Cell Cycle Kinetics and Metabolism of Ehrlich Ascites Tumor Cells in the Presence of Chloramphenicol as Inhibitor of Mitochondrial Protein Synthesis

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Z. Naturforsch. **39c**, 126–135 (1984); received July 29/October 4, 1983

Dedicated to Prof. P. Karlson on the Occasion of His 65th Birthday

Ehrlich Ascites Tumor Cells, Chloramphenicol, Cell Cycle Progression, Energy Metabolism

In the presence of 150 µg/ml of chloramphenicol, viability (dye exclusion test) of Ehrlich ascites tumor cells is not severely affected within 48 h; however the number of dead cells increases above this concentration; more than 10 mg/ml render all of the cells dye-positive within a culture period of 24 h. With the highest concentration tested (25 mg/ml) cells begin to lose viability 8 h after beginning of treatment.

In the first passage in the presence of the antibiotic, proliferation of the cells is reduced by about 50%; in the second passage cell growth was about 65% that of controls. As is shown by flow cytometric analysis and BrdU-H33258 technique of flow cytometry, the reduction of proliferation in the second passage is caused by retardation of cell cycle progression of about 8 h; in the third passage the cell cycle is delayed by further 8 h as compared to controls. On recultivation in the third passage in the absence of the inhibitor an increase of cell number of about 75% of controls was observed.

Lactate production and glucose uptake of the cells were stimulated by the inhibitor by about 20%; oxygen consumption was 60% that of controls after 24 h; however the ATP/ADP ratio of chloramphenicol treated cells was in the range of controls.

Over a time period of 24 h the incorporation of [2-^{14}C]thymidine is reduced to 34 ± 3%, incorporation of [2-^{14}C]uridine was 88 ± 4% that of controls, incorporation of [U-^{14}C]lysine was not significantly affected by 150 µg/ml of chloramphenicol; the same was found for the uptake of [2-^{14}C]-α-aminoisobutyric acid.

Electron micrographs of chloramphenicol treated cells reveal a high amplitude of swelling of all mitochondria with a translucent appearance of the inner compartment. The mitochondrial membranes remain largely intact but the number of cristae is drastically reduced.

Introduction

In a previous communication we have shown, that inhibition of oxidative ATP synthesis by oligomycin does not severely affect the ATP/ADP ratio of Ehrlich ascites tumor (EAT) cells but stopped the cell cycle progression resulting in an accumulation of the cells in the early S-phase [1]. In the presence of oligomycin a high electrochemical potential is built up across the mitochondrial membrane, which prevents the exchange of ATP_{ext} against ADP_{int} [2]. We therefore suggested, that impairment of mitochondrial ATP dependent processes as a result of blockade of energy supply from the cytoplasm is the main reason for the arrest of cell growth. First of all we think of protein synthesis because it was recently shown that ATP synthesized via oxidative phosphorylation, not GTP synthesized from substrate phosphorylation, limits the rate of mitochondrial protein synthesis [3]. The significance of mitochondrial protein synthesis for regular cell cycle progression may be tested by applying a specific inhibitor *e.g.* chloramphenicol. In the present work we therefore have investigated proliferation, cell cycle kinetics, energy metabolism and macromolecular synthesis of *in vitro* grown EAT cells in the presence of chloramphenicol. The results of our experiments suggest, that the inhibition of mitochondrial protein synthesis seems not to be responsible for the arrest of cell cycle progression in the early S-phase after blockade of oxidative phosphorylation by oligomycin, because chloramphenicol causes a retardation of cell cycle progression by prolongation of the duration of the G1- and S-phase of the cell cycle, which may be observed already in the first passage in the presence of the inhibitor.

Reprint requests to Prof. Dr. Fr. Schneider.

