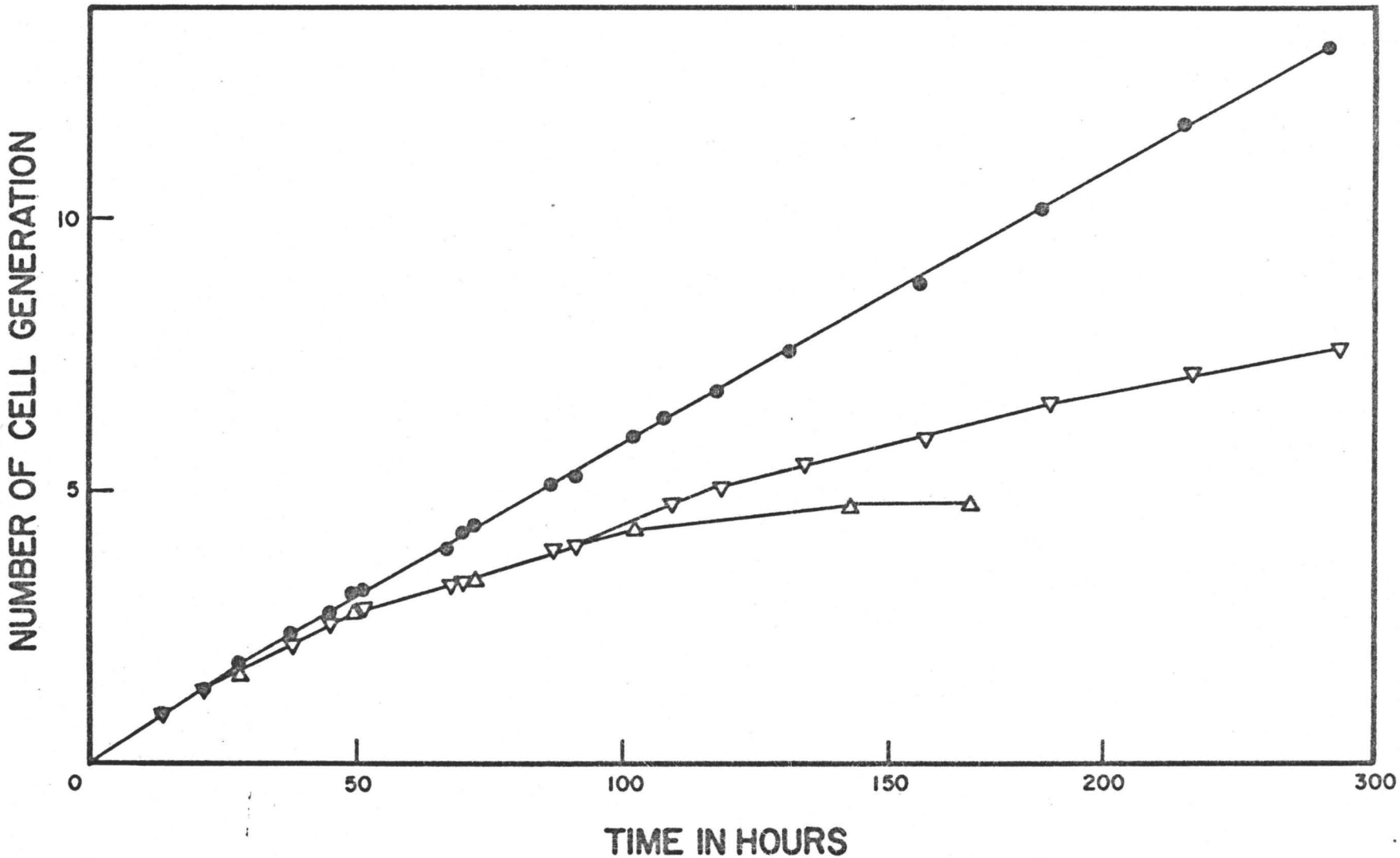
concentration of interest was 10 µg/ml since at such a concentration one would expect inhibition of mitochondrial protein synthesis by D-chloramphenicol (75% inhibition in rat liver mitochondria) and Tevenel but no effect on NADH oxidase by either D- or L-chloramphenicol or Tevenel. Furthermore this is the concentration of chloramphenicol which is often found in the serum of patients suffering side effects from chloramphenicol therapy. The effect of D-threo chloramphenicol and the L-threo isomer, both at 100 µg/ml, is shown in Fig. 4. The average standard deviations for the control, D-chloramphenicol and L-chloramphenicol treated cells were 0.1 ($N \geq 7$), 0.1 ($N \geq 3$) and 0.3 ($N=6$) generations respectively. If the cells which were growing in chloramphenicol were not diluted daily with fresh chloramphenicol-medium, they became acidic and there was a more pronounced inhibition of cell growth. It is apparent that D-chloramphenicol at this concentration was a strong inhibitor of cell growth. While the control continued to grow logarithmically the cells in D-chloramphenicol leveled off at 80 hr after having gone through 1.5 generations. In the presence of the L-isomer, cells grew indefinitely at about 66% of the control rate. It was found that the lower growth rate in the presence of L-chloramphenicol was not due to a reduction of the ATP level in these cells. An increase in generation time is usually due to an increase in the length of the G_1 period of the cell cycle (Johns, R.M., MSc. thesis, McMaster University, 1967) but it is not known if this is the case for the cells growing in L-chloramphenicol.

The effect of the sulfamoyl analogue (Tevenel) at 10 and 100 µg/ml is shown in Fig. 5. The average standard deviation of the number of cell generations was 0.2 ($N=7$) for cells grown in 100 µg/ml and 0.1 ($N \geq 4$) for those grown in 10 µg/ml Tevenel. Up to 90 hr they had a similar effect on growth but the cells in 100 µg/ml level off at 140 hr

Figure 4. Effect of D- and L-Chloramphenicol (100 µg/ml) on Growth of L Cells. ● , Control; ○ , L-chloramphenicol; □ , D-chloramphenicol.

Figure 5. Effect of Tevenel on Growth of L Cells. ▽ , 10 µg/ml; △ , 100 µg/ml.


NUMBER OF CELL GENERATIONS
10
5
0
50
100
150
TIME IN HOURS


NUMBER OF CELL GENERATION
10
5
0
50
100
150
200
300
TIME IN HOURS

after going through 4.75 generations whereas the cells in 10 µg/ml only began to level off at 11 days after going through 7.25 generations.

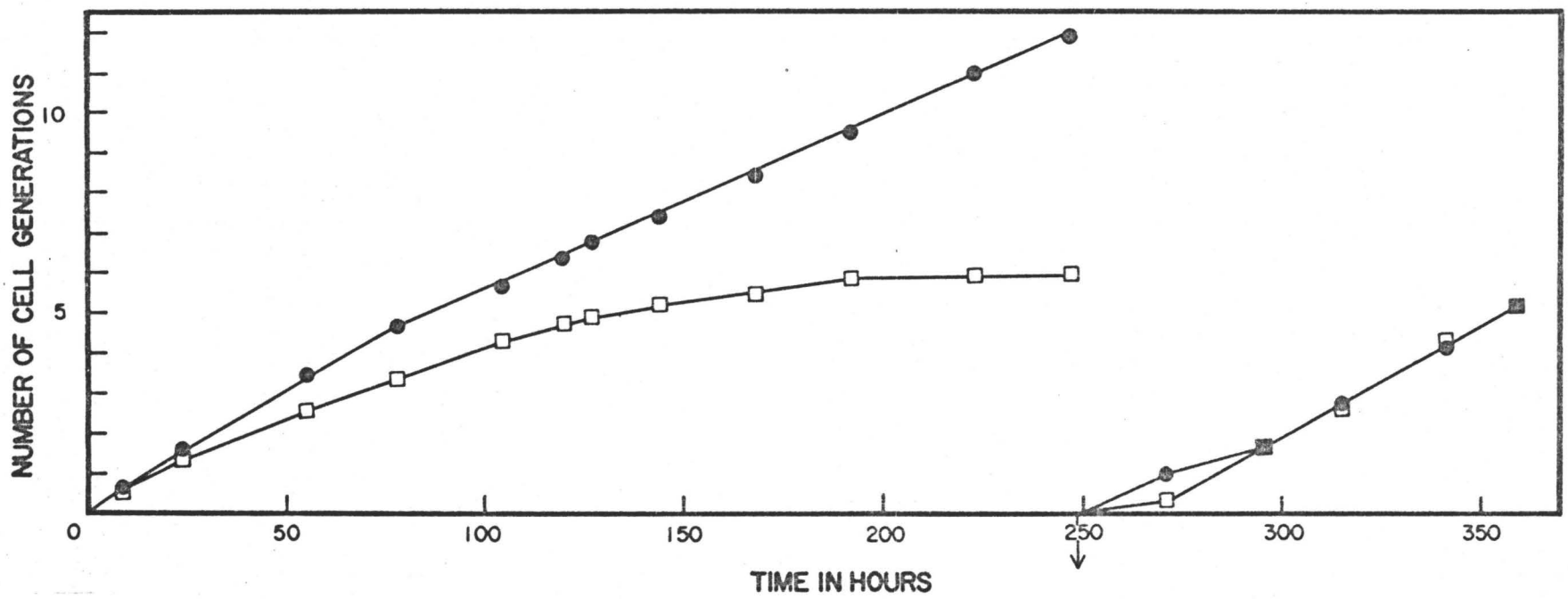
Since it is desirable to use the minimum inhibitory concentration, 10 µg/ml seemed a more reasonable concentration to use. In addition it was of interest to know if the cells could recover from the chloramphenicol treatment. Consequently a number of experiments were conducted in which the cells were grown in D-chloramphenicol (Fig. 6, 7 and 8), L-chloramphenicol (Fig. 7) and Tevenel (Fig. 8) each at 10 µg/ml for various periods of time and then resuspended in ordinary medium to follow recovery. The average standard deviation was 0.1 ($N \geq 4$) for cells grown in D-chloramphenicol and 0.2 ($N \geq 4$) for cells in L-chloramphenicol. L-chloramphenicol at 10 µg/ml did not have an inhibitory effect on cell growth, indicating that the reduced rate caused by D-chloramphenicol was specifically due to an inhibition of mitochondrial protein synthesis. Even after 10 or 11 days of treatment it was found that cells grown in D-chloramphenicol or Tevenel at 10 µg/ml could recover the normal growth rate. Although the growth of cells in 10 and 100 µg of Tevenel/ml was similarly inhibited, it obviously takes cells grown in the higher concentration a longer time to recover. The presence of 10^{-6}M cycloheximide prevented the recovery of D-chloramphenicol treated cells and had a reversible inhibitory effect on the growth of otherwise untreated cells (control cells).

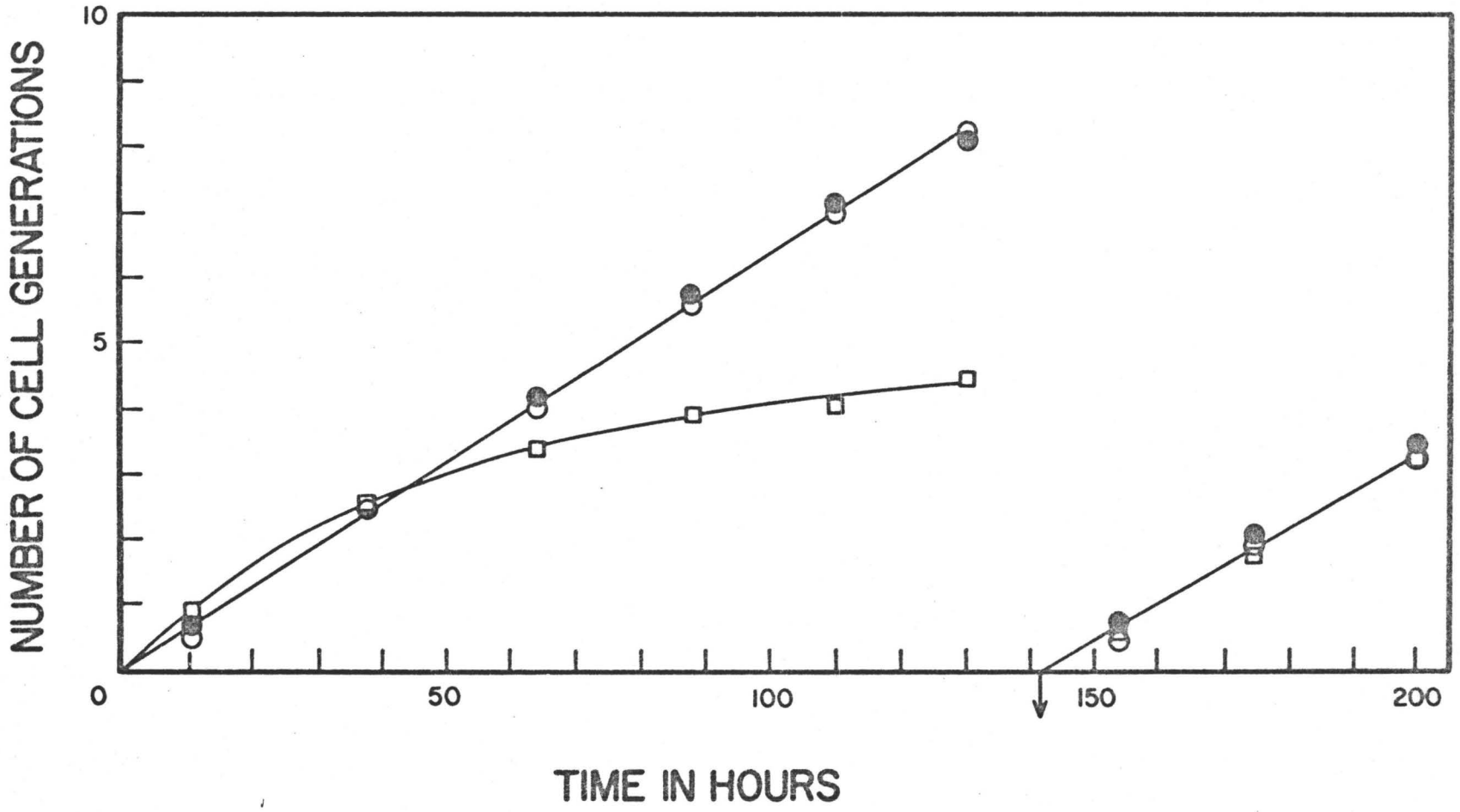
To determine the percentage of cells that were viable and hence could recover from a prolonged chloramphenicol treatment, the cells were examined for the ability to exclude the stain eosin Y. After 135 hr the percentage of viable cells in the presence of ordinary medium, L-CAP (10 µg/ml) or Tevenel (10 µg/ml) was 99%. The viability of cells grown in D-chloramphenicol (10 µg/ml) was 95%.

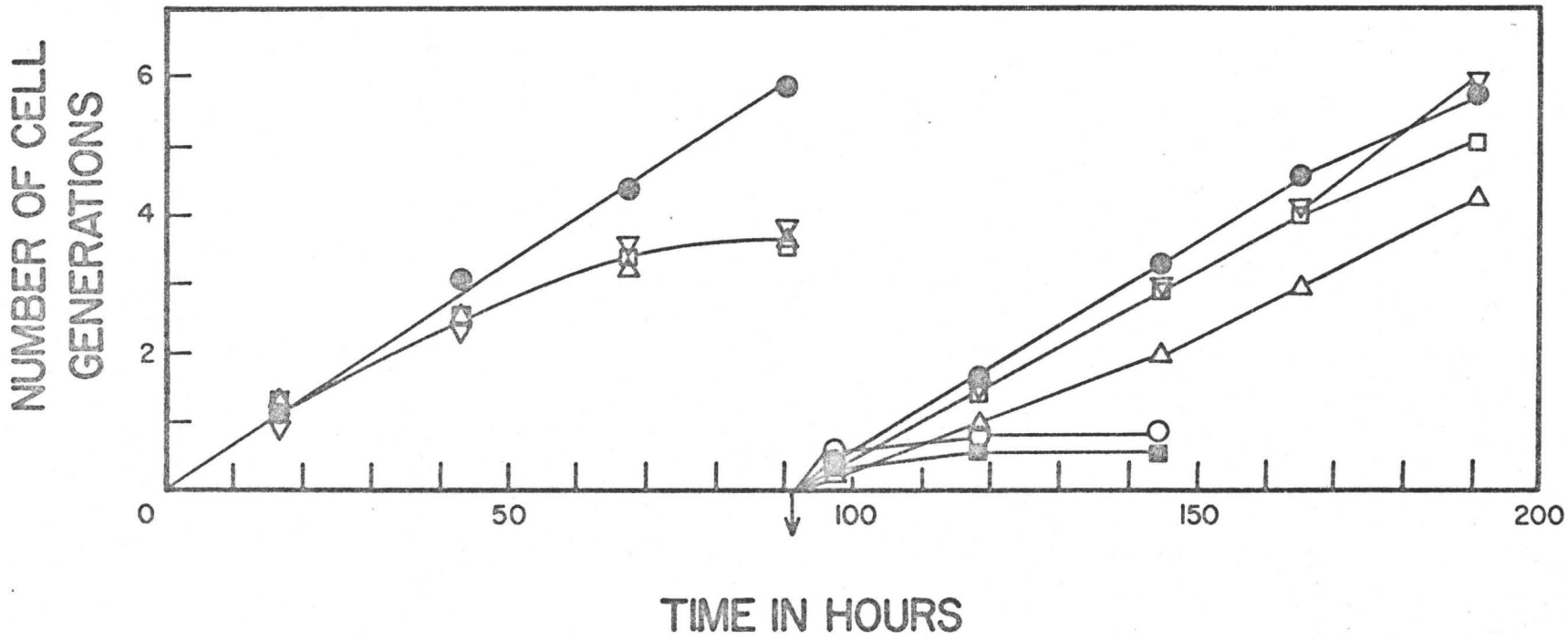
Figure 6. Inhibition and Recovery of Growth of L Cells in D-Chloramphenicol (10 µg/ml). The arrow indicates the change to ordinary medium. ●, Control; □, D-chloramphenicol.

