Bioenergetics (1973), 5, 85-105

Mitochondrial Biogenesis in Human Fibroblasts

Lester Packer, Janiece S. Nolan, Surendra Katyare and James Smith

Department of Physiology and Anatomy, University of California Berkeley, CA 94720
and the Physiology Research Laboratory, Veterans Administration Hospital Martinez, CA 94553, USA

Received 9 October 1973

Abstract

It is not known whether limitation of lifespan represents a programmed genetic event or is a result of environmental factors imposed by the conditions of culture. An investigation of the factors surrounding the limited *in vitro* lifespan of human diploid fibroblasts has been undertaken. We have investigated the role of mitochondria in the finite lifespan of WI-38 human lung fibroblasts. Mitochondrial function was depressed in a controlled manner by treating cells with ethidium bromide and chloramphenicol both of which inhibit normal biogenesis. These antibiotics decrease cytochrome oxidase activity, change cell ultrastructure, and inhibit growth at high concentration. At lower concentrations the antibiotics do not affect cell proliferation for several generations. However, their effect is cumulative and after several generations the cells enlarge, stop dividing and die. Removal of antibiotics from the culture media before death restores proliferative capacity. At still lower concentrations cytochrome oxidase activity was decreased but continuous growth in the presence of the antibiotics caused no decrease in *in vitro* lifespan. Thus, the potential for oxidative metabolism appears to be in excess of that needed for cell proliferation at all stages of the *in vitro* lifespan of a culture. The importance of cytoplasmic protein synthesis was evaluated using cycloheximide, a specific inhibitor of this process. Cycloheximide was used to try to distinguish between the effects due to general inhibition and that due to specific inhibition of mitochondrial biogenesis. Exposure of cultures to concentrations of cycloheximide which inhibited growth drastically caused no decrease in cytochrome oxidase activity.

Introduction

Most normal diploid cells that grow in culture manifest a limited *in vitro* lifespan. In particular, as shown by the pioneering work of Hayflick and Moorhead [1] and Hayflick [2], human diploid fibroblasts like WI-38 cells manifest 50 ± 10 population doublings *in vitro*. Since perhaps more is known about this particular human diploid cell population than any other, we have chosen it to study the factors that surround the limited capacity for cell proliferation. It is not known whether this limitation of lifespan is the result of environmental factors, peculiar to the conditions of culture imposed upon the cells, or whether this event is programmed, involving an intrinsic, genetically controlled limitation on the number of population doublings.

The first question we have investigated is whether the constraints of diffusion-limited *in vitro* culture conditions impose a critical dependence of cell proliferation upon mitochondrial oxidative metabolism. To study this problem we have depressed mitochondrial function in a controlled manner by treating cells with antibiotic reagents such as ethidium bromide (EB) and chloramphenicol (CAP), which selectively inhibit normal mitochondrial biogenesis. We have also studied some effects of cycloheximide (CHI), an antibiotic that inhibits the synthesis of cytoplasmic proteins. We have pursued this problem by investigating changes in the growth, morphology, and cytochrome oxidase activity of antibiotic treated and control cells as a function of their *in vitro* lifespan.

Materials and Methods

Cell Culture

WI-38 starter cultures were obtained at early passage (approximately 12) from the laboratory of Dr. Leonard Hayflick (Standford University) and maintained at 37°C. Cells were subcultivated with 0.25% trypsin routinely at a 1 : 4 split ratio on a weekly basis, keeping a record of population doublings (see Hayflick [1, 2], McHale and Packer [3]). Falcon Plastics 25 cm^2 and 75 cm^2 flasks were used.

A Coulter Counter Model B with a Model J automatic particle size distribution analyzer was used for cell counts and cell volume determinations. Replicate cultures were trypsinized, aspirated, and 0.5 ml used for counting, with an average of three counts presented in the growth curves. The screening experiments (Fig. 1) were carried out three more times on various early and intermediate (P-22–30) sets of cultures.

WI-38 cells were grown in Eagle's Basal Medium supplemented with fetal bovine serum (10%) and 2 mM glutamine. Flow Laboratories and

Grand Island Biological Co. reagents were used. The use of antibiotics was avoided to prevent masking of low levels of bacterial contamination.

Medium and cells were routinely monitored for bacteria or fungi. Cells and culture medium samples were analyzed for *Mycoplasma sp.* by Dr. L. Hayflick's laboratory. No *Mycoplasma* contamination was found.

When cell cultures were subjected to acute treatments, the appropriate concentration of antibiotic was added to the cultures 24 hours after subcultivation. When cells were exposed to the antibiotic for several subcultivation periods (chronic treatment) the appropriate concentration of antibiotic was added to the culture medium at the time of subcultivation.

Antibiotics

Ethidium bromide (EB), chloramphenicol (CAP), and cycloheximide (CHI) (Sigma Chemical Co.) were dissolved in Eagle's Basal Medium, filtered through a 0.2 μ Gelman filter and added to complete growth medium in appropriate concentrations. 100x stock solutions were kept at 10° C no longer than two weeks, and dilutions were made when needed. For acute and chronic experiments, cell medium was replenished every four days. Cells examined by electron microscopy were under treatment for four days only, except for chronic experiments in which a prolonged treatment with antibiotic was required with regular medium changes.

Acute Experiments

In order to screen for toxicity and establish minimum effective dosage of acute exposure to antibiotics, the following arbitrary distinctions were made for a single population doubling:

Acute—The minimum concentration of antibiotic which was lethal to the culture by the time controls reached confluency.

Threshold—The maximum concentration of antibiotic which allowed the treated cells to reach confluency at the same time controls did, and no morphological damage was apparent from observations of cells with a light microscope.

Chronic Experiments

For chronic exposure to antibiotics, cells were continually subcultivated in the stated concentrations. Parallel cultures were used to determine cytochrome oxidase activity at each subcultivation. At each subcultivation 4×10^5 cells were inoculated into 75 cm^2 flasks. Cultures were subcultivated at eight day intervals.