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Materials and Methods

All chemicals, buffer and media substances were of the purest grade available from Boehringer

Mannheim; Merck, Darmstadt; Serva, Heidelberg, and Sigma, München. [2-^{14}C]thymidine (spec. act. 60 Ci/mol), [2-^{14}C]uridine (spec. act. 60 Ci/mol), L-[U-^{14}C]leucine (spec. act. 340 Ci/mol) [2-^{14}C]-α-aminoisobutyric acid (spec. act. 51 Ci/mol) were from Amersham-Buchler, Braunschweig. Cocktails for scintillation counting were obtained from Roth, Karlsruhe. Ethidiumbromide, 5-bromo-deoxyuridine and chloramphenicol were from Serva, Bisbenzimide-H33258 was from Riedel de Haen, Hannover, Microcillin was a gift from Bayer, Leverkusen, horse serum was a gift from Behringwerke, Marburg.

Cell strain and growth conditions

A strain of hyperdiploid Ehrlich ascites tumor (EAT) cells subcultured by Karzel [4] was used for the experiments. Details of cell culture procedure and incorporation experiments are described in 5, 6, 7. Chloramphenicol was dissolved in medium (7.5 mg chloramphenicol/ml medium). For recultivation in the absence of chloramphenicol, cells were pelleted after 24 h by centrifugation, washed with culture medium and transferred into fresh medium without the inhibitor (third passage *in vitro*). The controls were treated in the same way.

Cell cycle analysis

Phase composition of the cultures was analyzed by flow-cytometry as described in [8] and [9]. The fluorescence of DNA bound dye was measured proportionally, stored and graphed with the flow cytometer ICP 11, Phywe Göttingen, according to Göhde and Dittrich [8]. The quantitative evaluation of the histograms to obtain the fractions of cells in the various compartments is illustrated in reference [9].

Applying the BrdU-H33258 technique [10], cells were grown in the presence of 5-bromo-deoxyuridine (40 mg/l culture medium) deoxycytidine (32 mg/l culture medium) was added to reduce cytotoxic effects due to induction of lack of deoxycytidine in the cells by BrdU. About 10^6 cells were fixed by suspending in 10 ml 96% ethanol. The fixed cells were collected by centrifugation (2 min, $500 \times g$) and treated with 0.1 ml (10 Kunitz Units) of RNase for 2 h at 37 °C (DNase free, Serva Heidelberg). After washing with NaCl solution the nuclei were stained by addition of 0.5 ml 0.2 M citric acid and 1.0 ml H33258 (10 mg/l in 50 mM TRIS/HCl pH 7.0 and 20% ethanol). Fluorescence was measured immediately after staining.

The principle of the method is based on the observation that after addition of BrdU the amount of DNA stainable with Bisbenzimide H33258 at pH 7 remains constant for each cell even if new DNA is synthesized. Thus, the cells of a certain subcompartment of the cell cycle remain in the same fluorescence compartment until they divide. After cell division, the progeny cells appear in the DNA histograms at half the original fluorescence intensity. For further details of the method see reference [11, 12].

Electron microscopy

Electron micrographs were obtained as described in [7].

Measurement of metabolic parameters

L-Lactate production of the cells was assayed as described by Hohorst [13], glucose consumption was measured with the glucose oxidase/perid-test from Boehringer (Mannheim), oxygen uptake was determined amperometrically with a Clark electrode [14]. Protein content of the cultures was estimated following the Folin method [15]. DNA content of cell cultures was determined fluorometrically as described by Labarca and Paigen [16]. The relative rates of DNA-, RNA- and protein synthesis and the uptake of amino acids were measured by incorporation of [2-^{14}C]thymidine, [2-^{14}C]uridine, L-[U-^{14}C]-leucine and [2-^{14}C]-α-aminoisobutyric acid as described in [1].

ATP and ADP levels of the cells were assayed by applying the luciferin/luciferase system [17] using a Bioluminat 9500 (Bertholdt). For further details see [1].