Figure 7. Growth of Cells in L-Chloramphenicol (10 µg/ml), Inhibition of Growth in D-Chloramphenicol (10 µg/ml) and Recovery after its Removal. The arrow indicates the change to ordinary medium. ●, Control; ○, L-chloramphenicol; □, D-chloramphenicol.

Figure 8. Inhibition of Growth of L Cells in D-Chloramphenicol (10 µg/ml), Tevenel (10 and 100 µg/ml) and Cycloheximide. The arrow indicates the change to ordinary medium or medium containing cycloheximide. ●, Control; □, D-chloramphenicol; ■, 1 x 10^{-6}M cycloheximide after 90 hr D-chloramphenciol; ○, 1 x 10^{-6}M cycloheximide; ▽, Tevenel at 10 µg/ml; △, Tevenel at 100 µg/ml.


NUMBER OF CELL GENERATIONS
10
5
0
50
100
150
200
250
300
350
TIME IN HOURS


NUMBER OF CELL GENERATIONS
10
5
0
50
100
150
200
TIME IN HOURS


NUMBER OF CELL GENERATIONS
6
4
2
0
50
100
150
200
TIME IN HOURS

It was possible that the reduced growth rate in the presence of D-chloramphenicol could be due to a decrease in the level of ATP. This did not appear to be the case. In cells grown in D-chloramphenicol (10 µg/ml) for 90-94 hr, the level of ATP was 76.6 ± 9.7 mµmoles/10^7 cells compared with 74.2 ± 9.0 in the control (N=3 for both).

III Enzymes

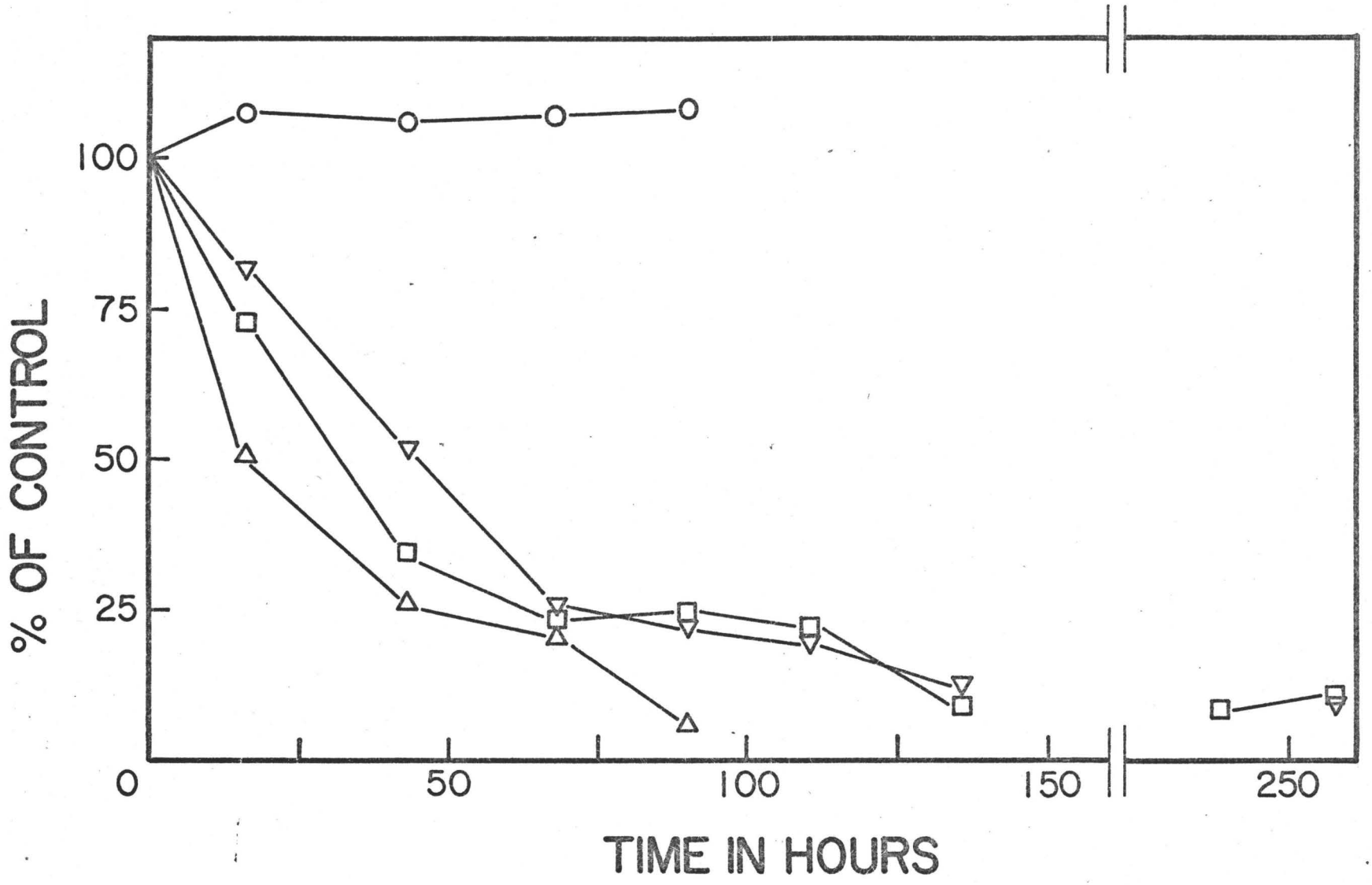
The next step was to examine the effect of chloramphenicol on the syntheses of individual mitochondrial proteins. There are reports of decreased cytochrome c oxidase activity in yeast in the presence of chloramphenciol. The effect on cytochrome oxidase activity in L cells grown in D-chloramphenicol, L-chloramphenicol and Tevenel at 10 µg/ml and Tevenel at 100 µg/ml is shown in Fig. 9. The results are expressed as percent of control specific activity, which is 1.56 ± 0.22 units where a unit is defined as a chnage of one E_{550} unit per mg protein.

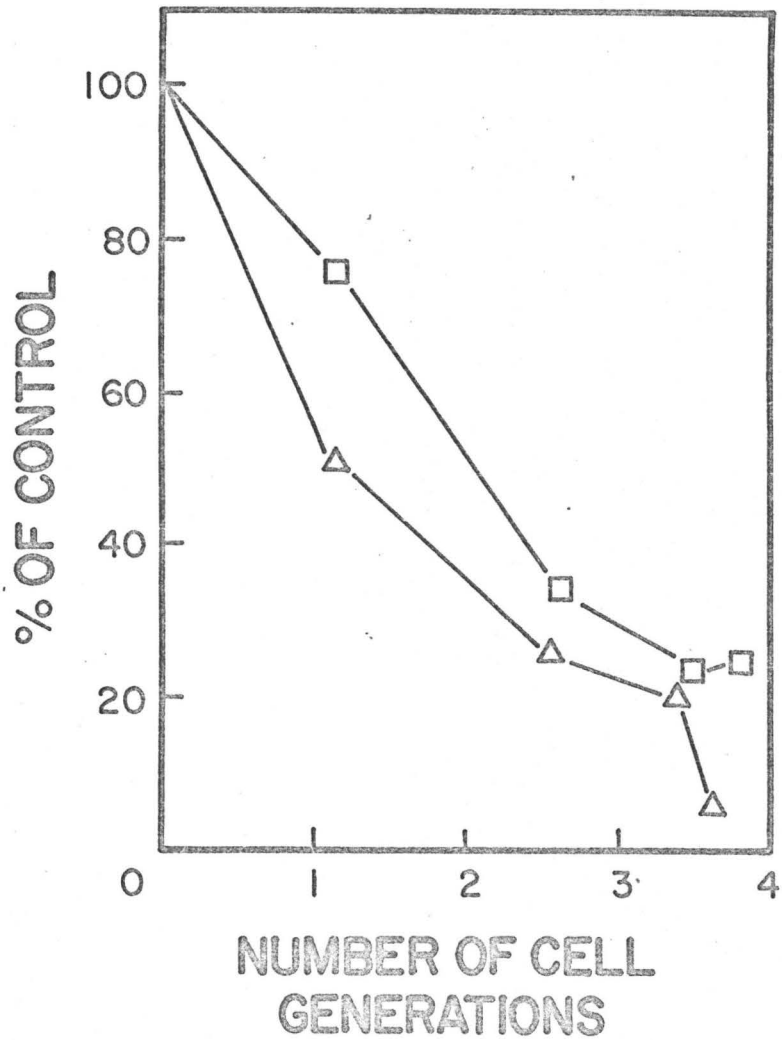
Although the results were expressed in terms of specific activity rather than total activity the former should approximate the latter since the presence of chloramphenicol (10 µg/ml) did not affect the total amount of mitochondrial protein after a 90 hr treatment. Nor was there a change in cyanide sensitivity of cytochrome oxidase at this time; 1 x 10^{-5}M potassium cyanide (freshly prepared) completely inhibited cytochrome oxidase activity in isolated mitochondria from both the control and D-chloramphenicol treated cells.

From the results represented in Fig. 7 it was apparent that L-chloramphenicol had no effect on cell growth. In Fig. 9 one sees that the level of cytochrome oxidase in L-chloramphenicol treated cells was slightly higher than the control values. The variation in the control is 1-4%.

Figure 9. Effect of D- and L-Chloramphenicol (10 μg/ml) and Tevenel (10 and 100 μg/ml) on the Level of Cytochrome Oxidase in Mitochondria from L Cells as a Function of Time. ○, L-Chloramphenicol; □, D-chloramphenicol; ▽, Tevenel at 10 μg/ml; △, Tevenel at 100 μg/ml.

Figure 10. Effect of D-Chloramphenicol (10 μg/ml) and Tevenel (100 μg/ml) on the Level of Cytochrome Oxidase as a Function of the Number of Cell Generations. □, D-Chloramphenicol; △, Tevenel.

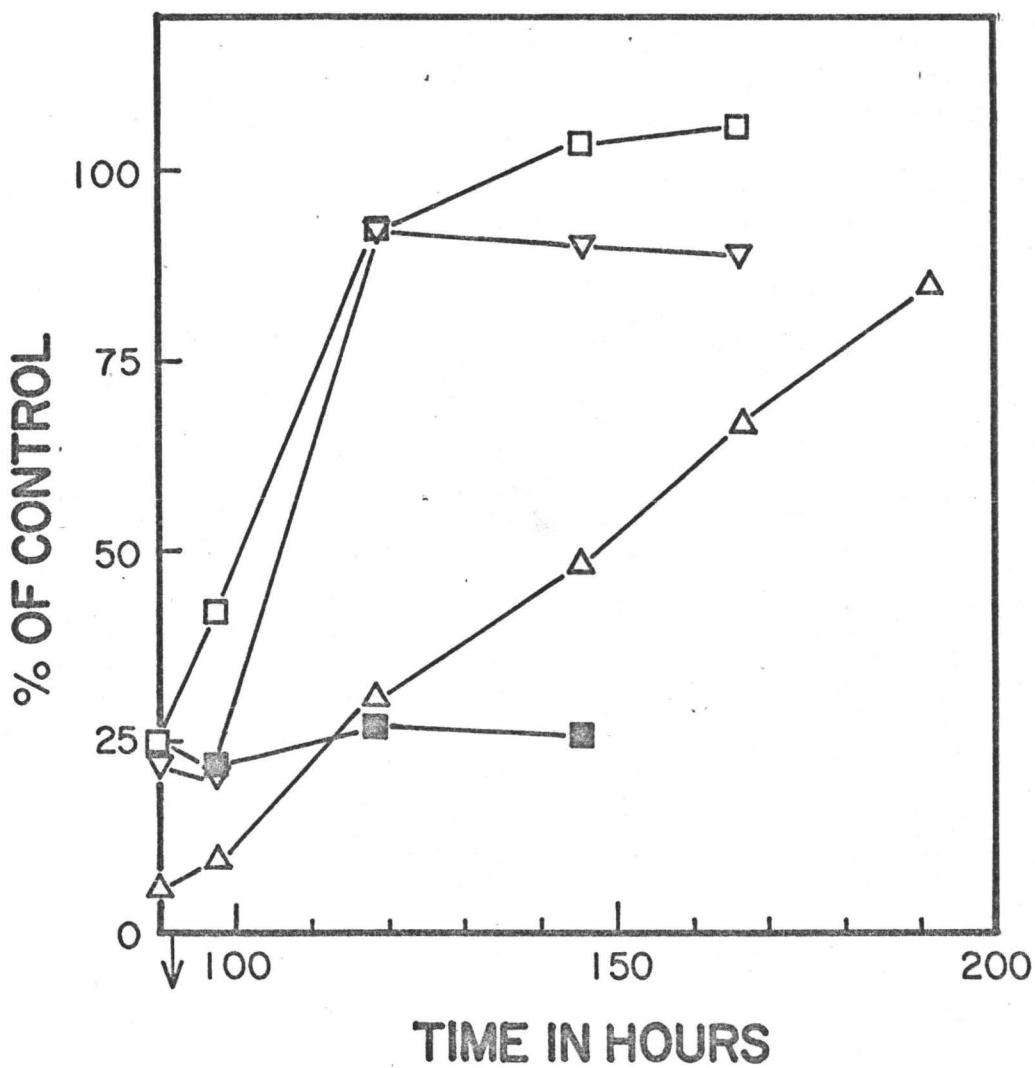

% OF CONTROL
100
75
50
25
0
50
100
150
250
TIME IN HOURS


100
80
60
40
20
0
1
2
3
4
% OF CONTROL
NUMBER OF CELL GENERATIONS

Although D-chloramphenicol at 10 µg/ml and Tevenel at 10 and 100 µg/ml had a similar effect on growth up to 90 hr, they differed in their effect on cytochrome oxidase. There was a gradual decrease in cytochrome oxidase activity with time (Fig. 9) and the corresponding increase in the number of cell generations (Fig. 10). However the cytochrome oxidase activity leveled off at about 25% after 68 hr in the presence of D-chloramphenicol at 10 µg/ml while it continues to drop to 6% by 90 hr in the presence of Tevenel at 100 µg/ml. The plateau region in the presence of D-chloramphenicol and Tevenel (10 µg/ml) was followed by a further decrease to 10% by about 135 hr (4.5 generations), followed by another plateau which continued at least until the 10th day of treatment (6 generations).

The decrease in the level of cytochrome oxidase in the presence of D-chloramphenicol and Tevenel indicated a definite mitochondrial role in the synthesis and/or assembly of cytochrome oxidase. The next problem was to determine whether the inhibition was reversible under conditions of normal and reduced synthesis of proteins in the cytoplasm. If the cells could recover in ordinary medium then prevention of recovery in the presence of an inhibitor of cytoplasmic protein synthesis in the medium would indicate a nuclear role in the synthesis of cytochrome oxidase. The recovery of cytochrome oxidase activity in mitochondria from cells grown for 90 hr in D-chloramphenicol (10 µg/ml) and Tevenel (10 and 100 µg/ml) is shown in Fig. 11. The level of cytochrome oxidase in cells treated with D-chloramphenicol and then transfered to ordinary medium increased to 42% of the control value after 6 hr and the recovery was virtually complete by 27 hr (1.7 generations). In the presence of 1×10^{-6}M cycloheximide there was no recovery of cytochrome oxidase activity.

Figure 11. Recovery of Cytochrome Oxidase Activity after Removal of D-Chloramphenicol (10 µg/ml) and Tevenel (10 and 100 µg/ml) after a 90 hr Treatment. The arrow indicates the change in medium. □, ordinary medium after D-Chloramphenicol; ■, 1 x 10^{-6}M cycloheximide after 90 hr D-chloramphenicol; ▽, ordinary medium after Tevenel at 10 µg/ml; △, ordinary medium after Tevenel at 100 µg/ml.


100
75
50
25
0
% OF CONTROL
100
150
200
TIME IN HOURS

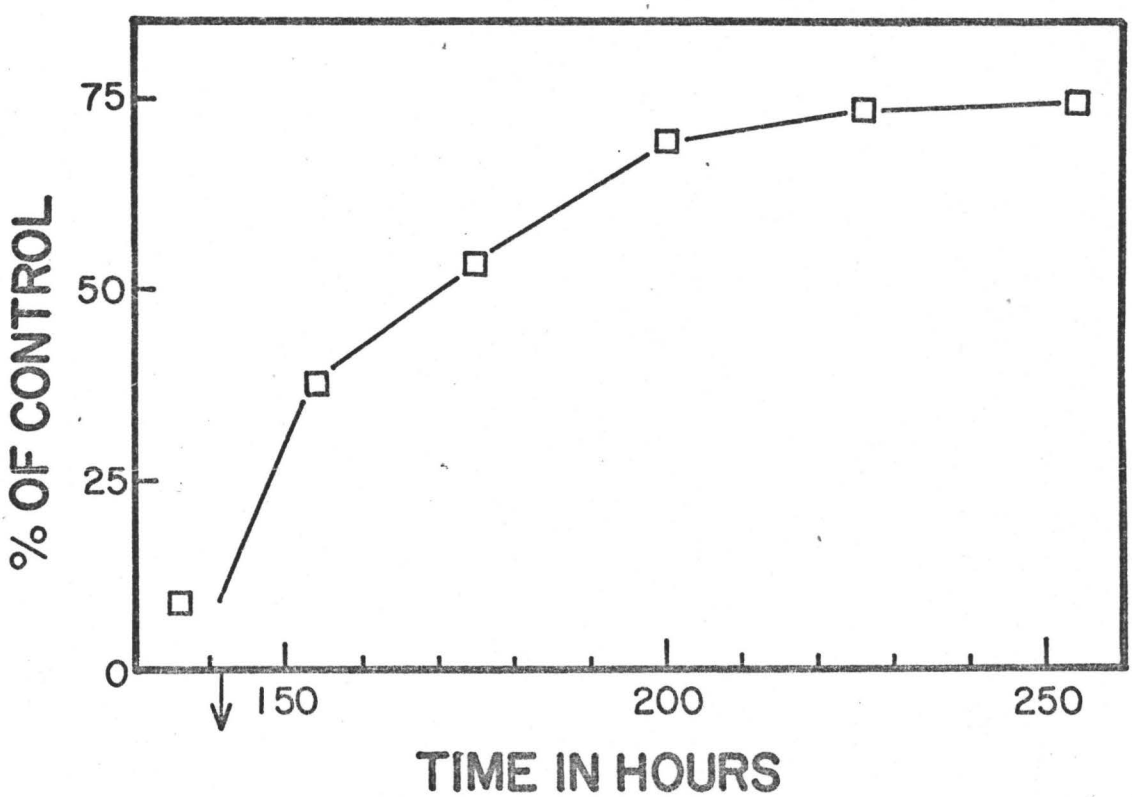
Recovery of cytochrome oxidase activity in motochondria of cells treated with Tevenel at 10 µg/ml was also almost complete at 27 hr (1.5 generations) whereas the level of cytochrome oxidase in cells treated with Tevenel at 100 µg/ml is only 31% of the control at this time (1 generation). After 100 hr (4.3 generations) in ordinary medium the level of cytochrome oxidase in the Tevenel (100 µg/ml) treated cells had increased to 85% of the control.

