RESPIRATORY ENZYMES AND MITOCHONDRIAL MORPHOLOGY OF HeLa AND L CELLS TREATED WITH CHLORAMPHENICOL AND ETHIDIUM BROMIDE

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ABSTRACT

Exposure of HeLa and L cells to chloramphenicol causes a progressive dose-dependent decrease in cytochrome oxidase and succinate–cytochrome *c* reductase activities, concomitant with an increase in the amount of cytochrome *c*. At 2–3 days, the specific activities of the enzymes have fallen to about one-half of control values; the mitochondria appear swollen. By day 5, enzyme activities are about one-quarter of control values; the mitochondria are more swollen, with disorientation and disintegration of cristae. By day 6–8, after three generations, growth has stopped, enzyme activities are approximately the same as on day 5, and cytochrome *c* content has reached 170% of control value. Mitochondria show severe changes, cristae being affected more than peripheral inner membrane. The number of profiles continues to be nearly normal. After 30 days, cytochrome oxidase activity remains low but now there are mitochondria in intermediate and condensed configuration. There is a gradual accumulation in the cytoplasm of smooth membrane elements. If chloramphenicol is removed, cells recover. Ethidium bromide treatment for up to 8 days yields results virtually identical to those obtained with chloramphenicol. Cells treated with 10^{-4} M KCN show a decrease in cytochrome oxidase activity to about one-third of control value and an elevated amount of cytochrome *c*. Only a small number of mitochondria appear damaged. Autochthonous mitochondrial syntheses appear to be essential for the organization of the cristae. When cytochrome oxidase activity is impaired, a regulatory mechanism for cytochrome biosynthesis geared to mitochondrial function may be lacking, resulting in an increase in cytochrome *c* content.

INTRODUCTION

Respiratory enzymes and mitochondrial membranes are normally synthesized in a coordinated fashion, the proportions of the various components being characteristic for a given cell (1). Regulation of the amounts of enzyme in mammalian cells depends on environmental conditions; for example, on oxygen tension (2–5) and on hormonal levels (6) but not on glucose levels (3, 5). In lower eukaryotes, mitochondrial lesions may result in an imbalance of cytochrome syntheses (7, 8) accompanied by defects in mitochondrial ultrastructure (9), as exemplified in the *petite* mutant of yeast. This mutant can be produced by treating normal yeast cells with acriflavin and ethidium bromide, drugs that interfere with the replication of mitochondrial DNA (10–12). The phenotype of the mutant appears during growth in the presence of antibiotics such as chloramphenicol and macrolide

compounds (e.g., erythromycin) which inhibit mitochondrial protein synthesis (13–17). Thus, the use of agents which selectively influence intramitochondrial synthesis of nucleic acid and/or protein can provide insight into the factors which regulate balanced synthesis of cytochromes. Cellular changes resembling the phenotype of the *petite* mutant have been observed in the regenerating liver of whole animals treated with chloramphenicol (18–20) and in cultivated cells growing in the presence of chloramphenicol (21–24) or of ethidium bromide (12, 25).

In the present study of the effects of chloramphenicol and ethidium bromide, we describe the extent of the imbalance of mitochondrial cytochromes in relation to the discernible changes in the mitochondrial membrane. Cytochrome oxidase activity is used as an assay for cytochrome aa_3. The activity of the complex of enzymes which includes an antimycin-sensitive cytochrome *b* reaction (6) is tested by assay of succinate–cytochrome *c* reductase. Cytochrome *c* is extracted and quantitated. The effect of chloramphenicol and ethidium, which restrict cell growth by interfering specifically with mitochondrial synthetic functions, is compared with that of other growth-inhibiting conditions.

MATERIALS AND METHODS

HeLa (CCL2) cells were grown in monolayer and HeLa (S3) and L cells were grown in suspension culture, each in the appropriate formula of Eagle's minimal essential medium containing additional glutamine at a final concentration of 4 mM, penicillin at 100 units/ml, streptomycin at 50 μg/ml, and 5% fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.). Cultures were diluted with fresh medium daily or every other day; when new medium was not added, glucose was supplemented (50 mg/100 ml). Glucose levels were determined by means of the Glucose Stat-Pack, Calbiochem (Los Angeles, Calif.). For amino acid deprivation tests, arginine was omitted from the medium. Actinomycin D, KCN, chloramphenicol, and ethidium bromide were sterilized by filtration and added to the medium as indicated.

For assay of enzyme activities, cells were lysed in 0.01 M potassium phosphate (pH 7.4) made 0.1% in deoxycholate. Cytochrome oxidase activity was determined as described (26) for L cells, and in 0.02 M phosphate buffer (pH 7.0) for HeLa cells. Both cytochrome oxidase and succinate–cytochrome *c* reductase (27) activities were determined in the Cary spectrophotometer (Cary Instruments, Monrovia, Calif.) at approximately 27°C. Cytochrome *c* was extracted, purified, and quantitated after the method of Margoliash and Walasek (28). DNA was determined by the diphenylamine reaction (29) and protein by the method of Lowry et al. (30).

For fixation for electron microscopy, cells in suspension culture were harvested by centrifugation and resuspended in warm, balanced salt solution. Cells in monolayer culture were rinsed with the warm salt solution and fixed *in situ*, or in the same manner as cells from suspension culture after brief trypsinization (0.125% trypsin in balanced salt solution made 0.15% in disodium ethylenediaminetetraacetate) and incubation in growth medium at 36.5°C for 60 min. Fixation was carried out either in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at 40°C for 60–90 min, or in a mixture of osmium tetroxide and glutaraldehyde (31) in 0.1 M cacodylate (pH 7.4). Subsequent buffer washes contained 2.5% sucrose. Glutaraldehyde was followed by postfixation in 2% osmium tetroxide. The glutaraldehyde–osmium tetroxide fixation was followed by treatment with saturated uranyl acetate in 0.1 M acetate buffer (pH 6.3). Fixed cells were pelleted in molten agar (31). The coherent cell pellets were dehydrated through a graded series of dilutions of ethanol and embedded in Epon 812 (Shell Chemical Co., St. Louis, Mo.). Thin sections, cut on the Cambridge ultramicrotome (Cambridge Instrument Co., Cambridge, England), were stained with saturated uranyl acetate in 50% ethanol followed by lead citrate, and were examined in the Siemens Elmiskop I electron microscope.

RESULTS

Cell Growth

Both chloramphenicol and ethidium bromide inhibit the proliferation of L cells and of HeLa cells in suspension culture. Fig. 1 shows the effect of chloramphenicol on the growth of L cells expressed as the cumulative cell count per culture. The cells are moderately enlarged, containing 26.8 μg DNA/mg protein as compared with the control value of 40 μg DNA/mg protein. Growth virtually ceases after three generations, although L cells can then be transferred from suspension culture to monolayer culture where they survive at least 34 days in the presence of chloramphenicol, 20 μg/ml. If the drug is removed, the cells resume a normal rate of growth within 5 days. HeLa cells treated with chloramphenicol, 5 μg/ml, multiply at a rate equal to that of the control for 5 days; at 20 μg/ml, they grow at a rate similar to that described for the treated L cells in Fig. 1.