Results

Viability and cell proliferation in the presence of chloramphenicol

Viability of the cells is not severely affected within 24 h by concentrations up to 200 µg/ml of chloramphenicol; above 500 µg/ml the number of nigrosin positive cells exceeds never 10% and in the presence of 10 mg/ml the cells were totally damaged within 24 h. Fig. 1 shows that even with the highest

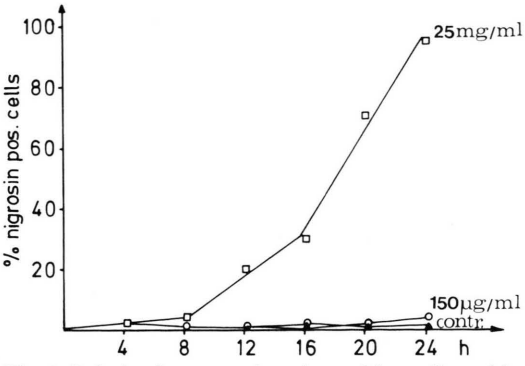


Fig. 1. Relation between nigrosin positive cells and incubation time at different concentrations of the inhibitor.

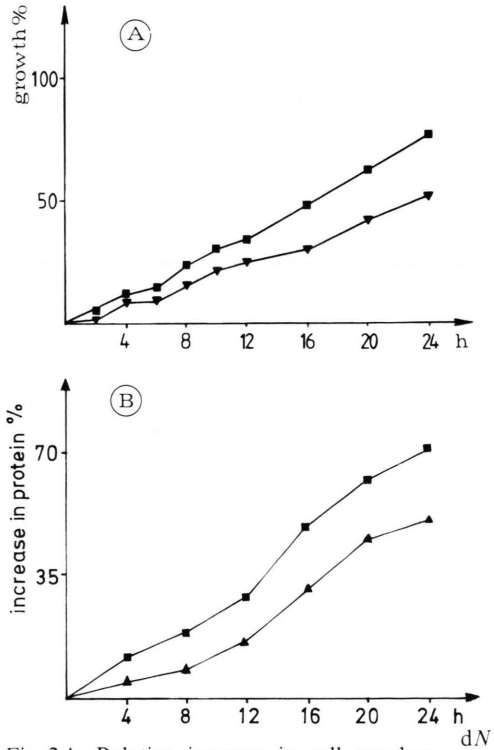


Fig. 2A. Relative increase in cell number $\frac{dN}{dt} \cdot \frac{100}{N} =$ percentage growth of cells grown in the presence of 150 µg/ml of chloramphenicol ▲—▲ and of controls ■—■. B. Increase of protein.

concentration of the inhibitor the number of nigrosin positive cells increases only 8 h after starting the incubation. For all further experiments a concentration of 150 µg/ml of the inhibitor was chosen. With this concentration the number of nigrosin positive cells never exceeded 5%.

Proliferation of the cells in the presence of chloramphenicol within 24 h is compared to the growth of control cultures in Fig. 2 A. Growth of controls within 24 h was 75% ± 8 (mean of 12 cultures); in the presence of chloramphenicol growth of the cells was reduced to 50% ± 5 (mean of 36 cultures). The DNA-content of the controls increased from 4.1 µg/ml to 10.5 µg/ml, in chloramphenicol treated cells from 4.1 µg/ml to 8.75 µg/ml with 24 h.

Within the same time period the protein content of the control cultures increased from 90 µg/ml to 160 µg/ml (Fig. 2B); this is compatible with the growth rate of the cells. The corresponding values in the presence of chloramphenicol were 90 µg/ml and 130 µg/ml respectively. The protein content per cell is not significantly affected by the inhibitor; no unbalanced growth could be detected. The ratio µg DNA/mg protein was 45 at the beginning, 70 after 12 h and 44 after 20 h for the controls as well as the chloramphenicol treated cells.

Proliferation kinetics

Cell cycle distribution of chloramphenicol treated populations in the second passage *in vitro* is compared to phase composition of control cultures in Fig. 3A and B. The most prominent feature of the cytokinetics of chloramphenicol treated cells is a retardation of the cycle progression of about 8 h within a period of 20 h as compared to control cultures. This conclusion may be drawn from the following observations: G1 cells of chloramphenicol treated cultures decrease and consequently S cells increase 4 h later than the corresponding cells of the control cultures.