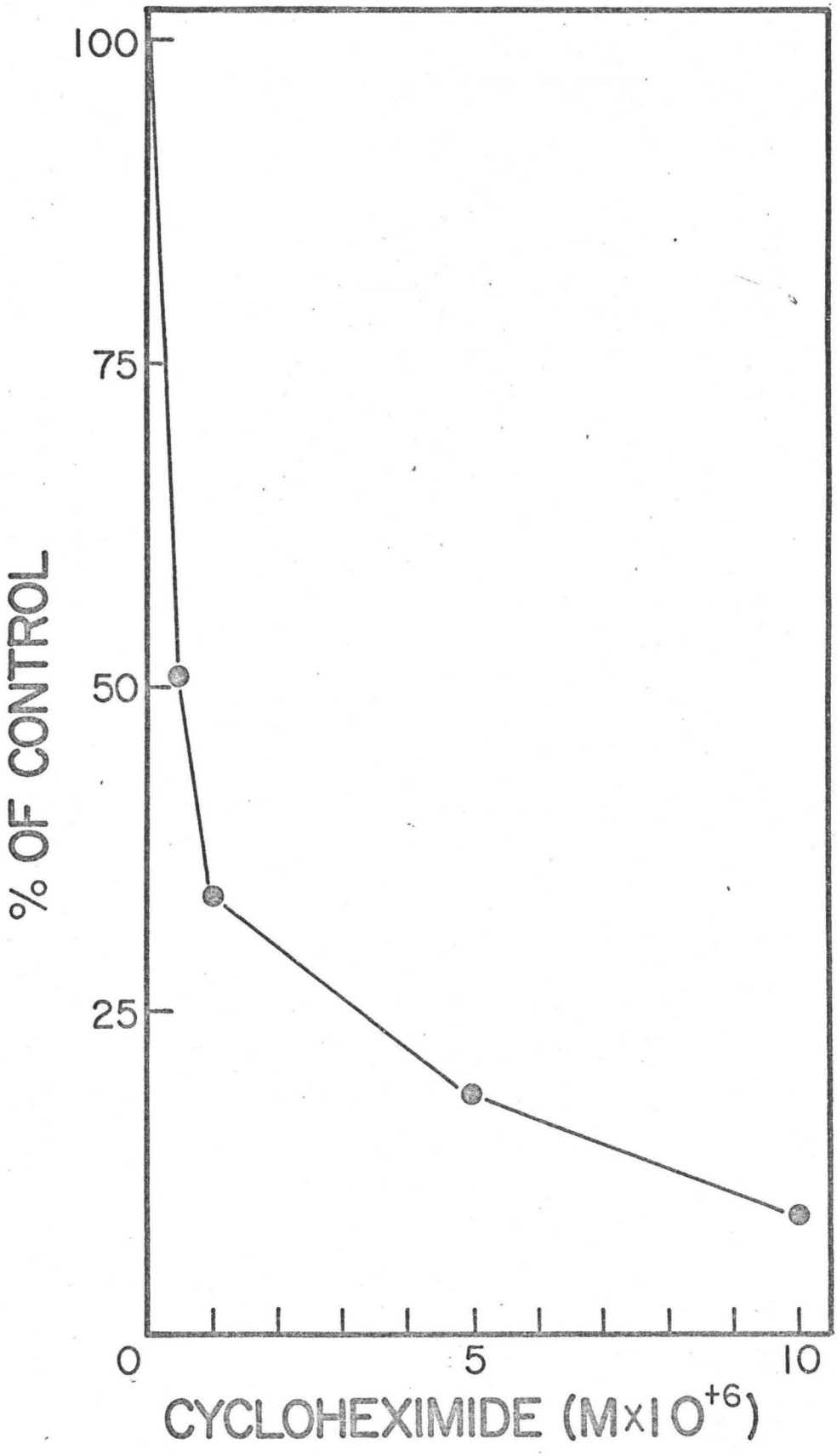
The recovery of cytochrome oxidase activity in mitochondria from cells treated with D-chloramphenicol for a longer period of time (140 hr) is shown in Fig. 12. After a longer period of inhibition it appeared to take a longer time to recover and as in this case (1 expt.) recovery may not be complete.

Since cycloheximide at 1×10^{-6}M prevented recovery of cytochrome oxidase activity in D-chloramphenicol treated cells, some synthesis of protein in the cytoplasm must be required for the production of a functional cytochrome oxidase enzyme. To obtain some idea of the importance of this it was necessary to investigate the effect of various concentrations of cycloheximide on cellular protein synthesis and cytochrome oxidase activity. The effect on cellular protein synthesis was examined by following the incorporation of C^{14}-leucine after a 20 min incubation in the presence of cycloheximide. The results are shown in Fig. 13. It is apparent that 1×10^{-6}M cycloheximide, which was sufficient to prevent recovery of cytochrome oxidase activity in D-chloramphenicol treated cells, caused about 66% inhibition of cellular protein synthesis. The concentration of cycloheximide does not consistently inhibit the synthesis of cytochrome oxidase in cells not treated with chloramphenicol. In four experiments the specific activity of cytochrome oxidase in mitochondria from cells treated with 1×10^{-6}M cycloheximide for 27 hr ranged from 36.6 to 100% of the control, with the average being 63.8.

Figure 12. Recovery of Cytochrome oxidase Activity after Removal of D-Chloramphenicol (10 μg/ml). The arrow indicates the change to ordinary medium.

Figure 13. Effect of Cycloheximide on Protein Synthesis in L Cells.


75
50
25
0
% OF CONTROL
150
200
250
TIME IN HOURS


100
75
50
25
0
5
10
% OF CONTROL
CYCLOHEXIMIDE (M×10+6)

There was no effect on mitochondrial malate dehydrogenase at this concentration (101.6% of control value). The level of cytoplasmic malate dehydrogenase was 75.6 ± 3.1% of the control value. At a much higher concentration of cycloheximide (1×10^{-5}M) a consistent inhibition of cytochrome oxidase activity (35.0% of control value after 36 hr treatment) but no change in mitochondrial malate dehydrogenase (99.0% of control). This inhibition was found to be reversible (75% of control after 143 hr recovery). In an experiment in which cells were grown for 12 hr in the presence of an intermediate concentration of cycloheximide, 5×10^{-6}M, the values obtained for cytochrome oxidase and mitochondrial and cytoplasmic malate dehydrogenase were respectively 85.4, 114.7 and 79.9% of the control. After a 2 1/2 hr recovery period both the cytochrome oxidase and cytoplasmic malate dehydrogenase values overshot the control value to 145.8 and 130.2% respectively. If however Tevenel at 100 µg/ml was present during the recovery period the value for cytochrome oxidase went down to 44.8 but the cytoplasmic malate dehydrogenase was relatively unchanged (74.4%).

In the cycloheximide experiments, which were relatively short term, there was no decrease in the specific activity of mitochondrial malate dehydrogenase. Failure to observe a decrease in the specific activity however, does not indicate that synthesis of the enzyme was not inhibited since it is likely that cycloheximide would inhibit the synthesis of the bulk of the mitochondrial proteins, leaving the specific activities unchanged. The levels of malate dehydrogenase in the longer term chloramphenicol experiments are given in Table I. In the presence of D-chloramphenicol there may be no inhibition or a slight inhibition. In the presence of Tevenel at 10 or 100 µg/ml there was a decrease in mitochondrial malate dehydrogenase after 90 hr of treatment. Although cycloheximide had an inhibitory effect on

Table I

Percent of Control Specific Activity during 90 hr. Treatment with Chloramphenicol

Enzyme	16 1/2 hr			43 hr			68 hr			90 hr		
	D-CAP† (10)	Tev† (10)	Tev (100)	D-CAP (10)	Tev (10)	Tev (100)	D-CAP (10)	Tev (10)	Tev (100)	D-CAP (10)	Tev (10)	Tev (100)
Cytochrome c oxidase	72.8 ±27.3 N=4	81.7 ±12.6 N=3	51.1 ±19.2 N=3	34.5 ±9.4 N=4	51.6 ±7.5 N=3	26.1	23.6 ±5.5 N=3	25.9 ±3.4 N=3	20.8	25.0 ±7.0 N=7	22.3 ±5.1 N=4	6.1
Succinic dehydrogenase				96.5*						107.9*		
NADH dehydrogenase	80.2	109.6*	78.5	89.0	91.0	86.0	98.0	96.7	98.5	97.5		
ATPase				75.6*						68.9 ±2.2 N=3		
malate dehydrogenase (mitochondrial)	97.6 ±34.3 N=3	95.0	96.1*	80.7	82.7*	87.3	83.0 ±16.1 N=3	95.8	95.0	85.8 ±7.4 N=5	84.7	64.2
malate dehydrogenase (cytosol)	104.0 ±9.2 N=3	91.2	124.4	108.8 ±16.0 N=4	113.8	103.1	116.0	123.2	128.0*	103.4 ±10.0 N=6	123.7	103.7
lactate dehydrogenase	99.4 ±5.2 N=3	105.1	102.7	93.3 ±17.7 N=3	102.3 ±8.3 N=3	103.3*	116.5	106.6	113.4*	75.8 ±8.5 N=5	96.6	83.4
glucose-6-phosphate dehydrogenase	96.9 ±7.0 N=3	99.0	99.5	98.0 ±3.2 N=3	91.0	101.5	102.9	108.3	109.7*	85.1 ±12.8 N=5	103.7	91.0
aldolase										75.4		
phosphofructokinase										129.8*		

* These values indicate 1 experiment. † CAP - Chloramphenicol; † Tev-Tevenel

the synthesis of cytoplasmic malate dehydrogenase, D-chloramphenicol and Tevenel did not.

The percent of control specific activity of other enzymes examined are also given in Table I. There was no decrease in the level of the mitochondrial enzymes succinic dehydrogenase and NADH cytochrome c reductase but there was about 30% decrease in the ATPase activity after a 90 hr treatment. The NADH cytochrome c reductase enzyme studied was found to be insensitive to rotenone in the concentration range 2-15 µg/ml even after purification of the mitochondria (from the control) in an isopycnic gradient. Part of the insensitivity may be due to the presence of triton since it was found that rotenone at 5 µg/ml caused 94% inhibition of activity in frozen-thawed beef heart mitochondria which were not disrupted with triton and only 34% inhibition in those which were disrupted with triton.

There appeared to be a slight decrease in the levels of lactate dehydrogenase and glucose-6-phosphate dehydrogenase after 90 hr in the presence of D-chloramphenicol. There was also a decrease in aldolase activity at this time. The only enzyme in which there was an apparent increase after D-chloramphenicol treatment was phosphofructokinase. The value given is a minimal percentage since it was found that the enzyme was unstable in extracts of D-chloramphenicol treated cells. One hr after separation of the mitochondrial-free supernatant the phosphofructokinase activity had decreased to 46.7% of its original value whereas the control remained unchanged.

Upon removal of D-chloramphenicol and Tevenel at 10 µg/ml one sees from Fig. 11 and in Table II that after 27 hr, recovery of cytochrome oxidase activity was virtually complete. The level of activity of the cytoplasmic enzymes examined (malate dehydrogenase, lactate dehydrogenase and glucose-6-

Table II

Percent of Control Specific Activity during Recovery from 90 hr. Treatment with Chloramphenicol

Enzyme	27 hr				54 hr				75 hr*			100 hr*		
	†D-CAP (10)	D-CAP †Cyclo	†Tev (10)	Tev (100)	D-CAP (10)	D-CAP Cyclo	Tev (10)	Tev (100)	D-CAP (10)	Tev (10)	Tev (100)	D-CAP (10)	Tev (10)	Tev (100)
cytochrome c oxidase	92.2 ±27.4 N=4	27.6 ±7.9 N=4	92.2*	47.3	103.5 ±19.1 N=3	26.1	90.6*	48.5*	105.5	88.9	66.8	113.6	89.2	85.0
malate dehydrogenase (mitochondrial)	110.6 ±17.0 N=4	92.9	138.5*	125.7*	116.6	76.5*	97.1*	-	84.7	68.2	76.9			
malate dehydrogenase (cytosol)	98.9	113.1 ±15.4 N=3	91.4*	95.4*	113.2	140.8	108.7*	133.3*	103.9	144.2	133.9	113.7	121.7	133.1
lactate dehydrogenase	94.9 ±17.9 N=3	75.2 ±13.9 N=3	100.9*	102.7*	120.9	98.5	83.1*	142.3*	118.8	121.0	143.6	102.7	100.5	111.0
glucose-6-phosphate dehydrogenase	109.3 ±13.0 N=3	85.8 ±9.8 N=3	109.9*	105.5*	121.2	68.9	132.4*	144.6*	107.3	107.3	123.1	94.2	105.8	95.2

* These values indicate 1 experiment . †CAP-chloramphenicol; † Tev- Tevenel; †cyclo-cycloheximide

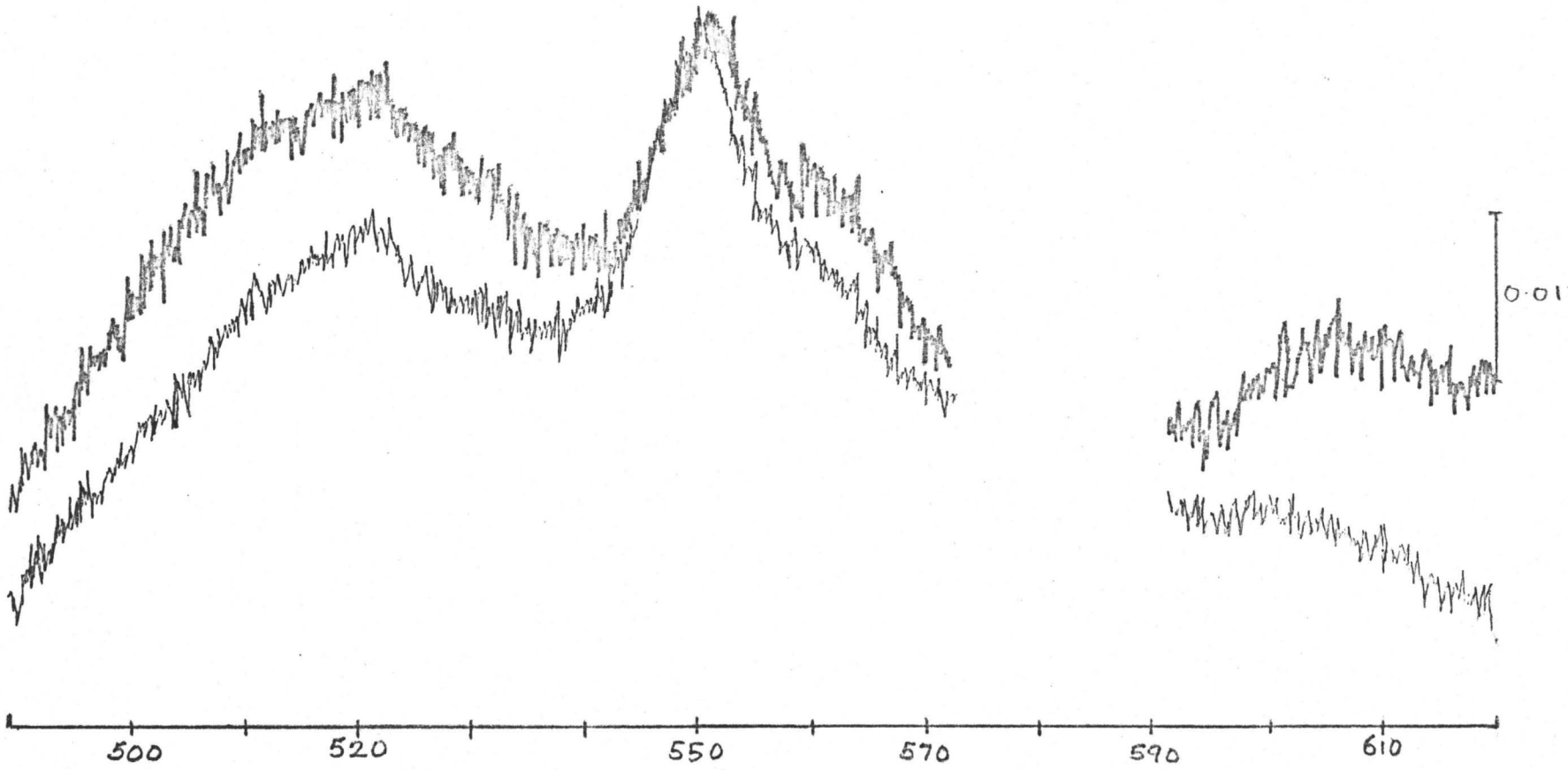
phosphate dehydrogenase) were also close to the control value at this time (Table II) except in those cells treated with D-chloramphenicol and then resuspended in 1 x 10^{-6}M cycloheximide where the level of lactate dehydrogenase and glucose-6-phosphate dehydrogenase remained unchanged at 27 hr. As the recovery was allowed to continue there was an increase in cytoplasmic malate dehydrogenase activity. The level of mitochondrial malate dehydrogenase appeared to overshoot the control and then fell off slightly below the control level.