L cells treated with ethidium bromide, 1.0 μg/

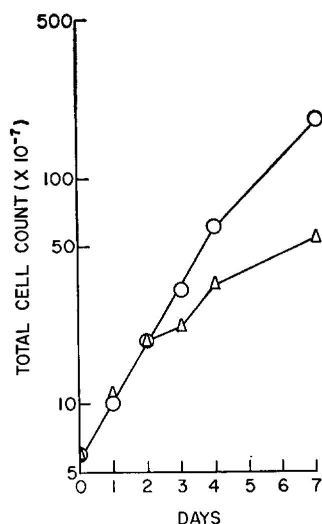


FIGURE 1 Cumulative growth of L cells in suspension culture: control, circles; chloramphenicol-treated, 15 μg/ml, triangles.

TABLE I

A Comparison of Some Respiratory Enzymes in the HeLa and the L Cell

	HeLa	L cell
Cytochrome oxidase*	30 ± 6	80 ± 23
Succinate–cytochrome *c* reductase‡	20 ± 2	24 ± 5

* Nanomoles of cytochrome *c* oxidized per milligram protein per minute. Number of independent determinations: HeLa, 6; L cell, 36.

‡Nanomoles of cytochrome *c* reduced per milligram protein per minute. Number of independent determinations: HeLa, 10, L cell, 19.

ml (2.5×10^{-6} M), grow at 37% of the control rate for as long as a week; L cells treated with 0.039 μg/ml (10^{-7} M) grow normally for 5 days, then abruptly cease growth.

Respiratory Enzyme Activity

Cytochrome oxidase and succinate–cytochrome *c* reductase activities of HeLa and L cells are shown in Table I. During growth of the HeLa cell in suspension culture in the presence of chloramphenicol (Table II) the total protein of the control culture increases about eightfold during 4 days, from 50 μg protein/ml culture to 412 μg protein/ml culture, while the total cytochrome oxidase activity (protein per milliliter times specific activity) per milliliter culture increases from 1.8 units to 14.4 units. The total protein of a culture exposed to chloramphenicol, 50 μg/ml, increases about threefold during 4 days, from 50 μg protein/ml culture to 145 μg protein/ml culture, while the total cytochrome oxidase activity remains approximately constant, despite the considerable augmentation of protein.

Essentially the same situation obtains in a typical experiment in which L cells were examined (Table III). The total protein of a culture exposed to chloramphenicol, 5 μg/ml, increases to 660 μg protein/ml culture after 4 days as compared with the control of 675 μg protein/ml culture. Total cytochrome oxidase activity in the treated culture, however, is 21.8 units/ml as opposed to the control of 39.1 units/ml. After exposure to chloramphenicol, 25 μg/ml, despite a moderate increase in total protein to 405 μg/ml culture, total cytochrome oxidase activity does not increase. Similar observations were made in the case of succinate–cytochrome *c* reductase (Table III). For example, cells treated with chloramphenicol, 25 μg/ml, contain a final total of 3.2 units of activity/ml as compared with the control of 17.5 units of activity/ml. Cells treated chronically with chloramphenicol contain about 20% of the control cytochrome oxidase activity.

Growing of L cells in the presence of ethidium bromide, 1.0 μg/ml (2.5×10^{-6} M), results in a reduced rate of protein synthesis (Table IV) while treatment with 0.039 μg/ml (10^{-7} M) does not affect the rate of total protein synthesis until day 5, after which no further increase in protein occurs. Total cytochrome oxidase activity and succinate–cytochrome *c* reductase activity remain at the initial value with either dosage.

Cytochrome c Content

The cytochrome *c* content of L cells is 28 ± 4 (13 determinations) nmoles/g protein. Cultivation of the L cell in the presence of chloramphenicol 20 μg/ml, for 3 days (Table V) results in a decline of cytochrome oxidase activity to 73% and in a value for cytochrome *c* content equal to 113% of the corresponding control values. At 8 days, total protein increases fourfold, cytochrome oxidase declines to 24% of control, and cytochrome *c* content increases to 170% of control. Intermediate values are observed with a dose of chloramphenicol of 5 μg/ml. Cultivation for 7 days in the presence of a large dose of ethidium bromide (1.0 μg/ml)

TABLE II

Effect of Chloramphenicol (CAP), 5 μg/ml and 50 μg/ml, on the Protein Synthesis and Cytochrome Oxidase Activity of HeLa Cells in Suspension Culture

	Protein (cumulative), μg/ml			Specific activity cytochrome oxidase*			Total activity‡ (protein times specific activity)		
		CAP/ml			CAP/ml			CAP/ml	
Day	Control	5 μg	50 μg	Control	5 μg	50 μg	Control	5 μg	50 μg
0	50	50	50	35	35	35	1.8	1.8	1.8
2	144	122	86	29	18	18	4.2	2.2	1.5
4	412	294	145	35	17	12	14.4	5.0	1.7

* Nanomoles of cytochrome *c* oxidized per milligram protein per minute.
‡ Nanomoles of cytochrome *c* oxidized per milliliter culture per minute.

TABLE III

Effect of Chloramphenicol, 5 μg/ml and 25 μg/ml, on the Protein Synthesis, Cytochrome Oxidase Activity and Succinate–Cytochrome c Reductase Activity of L Cells in Suspension Culture

	Protein (cumulative), μg/ml			Specific activity cytochrome oxidase*			Total activity‡ (protein times specific activity)			Specific activity succinate–cytochrome *c* reductase§			Total activity‖ (protein times specific activity)		
		CAP/ml			CAP/ml			CAP/ml			CAP/ml			CAP/ml	
Day	Control	5 μg	25 μg	Control	5 μg	25 μg	Control	5 μg	25 μg	Control	5 μg	25 μg	Control	5 μg	25 μg
0	131	131	131	64	64	64	8.4	8.4	8.4	26	26	26	3.4	3.4	3.4
1	218	218	206												
2	293	307	274	51	29	19	14.9	8.9	5.2	26	17	17	7.6	5.2	4.7
4	675	660	405	58	33	5	39.1	21.8	2.0	26	10	8	17.5	6.6	3.2

* Nanomoles of cytochrome *c* oxidized per milligram protein per minute.
‡ Nanomoles of cytochrome *c* oxidized per milliliter culture per minute.
§ Nanomoles of cytochrome *c* reduced per milligram protein per minute.
‖ Nanomoles of cytochrome *c* reduced per milliliter culture per minute.