S-cells attain a maximum 20 h after beginning of the growth in the presence of the inhibitor, while the controls reach the same value already after 12 h, which implies that passage through the S-compartment retards the chloramphenicol treated cells by further 4 h. Consequently the number of S cells decreases 8 h later than the S cells of the controls; the G2 cells increase after 20 h, 8 h later than the G2-cells grown in the absence of the inhibitor. Up to 20 h the chloramphenicol treated cells apparently accumulate in the S-phase of the cell cycle: The number of G1-cells decreased from about 44% to 10%, the fraction of S-cells increased from 27% to 50% and the portion of G2 cells dropped from 29% to 21%.

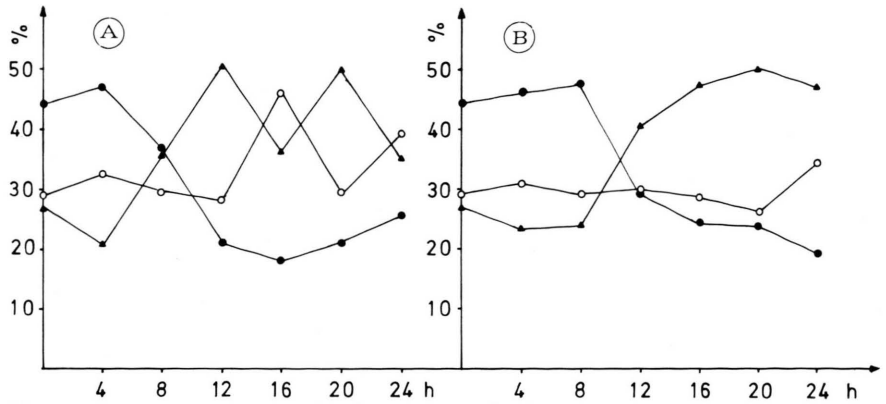


Fig. 3. Cell cycle distribution of cultures grown in the second passage *in vitro* over a period of 24 h. G1-cells ●—●, S-cells ▲—▲, G2-cells ○—○ Ⓐ control cultures Ⓑ in the presence of chloramphenicol.

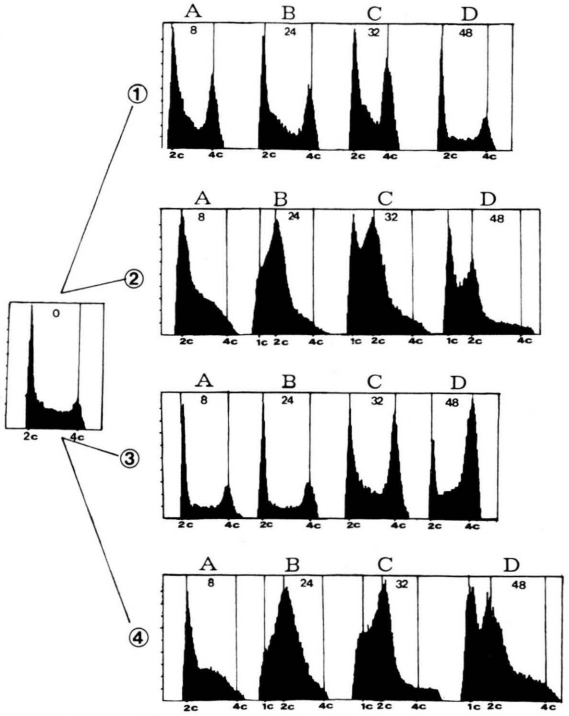


Fig. 4. DNA histograms of ① control cultures ② control cultures grown in the presence of BrdU ③ cultures grown in the presence of chloramphenicol ④ cultures grown in the presence of chloramphenicol + BrdU.