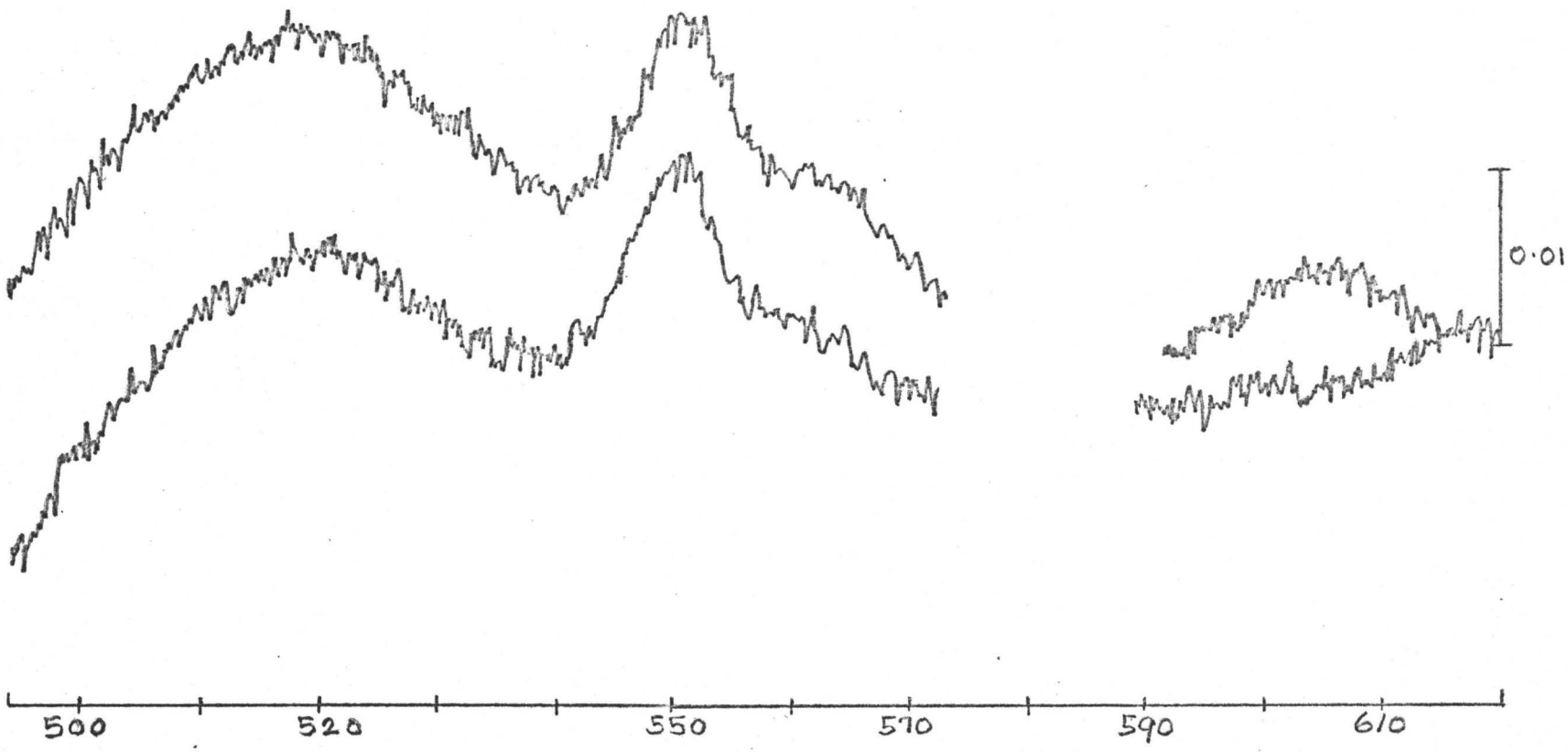
IV Mitochondrial Cytochromes

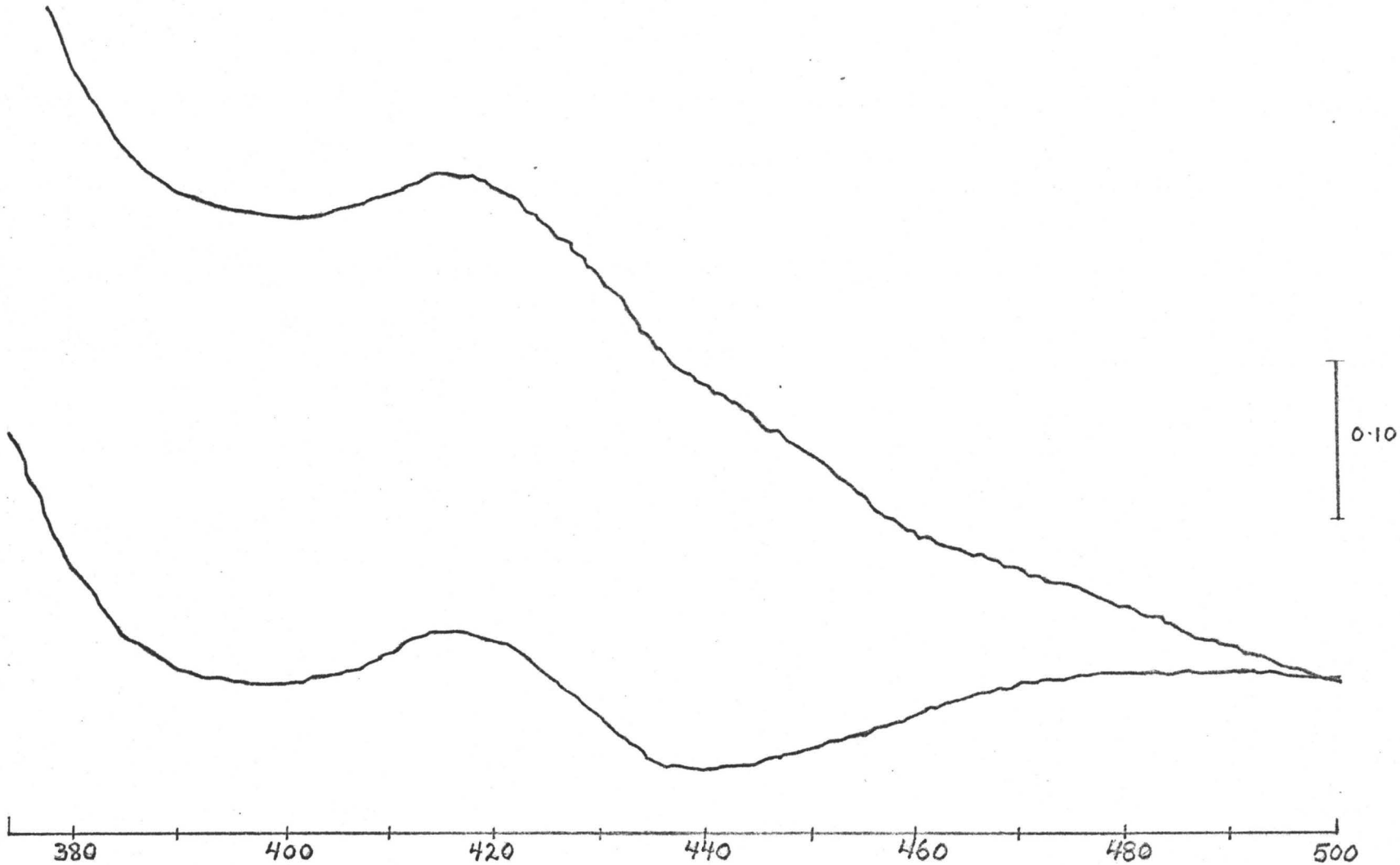
The respiratory ability of cells grown in D-chloramphenicol (10 µg/ml) was found to be decreased. Untreated cells had an oxygen consumption of 11.81 ± 1.44 mµmoles O_2/min for 10^7 cells (N=4), which was completely inhibited by 2 x 10^{-4}M potassium cyanide. Cells grown in D-chloramphenicol for 70 hr had an oxygen consumption of 4.43 mµmoles/min for 10^7 cells (37.5% of control). The cytochrome oxidase activity in the presence of chloramphenicol was 23-25% of the control at this time and after 90 hr treatment. It was of interest then to examine the spectra of mitochondrial cytochromes to see if there was a corresponding decrease in the cytochrome a and a_3 peak and to see if there was a change in content in any of the other cytochromes which might also lead to a decreased respiratory ability in D-chloramphenicol treated cells.

The difference spectra of mitochondrial cytochromes from control cells and cells grown in D-chloramphenicol for 90 hr are shown in Fig. 14, 15 and 16. Because a greater reduction of the cytochromes (especially cytochromes b, a and a_3) was obtained with dithionite than with succinate, the spectra given are where dithionite was used as the reducing agent. Fig. 14 represents an experiment in which the concentration of mitochondrial protein was 5.55 mg/ml (control cells) and 5.40 mg/ml (D-chloramphenicol-treated cells). After

Figures 14, 15 and 16. Effect of D-Chloramphenicol (10 µg/ml for 90 hr) on Difference Spectrum of Mitochondrial Cytochromes of L Cells. The upper spectra are those of the control.


0·01
500
520
550
570
590
610


0·01
500
520
550
570
590
610


0·10
380
400
420
440
460
480
500

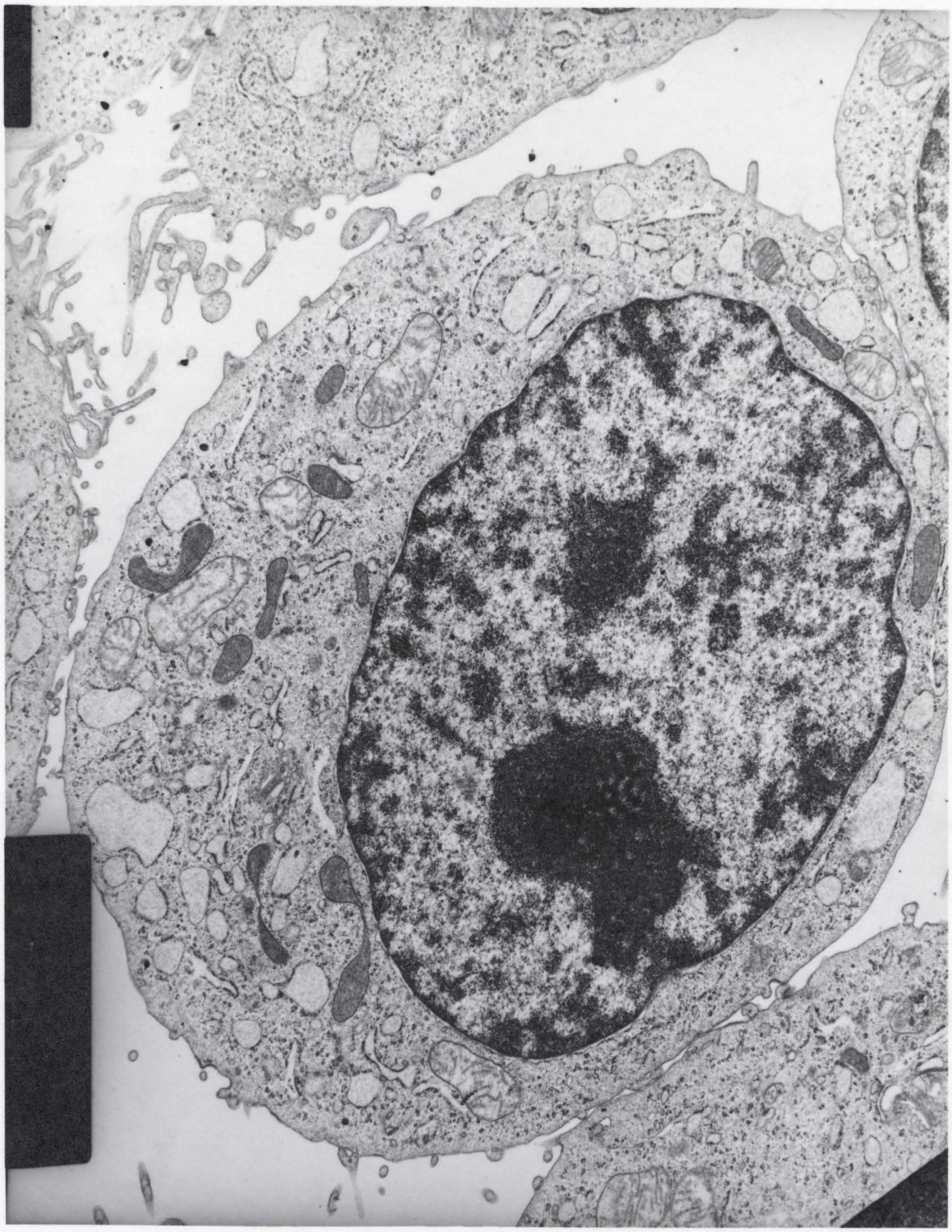
a 90 hr treatment the absorption of cytochrome c at 550 nm is unchanged, there is a slight decrease in absorption of cytochrome c, at 553 nm, a larger decrease in the cytochrome b band at 563 nm and almost no absorption by cytochromes a and a_3 at 605 nm. Although the protein concentrations were lower (control=3.6 mg/ml, D-chloramphenicol=2.3 mg/ml) in the experiment shown in Fig. 15 and 16, a reduced absorption, and hence content, of cytochrome b (563 and 430 nm) and absence of cytochromes a and a_3 (605 and 450 nm) is still apparent. It is very difficult to obtain an accurate base line in these experiments because of the problem of light-scattering, however a base line was approximated by using the standard maximum and minimum absorption ratios of cytochrome c and cytochrome a and a_3. From this base line a value for cytochromes c_1 and b were obtained and found to be 93 and 54% of the control respectively. There was an immeasurable amount of absorption in the region of cytochromes a and a_3 in mitochondria from cells treated with D-chloramphenicol for 90 hr.

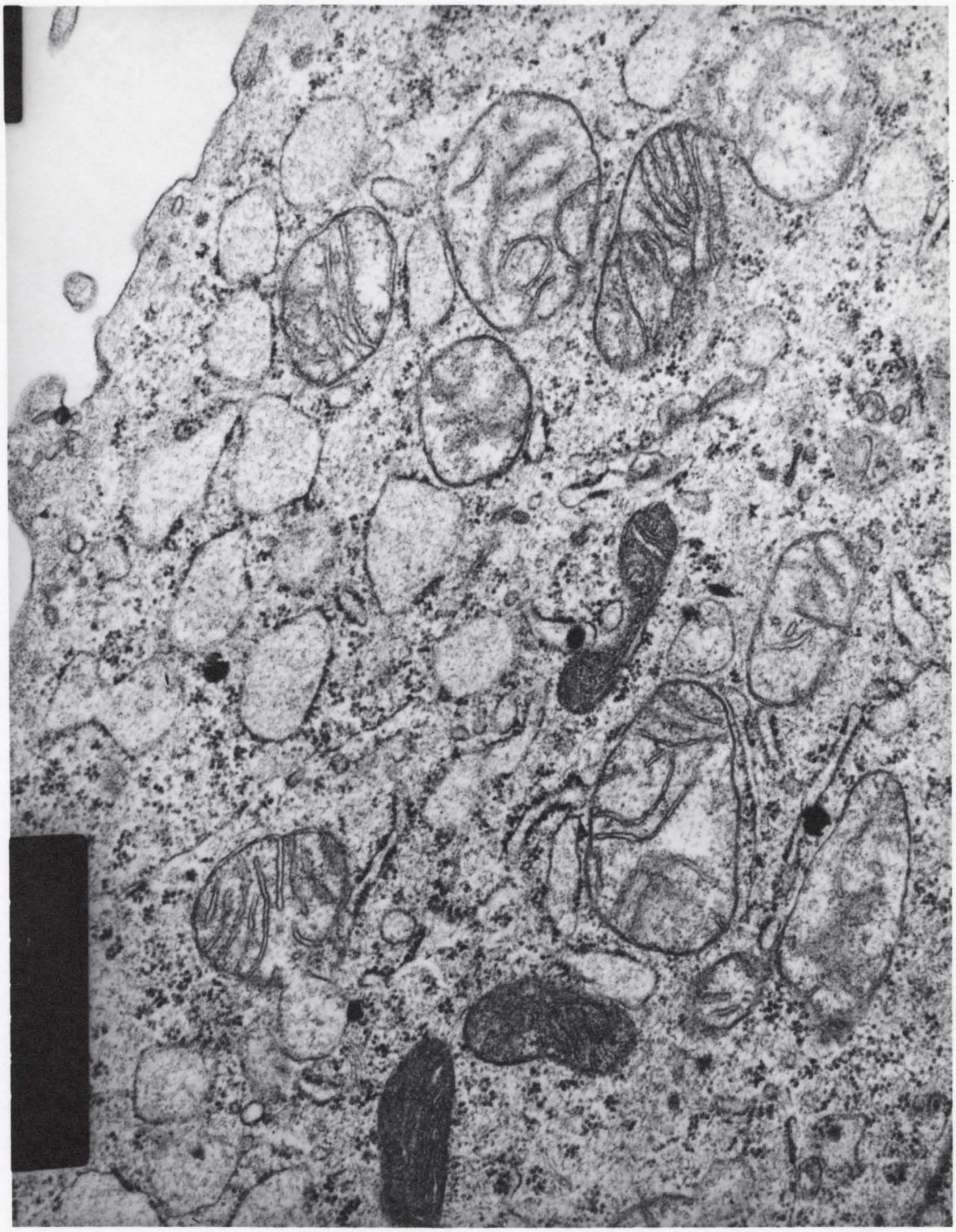
V Electron Microscopy

The electron microscope appearance of a normal L cell is shown in Fig. 17. Mitochondria of various shapes and sizes are apparent. A higher magnification of a part of a normal cell is shown in Fig. 18, Numerous cristae are visible at both magnifications. Although mitochondrial ribosomes are not readily visible in these reproductions they were apparent in the original electron micrographs as small black dots (compare Haldar, Hreeman and Work, 1967). After a 90 hr D-chloramphenicol (10 µg/ml) treatment, 10% of the cell population showed increased cytoplasmic vacuolation, apparent in the upper-most cell in Fig. 19 and 20. The affected (heavily vacuolated) cells usually, but not always, have mitochondria with cristae condensed at the ends of the rod-like shapes, leaving a clear area in the central region (Fig. 20 and 21).