TABLE IV

Effect of Ethidium Bromide, 1.0 μg/ml (2.5×10^{-6} M), on the Protein Synthesis, Cytochrome Oxidase Activity, and Succinate–Cytochrome c Reductase Activity of L Cells in Suspension Culture

	Protein (cumulative), μg/ml		Specific activity cytochrome oxidase*		Total activity‡ (protein times specific activity)		Specific activity succinate–cytochrome *c* reductase§		Total activity‖ (protein times specific activity)	
Day	Control	Exp¶	Control	Exp¶	Control	Exp¶	Control	Exp¶	Control	Exp¶
0	145	145	57	57	8.3	8.3	27	27	3.9	3.9
2	320	182	64	48	20.5	8.7	20	16	6.4	2.9
5	518	246	71	32	36.8	7.9	17	6	8.8	1.5

* Nanomoles of cytochrome *c* oxidized per milligram protein per minute.
‡ Nanomoles of cytochrome *c* oxidized per milliliter culture per minute.
§ Nanomoles of cytochrome *c* reduced per milligram protein per minute.
‖ Nanomoles of cytochrome *c* reduced per milliliter culture per minute.
¶ Ethidium bromide, 1.0 μg/ml (2.5×10^{-6} M).

TABLE V

Effect of Chloramphenicol and Ethidium Bromide on Some Cytochromes of the L Cell

	Dose		Time	Cytochrome oxidase*	Succinate–cytochrome *c* reductase‡	Cytochrome *c*¶
	μg/ml	*M*	*days*			
Chloramphenicol	20	6.2×10^{-5}	3	73	—	113
	5	1.6×10^{-5}	8	47	39	140
	20	6.2×10^{-5}	8	24	22	170
Ethidium bromide	1.0	2.5×10^{-6}	7	20	17	129
	0.039	10^{-7}	6	25	14	166
KCN	6.5	10^{-4}	4	32	100	119
	6.5	10^{-4}	6	40	100	127

* Nanomoles of cytochrome *c* oxidized per gram protein per minute, as per cent control.
‡ Nanomoles of cytochrome *c* reduced per gram protein per minute, as per cent control.
¶ Nanomoles per gram protein, as per cent control.

permits no more than a doubling of total protein. There is a reduction in cytochrome oxidase and succinate–cytochrome *c* reductase values to about one-fifth of control values (Table V). Growth for 6 days in the presence of ethidium bromide, 0.039 μg/ml, results in a sevenfold increase in total protein, a severe reduction in enzyme specific activities and an increase in cytochrome *c* content to 166% of control values (Table V).

Effect on Cytochromes of Agents Restricting Cell Growth

In order to compare the effect on cytochrome *c* content of cultivation in the presence of chloramphenicol and ethidium bromide with the effect of other growth-restricting agents, experiments were performed in which either arginine, the required amino acid, was omitted, or actinomycin D was introduced. No changes occur in the specific activity of cytochrome oxidase and of succinate–cytochrome *c* reductase after arginine restriction (5) or after treatment with actinomycin D (32). Arginine deprivation, tested in suspension medium over a period of 3 days, results in cessation of growth accompanied by a decrease in the amount of cytochrome *c* to 80% of the control value when expressed as nanomoles of cytochrome *c* per gram protein, and to 90% of the control value when expressed as nanomoles of cytochrome *c* per milligram DNA. Treatment of L cells with actinomycin D diminished uptake of uridine-^{14}C into RNA by 85% in 2 hr (32), arrested cell division, but permitted unbalanced cytoplasmic growth with an increase in total protein of 139% during the first 28 hr. These enlarged cells, which did not show further increase in protein content, contained 108% (average of six experiments) of the control amount of cytochrome *c*.[1]

In order to distinguish the effects on cells of conditions in which cytochrome oxidase is initially directly inhibited from those of conditions in which the formation of this enzyme and other mitochondrial components is believed to be primarily interfered with (e.g., reference 13), cells were treated with KCN. Growth of the L cell was completely inhibited in 10^{-3} M KCN, was unaffected in 10^{-5} M KCN, and was 70–80% of control in 10^{-4} M KCN. At the latter concentration, there was a threefold increase in cells in 4 days and a fourfold increase in 6 days. The cells were slightly enlarged, containing 32 μg DNA/mg protein compared with the control value of 40 μg DNA/mg protein. Cytochrome oxidase activity in twice-washed preparations was 30–40% of control activity. Succinate–cytochrome *c* reductase activity was at control levels. Cytochrome *c* content was elevated to 119–127% of control (Table V).

Morphological Observations

The ultrastructure of HeLa and L cells has been described (e.g., references 33 and 34). Their mitochondria are almost always in the orthodox (typical) configuration (Fig. 2). The matrix of the inner mitochondrial chamber is normally denser than

[1] Godman, G. C., T. P. Keneklis, and M. E. King. Respiratory enzyme activity and mitochondrial volume of L cells treated with actinomycin D. Manuscript in preparation.

the ground cytoplasm, especially in L cells. A quantitative estimation of the relative distribution of mitochondrial components, as determined from lineal analysis of electron micrographs (35, 36) of a normal nonsynchronous population of 50 cells, is given in the following tabulation (the parameters defined in reference 36 are used):

	HeLa	L cell
Mean volume density Mitochondrial volume (cm^3) per cytoplasmic volume (cm^3),	8.9% (7.3–11.0)	7.1%
Mean surface density Mitochondrial membrane surface (cm^2) per cytoplasmic volume (cm^3),	2.4	1.9
Ratio of outer mitochondrial membrane surface (cm^2) to mitochondrial volume (cm^3)	6.8	7.8
Ratio of inner mitochondrial membrane (with cristae) surface (cm^2) to mitochondrial volume (cm^3),	27.5	30.5
Ratio of inner membrane surface (cm^2) to outer membrane surface (cm^2),	4	4
Median mitochondrial diameter, μ	0.39	0.36

Both HeLa and L cells respond similarly to each of the agents employed in this study, and the most striking changes are in the mitochondria.

CHLORAMPHENICOL: After exposure of cells to concentrations of chloramphenicol in the range of 20–40 μg/ml for 3 days, most of the mitochondria are moderately swollen; in the inner chamber, irregular electron-lucent zones replace the denser matrix. In the more swollen mitochondria, the entire matrix space of the inner chamber has lost density (Fig. 3). The cristae are reduced in number, and are displaced by the expanded lucent areas in the inner chamber; the usual transverse orientation of the cristae is distorted.

At 3 days, the mean volume density of chloramphenicol-treated HeLa cells is about 13.3%, an elevation over the control value of 10.4%. This increase may be attributed to the larger volume per unit volume cytoplasm occupied by the swollen mitochondria, which also give a lower range of ratios of mitochondrial outer surface to mitochondrial volume (mean = 5). The mean and median number of mitochondrial profiles per 100 μ^2 of cytoplasm (34.6) is not, however, significantly lower than that of the controls (mean 35.0, median 35.7).