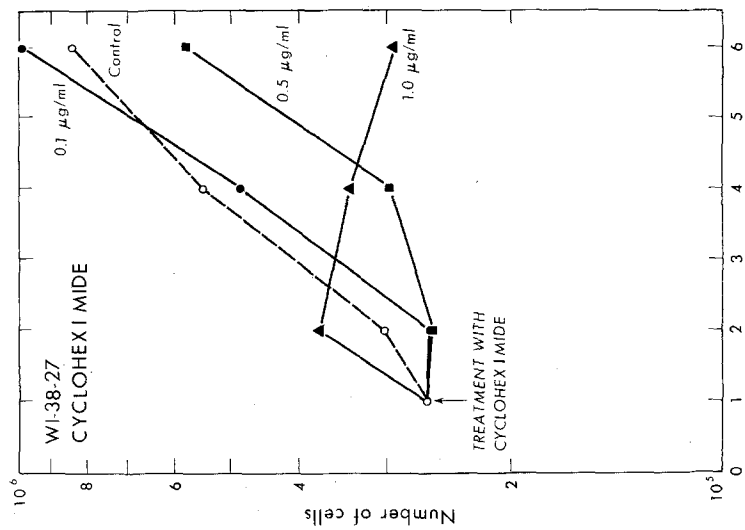
WI-38-27
CYCLOHEXIMIDE
Control
0.1 μg/ml
0.5 μg/ml
1.0 μg/ml
TREATMENT WITH CYCLOHEXIMIDE
Number of cells
Days

Fig. 1b.

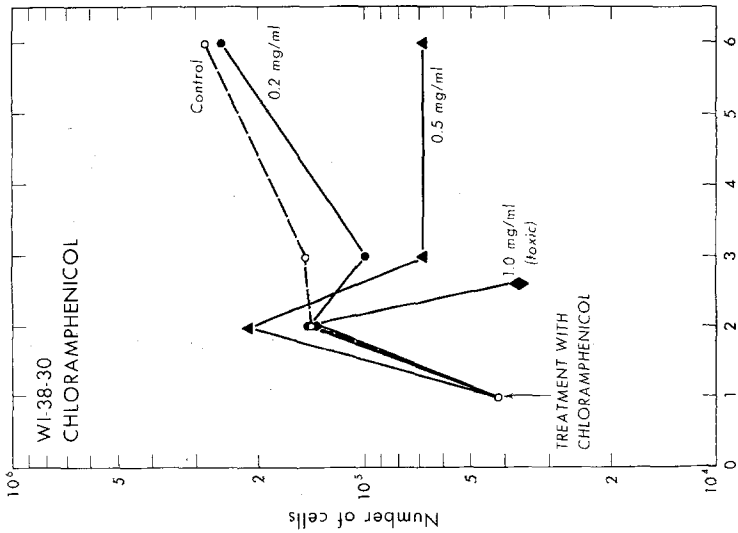
WI-38-30
CHLORAMPHENICOL
Control
0.2 mg/ml
0.5 mg/ml
1.0 mg/ml (toxic)
TREATMENT WITH CHLORAMPHENICOL
Number of cells
Days

Fig. 1a.

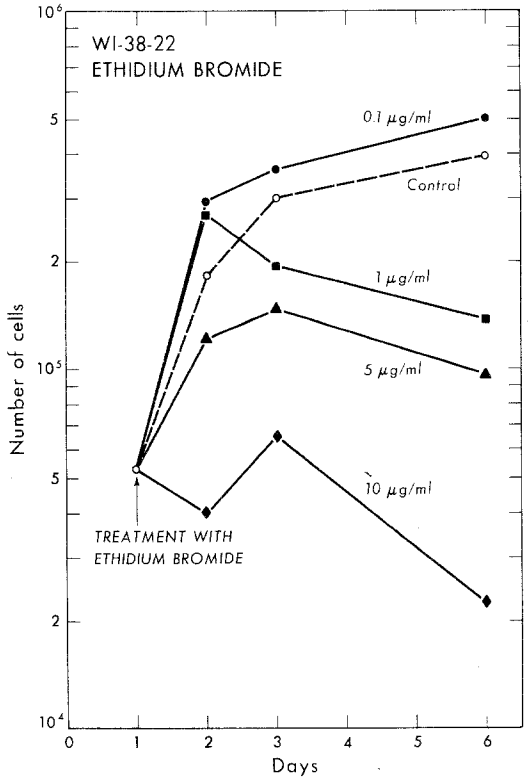

Fig. 1c.

Figure 1. Abscissa: Time after subcultivation (days); Ordinate: Cell number per 25 cm^2 flask. Proliferation of early passage WI-38 cells in the presence of different antibiotics: CAP, CHI and EB. Antibiotic treatment began on day one. (a) Effect of chloramphenicol concentrations on growth on WI-38 cells: ○——○, 0; ●——●, 0.2; ▲——▲, 0.5; and ◆——◆, 1.0 mg/ml. (b) Effect of cycloheximide concentrations on growth of WI-38 cells: ○——○, 0; ●——●, 0.1; ■——■, 0.5; and ▲——▲, 1.0 μg/ml. (c) Effect of ethidium bromide concentrations on growth of WI-38 cells: ○——○, 0; ●——●,0.1; ■——■, 1.0; ▲——▲, 5; and ◆——◆, 10 μg/ml.

Electron Microscopy

Cells growing in plastic 25 cm^2 flasks were fixed with 2% buffered glutaraldehyde after four days treatment with antibiotics. The cells were subsequently post-fixed with 1% buffered osmium tetroxide, stained with 0.5% aqueous uranyl acetate, dehydrated in a graded series of ethanol concentrations, and embedded in Epon using a modified technique of Brinkley *et al.* [4]. Thin sections were obtained using a

MT-2 ultramicrotome, and stained with 0.5% uranyl acetate and lead citrate. Sections were examined with a Siemens IA electron microscope.

For determination of mitochondrial area, photographs were Xeroxed, mitochondrial areas, cut out and weighed. Mitochondrial areas could be calculated by this procedure.

Cytochrome Oxidase

For determination of cytochrome oxidase activity, the cells were gently scraped from 75 cm^2 flasks and washed once in 0.25 M sucrose containing 2 mM EDTA and 0.1% ethanol, pH 7.5. The cells were suspended in the same medium, homogenized and sonicated for 2 sec using the microprobe of Biosonik III sonicator and salt-ice water bath. Cytochrome oxidase activity was measured according to a micro-procedure developed by Sun and Poole [5]. The assay medium consisted of 100 mM Tris-HCL, pH 7.5, 1 mM EDTA, 0.05 mM reduced cytochrome c and 0.1% emasol. Cytochrome c was reduced with sodium dithionite just before use. The assay was carried out at 37°C. Oxidation of cytochrome c was followed at 550 nm using a Cary 14 recording spectrophotometer. The activities are expressed as nmoles cytochrome c oxidized/min/mg protein.