Figure 17. Thin-Sectioned Normal L Cell x 14,560.

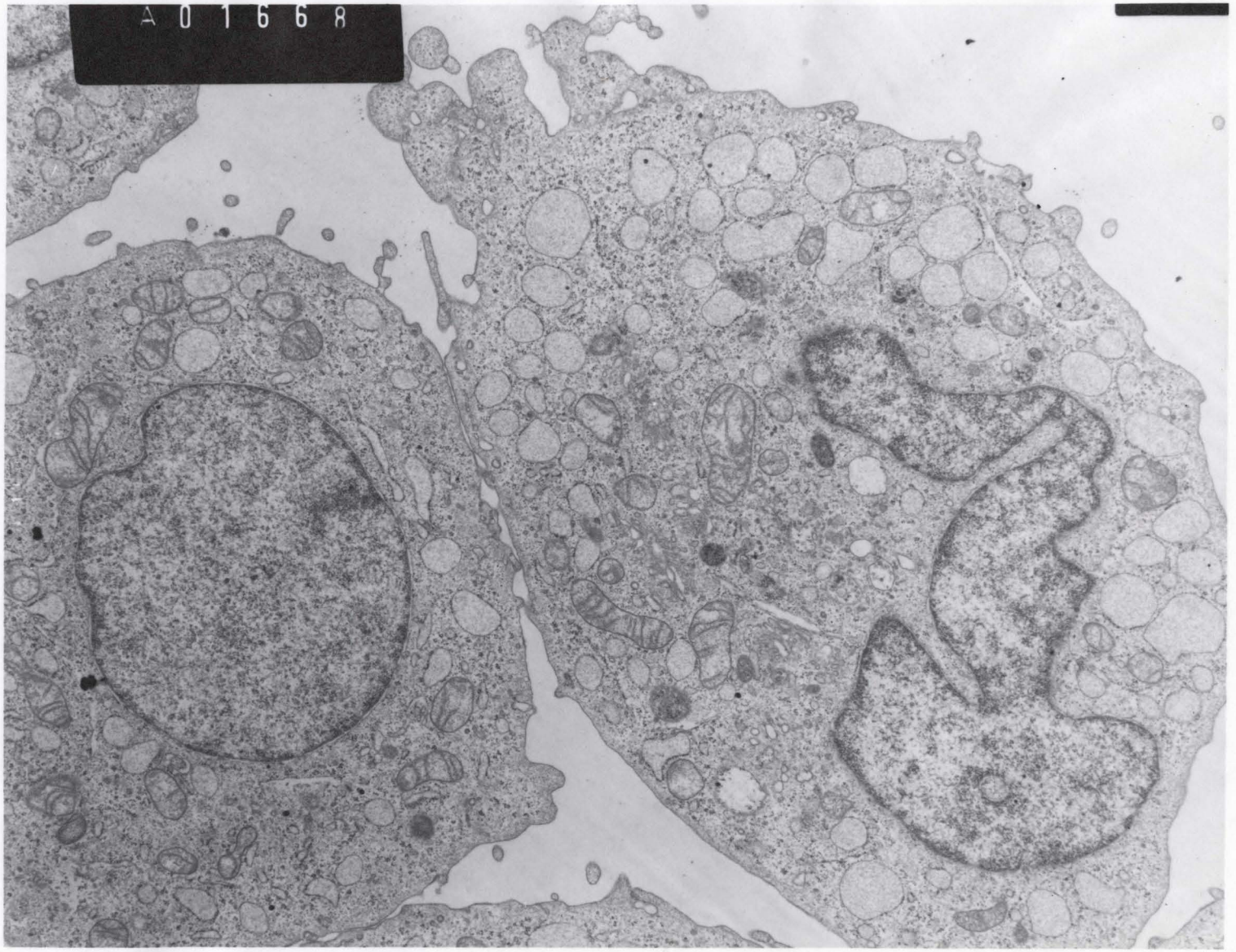
Figure 18. Mitochondria of Thin-Sectioned Normal L Cell x 54,600.

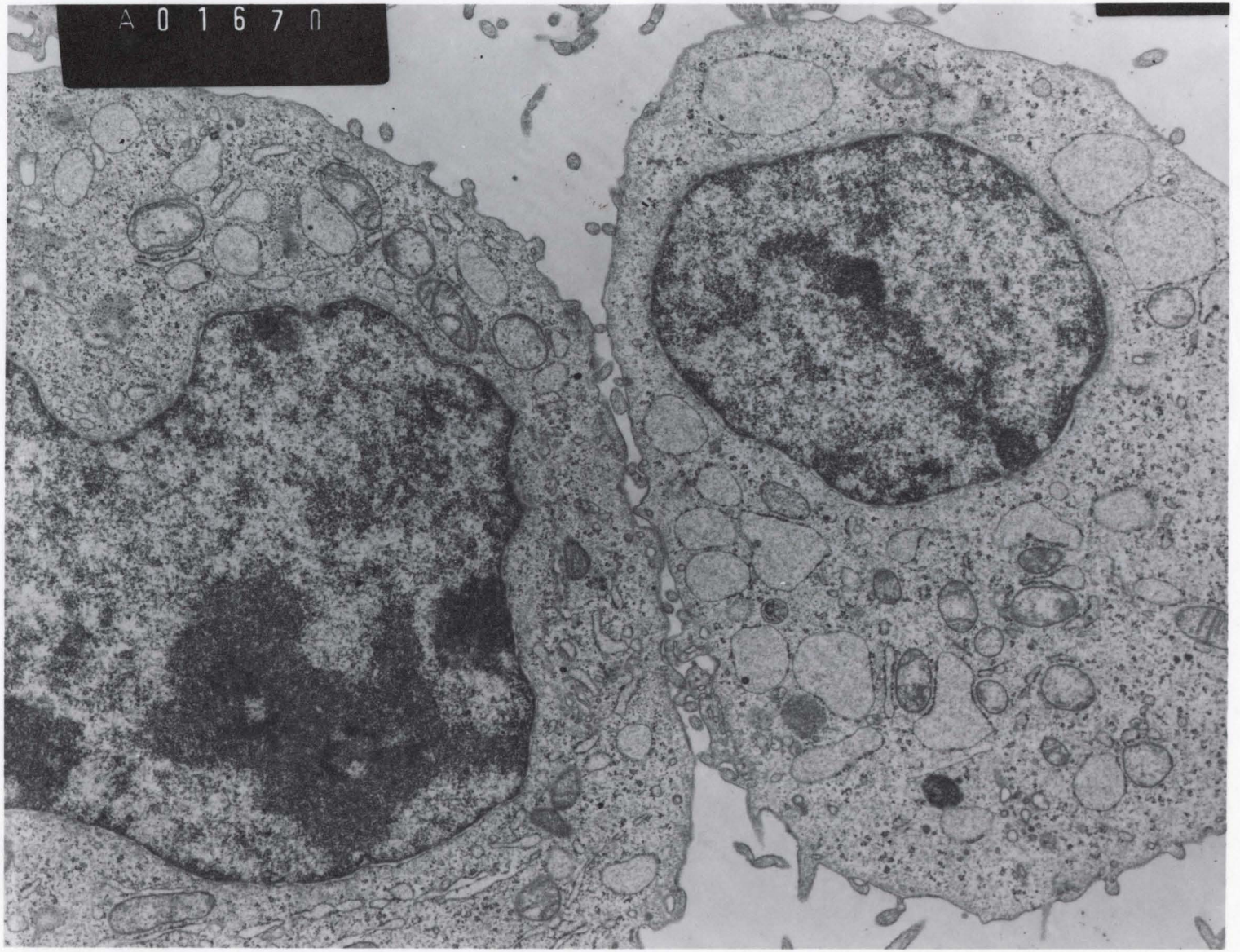


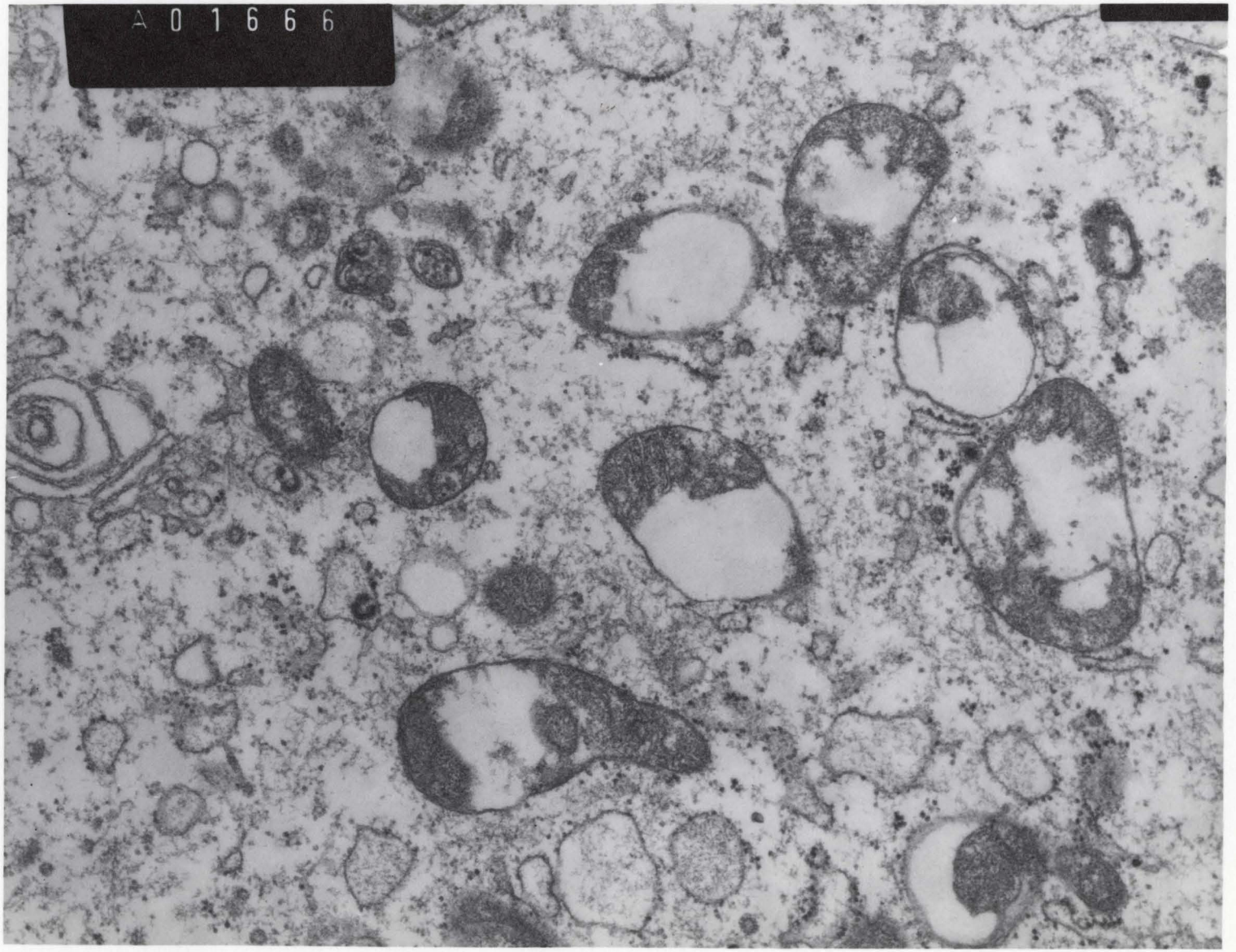


Figures 19 and 20. Thin-Sectioned L Cells after 90 hr Growth in D-Chloramphenicol (10 µg/ml) x 14,560.

Figure 21. Mitochondria of Thin-Sectioned L Cells after 90 hr Growth in D-Chloramphenicol (10 µg/ml) x 54,600.


A 0 1 6 6 8


A 0 1 6 7 0


A 0 1 6 6 6

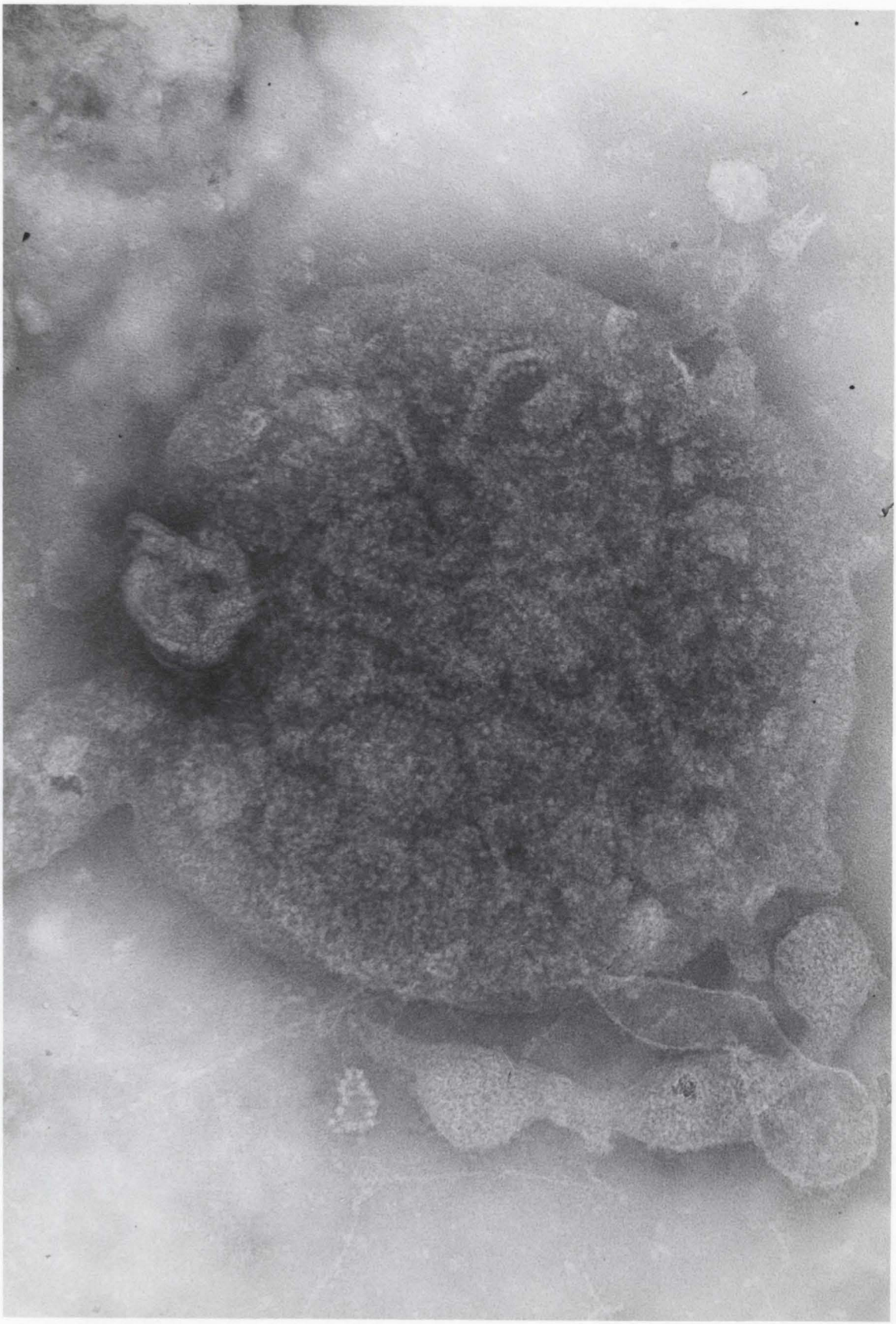
A negatively stained mitochondrion from a normal cell is shown in Fig. 22. The cristae are covered with knobs (ATPase particles). These particles are even more apparent in Fig. 23, which shows cristae from a disrupted mitochondrion. Although a 31% decrease in ATPase activity was observed in mitochondria from cells treated with D-chloramphenicol (10 µg/ml) for 90 hr, a reduction in the number of ATPase particles in these mitochondria was not observed (Fig. 24 and 25).