By the 4th and 5th days of exposure to these concentrations, in L cells, the mitochondria not visibly affected by chloramphenicol are present in a form intermediate between the condensed and the orthodox conformation (37, 38), characterized by dense inner chamber matrix and normal outer chambers. In both HeLa and L-cell mitochondria, many cristae are shortened and exhibit degenerative changes usually affecting their more distal parts (Figs. 3, 4). These changes most often take the form of a marked loss of density of a segment of cristal membrane, sometimes coincident with dilatation of the same segment of intracristal space, resulting in a divergence of the membranes (Fig. 5). Each of these unit membranes preserves, at first the same thickness (about 55 A) and the same tripartite structure as the unit membranes of normal cristae fixed in this way. As the membranes lose density, they become attenuated and are finally represented by discontinuous wisps lacking definite structure (Figs. 4–6). As the destruction of the cristae proceeds, in addition to saccules of membranous elements, a fine fibrillogranular debris accumulates in the dilated inner chambers. In some instances the detached cristal membranes form laminate or whorled constructions. That part of the inner membrane facing the outer mitochondrial membrane usually remains morphologically intact until cristal degeneration is far advanced. Mitochondrial ribosomes remain recognizable.

The HeLa cells appear to show somewhat more advanced and more uniform changes than do the L cells, although both cell types are now visibly enlarged. In the juxtanuclear cell center, the stacks of lamellar components of Golgi apparatus are dilated and decreased in length and in number. They are apparently displaced by many vesicles (ranging from 35 to 80 mμ in diameter) and vacuoles, and by tubular proliferations of smooth endoplasmic reticulum (Fig. 7). The mean count of mitochondrial profiles per 100 μ^2 of cytoplasm is 30.3 (median 28.3). This doe not represent an important reduction compared with the controls (mean 35), in view of the fact that the cells will have divided at least twice since administration of chloramphenicol.

At 7–10 days, in HeLa cells, there are a few mitochondria in the condensed or intermediate

configuration (Fig. 8). The most severely altered mitochondria are swollen, in some segments up to four times normal diameter. In some sections, no normal cristae are recognizable, but there are detached profiles of doubled membrane, or wisps of unit membrane of reduced density, derived from cristae (Fig. 5). In most of these mitochondria, the peripheral inner membrane still appears to be intact over most sectors, but, as the lesion progresses, inner membrane may also disappear. The outer chamber may then be ballooned out. Sections through dilated segments devoid of inner membrane would, of course, give an appearance in thin section indistinguishable from the large hydropic smooth-walled vacuoles of Golgi or endoplasmic origin (Fig. 9). However, most of the chloramphenicol-affected mitochondria persist with recognizable relics of inner membrane and cristae. The close spatial relationship of rough endoplasmic reticulum to the mitochondrion, well known in normal cells (e. g., reference 39), persists in the chloramphenicol-treated cells. As exposure to chloramphenicol is continued, the mitochondrial lesions do not advance significantly beyond what is seen at 7–10 days.

At 7–10 days, the cells are more obviously enlarged. In some cells, the polyribosomes are reduced in number, and many monoribosomes and

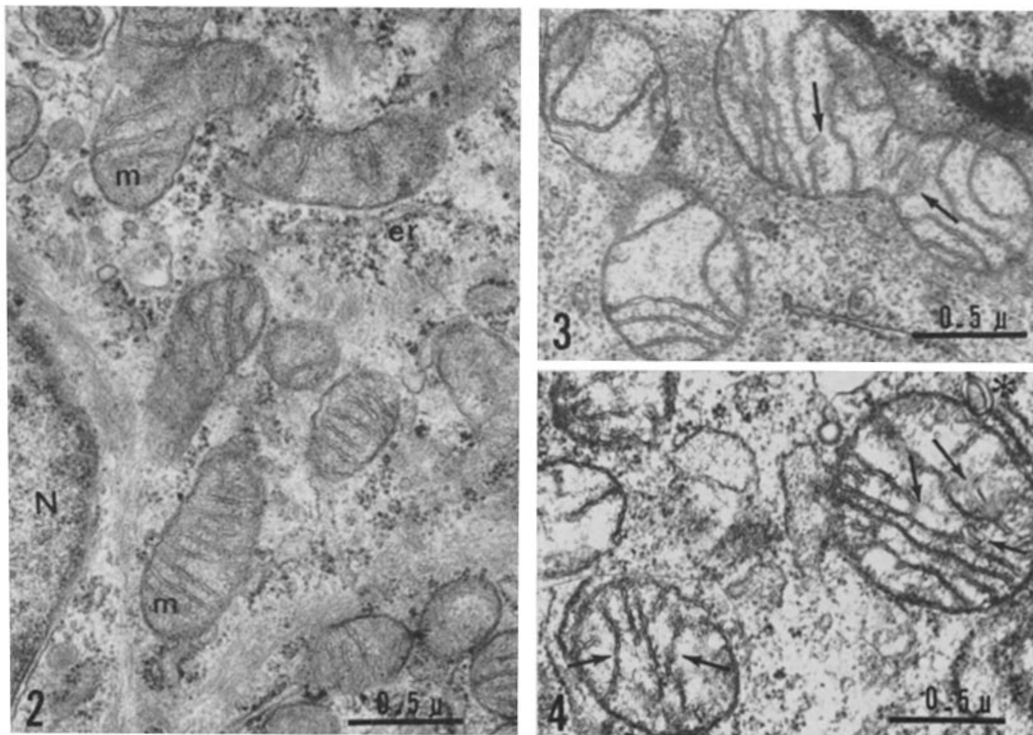


FIGURE 2 An area of cytoplasm near the nucleus (*N*) of a HeLa cell. The mitochondria (*m*) are in the orthodox or expanded configuration, usual in the normal cell. Segments of rough endoplasmic reticulum (*er*) are marked. × 30,000.

FIGURE 3 Early changes in mitochondria of a HeLa cell 3 days after exposure to chloramphenicol, 40 μg/ml. The inner (matrix) chamber is swollen and more electron-lucent, the cristae are separated, and a few focal segments of cristal membrane have lost density (arrows). × 30,000.

FIGURE 4 Mitochondria of an L cell after 4 days of exposure to chloramphenicol, 20 μg/ml. The inner chamber is hydropically distended, and electron-lucent spaces displace the remaining matrix material. Regressive changes, evident in some of the cristae, include apparent detachment or segmentation, loss of density of the membrane, and disappearance of definite structure (arrows). A cytoplasmic vesicle is in contiguity with a mitochondrion (asterisk) and appears to be invaginating into it. × 30,000.

smaller (13–15 mμ) particles, interpreted as ribosomal subunits, appear.

The cytoplasm of cells continuously exposed to chloramphenicol for 20 or 30 days is filled with many closely spaced vesicles and vacuoles bounded by smooth membranes (Fig. 7), the largest of which may exceed 1 μ in diameter. There are now present in the cytoplasm many structurally intact mitochondria, most of which are in a condensed conformation, with dense inner chamber matrix and dilated intracristal spaces, or in intermediate conformation, in which the intracristal space is of normal dimensions (Fig. 10).