Protein was determined by a modification of the method of Lowry *et al.* [6] using crystalline bovine serum albumin as a standard.

Results

Cell Proliferation—Acute Treatment with Antibiotics

Typical growth curves and the effects of CAP, CHI, and EB on proliferation of human diploid fibroblasts is shown in Fig. 1a, b, and c respectively. Each antibiotic, at the concentrations used in this series of experiments, manifests inhibition of cell proliferation.

The threshold concentrations of antibiotics, i.e., which arrest growth, may cause a slight enlargement of cell size as judged by mean cell volume registered in a Coulter Counter or qualitatively by light microscopy.

Gross Morphology and Mitochondrial Content

Morphological examination was carried out on cells grown at the antibiotic concentration for the threshold level for inhibition of cell proliferation. Antibiotic treatments at the threshold of growth inhibition have marked effects on cellular and mitochondrial structure. Results are summarized below:

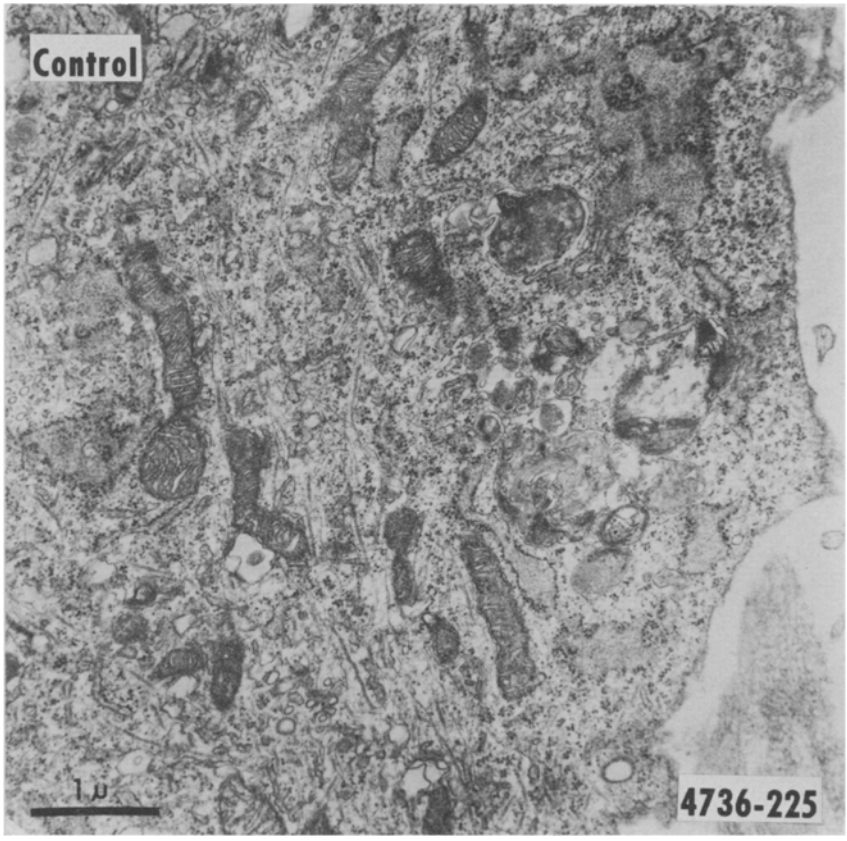

Figure 2. High magnification electron micrograph of untreated early passage WI-38 cells, showing distribution of mitochondria in peripheral region.

Control

Electron micrographs of peripheral regions show normal mitochondria (Fig. 2). The inner and outer membranes and the cristae are evident.

Ethidium bromide

In cells treated with 2 μg/ml (Fig. 3) and 5 μg/ml of EB (Fig. 4) marked changes in mitochondrial structure are observed. Increasing the concentration of EB progressively causes the appearance of smaller mitochondria which seem to be fragmented. At higher concentrations of EB, mitochondrial structure is not well defined; the inner membrane and cristae are disorganized (Fig. 4). The effect of EB on cell structure is

Figure 3. High magnification electron micrograph of early passage WI-38 cells treated with 2 μg/ml of ethidium bromide. An area peripheral to the nuclear region is shown.

pleomorphic. There seems to be an enhanced appearance of microfilamentous structures. The loss of mitochondria may reveal these structures more clearly, or there could be a real increase in their occurrence.

Changes in the endoplasmic reticulum following EB treatment were also noted. The cisternal diameter is larger (Fig. 4) and the occurrence of Golgi regions is not very evident after higher EB treatment.

Chloramphenicol

Treatment with CAP at 200 μg/ml has the remarkable effect of increasing the number of mitochondria and mitochondria-like structures.

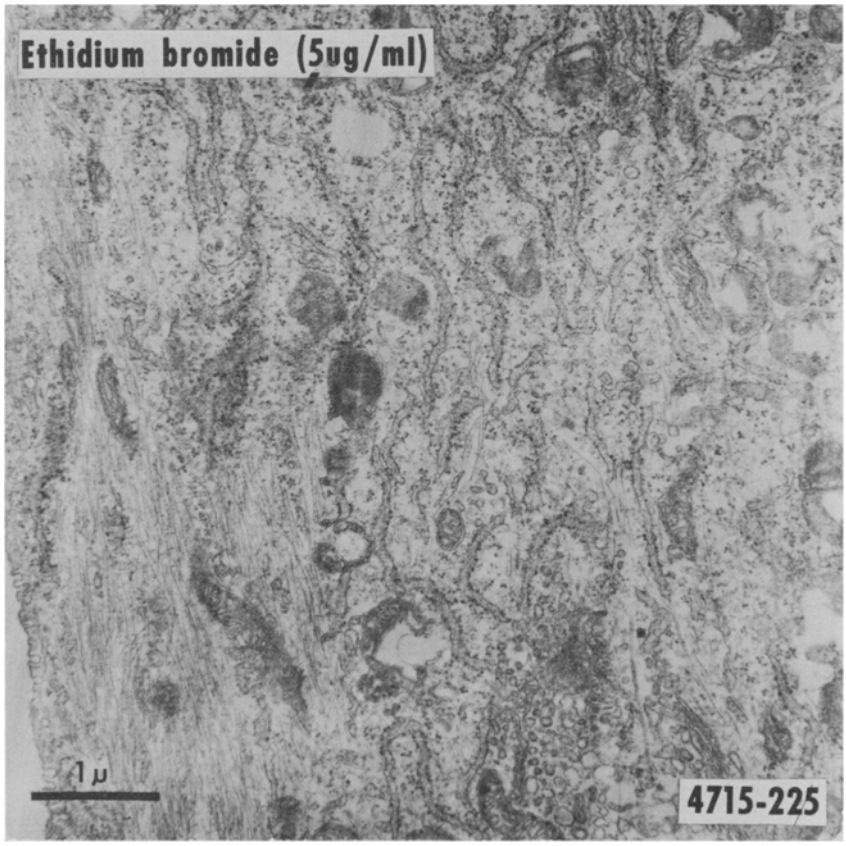

Figure 4. High magnification electron micrograph of early passage WI-38 cells treated with 5 μg/ml of ethidium bromide. An area peripheral to the nuclear region is shown.