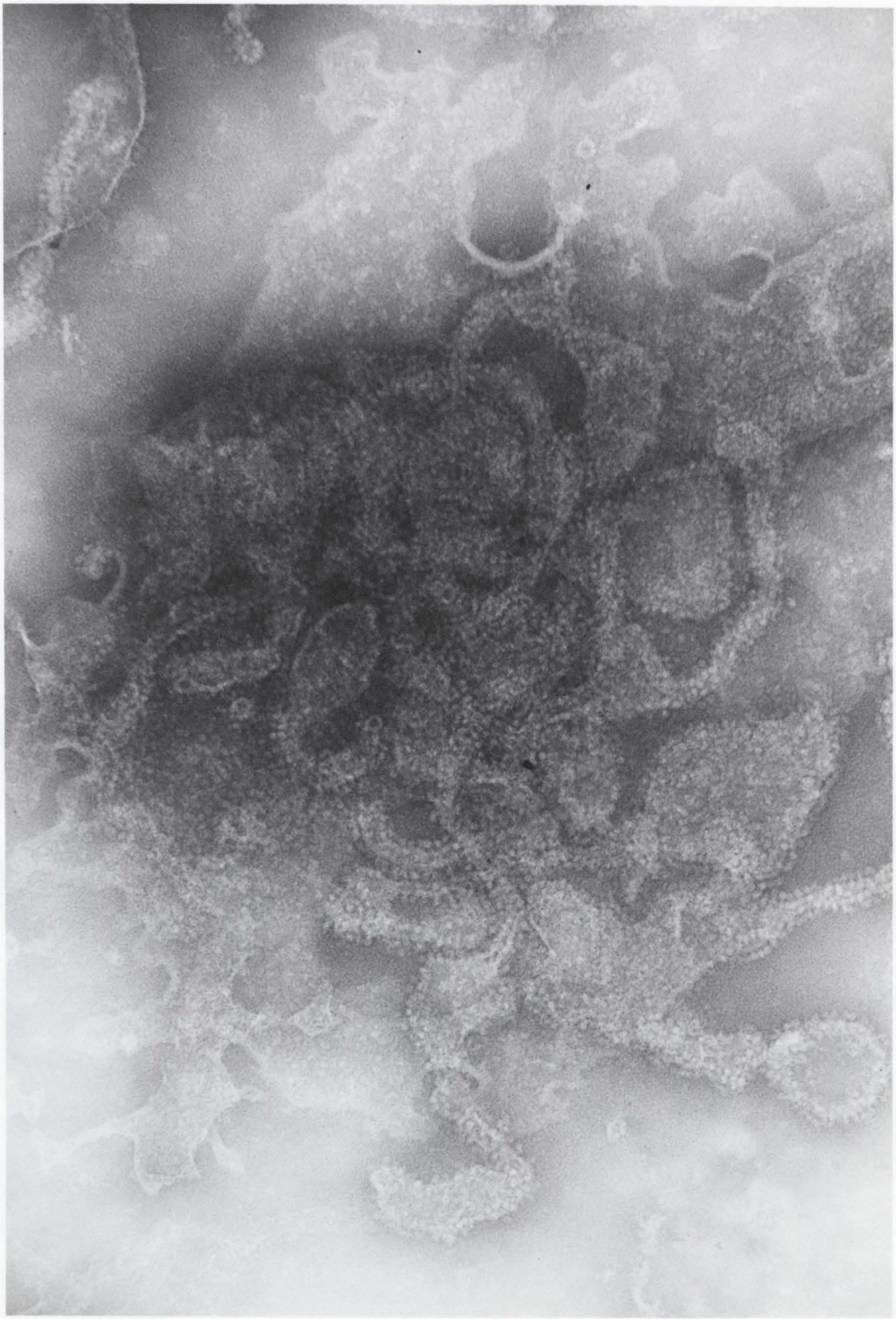
Freeze-etched mitochondria from normal cells and cells treated with D-chloramphenicol (10 µg/ml) for 90 hr are shown in Fig. 26, 28, 30 and Fig. 27, 29 and 31 respectively. The particles (≈100Å in diameter) which are seen in an hexagonal array in Fig. 26, are probably on the outer surface of the inner membrane. This type of arrangement was seen in concave views of isolated inner membranes of rat liver and L cell mitochondria. The patchwork appearance shown in Fig. 27 is often seen in unfixed freeze-etched material. Some particles from the inner membrane can be seen to protrude the relatively smooth outer membrane of the mitochondrion. The particulate inner membrane also has pits or indentations which are visible in Fig. 28 and 29. These were also observed in purified inner membranes. The pits probably indicate areas where there is an infolding to form cristae. In Fig. 30 and 31 the particles, pits and cristae of the inner membrane are seen. The cristae, which appear as a network, are more clearly seen in mitochondria from freeze-etched cells. As seen in Fig. 32 (within a mitochondrion from a normal cell) the cristae are covered with small particles about 20Å in diameter. Although there is at present not a complete understanding of the structures seen in freeze-etched mitochondria there does not appear to be a difference in mitochondria from normal and D-chloramphenicol treated cells.

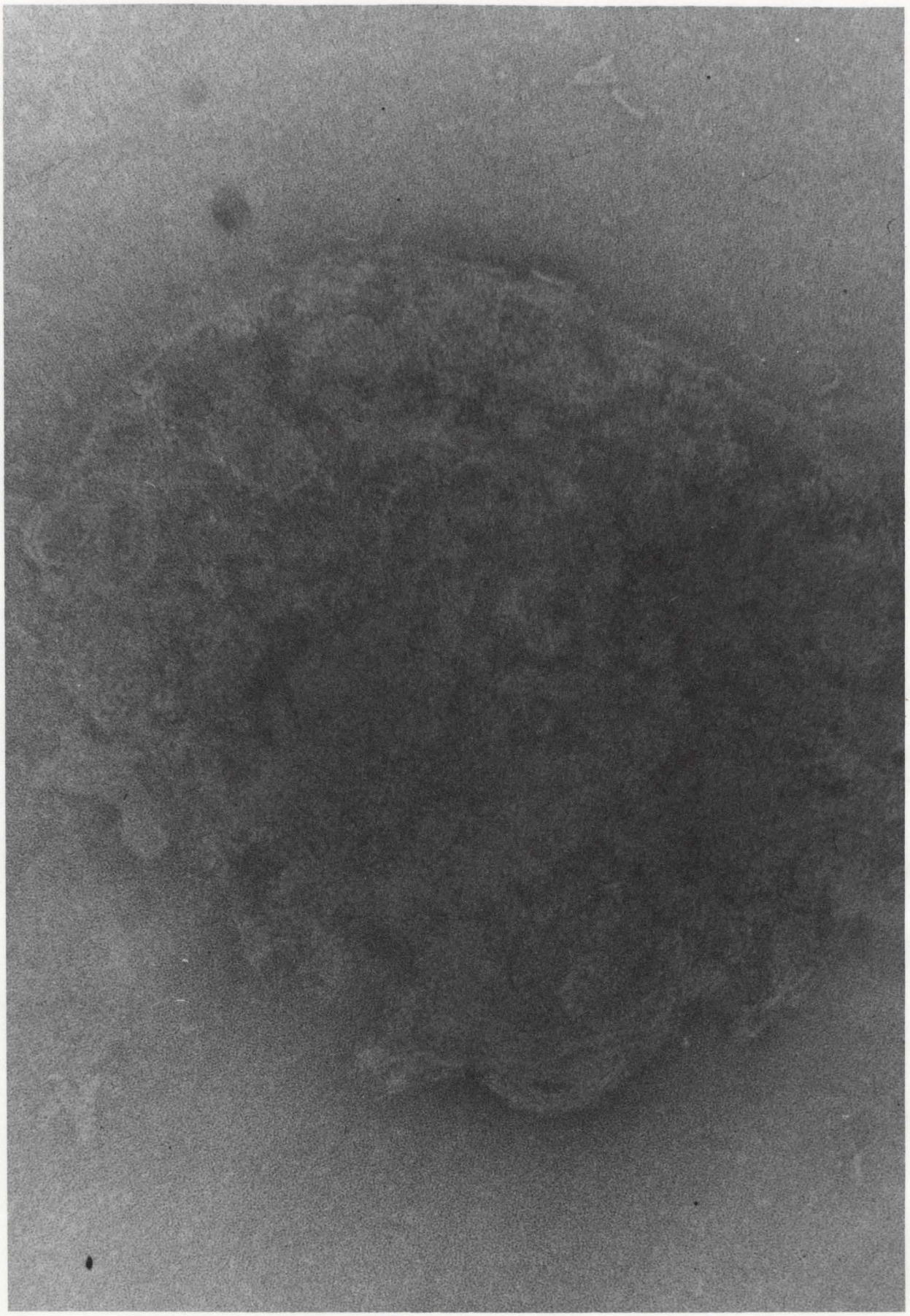
Figures 22 and 23. Negatively-Stained Mitochondria from Normal L Cells Fig. 22 ≃ x 156,000. Fig. 23 ≃ x 125,000.

Figure 24. Negatively-Stained Mitochondria Isolated from L Cells after 90 hr Growth in D-Chloramphenicol (10 μg/ml) ≃ x 120,000.

Figure 25. Cristae of Negatively-Stained Mitochondria Isolated from L Cells after 90 hr Growth in D-Chloramphenicol (10 μg/ml) ≃ x 250,000.







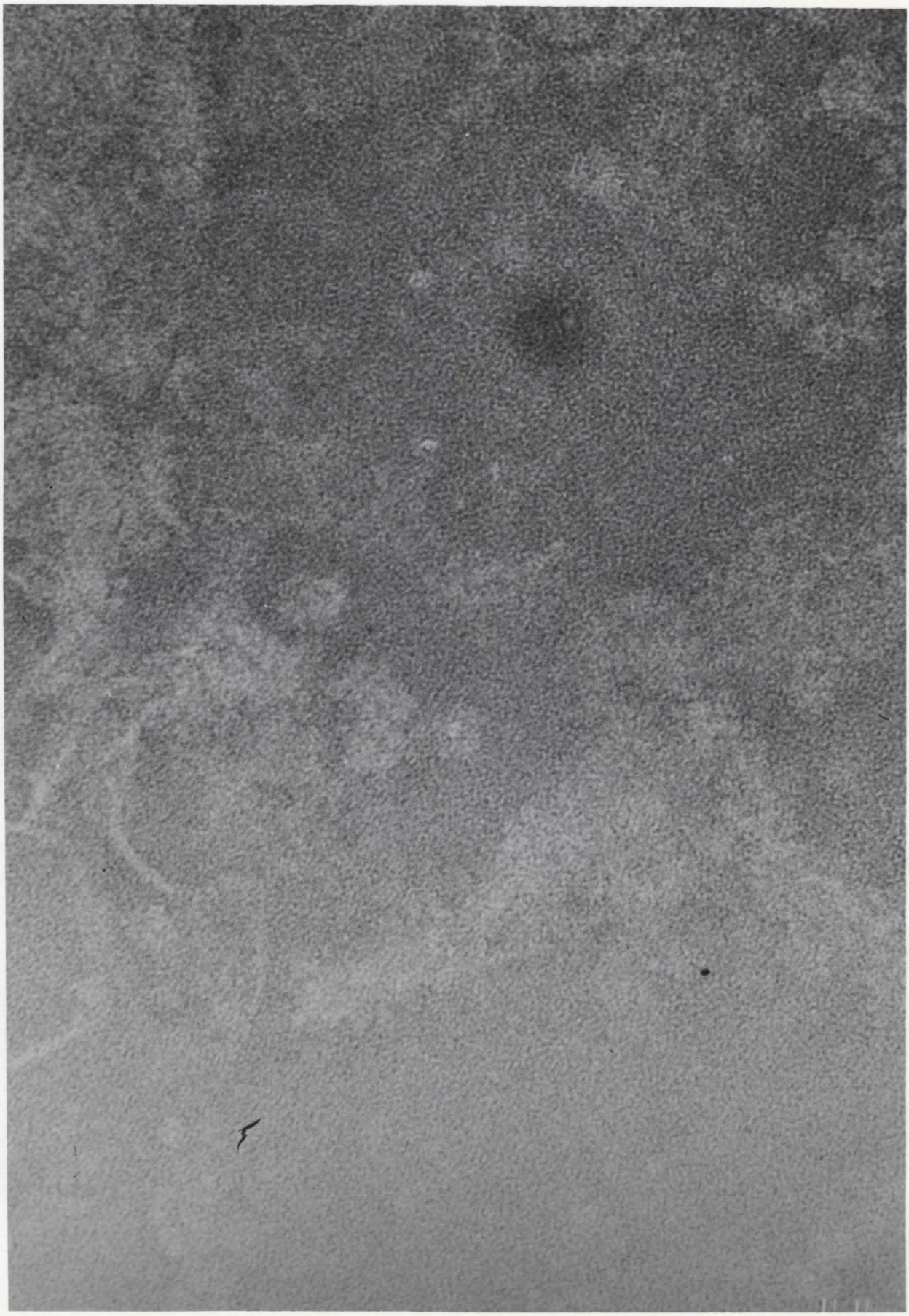


Figure 26. Freeze-Etched Mitochondria from Normal L Cells shows Hexagonal Array of Particles ≈ x 104,000.

Figure 27. Freeze-Etched Mitochondria Isolated from L Cells after 90 hr Growth in D-Chloramphenicol (10 µg/ml) showing Patchwork Appearance. ≈ x 104,000.

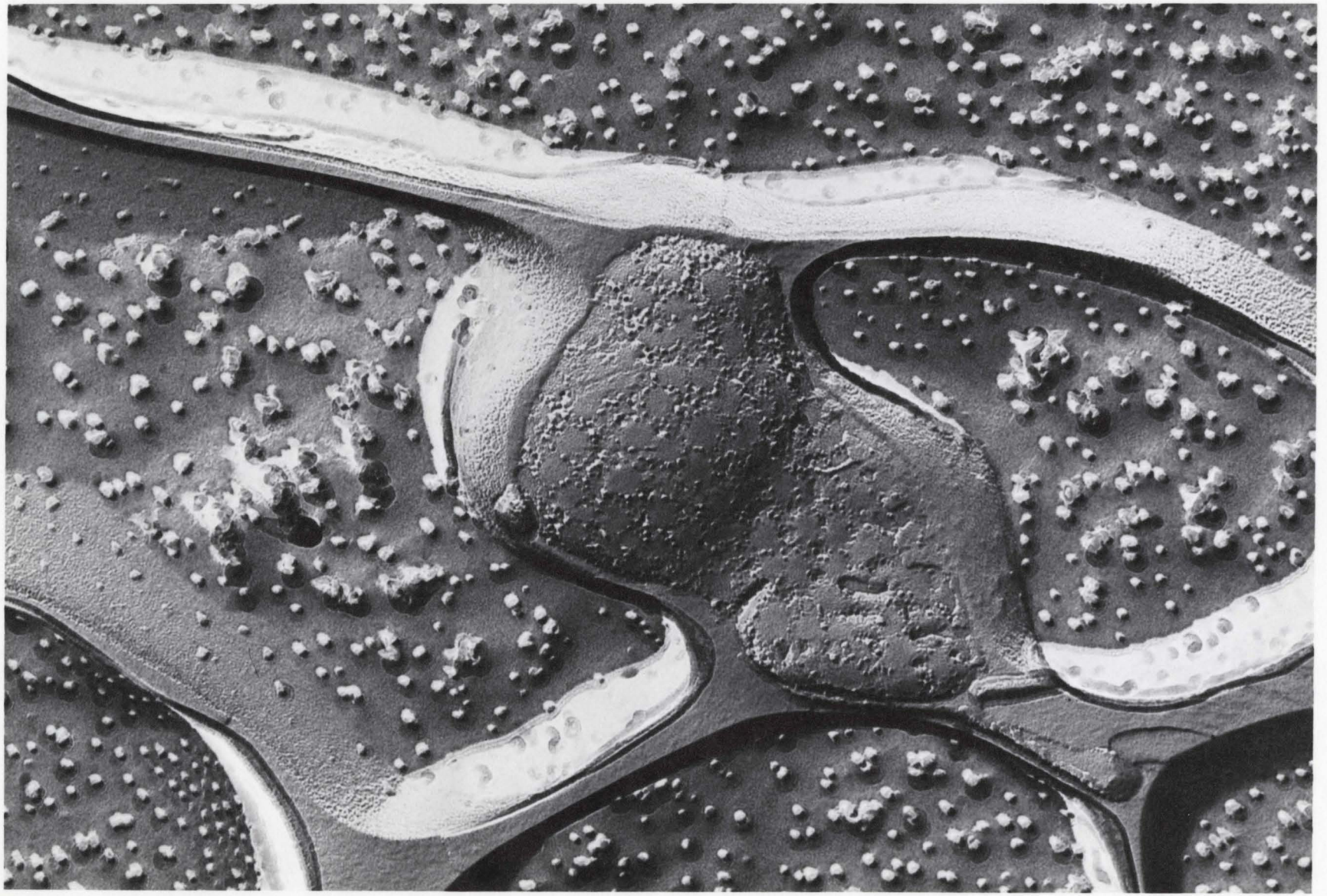
Figure 28. Freeze-Etched Mitochondrion from Normal L Cell showing Particles and Indentations ≈ x 78,000.

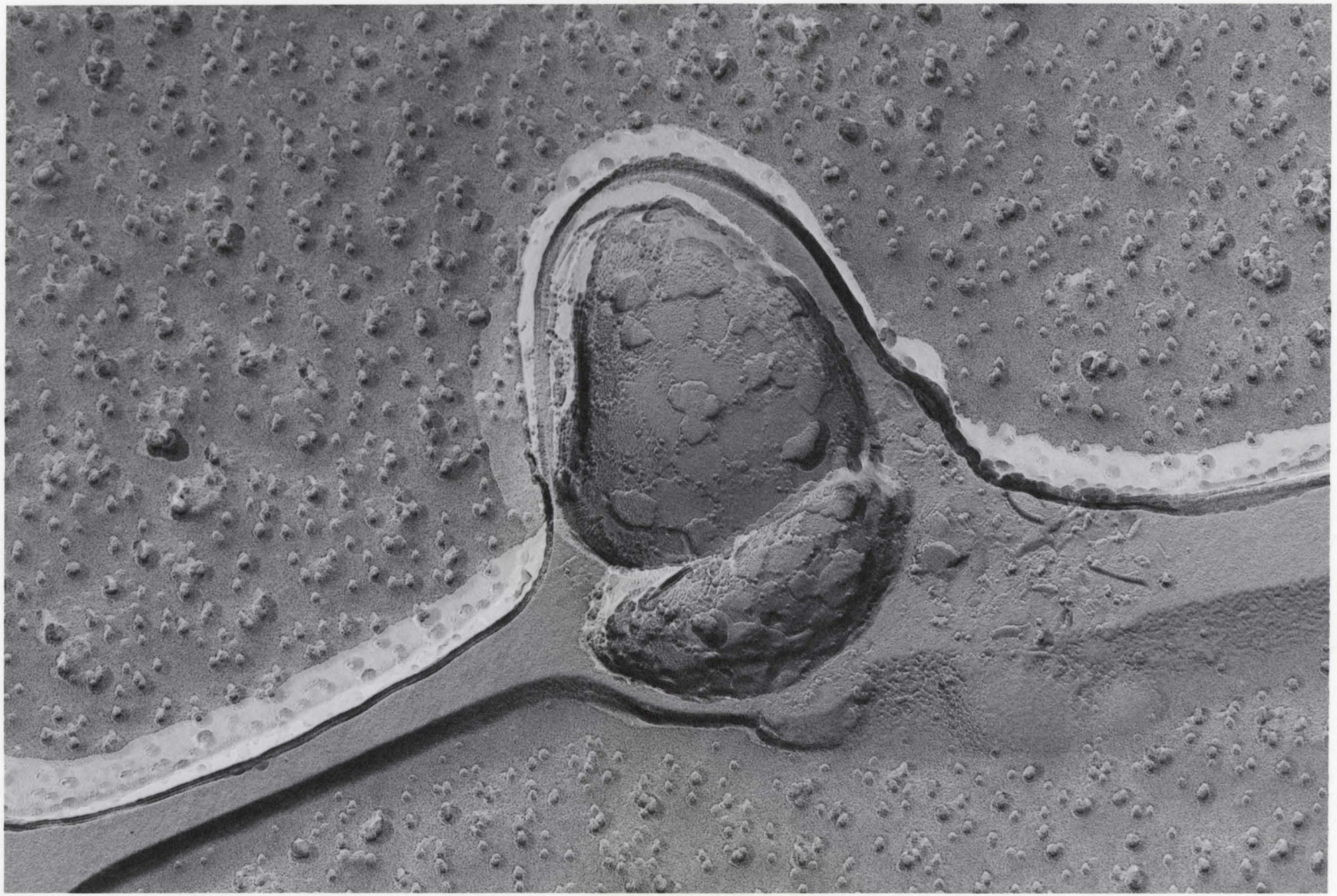
Figure 29. Freeze-Etched Mitochondrion from L Cells after 90 hr Growth in D-Chloramphenicol (10 µg/ml) showing Particles and Indentations ≈ x 104,000.