Among the accumulations of vesicles and vacuoles in the Golgi zone are bodies containing filamentous or membranous material (Fig. 11). In many cells, compact masses of membrane, vesicles, or cytoplasm are enveloped by thecal formations of endoplasmic reticulum, in a kind of autophagic body (Figs. 7, 11). Between the many membrane-bounded elements, the ground cytoplasm lacks electron opacity, few microfilaments are seen (Fig. 10). The nuclei of the chronically treated cells have enlarged nucleoli and may contain bodies of low density similar to those described by Bouteille et al. (40).

Cells exposed to 20–40 μg of chloramphenicol for as long as 34 days recover rapidly. 4 days after being washed and restored to fresh medium, such HeLa cells are almost completely recovered morphologically. Only an increased number of vesicles and myelin-like bodies, an increased prominence of the Golgi lamellae, and a somewhat diminished ratio of inner membrane surface area to mitochondrial volume (23.8 μ^2/μ^3 mitochondrion in recovered cells, compared to 27.5 in controls) testify to former injury by chloramphenicol. L cells are apparently slower to recover than HeLa cells.

ETHIDIUM BROMIDE: The changes seen after 5 days of treatment with 1.0 μg/ml of ethidium bromide are in most respects qualitatively similar to those described for chloramphenicol (Fig. 12). There is, however, more heterochromatization in the nuclei, and a much greater accumulation of vesicles, vacuoles, and tubules of smooth endoplasmic reticulum in the cell center. The lesions in the mitochondria are more advanced and uniform than those encountered after a comparable period of treatment with chloramphenicol.

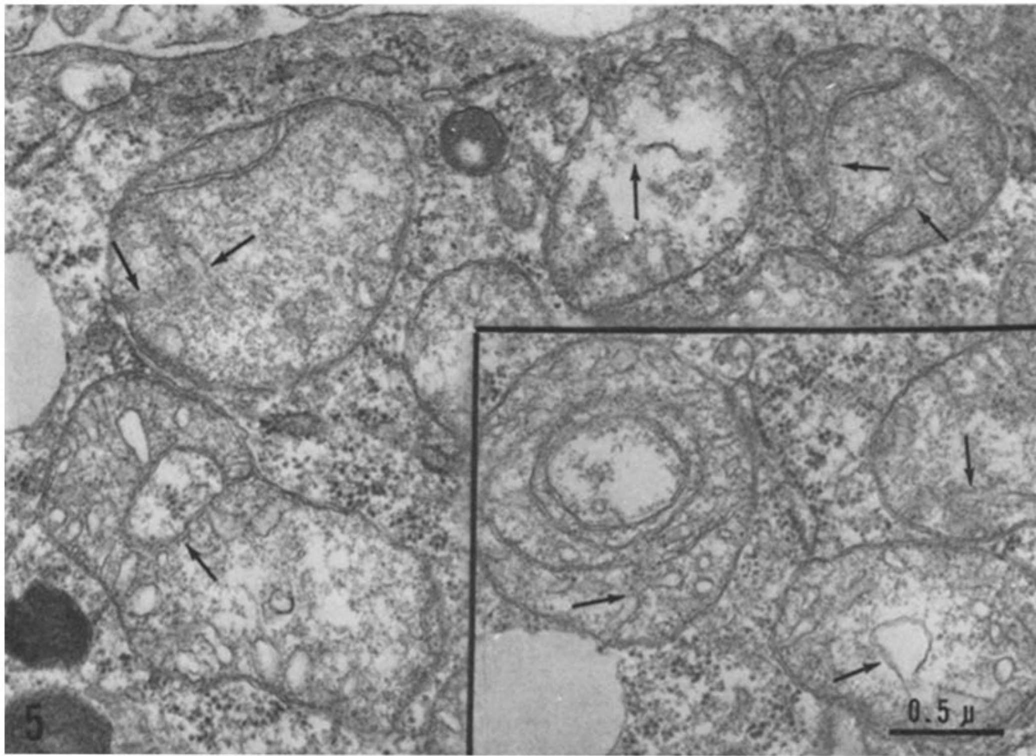
CYANIDE: At 10^{-5} M, KCN was without visible effect. At 10^{-4} M, a variable number of mitochondria, in most cells a minority, exhibited distinct changes at 5 days. These consist of focal or generalized swelling of the inner chamber, usually of moderate degree, and segmental or focal degeneration of inner membrane. In the juxtanuclear cell center there are increased numbers of vesicles and vacuoles, although not so numerous as after the other agents described herein.

DISCUSSION

Treatment of mammalian cells in culture with chloramphenicol and ethidium bromide, which interfere selectively with intramitochondrial macromolecule synthesis, results in an abnormal pattern of cytochrome production and in disorganization of mitochondrial structure. That cultivated cells would respond to these agents in a manner similar, at least in part, to cells of lower eukaryotes was indicated by observations of a decrease in cytochrome oxidase activity in treated HeLa (21), human fibroblast (25), and rat heart

FIGURE 5 Advanced changes in the mitochondria of a HeLa cell exposed to chloramphenicol, 40 μg/ml, for 10 days. The inner chamber is greatly expanded. There has been a marked loss of cristae, and many remaining cristae are disoriented and appear as loops or whorls. Degeneration of cristal membrane (arrows), which first affects the more distal segments, is recognized by diminished density, then by loss of definite structure and outline, and by detachment. The peripheral inner membrane is generally intact. × 30,000.