Figure 5 shows the appearance of numerous mitochondria, which retain their normal appearance in some instances, but in others, are closely associated with myelin-like structures which appear morphologically to be derived from mitochondria. There is a large increase in the proportion of mitochondria to other organelles in CAP treated cells. Other cellular structures following CAP treatment appear normal.

Cycloheximide

Cycloheximide treatment at 1 μg/ml does not markedly affect gross morphology (Fig. 6) or the number of mitochondria as compared to untreated cultures. The most remarkable effect appears on the smooth

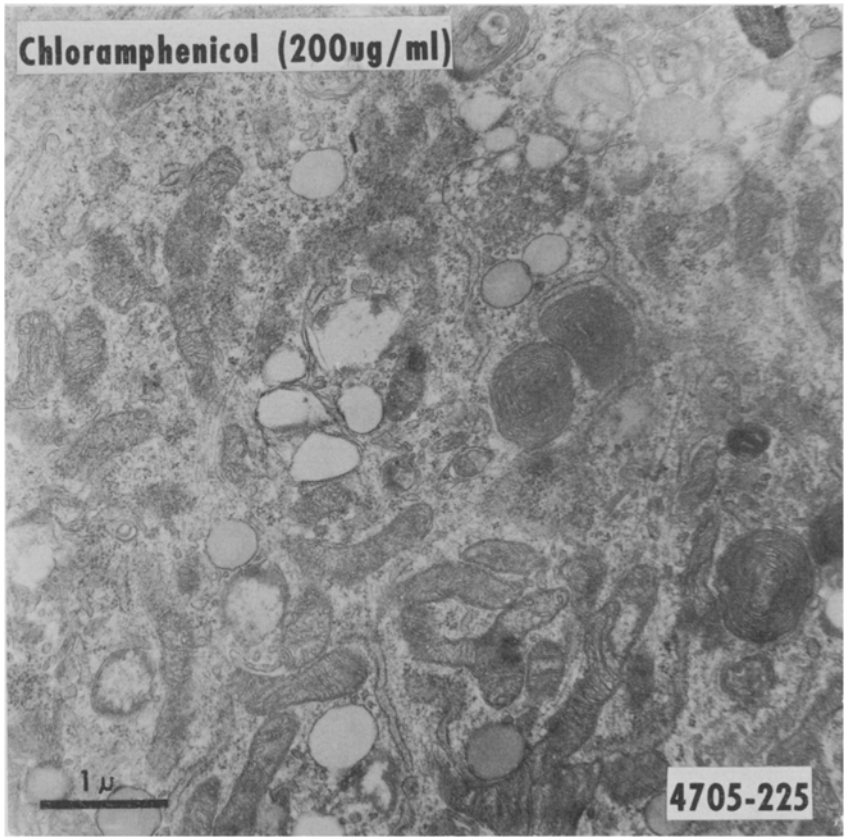

Figure 5. High magnification electron micrograph of a peripheral region of early passage WI-38 cells treated with 200 μg/ml of chloramphenicol. Myelinated mitochondrial structures are evident.

surfaced elements of the endoplasmic reticulum which are more numerous. A more elaborate development of Golgi vesicles is also observed; these vesicles are not filled with electron dense material. These effects may be related to the known inhibition of cytoplasmic protein synthesis by cycloheximide.

Mitochondrial Content

The effect of the various antibiotic treatments on the occurrence of mitochondria is summarized in Table I. The mitochondrial content was evaluated by determining the relative areas they occupied in photographs (cf. Methods) of regions of WI-38 cells peripheral to the nucleus.

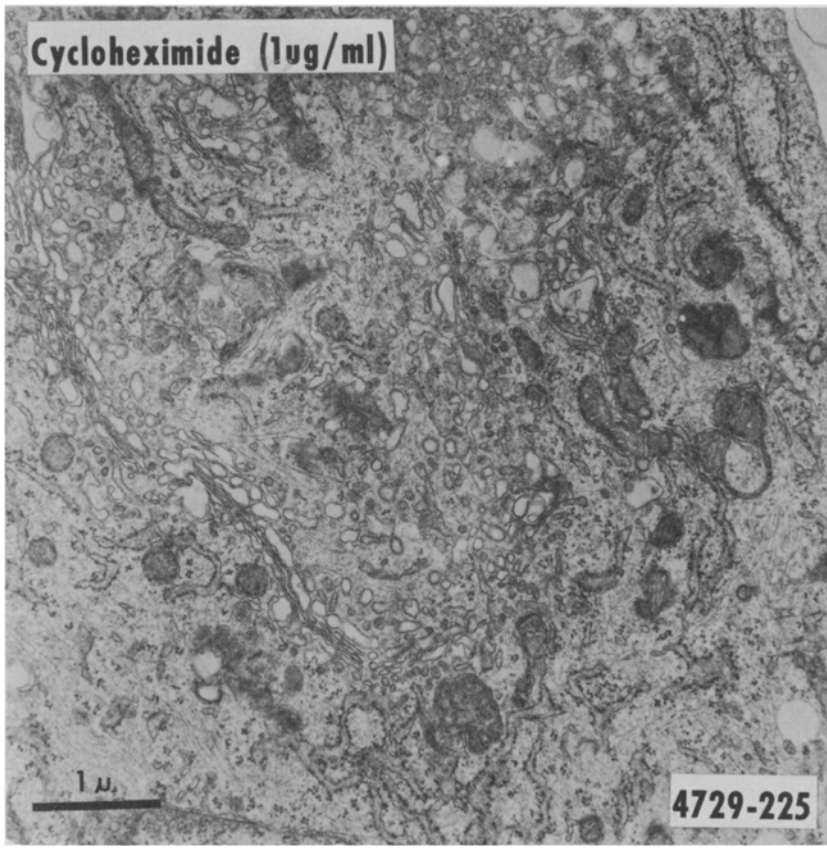

Figure 6. High magnification electron micrograph of a peripheral region of early passage WI-cells treated with 1 μg/ml of cycloheximide.