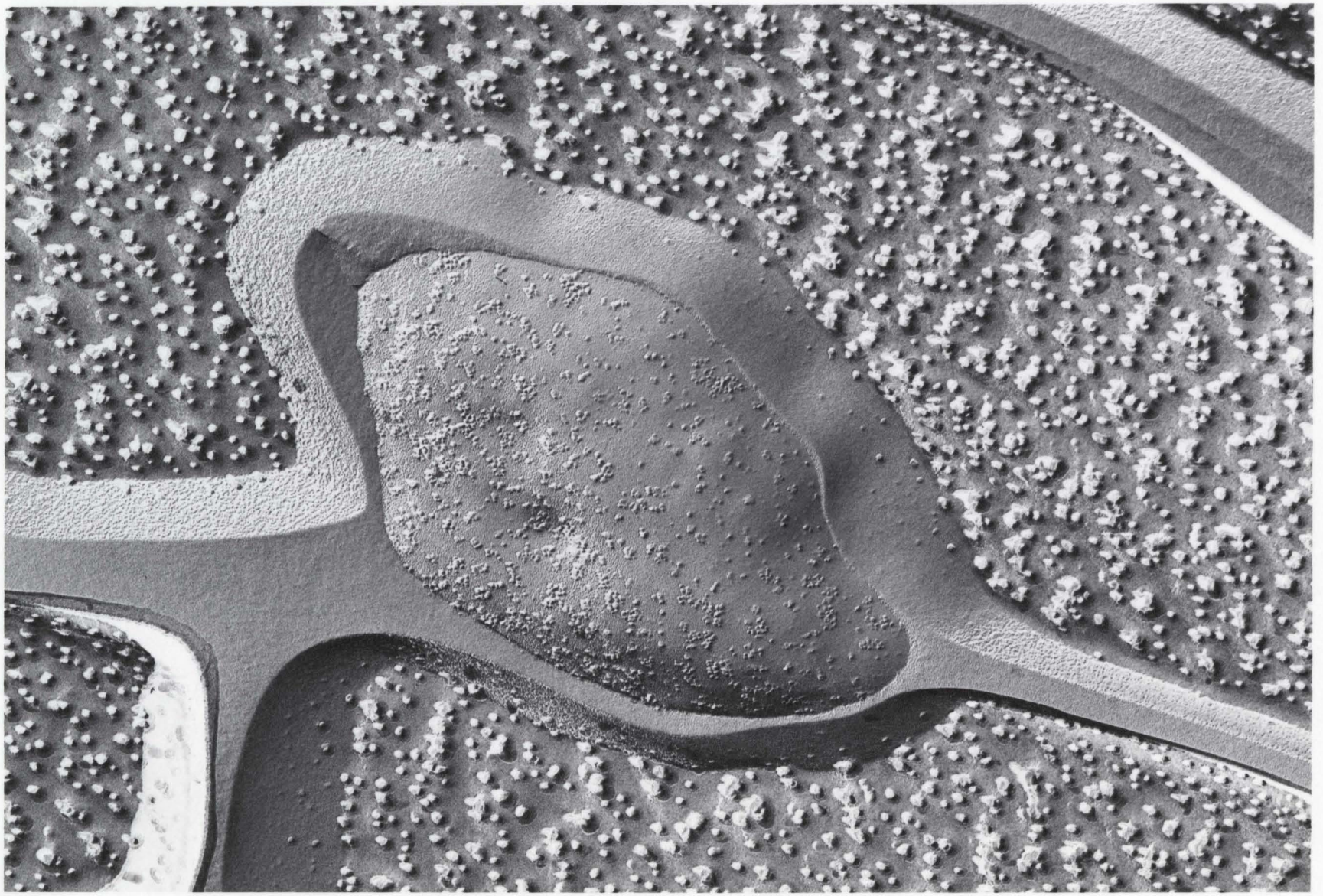
Figure 30. Freeze-Etched Mitochondrion from Normal L Cell showing Particles, Indentations and Cristae ≈ x 104,000.

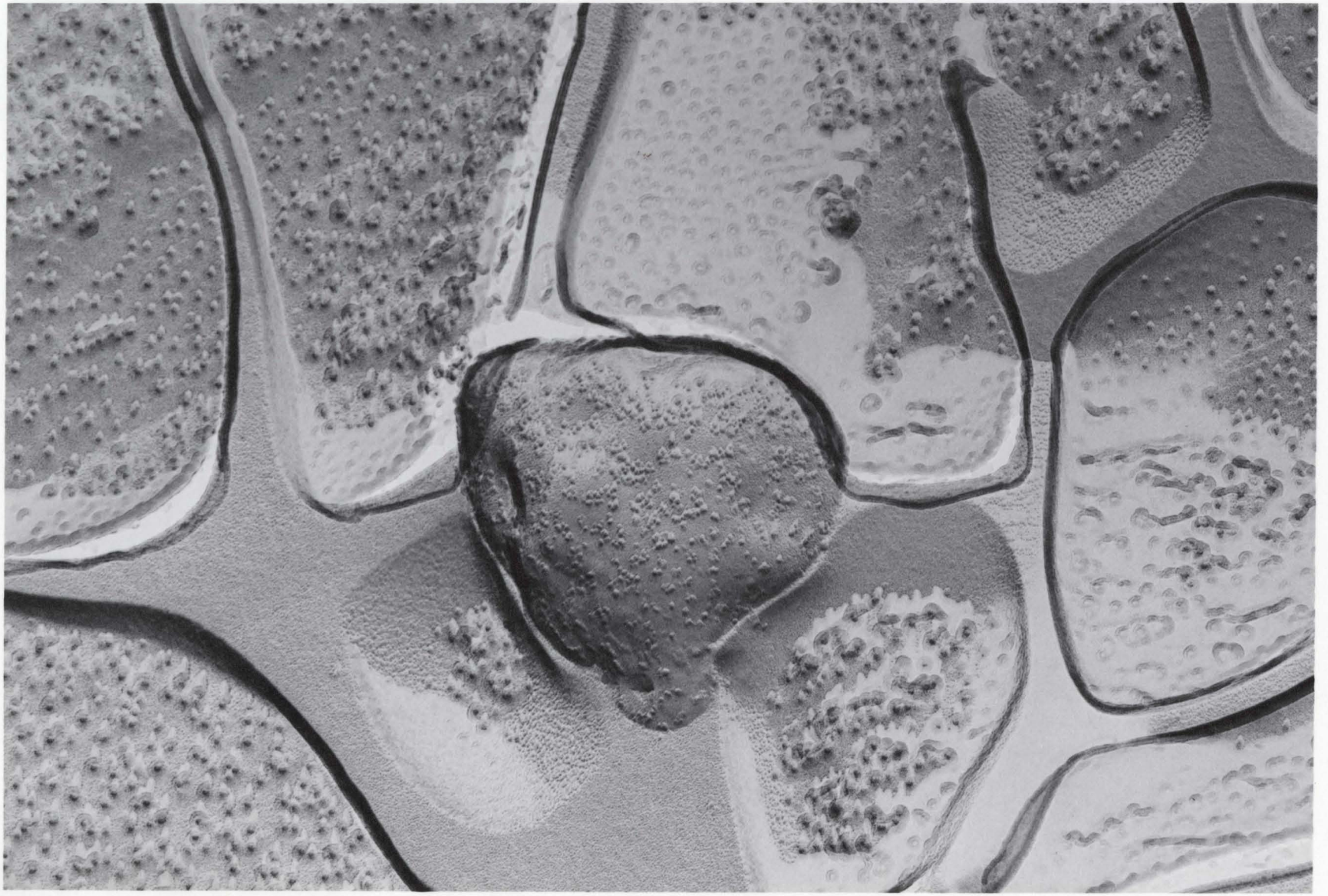
Figure 31. Freeze-Etched Mitochondrion from L Cells after 90 hr Growth in D-Chloramphenicol (10 µg/ml) showing Particles, Indentations and Cristae ≈ x 96,000.

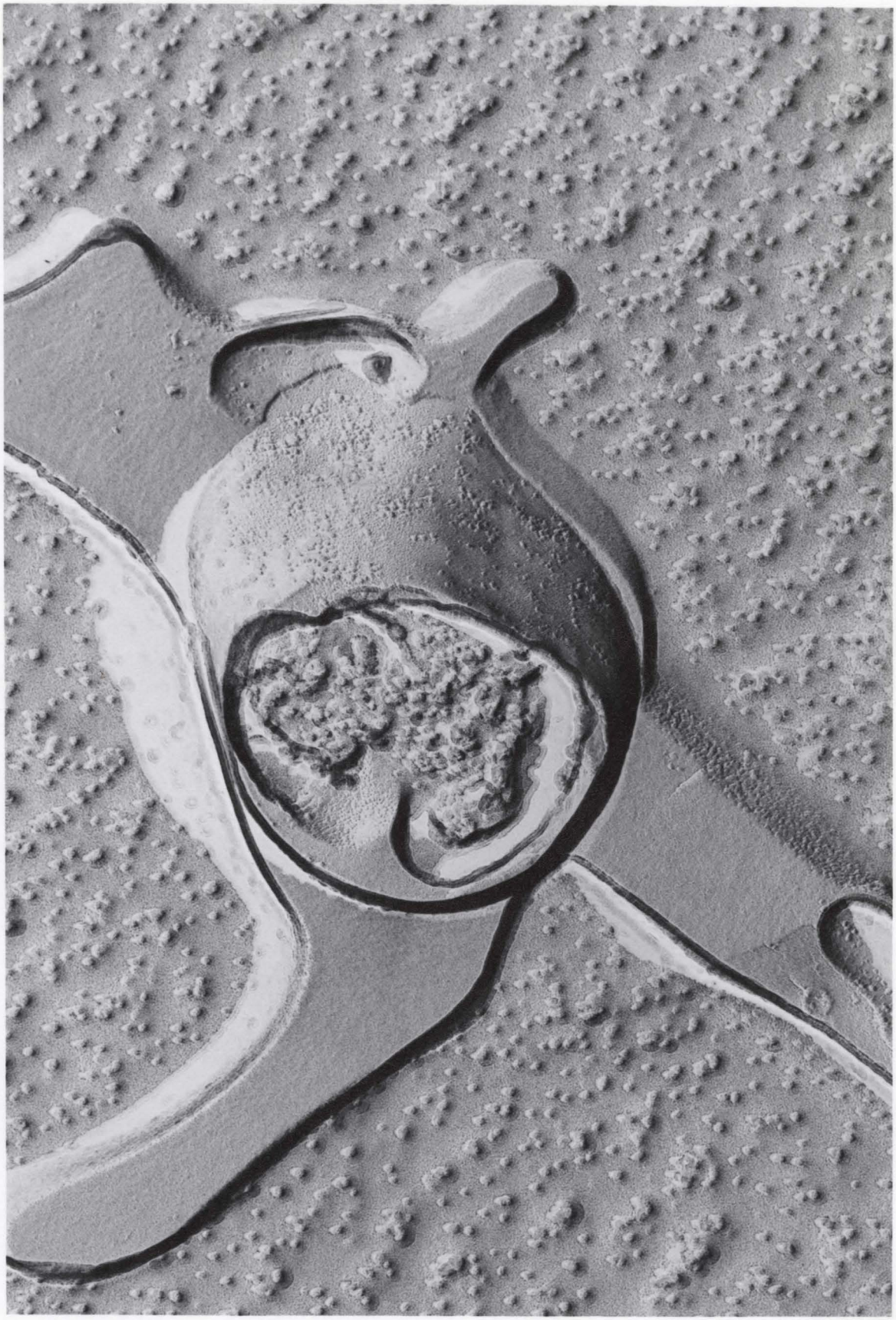
Figure 32. Interior of Mitochondrion from Freeze-Etched Normal L Cell showing cristae ≈ x 260,000.

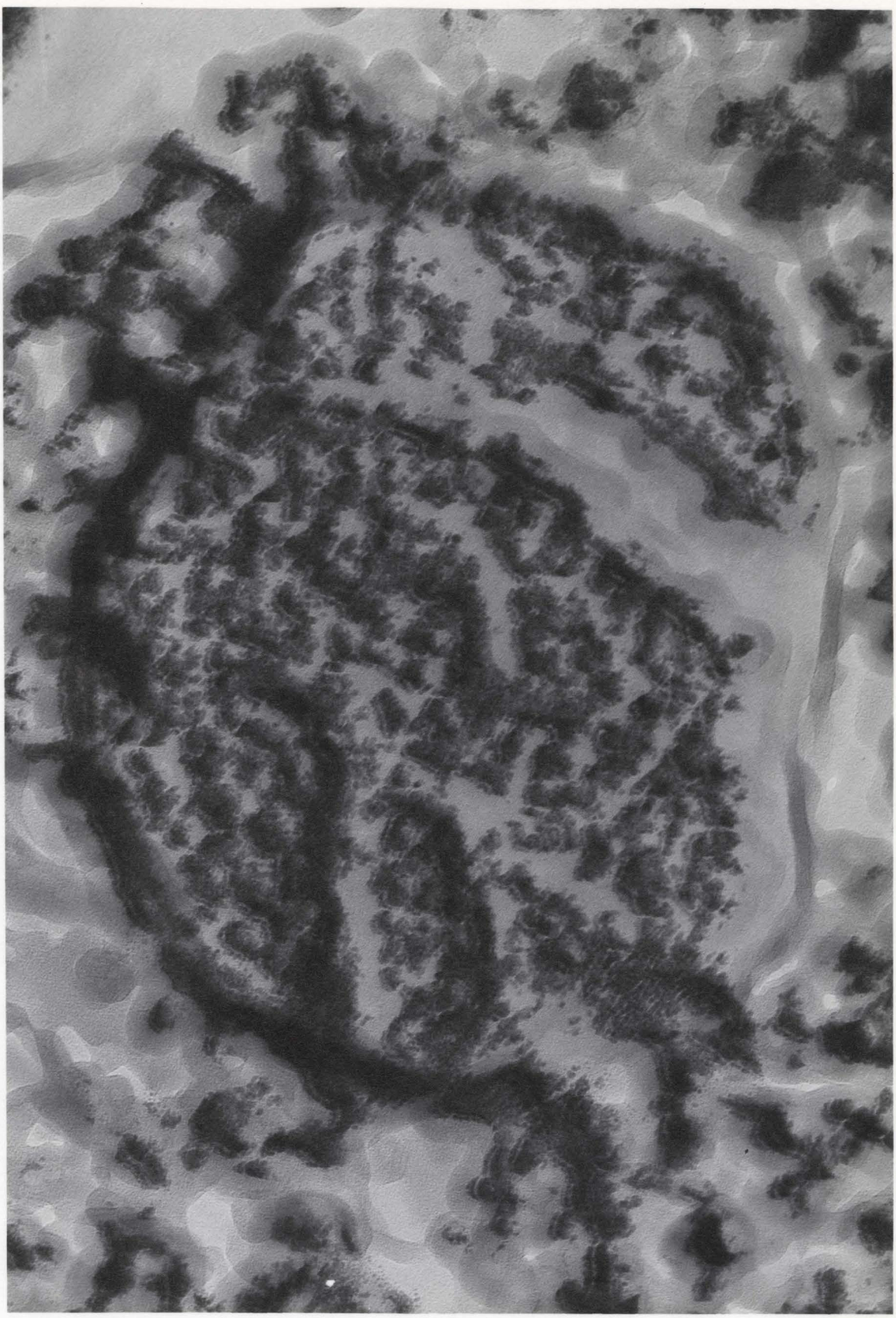












Chapter 3

Discussion

I Protein Synthesis in Isolated Mitochondria

D-threo-Chloramphenicol, but not its isomers inhibits the synthesis of protein by bacteria, chloroplasts (Ellis, 1969) and mitochondria. It has been suggested that in bacteria it may act by inhibiting the peptidyl transferase reaction (Trout and Monro, 1964; Coutsogeorgopoulus, 1967; Condliffe and McQuillen, 1967). More recently, Pestka (1969) has presented evidence that chloramphenicol inhibits protein synthesis by interfering with the binding of the aminoacyl-end of aminoacyl tRNA to ribosomes. Its mechanism of action in mitochondria is not known. Freeman (1970) has shown similar structural requirements of chloramphenicol for inhibition of protein synthesis in bacteria and mitochondria, which indicates that bacteria and mitochondria have a similar binding site on their ribosomes for chloramphenicol. This supports the suggestion that mitochondria have evolved from symbiotic bacteria. He obtained 50% inhibition of protein synthesis in rat liver mitochondria with 15 μM of D-chloramphenicol and its methylthio, methylsulfonyl and sulfamoyl (Tevenel) analogues. In the experiments reported here a higher concentration of chloramphenicol was required for inhibition of protein synthesis in mitochondria isolated from mouse liver, mouse L cells and human KB cells. D-chloramphenicol at 28, 38 and 90 μM respectively was required for 50% inhibition in the three systems studied. As in the case of rat liver mitochondria, one can obtain almost complete inhibition of protein synthesis in mouse liver mitochondria by increasing the chloramphenicol concentration. A leveling off around 50-35% of the control was observed in the inhibition of protein synthesis by isolated mitochondria from cells in tissue culture. It is not known why this

plateau occurs. It cannot be accounted for by cytoplasmic contamination since the addition of cycloheximide did not decrease the chloramphenicol-resistant incorporation. The presence of a plateau may account in part for the apparently high concentration of chloramphenicol required for 50% inhibition in these systems as compared with rat liver mitochondria.