FIGURE 6 Mitochondria of an L cell after 19 days of exposure to chloramphenicol, 20 μg/ml, illustrating in greater detail regressive changes affecting the cristae. The inner chambers are hydropically dilated and electron lucent except for patches of granulofibrillar material in which there are a few dense 15-mμ granules, probably mitochondrial ribosomes (arrowheads). Segments of cristal membrane are of greatly reduced density although still of normal dimension (56–58 A) (arrows). At these loci, the intracristal space is dilated. In more advanced stages of deterioration, the unit membrane structure is lost, then the cristae disintegrate into apparently amorphous material. The peripheral part of the inner membrane is preserved intact in these mitochondria in which deterioration of cristae is advanced. × 60,000.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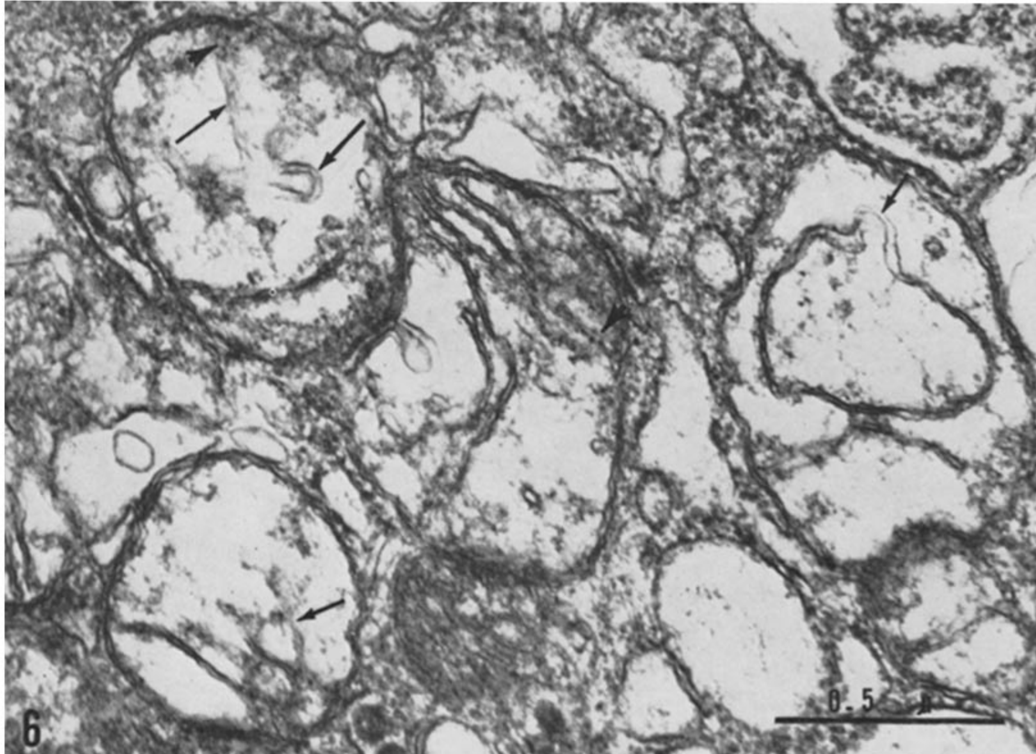
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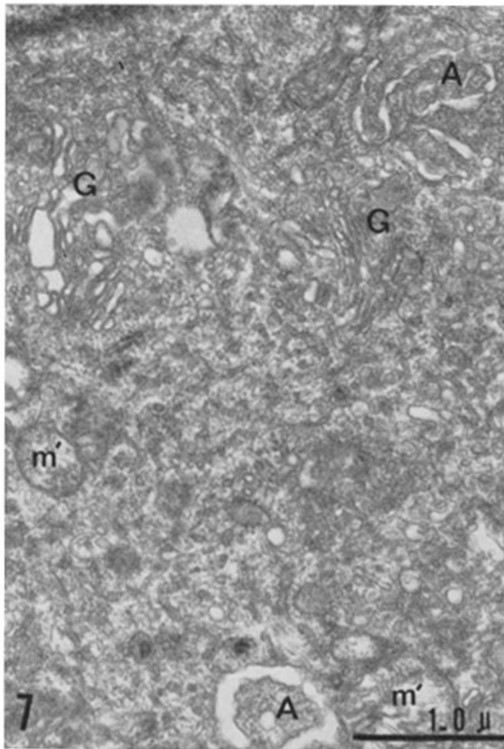
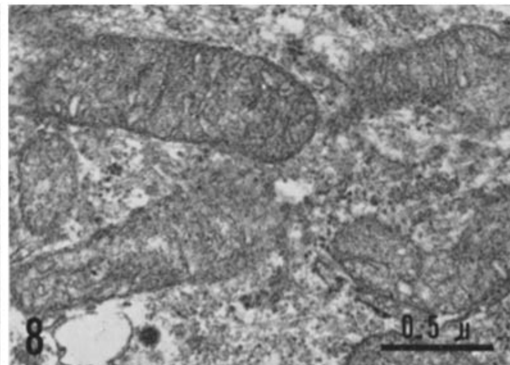




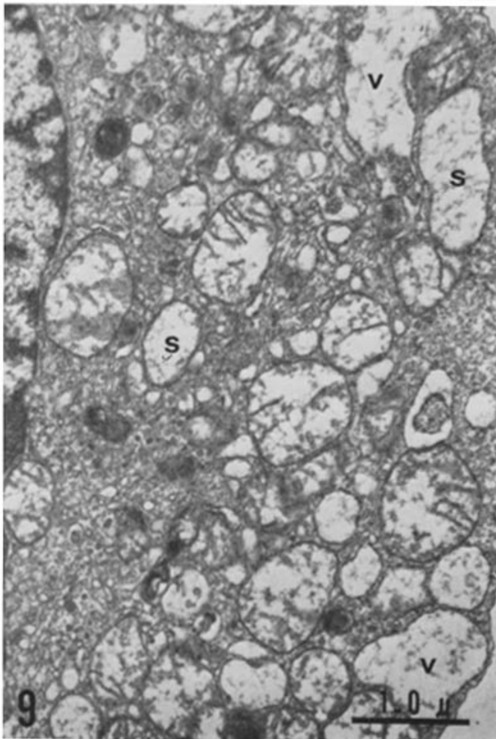
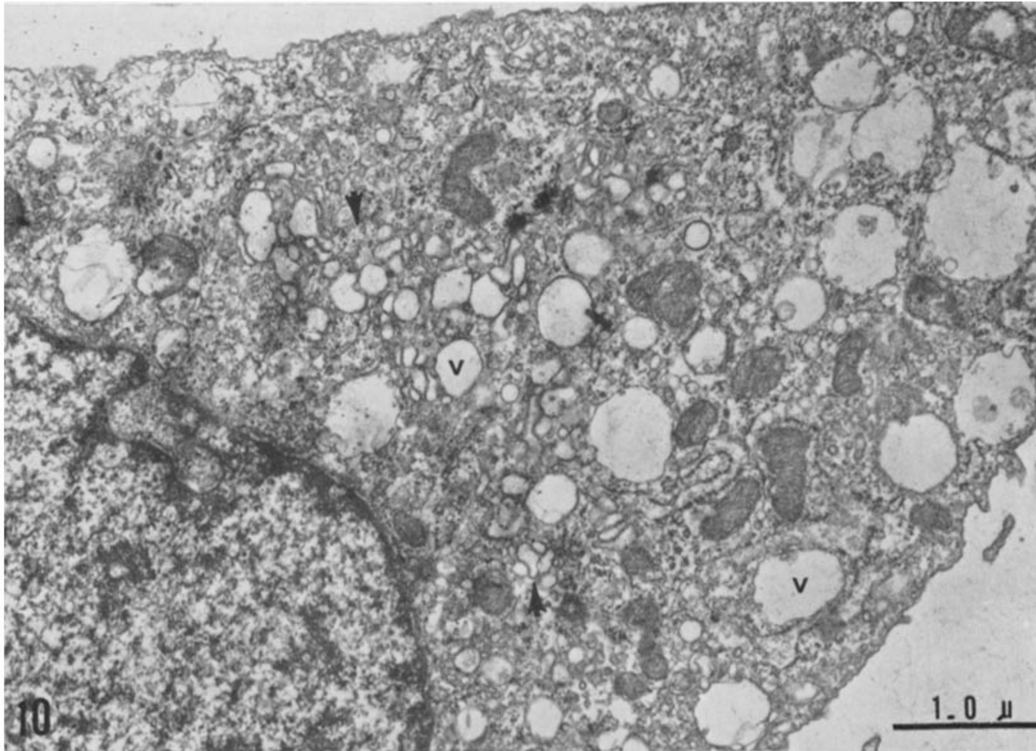


FIGURE 7 Area of juxtanuclear cytoplasm of an L cell, treated for 19 days with chloramphenicol, 20 μg/ml, within which there are myriad small branching tubular elements and small vesicles of proliferating smooth endoplasmic reticulum. Dilated Golgi saccules appear at *G*. Altered mitochondria are marked *m'*. Small peninsulas of cytoplasm are enveloped by channels with smooth membranes at *A*. $\times$ 20,000.