Mitochondria decreased both in number and size with EB; with CAP, at the concentration employed, the number of mitochondria increased, even apart from the occurrence of myelin figures which may also be attributed to mitochondria. A small decrease in the number of mitochondria occurred with cycloheximide.

Effect of Ethidium Bromide, Chloramphenicol and Cycloheximide on long-term growth potential and cytochrome oxidase activity of fibroblasts

In order to answer the question as to whether mitochondria *per se* are important to the limited *in vitro* lifespan in human fibroblasts,

TABLE I. Effect of ethidium bromide, chloramphenicol, and cycloheximide on biogenesis of mitochondria in human diploid (WI-38) cells

Conditions	Cell area (μ^2)	Number of mitochondria	Mitochondrial area μ^2	Mitochondrial area % of total
Control	66.67	14.5	3.10	4.65
Ethidium bromide				
(2 μg/ml)	68.38	9.7	2.53	3.71
(5 μg/ml)	61.30	9.0	0.96	1.57
Chloramphenicol				
(200 μg/ml)	68.44	19.6 3.3[a]	6.21 0.88[a]	9.08 1.29[a]
Cycloheximide				
(1 μg/ml)	64.62	13.3	2.13	3.30

[a] Proportion of mitochondrial "myelin figures" present.

antibiotics that interfere with the normal biogenesis of mitochondria were tested under chronic conditions of treatment (cf. Methods). Concentrations considerably lower than threshold levels had to be used for chronic experiments. As shown in Fig. 7, when the antibiotics were present in the growth medium at sufficiently low levels, there was no effect on the cell yield after eight days of growth nor on the *in vitro* lifespan. However, when the antibiotic concentration was increased by a

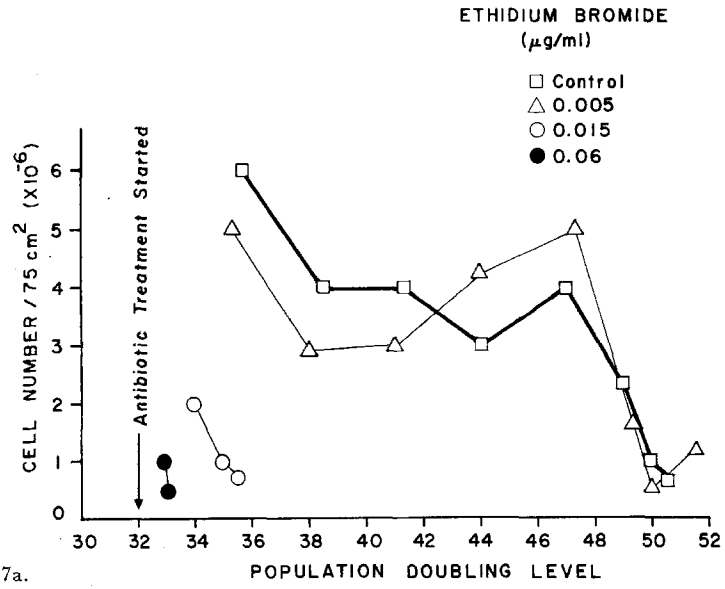

Fig. 7a.

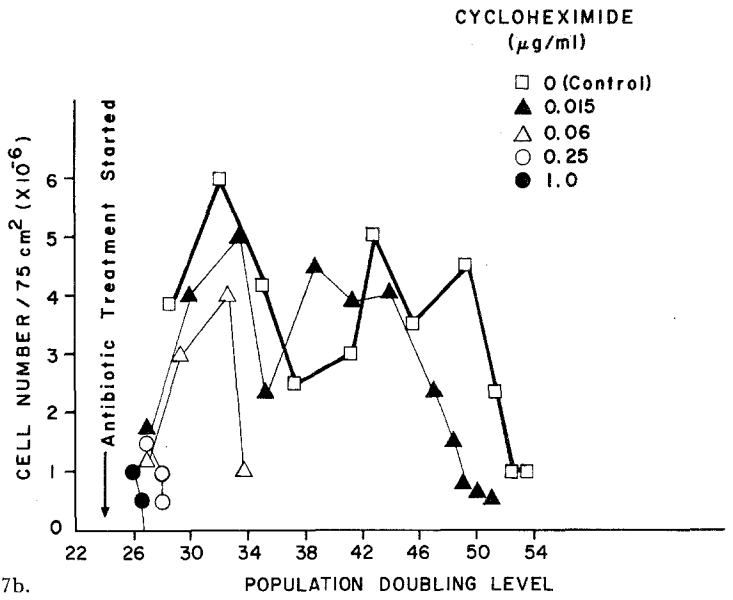

Fig. 7b.

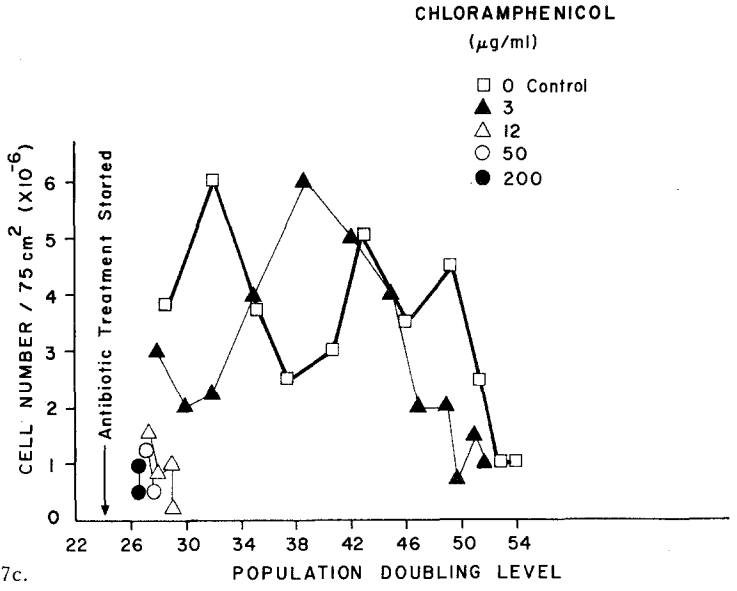

Fig. 7c.