The inhibition of mitochondrial protein synthesis by chloramphenicol has been observed in many systems, including human bone marrow and it is thought to be the biochemical basis for bone marrow depression observed in patients receiving chloramphenicol. It has also been offered as an explanation for the inhibition of the primary antibody response observed with chloramphenicol.

II Effect of Chloramphenicol on Cell Growth

D-Chloramphenicol and its sulfamoyl analogue (Tevenel) have been found to inhibit mitochondrial protein synthesis. D-Chloramphenicol also inhibits NADH oxidase but Tevenel does so only at very much higher concentrations. The L-isomer does not inhibit protein synthesis, but like D-chloramphenicol it inhibits NADH oxidase (Freeman and Haldar, 1968; Freeman, 1970) and so it was used to help distinguish between direct inhibition of mitochondrial protein synthesis and any indirect effect due to inhibition of NADH oxidase.

Since the inhibition of cell growth caused by D-chloramphenicol at 100 µg/ml was greater than the inhibition caused by either Tevenel or L-chloramphenicol at the same concentration, the observed effect is likely due to a combined inhibition of NADH oxidase and mitochondrial protein synthesis. At 10 µg/ml, L-chloramphenicol had no effect on the growth of L cells. In a 90 hr period D-chloramphenicol and Tevenel at 10 µg/ml and Tevenel at 100 µg/ml had a similar inhibitory effect on growth. In longer periods of treatment the

decreasing order of inhibition was Tevenel (100 µg/ml), D-chloramphenicol (10 µg/ml) and Tevenel (10 µg/ml). Due to the difference in molecular weight 10 µg D-chloramphenicol/ml is a slightly higher molarity (31 µM) than 10 µg Tevenel/ml (28 µM) and this may account for the slightly greater inhibition caused by D-chloramphenicol. It was found that even after exposure to D-chloramphenicol for 10-11 days the cell growth rate returned to normal after removal of the chloramphenicol.

There was not a significant change in cell viability or level of ATP in cells treated with D-chloramphenicol for 90 hr. Because there was no effect on cell growth at 10 µg of L-chloramphenicol/ml and because the effect caused by D-chloramphenicol was similar to that caused by Tevenel, the observed inhibition of growth is probably due to a specific inhibition of mitochondrial protein synthesis. Since the serum chloramphenicol concentration in patients suffering from the chloramphenicol-induced bone marrow depression is about 10 µg/ml, this lends further support to the idea that inhibition of mitochondrial protein synthesis is the basis of this depression.

The results described demonstrate a lesser inhibition of cell growth than was reported by Firkin and Linnane (1968) with HeLa cells and by Nass (1969) with L cells. These workers stated that D-chloramphenicol at 20-25 µg/ml caused a cessation of cell growth after 2 generations. As reported here, it was found that even in the presence of Tevenel at 100 µg/ml, L cells went through 4.8 generations if the cells were diluted daily.

The results also demonstrate the importance of using low concentrations or chloramphenicol or alternatively Tevenel at higher concentrations to ensure an inhibition of mitochondrial protein synthesis without causing an inhibition of NADH oxidase. Pious (1970) has recently reported that chloramphenicol inhibits the recovery of cytochromes in anaerobically grown

cells (human) exposed to oxygen. However this was only observed at concentrations greater than 100 µg/ml and only partial inhibition was observed at 40 µg/ml. The results must therefore be treated with caution.

III Effect of Chloramphenicol on the Synthesis of Enzymes

Chloramphenicol selectively inhibits the synthesis of protein by mitochondria. Those proteins whose appearance is inhibited must be those which are in part synthesized in the mitochondria or the synthesis and/or assembly of the protein is controlled by a product of mitochondrial protein synthesis. Similarly, if the appearance of a protein is inhibited by the presence of cycloheximide, which is a specific inhibitor of cytoplasmic protein synthesis, this would suggest that the protein is synthesized on the cytoplasmic ribosomes.

If protein synthesis in the mitochondria is completely inhibited by chloramphenicol and provided there is no turn-over of protein, one would expect a decrease in the protein to 50% of the control after 1 generation, to 25% after 2 generations and to 12% after 3 generations. If there is not complete inhibition the decrease would be less. If there was degredation in addition to the inhibition of protein synthesis, the decrease would be greater.

The only enzyme examined whose synthesis was largely affected by chloramphenicol was cytochrome oxidase. Under conditions in which there was a gradual decrease in growth rate, with no change in total mitochondrial protein, the level of cytochrome oxidase was found to decrease with time and the corresponding increase in the number of cell generations. This effect was specific for D-chloramphenicol and Tevenel. L-Chloramphenicol did not decrease the rate of growth or the specific activity of cytochrome oxidase. Since the cytochrome oxidase activity was reduced to 55% of the

control in 1 generation and to 35% in 2 generations in the presence of Tevenel at 100 µg/ml and only to 80% of the control in 1 generation and to 40% in 2 generations in the presence of D-chloramphenicol at 10 µg/ml, it is likely that this concentration of D-chloramphenicol causes only partial inhibition of protein synthesis, while Tevenel at 100 µg/ml causes almost complete inhibition. The experiments with isolated L cell mitochondria indicated a 40% inhibition of protein synthesis by D-chloramphenicol at about 10 µg/ml. If this is true for mitochondria in situ one might expect a level of cytochrome oxidase about 80% of the control after 1 generation, 40% after 2 generations and 20% after 3 generations. The observed specific activities were 50 and 19% respectively after 2 and 3 generations in the presence of D-chloramphenicol. Since there was no decrease in total mitochondrial protein these specific activities can be considered as equivalent to absolute amounts. There was no evidence of degradation of pre-existing cytochrome oxidase.

After a 90 hr treatment (3.6 - 3.8 cell generations) the level of cytochrome oxidase was 25% of the control in D-chloramphenicol treated cells and 6% of the control in cells grown in Tevenel at 100 µg/ml. The plateau regions (at 68 - 125 hr and 125 - 250 hr) observed during prolonged treatment with D-chloramphenicol might be explained by the very slow growth rate which might result in a longer turn-over time for cytochrome oxidase. Failure to observe a 100% decrease in cytochrome oxidase activity even after prolonged treatment with D-chloramphenicol (10 or 11 days) suggests that D-chloramphenicol at 10 µg/ml still allows some mitochondrial protein synthesis to continue.

The decrease in cytochrome oxidase activity was found to be completely reversible after a 90 hr treatment with D-chloramphenicol. Twenty-seven hr (1.7 generations) after the removal of D-chloramphenicol

the level of cytochrome oxidase had increased to 92% of the control. If the level of cytochrome oxidase had been recovered by the usual rate of synthesis one would expect a value 78% of the control after 1.7 generations. Thus the rate of synthesis during recovery is probably similar to the normal rate of synthesis. The recovered levels of cytochrome oxidase for cells treated with Tevenel at 10 and 100 µg/ml for 90 hr were 90 and 85% respectively. The recovered level of cytochrome oxidase for cells treated with D-chloramphenicol for 140 hr was 75%. It is possible that complete (ie. 100%) recovery may have been achieved after a much longer period of time or on the contrary, the failure of complete recovery may reflect some irrepairable damage due to a secondary effect. One might expect more secondary effects in longer periods of treatment or at higher concentrations of chloramphenicol.

No change in the total mitochondrial protein was observed during the chloramphenicol treatment. From this it is concluded that the bulk of the mitochondrial protein is synthesized in the cytoplasm, as has been found in yeast (Schweyen and Kaudewitz, 1970). If this is true and if there is no turn-over of protein, then addition of cycloheximide would not affect the specific activity of any mitochondrial enzyme. If a decrease is observed it means that this enzyme is synthesized in part at least in the cytoplasm and turns over more rapidly than the bulk of mitochondrial enzymes.

Recovery of cytochrome oxidase activity in cells treated with D-chloramphenicol (10 µg/ml) for 90 hr was found to be inhibited by the presence of 1×10^{-6}M cycloheximide, a concentration which causes 66% inhibition of cellular protein synthesis. At this concentration a consistent inhibition of synthesis of cytochrome oxidase was not obtained. A higher concentration of chloramphenicol was usually required to cause a decrease in cytochrome oxidase activity. Cycloheximide at 1×10^{-5}M

caused a reversible decrease in the level (specific activity) of cytochrome oxidase to 35% of the control after 36 hr treatment. After 12 hr in the presence of 5×10^{-6}M cycloheximide the cytochrome oxidase level had dropped to 85% of the control but had completely recovered within 2·5 hr after removal of the cycloheximide.

The experiments described in which chloramphenicol was used as an inhibitor of mitochondrial protein synthesis are in agreement with those of Kroon (1968) and Firkin and Linnane (1969) in indicating that some mitochondrial protein synthesis is required for the formation of a functional cytochrome oxidase enzyme. The inhibition of recovery of cytochrome oxidase activity in the presence of cycloheximide and the decrease in cytochrome oxidase activity caused by cycloheximide in the absence of chloramphenicol strongly indicates that some cytoplasmic protein synthesis is required for formation of a functional cytochrome oxidase enzyme. This is in agreement with the experiments of Rouslin and Shatz (1969) in which they found that both cytoplasmic and mitochondrial protein synthesis were required for respiratory adaptation in baker's yeast. The finding by Tuppy and Birkmayer (1969) of the apoprotein of cytochrome c oxidase in the respiratory deficient mitochondrial "petit" mutants of yeast suggest that the apoprotein is synthesized in the cytoplasm. The apoprotein was devoid of cytochrome oxidase activity until it was reconstituted with cytohemin from mitochondria of wild type yeast. It is obvious then that the nucleus and mitochondria have joint control over the formation of a functionally active cytochrome oxidase enzyme. It is likely that the apoprotein of cytochrome oxidase is synthesized outside the mitochondrion but that at least part of the synthesis of the functional enzyme and its assembly is controlled by a product of mitochondrial protein synthesis. Labeling studies will be necessary to determine the exact role of mitochondrial and cytoplasmic protein synthesis in the formation of cytochrome oxidase.

The experiments of Rouslin and Shatz on yeast indicated that there was an accumulation of cycloheximide-insensitive precursors which required some cytoplasmic protein synthesis for formation into the final functional product. There was no evidence of a similar accumulation of chloramphenicol-insensitive precursors in their experiments. There was no evidence of precursors of either type in the experiments described here. The presence of a precursor is judged by a partial recovery of activity in the presence of the other inhibitor.

The inhibition of the synthesis of cytochrome oxidase in the presence of chloramphenicol gave no indication of degradation of this enzyme. The results with cycloheximide however, in which there was as much as a 65% decrease in the level of cytochrome oxidase with no change in mitochondrial malate dehydrogenase, indicate that cytochrome oxidase is turned over much more rapidly than other mitochondrial enzymes. It is possible that there is constant turn-over of the cytoplasmic component but not of the mitochondrial component of cytochrome oxidase.

No change was observed in the level of the mitochondrial enzymes succinic dehydrogenase and NADH cytochrome c reductase during chloramphenicol treatment. A small decrease was observed in mitochondrial malate dehydrogenase and ATPase after a 90 hr treatment.

If chloramphenicol has no effect on the level of any particular mitochondrial enzyme, it can be concluded that the enzyme is synthesized in the cytoplasm. A small decrease in the presence of chloramphenicol is probably a secondary effect but perhaps indicates that continued synthesis of protein by mitochondria is necessary to maintain the full complement of the enzyme either in situ or during isolation of mitochondria.

In both regenerating rat liver (Firkin and Linnane, 1969) and yeast (Vary et al, 1969) the synthesis of the mitochondrial enzymes succinic dehydrogenase and malate dehydrogenase were found to be insensitive to chloramphenicol. There is

genetic evidence in man (Davidson and Cortner, 1967) and in Neurospora crassa (Munkres, 1968) that the gene for mitochondrial malate dehydrogenase is nuclear. The work of Tzagoloff (1969) indicates that ATPase is synthesized on the cytoplasmic ribosomes of yeast, and thus the gene for this enzyme must also be nuclear.

The degree of change in the cytoplasmic enzymes in the presence of chloramphenicol should give some indication of the extent of the secondary effects of chloramphenicol on the cell. If, as has been indicated by Hales (1967), aldolase and phosphofructokinase are rate-limiting enzymes in glycolysis, one might expect a large increase in the specific activity of these enzymes to compensate for the decrease of mitochondrial energy production in the presence of chloramphenicol. However only a small increase in phosphofructokinase was observed and there was a slight decrease in the specific activity of aldolase. The levels of lactate dehydrogenase and glucose-6-phosphate dehydrogenase also showed a small decrease.

IV Effect of Chloramphenicol on Mitochondrial Cytochromes

The decrease in the level of cytochrome oxidase activity observed in L cells treated with D-chloramphenicol (10 μg/ml) for 90 hr is associated with a decrease in the respiratory ability of the cell and a corresponding decrease in content of cytochromes *a* and a_3. The reduction in the level of cytochromes *a* and a_3 observed is in general agreement with the reduction observed in HeLa cells (Firkin and Linnane, 1968), in regenerating rat liver (Firkin and Linnane, 1969), with the chloramphenicol-induced inhibition of synthesis of cytochromes *a* and a_3 in yeast (Clark-Walker and Linnane, 1966 and 1967) and in the fungus Pythium ultimum (Marchant and Smith, 1968). A reduction in the amount of cytochrome *b* has also been observed in L cells, HeLa cells and regenerating rat liver in the presence of chloramphenicol.

The decrease in cytochrome c_1 was larger in HeLa cells and regenerating rat liver than in L cells. However cytochrome c_1 is difficult to measure because its peak absorption is so close to that of cytochrome c. Yeast cells grown in the presence of chloramphenicol were devoid of cytochromes c_1 and b and P. ultimum hyphae were devoid of cytochrome b.

In yeast the observations have been interpreted as indicating that the four insoluble cytochromes of the electron transport chain are synthesized in the mitochondrion. This also seems to be true for cytochromes a and a_3 in mammalian cells. The smaller reduction observed for cytochromes c_1 and b indicates that they are probably synthesized on the cytoplasmic ribosomes, as has been suggested for the other insoluble mitochondrial proteins succinic dehydrogenase, NADH cytochrome c reductase and ATPase. The absence of an effect on the level of cytochrome c in the presence of chloramphenicol confirms many previous demonstrations that cytochrome c is synthesized on cytoplasmic ribosomes.

V Effect of Chloramphenicol on Mitochondrial Ultrastucture

Aside from inhibition of mitochondrial protein synthesis, cytoplasmic vacuolation is one of the most characteristic features of a chloramphenicol treated cell. Vacuolated erythroblasts are a general finding in the bone marrow of patients on chloramphenicol therapy (Saide et al, 1961). In the regenerating rat liver experiments of Firkin and Linnane (1969), 15% of the total population showed such vacuolation but the distribution of the vacuolated cells was uneven, with the periportal zone being the most profoundly affected (50% vacuolated cells). When L cells are treated with D-chloramphenicol (10 µg/ml) for 90 hr, 10% of the total population is heavily vacuolated. Because the percentage of the total population which shows vacuolation is initially small but increases with time (25% showed extensive vacuolation and/or long distentions of the endoplasmic reticulum after 186 hr treatment) vacuolation is thought to be a secondary effect of chloramphenicol.

In L cells most of the heavily vacuolated cells also have abnormal looking mitochondria. The cristae appear to be condensed at the ends of the mitochondria in these cells. This may reflect a change in configuration or an actual loss of cristae. The mitochondria were not swollen at this time but after 186 hr all mitochondria were swollen and appeared to contain less cristae. Firkin and Linnane reported swollen mitochondria and a loss of cristae in regenerating rat liver in the presence of chloramphenicol. A marked reduction of mitochondrial cristae formation has also been observed in yeast grown in the presence of chloramphenicol (Clark-Walker and Linnane, 1967).

No changes were observed in negatively-stained mitochondria isolated from D-chloramphenicol treated cells (10 µg/ml for 90 hr). Although a 31% reduction of ATPase activity was observed there did not appear to be a reduction in the number of knobs (ATPase particles) on the cristae. In the presence of a much higher concentration of chloramphenicol (100 µg/ml for 7 days) the mitochondria of P. ultimum were found to be devoid of knobs (Marchant and Smith, 1968).

Most of the freeze-etched preparations of L cell mitochondria showed the inner membrane and no changes were apparent in mitochondria from D-chloramphenicol treated cells.

VI General

Chloramphenicol was found to cause a decrease in growth rate and a corresponding decrease in the synthesis and hence specific activity of cytochrome oxidase. Cycloheximide was also found to inhibit cytochrome oxidase activity. These results indicate that the mitochondria and the nucleus have joint control over the synthesis and assembly of cytochrome oxidase.

The small reductions observed in the specific activities of the mitochondrial enzymes malate dehydrogenase, succinic dehydrogenase, NADH cytochrome c reductase and ATPase indicates that these enzymes are synthesized in the cytoplasm before assembly in the mitochondrion. The same appears to be true for cytochromes c_1 and b and c.

Under conditions in which the level of cytochrome oxidase was reduced to 25% of the control, tne growth rate was about 30% of the control and only 10% of the cell population showed vacuolation and the presence of some abnormal mitochondria. Therefore even with a considerable decrease in the respiratory ability of cells they can still continue to grow and show no overt cytological changes.

The inhibition caused by D-chloramphenicol (10 µg/ml for 90 hr) was specific for this isomer, indicating a specific inhibition of mitochondrial protein synthesis. This lends support to the idea that this is the biochemical basis for the bone marrow depression observed in patients on chloramphenicol therapy. In 90% of the cell population, the cells were structurally unaffected by this treatment. Only 10% of the cells showed extensive vacuolation after 90 hr and 25% showed extensive vacuolation and/or distentions of the endoplasmic reticulum after 186 hr. It is suggested that vacuolation is a secondary effect since it is initially small but increases with the length of treatment.

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