FIGURE 8 Some mitochondria of normal structure in a HeLa cell, after 10 days of treatment with chloramphenicol, 40 μg/ml. They have dense matrices, and are in a form intermediate between the expanded and the extremely condensed configurations. $\times$ 30,000.

FIGURE 9 An area of cytoplasm of an L cell treated with chloramphenicol, 20 μg/ml, for 20 days. The mitochondria are markedly swollen and can be identified by the remaining cristae, relics of cristal membrane, and, in most cases, persisting peripheral inner membrane. Sections of some of the dilated segments of mitochondria in which inner membrane and cristal remnants are no longer identifiable are marked (*s*). Hydropic vacuoles or dilated cisterns of endoplasmic reticulum are shown at (*v*). $\times$ 15,000.



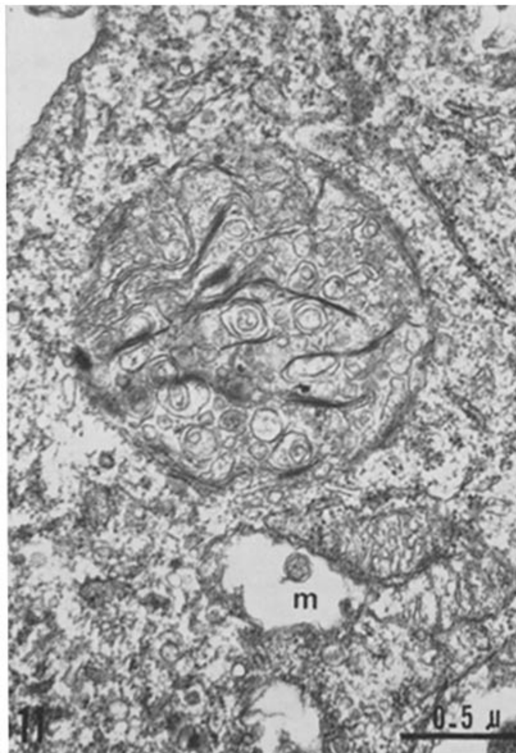


FIGURE 10 Area of an enlarged L cell after 30 days of exposure to chloramphenicol, 20 μg/ml. Most of the recognizable mitochondria are in the condensed or intermediate form. A few of the large vacuoles with single membranes may represent relics of deteriorated mitochondria, but distinction between these and hydropically dilated vacuoles (*v*) resulting from fusion of swollen vesicles (arrowheads) of smooth endoplasmic reticulum or Golgi origin is difficult. The cytoplasm is replete with vesicles and vacuoles, and the intermembranous ground cytoplasm is of reduced density. × 20,000.

FIGURE 11 An autophagic sequestrum in an L cell treated with 20 μg/ml chloramphenicol for 19 days. Its membranous contents may be derived from both mitochondria altered by the drug (*m*) and proliferated cytoplasmic vesicles. × 30,000.

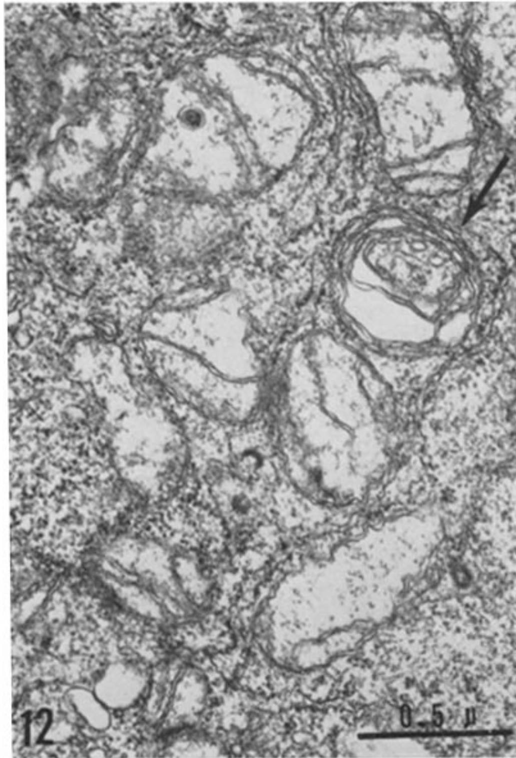


FIGURE 12 Cytoplasmic area of an L cell treated for 5 days with ethidium bromide, 1.0 μg/ml. The mitochondria show membrane changes similar to those resulting from chloramphenicol. Note the whorled formation of cristae (arrow) × 30,000.

(22) cells. The agents that act to prohibit autonomous mitochondrial protein synthesis affect mitochondrial structure similarly in yeast (15, 16, 41), algae (42), protozoa (43), and mammalian cells (18, 23, 24). The essential ultrastructural lesion is a loss of the cristae mitochondriales and, in animal cells, some change in permeability leading to distension of the inner chamber. Concomitant with the deterioration of cristal membrane, there is a loss of the structure-bound insoluble cytochromes from the mitochondria. HeLa and L cells have been systematically examined to study the evolution of a pattern of cytochrome imbalance and the correlation between the enzymatic and morphological abnormalities.

After 3 days of treatment of the cells with chloramphenicol or ethidium bromide, when the specific activity of cytochrome oxidase is at about one-half of the control value, the earliest ultrastructural change seen in the mitochondria is swelling of the inner chamber matrix space (Fig. 3). The outer chamber usually is unaffected. In cells treated for prolonged periods with chloramphenicol in doses smaller than those which directly affect electron transport (21, 44), mitochondria appear in the condensed or intermediate form (Figs. 8, 10). In terms of current concepts of conformational changes accompanying changes in electron transport (37, 38), the condensed conformation is associated in vitro with State III, in which adenosine diphosphate (ADP) and substrate are present at high levels, and the rate of respiration is rapid and limited by the capacity of the respiratory chain. An analogous situation might well obtain in vivo in the cells treated with chloramphenicol for long periods, in which the specific activity of cytochrome oxidase is less than 20% of control and in which, therefore, the respiratory chain must limit respiration. Mitochondria in this condensed configuration have also been depicted (45) in bone marrow cells of patients whose serum contained levels of chloramphenicol of 30 μg/ml.