Figure 7. Abscissa: population doubling level; ordinate: cell number/75 cm^2 (x 10^{-6}). Effect of prolonged treatment of antibiotics, EB, CHI and CAP on the growth of WI-38 cells. (a) Concentration of ethidium bromide (μg/ml): □——□, 0; △——△, 0.005; ○——○, 0.015; and ●——●,0.06. (b) Concentration of cycloheximide (μg/ml): □——□, 0; ▲——▲, 0.015; △——△, 0.06; ○——○, 0.25; and ●——●, 1.0. (c) Concentration of chloramphenicol (μg/ml): □——□, 0; ▲——▲, 3; △——△, 12; ○——○, 50; and ●——●, 200.

factor of four there was an immediate effect on growth rate and the cultures were lost after only a few subcultivations. Cultures treated with CHI behaved somewhat differently from those treated with EB or CAP. When treated with 0.06 μg/ml CHI (four times the concentration which had no influence on growth) the cultures showed a nearly normal growth rate for several generation doublings and the culture was able to survive for ten population doublings. A summary of the effect of various concentrations of the antibiotics on lifespan is given in Table II. In addition to the number of population doublings accrued during treatment, we also determined the total protein as an alternative measure of the amount of growth achieved during treatment. From Table II it is seen that the total protein is roughly proportional to the number of population doublings.

At each subcultivation during the antibiotic treatment cytochrome oxidase activity was determined in cultures parallel to the subcultivation series. The results of these experiments are shown in Fig. 8. Cultures treated with a concentration of CAP which did not inhibit growth or decrease lifespan (3.0 μg/ml) possessed only 50% of the cytochrome

TABLE II. Effect of chronic antibiotic treatment on lifespan, cellular proteins and cytochrome c oxidase activity of WI-38 cells

Medium concentration (μg/ml)	Population doublings accrued	Total protein (mg)	Average cytochrome oxidase (% of control)
Cycloheximide			
control	30	7.6	100
0.015	27	7.3	98
0.06	10	2.2	103
0.25	4	1.1	92
1.0	2	0.5	77
Ethidium bromide			
control	19	4.9	100
0.005	20	3.7	89
0.015	3	0.5	30
0.06	1	–	–
Chloramphenicol			
control	30	7.6	100
3.0	28	6.5	51
12.0	5	0.9	42
50.0	3	0.6	48
200.0	2	0.2	32

The cells were grown on different antibiotic concentrations as described in Materials and Methods section and as shown in Figs. 7 and 8. Cytochrome c oxidase activity is expressed as % of control. As indicated in Fig. 8a, the activity in control remained more or less constant at different passage levels.

oxidase activity of the untreated cultures (Fig. 8c). At higher concentrations the activity was somewhat lower, but even at concentrations which inhibited growth very quickly the cytochrome oxidase activity was still about 30% of the control. By contrast, concentrations of CHI (Fig. 8b) which did not allow cell growth had very little effect on the cytochrome oxidase activity.

Treatment with EB (Fig. 8a) at a concentration which did not inhibit growth appeared to cause an initial decrease in cytochrome oxidase activity. After a few population doublings, cytochrome oxidase activity was regained and remained at about the control level until the culture neared the end of its *in vitro* lifespan when it apparently decreased again. Also shown in Fig. 8a is the specific activity of untreated cultures as a function of population doubling level. There was no decrease in activity as the culture approached the end of its *in vitro* lifespan. The average cytochrome oxidase activity during treatment with various concentrations of antibiotics is given in Table II.

Recovery of Cytochrome Oxidase Activity and Growth Potential after Antibiotic Treatment

Table III shows the effect of treating cultures with antibiotics for two or four subcultivation periods on the lifespan of the cultures. In the case of CHI and EB, growth for two subcultivation periods in concentrations that drastically inhibit growth does not decrease the lifespan of the cultures. Soon after the antibiotics were removed the growth rate returned to that of cultures which had not been treated.

However, when cultures were treated for two subcultivation periods

TABLE III. Effect of removal of the antibiotics on the growth potential of WI-38 cells

Medium concentration (μg/ml)		Number of population doublings accrued[a]	Length of antibiotic treatment (transfers)[b]
Cycloheximide	1.0	29	2
	0.25	34	2
	0.06	30	4
	0.015	31	4
Chloramphenicol	3.0	30	2
	3.0	26	4
	12.0	19	4
Ethidium bromide	0.015	27	2
Control	0	30	0

[a] Sum of population doublings during and after antibiotic treatment.
[b] 4×10^3 cells/cm^2 inoculated at eight day intervals.

The details are as described in the Materials and Methods section.

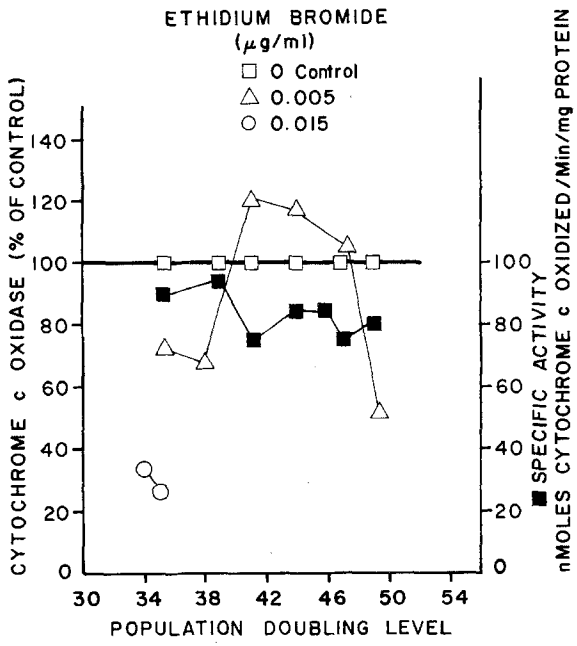
ETHIDIUM BROMIDE
(μg/ml)
□ 0 Control
△ 0.005
○ 0.015
CYTOCHROME c OXIDASE (% OF CONTROL)
SPECIFIC ACTIVITY
nMOLES CYTOCHROME c OXIDIZED/Min/mg PROTEIN
0
20
40
60
80
100
120
140
30 34 38 42 46 50 54
POPULATION DOUBLING LEVEL

Fig. 8a.

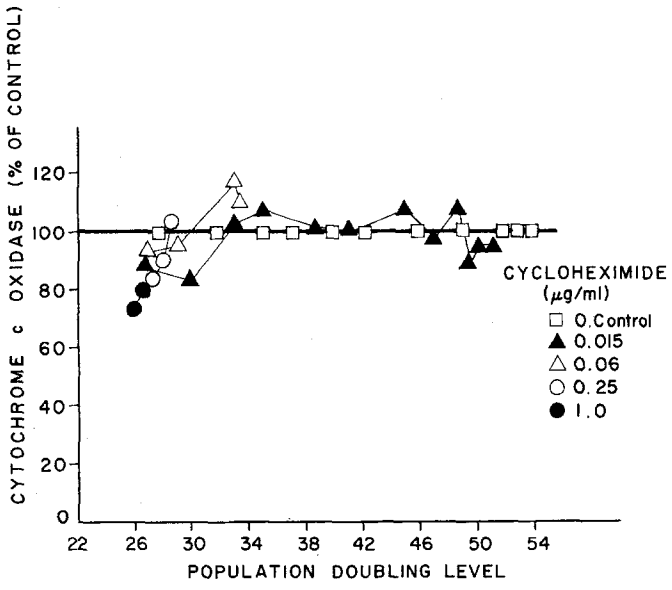
CYTOCHROME c OXIDASE (% OF CONTROL)
0
20
40
60
80
100
120
22 26 30 34 38 42 46 50 54
POPULATION DOUBLING LEVEL
CYCLOHEXIMIDE
(μg/ml)
□ 0.Control
▲ 0.015
△ 0.06
○ 0.25
● 1.0

Fig. 8b.

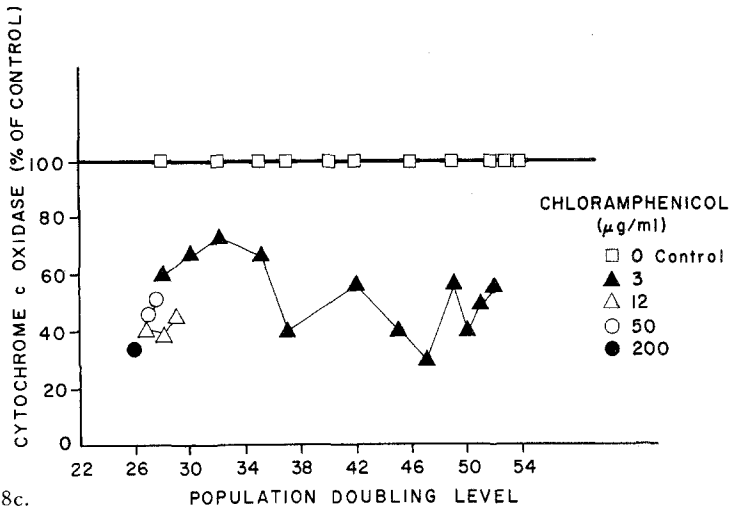

Fig. 8c.

Figure 8. Abscissa: population doubling level; ordinate (left): cytochrome c oxidase activity (% of control), (right in Fig. 8a): cytochrome c oxidase activity of control, n moles cytochrome c oxidized/min/mg protein; ■——■. (a) Concentration of ethidium bromide (μg/ml): □——□, 0; △——△, 0.005; ○——○, 0.015. (b) Concentration of cycloheximide (μg/ml): □——□, 0; ▲——▲, 0.015; △——△, 0.06; ○——○, 0.25; and ●——●, 1.0. (c) Concentration of chloramphenicol (μg/ml): □——□, 0; ▲——▲, 3; △——△, 12; ○——○, 50; and ●——●, 200.

with high concentrations of CAP (50 or 200 μg/ml) they were unable to recover after removal of the antibiotic. Cultures treated during four subcultivation periods with 12 μg/ml of CAP recovered from the antibiotic treatment slowly and the growth rate never became as high as that of untreated cultures. Furthermore, these cultures were unable to undergo as many population doublings as untreated cultures.

Treatment with CAP (12 μg/ml) and EB (0.015 μg/ml) resulted in cytochrome oxidase activities 40% and 25% respectively of that in untreated cultures (see Table IV). When the antibiotics were removed from the culture medium the cytochrome oxidase activity returned to that of untreated cultures within one or two sucultivation periods. The cytochrome oxidase activity remained high throughout the lifespan of these cultures.

Discussion

Biogenesis of Mitochondria in Human Cells

Earlier studies with EB and CAP in yeast [7], regenerating rat liver [8] and L-cells [9] have shown that these antibiotics inhibit the

TABLE IV. Recovery of cytochrome c oxidase activity in WI-38 cells after removal of the antibiotics

Medium concentration (μg/ml)	Cytochrome oxidase activity (% of control)	No. of transfers after antibiotic removal
Chloramphenicol 12.0 (4)[a]	40	–
	110	1
	80	2
	90	3
Ethidium bromide 0.015 (2)[a]	25	–
	114	2
	123	6
	105	9
	90	10

[a] Number of transfers in presence of antibiotic.

synthesis of functional cytochromes a, a_3, b and c_1. The effects of EB and CAP on growth, cytochrome oxidase and morphology in this investigation indicate that qualitatively similar effects occur in human diploid fibroblasts. In particular, the morphological effects of these antibiotics reagents are interesting because they indicate the selectivity of their effects. Ethidium bromide treated fibroblasts show the presence of fragmented mitochondria, similar to changes brought about by EB in *Tetrahymena* [10]. The effect of CAP on mitochondrial structure in human fibroblasts and other systems also seems similar. Prolonged CAP treatment caused the appearance of fewer cristae in He La cells [11, 12], *Polytomella* [13] and regenerating rat liver [8]. In *Ochromonas,* CAP treatment leads to the presence of prolamellar bodies and of abnormal thylakoid membranes [14]. The presence of myelin-like bodies observed in the present study have also been seen in other systems [15]; they may represent degenerating structures or non-functional mitochondria.

The occurrence of myelin-like figures is usually associated with the formation of lamellar membranous structures which are low in protein content. They are characteristic of myelin itself and the myelin-like membranes found in the surfactin producing type II lung alveolar cells, and perhaps other abnormal conditions where protein synthesis has been inhibited. These results suggest "decoupling" of the membrane protein synthesis from membrane lipid synthesis leading to the production of multi-layered myelin-like structures by CAP.