A rapid and progressive decrease in the specific activity of the structure-bound cytochromes accompanies the early ultrastructural changes seen with chloramphenicol. The decline in total cellular enzyme activity is dose-dependent (Tables II and III). The rapidity of the drug action is evidenced by examination of the effect of the larger doses: total initial cytochrome oxidase activity of a culture fails to increase at the same time that total protein is augmented. In our experiments the original amount of enzyme activity per culture is generally conserved and the fall in enzyme specific activity may be accounted for by dilution. The content of cytochrome *a* and a_3 (by 605- and 444-mμ absorption bands, respectively) and the cytochrome oxidase activity were found to be decreased in mitochondria of HeLa cells treated with chloramphenicol (21). The content of mitochondrial cytochrome *b* was also found to be decreased. These results are in harmony with our finding of decreased activity of succinate–cytochrome *c* reductase, the complex which requires the presence of cytochrome *b* for the activity to be manifest.

Findings similar to those observed after chloramphenicol treatment were obtained in L cells treated with ethidium bromide at 1.0 μg/ml (Table IV). A dose of 0.039 μg/ml (or 10^{-7} M), though it is not inhibitory to growth until day 5 when growth ceases abruptly after a sevenfold increase in protein, entirely prevents increase in

total cytochrome oxidase and succinate-cytochrome *c* reductase activity. Treatment of diploid human fibroblasts in culture with the same dose resulted in a marked decrease in cytochrome oxidase activity and a decrease in cellular content of cytochrome aa_3 as studied in whole cells by direct spectroscopy (25).

In contrast to the formation of firmly bound mitochondrial cytochromes (21, 25), the synthesis of cytochrome *c* and its continued transfer into and accumulation in the mitochondria is augmented by suppression of intramitochondrial protein synthesis (18, 21, 46–53). We find that the fall in cytochrome oxidase activity and in succinate-cytochrome *c* reductase activity following either chloramphenicol or ethidium treatment of L cells is accompanied by a rise in the total cellular content of cytochrome *c* (Table V). Since cellular growth has ceased by the time a maximal elevation of cytochrome *c* content is observed, conditions leading to the arrest of growth, namely, arginine deprivation and actinomycin D treatment, were examined for their effect on cytochrome *c* accumulation. These conditions do not significantly affect the amount of cytochrome *c* per gram of cell protein.

As cytochrome oxidase activity is diminished, an increase cytochrome *c* is observed. Mahler et al. (54) indicate that the interaction of ethidium bromide with mitochondrial DNA immediately impedes transcription of a component required for cytochrome oxidase activity, and perhaps of a component having a similar function for NADH: cytochrome *c* reductase; conversely, these authors found no effect on any of the steps required for the synthesis of all other enzymes, including the complete respiratory chain between succinate and cytochrome *c*. The subsequent increase in cytochrome *c* may represent a compensatory mechanism making up for an adenosine triphosphate (ATP) generating deficiency. Such an hypothesis was proposed (55) to account for the increase in chondriome observed in a unique human case of mitochondrial dysplasia with "loose coupling" of oxidation and phosphorylation (56) and in rat myocardium after dinitrophenol treatment (57). Beating rat heart cells in culture treated with chloramphenicol were indicated to be deficient in ATP (22). Thus, in the instance under consideration in our study, a marginal lack of ATP might serve as a stimulus for augmentation of all the mitochondrial constituents in the presence of low doses of the drug. As intramitochondrial processes are further inhibited with larger doses of drug, the synthesis, eventually, of more and more proteins may be compromised with the result that proteins essential for the integration of the firmly bound cytochromes are lacking, causing these cytochromes to disappear, while the loosely bound cytochrome *c* remains elevated in amount.

With respect to the decrease in growth and the fall in cytochrome oxidase activity, the effects of KCN resemble those of chloramphenicol and ethidium. Consistent with the idea that an increase in cytochromes is part of a generalized response to lowered ATP production, an increase in cytochrome *c* is observed in KCN-treated cells at 6 days.

Both the enzyme deficiences and the mitochondrial lesions appear the same in animal cells whether autonomous mitochondrial protein synthesis is inhibited by direct interference with translation (18–24), as in the presumed interaction or chloramphenicol with the mitochondrial ribosome, or indirectly, as a result of the impediment to transcription afforded by the intercalation of ethidium bromide into mitochondrial DNA (12, 24, 54) or inhibition of the mitochondrial RNA polymerase by rifamycin derivatives (58). In the presence ot 20–40 μg/ml of chloramphenicol (Fig. 4), the apparent concentration (i.e. number per 100 μ^2 of cytoplasm) of mitochondria does not fall drastically, although the number and approximate cell mass will have increased four- to eightfold. At least up to this point, then, organelles identifiable as mitochondria are not gradually diluted out and, although imperfect, continue to proliferate in the virtual absence of autochthonous mitochondrial protein synthesis. Later, visible deterioration of the cristae results (Figs. 5, 6, and 9).

Loss of cristae occurs *pari passu* with the fall in cytochrome oxidase and succinate–cytochrome *c* reductase activity (Fig. 3 and 4 and Tables II–V). Autonomous mitochondrial protein synthesis apparently does not play a direct role in the formation of the apoprotein of cytochrome aa_3, since this cytochrome was not labeled in isolated rat liver mitochondria (47), and because the apoprotein was present in mitochondria from yeast bearing the *petite* mutation (59). It has been suggested that a product of intramitochondrial synthesis is necessary for the integration of some of the constituents of the mitochondrial particle (54, 60–64). The ultrastructural changes of cristal

disintegration and swelling of the inner matrix chamber which precede disorganization of the peripheral inner membrane and of its attachment sites to the outer membrane (Fig. 6) are thought to result from lack of this intramitochondrial product. The changes observed are probably not secondary to decreased cytochrome oxidase activity since morphological changes of such magnitude are not observed after treatment with KCN.

The intracellular level of active drug may be reduced in the chronically treated cultures as a result of the activity of the vesicles, vacuoles, and proliferated elements of smooth endoplasmic reticulum accumulated in the cytoplasm during 5–7 days of exposure to chloramphenicol or ethidium bromide (Figs. 7, 10, and 12). They are most probably part of a cellular detoxification or sequestration reaction analogous to the well-known proliferations of microsomal membrane in hepatocytes after treatment with barbiturates. To some degree this process apparently also occurs in liver after systemic administration of chloramphenicol (20). Considerable numbers of mitochondria emerge in the continued presence of chloramphenicol after treatment for 20–30 days (Fig. 10). They may lie in the cytoplasm beside numerous mitochondria exhibiting a gamut of toxic changes; this suggests that there is heterogeneity of the mitochondrial population with respect to drug responsiveness.

These ultrastructural changes reflect a derangement of the metabolism of the entire cell by chloramphenicol or ethidium bromide. The most specific lesion is that of the inner mitochondrial membrane accompanied by a marked decrease in the specific activity of cytochrome oxidase and succinate–cytochrome *c* reductase and a large increase in the content of cytochrome *c*. That normally, despite conditions fully permitting oxygen induction, mitochondrial cytochromes do not appear in excess suggests that, besides known environmental factor, there is an additional regulatory mechanism for cytochrome synthesis, apparently geared to mitochondrial function.

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