Lifespan of Human Fibroblasts

Normal human cells manifest a finite lifespan when serially subcultivated *in vitro*. The factors which limit the *in vitro* lifespan of

such cells are presently unknown. Limitation of lifespan may relate to the state of differentiation [2] or alternatively, environmental conditions (e.g. the constraints of diffusion-limited *in vitro* culture conditions may impose a critical dependence on mitochondrial oxidative metabolism for cell proliferation). In this investigation, we have studied the effect on lifespan of mitochondrial oxidative metabolism. The results clearly show that cells treated with EB and CAP manifest an inhibition of synthesis of normal mitochondria as shown by decrease in cytochrome oxidase and morphological changes; while CHI, an inhibitor of cytoplasmic protein synthesis, had no effect on cytochrome oxidase activity even at levels which greatly inhibited growth.

Treatment of WI-38 cultures with 0.005 μg/ml of EB caused an initial decrease in cytochrome oxidase activity, but on continued subcultivation in its presence the activity returned to the control value. When the EB treated cultures entered Phase III the cytochrome oxidase activity again decreased. It may be that during continuous growth in EB the mitochondrial population became resistant to its action, but that resistance was lost when the culture reached the end of its *in vitro* lifespan. EB resistance has been reported in SV-40 transformed WI-38 cells [16] and CAP resistance has been reported in He La cells [17]. Also irreversible morphological changes in mitochondria of He La cells have been obtained by growth in EB [18].

Although EB and CAP, at low concentrations, cause a decrease in cytochrome oxidase activity they exert no effect on the proliferative capacity. This is especially apparent in cultures treated with 3,μg/ml CAP. During the last half of their *in vitro* life span the cytochrome oxidase activity was maintained at 50% of the normal level but there was no decrease in the number of population doublings achieved in culture (Table II).

EB has been shown to cause the disappearance of the circular form of mitochondrial DNA in several mammalian cell lines [19]. This change was reversible by subsequent growth in EB-free medium. This is similar to the behavior of cytochrome oxidase activity in our cultures.

EB when present at 0.015 μg/ml caused a 75% decrease in cytochrome oxidase activity and inhibited cell division. However, the effects of this antibiotic were reversible and both the cytochrome oxidase activity and proliferative capacity returned to normal when the drug was removed. Similar results have been reported after periods of anaerobic incubation of TC 12266 human diploid fibroblasts by Hakami and Pious [20]. In contrast, CAP when present in the culture medium for four subcultivation periods at 12 μg/ml did cause a decrease in the *in vitro* lifespan. However, the inhibition of cytochrome oxidase activity was not as great with CAP as with EB and the activity returned to the control level within one subcultivation period after removal of CAP. We also found that the specific activity of cytochrome oxidase did not change

during the last half of the *in vitro* lifespan of untreated cultures (Fig 8a). This is in agreement with the findings of Hakami and Pious [21].

It appears that the potential for oxidative metabolism is more than sufficient to promote cell proliferation throughout the lifespan of human diploid cell cultures. Since decreasing the cytochrome oxidase activity by 50% during the last half of the *in vitro* lifespan has no effect on the lifespan, we conclude that older cultures are not dependent on oxidative metabolism to any greater extent than young cells.

It is possible, however, that mitochondria may be indirectly involved in limiting the *in vitro* lifespan. Cytoplasmic damage may arise as a result of the continual turnover of mitochondria since the highest concentrations of free radical generating reactions and unsaturated fatty acids which are susceptible to lipid peroxidation, and calcium activated phospholipase, occur in mitochondrial membranes. In damaged membranes, phospholipase causes the further release of unsaturated fatty acids, which themselves are damaging to membranes. These reactions are normally latent in mitochondria until membrane damage occurs during mitochondrial turnover [22].

References

1. L. Hayflick and P. S. Moorhead, *Exptl. Cell Res.*, 25 (1961) 585.
2. L. Hayflick, *Exptl. Cell Res.*, 37 (1965) 614.
3. J. S. McHale and L. Packer, *Methods in Enzymology, Biomembranes* (1973) Vol. I, in press.
4. B. R. Brinkley, P. Murphy and L. C. Richardson, *J. Cell. Biol.*, 35 (1967) 279.
5. A. S. K. Sun and B. Poole, *Fractionation of mitochondria, lysosomes, plasma membrane and peroxisomes from rat embryo fibroblasts* (in preparation).
6. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
7. G. M. Kellerman, D. R. Biggs and A. W. Linnane, *J. Cell Biol.*, 42 (1969) 378.
8. F. C. Firkin and A. W. Linnane, *Exptl. Cell Res.*, 55 (1969) 68.
9. M. E. King, G. C. Godman and D. W. King, *J. Cell Biol.*, 53 (1972) 127.
10. D. C. Rein and R. R. Meyer, *J. Cell Biol.*, abstracts (1971) 478.
11. R. Lenk and S. Penman, *J. Cell Biol.*, 49 (1971) 541.
12. N. Kislev, C. M. Spolsky and J. M. Eisenstadt, *J. Cell Biol.*, 57 (1973) 571.
13. D. A. Evans and D. Lloyd, *Biochem J.*, 103 (1967) 22P.
14. H. Smith-Johannsen and S. P. Gibbs, *J. Cell Biol.*, 52 (1972) 598.
15. A. Adoutte, M. Balmefrezol, J. Beisson and J. Andre, *J. Cell Biol.*, 54 (1972) 8.
16. W. Klietmann, K. Kato, N. Sato and H. Koprowski, *Fed. Proc.*, 31 (1972) 620 (abst.).
17. W. Klietmann, K. Kato, B. Gabara, H. Koprowski and N. Sato, *Exptl. Cell Res.*, 78 (1973) 47.
18. C. M. Spolsky and J. M. Eisenstadt, *FEBS Lett.*, 25 (1972) 319.
19. M. M. K. Nass, *Exptl. Cell Res.*, 72 (1972) 211.
20. N. Hakami and D. A. Pious, *Nature*, 216 (1967) 1087.
21. N. Hakami and D. A. Pious, *Exptl. Cell Res.*, 53 (1968) 135.
22. A. Scarpa and J. G. Lindsay, *Eur. J. Biochem.*, 27 (1972) 401.

Acknowledgements

The authors are grateful to Pauline Sallata for technical assistance with experiments, to Kathy Donovan for assistance with cell culture, and to Susan L. Tinsley and Lou Worthington for assisting with electron microscopy. This research was supported by USPHS Grant HD 05875 and the Veterans Administration.