Further information on the cycle progression of G1-, S- and G2-cells were obtained by applying the BrdU-H33258 technique of flow cytometry. DNA histograms of controls and chloramphenicol treated cells grown in the absence and presence of BrdU are compared in Fig. 4. These histograms demonstrate that cells being in G2 at the beginning of the experiments have divided after 8 h: no G2 peak in A2 and A4; at the same time no significant number of S-cells have passed mitosis: no fluorescence signals below 2c in A2 and A4. 16 h later the main fraction of S- and late G1-cells have passed the cell cycle: fluorescence signals between 1c and 2c in B2 and B4. Retardation of cell cycle progression of chloramphenicol treated cells is confirmed by a comparison of the histograms B2 and B4: differences in fluorescence signals below 2c. Obviously some S-cells have not entered mitosis at this time: signals below 4c. 32 h after starting the cultures most of the G1-cells of the controls have divided: 1c peak in C2, while in chloramphenicol treated populations by far less G1-cells have passed mitosis. Retardation of cycling in the presence of chloramphenicol is revealed from differences in the G1/2 = 1c peak and the relative fluorescence intensity between 1c and 2c as compared to controls (compare C2 and C4). There is further on a significant fraction of S-cells which did not leave their fluorescence compartment (fluorescence signals < 4c in C2 and C4). 48 h after growth of the cells in the presence of the inhibitor, the cell cycle distribution was found to be 17% G1-, 28% S- and 55% G2-cells, that is to say more than 80% of the cells are in S + G2; in the control cultures, the corresponding values were 44% G1-, 30% S- and 26% G2-cells: the main fraction of cells is in G1 (see D1 and D3). From the BrdU histograms after 42 h it becomes evident, that in the presence and absence of chloramphenicol about 20% of the cells do not

cycle and that most of the chloramphenicol treated cells are in S + G2 (see above and D4).

DNA-, RNA- and protein synthesis

Incorporation of [2-^{14}C]thymidine into acid insoluble precipitate of controls and chloramphenicol treated cells is depicted in Fig. 5A. A typical incorporation pattern with a maximum at about 6 h was obtained under normal conditions. In the presence of the inhibitor the rate of DNA synthesis is about 70% of controls after 12 h and about 50% after 20 h. The results of incorporation studies with [2-^{14}C]uridine are depicted in Fig. 5B. In accordance with a retardation of the cell cycle progression RNA-synthesis is reduced by about 20% of controls on an average.

Incorporation of radioactivity from [^{14}C]leucine into acid insoluble fraction of the whole cells is not significantly affected on treatment of the cells with chloramphenicol. The same is true for the transport of α-amino isobutyric acid.

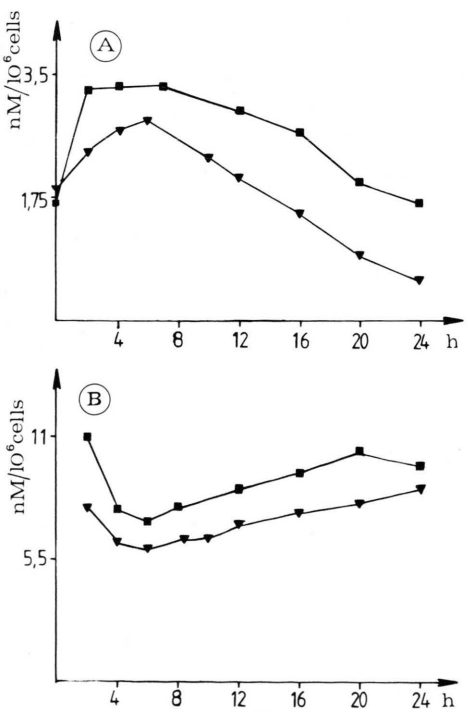


Fig. 5. Incorporation of [^{14}C]thymidine Ⓐ [^{14}C]uridine Ⓑ into acid insoluble precipitate of the cells grown in the presence of chloramphenicol ▲—▲, controls ■—■.

Energy metabolism

Inhibition of mitochondrial protein synthesis by chloramphenicol causes a significant increase of lactate production by about 20% (controls 1.01 ± 0.093 µmol/10^6 cells × 24 h, chloramphenicol treated cells 1.23 ± 0.15 µmol/10^6 cells × 24 h). Under the same conditions the glucose consumption of the controls within 24 h was found to be 0.555 µmol/10^6 cells. The corresponding values for the 3. passage were: Controls 1.0 µmol lactate and 0.680 µmol glucose, chloramphenicol treated cells 1.091 µmol lactate and 0.702 µmol glucose per 10^6 cells within 24 h.

At chloramphenicol concentrations of 150 µg/ml a reduction of oxygen assumption of the cells from 165 nmol/10^6 cells × h to 93 nmol was observed after 24 h, that is a reduction by about 40%.

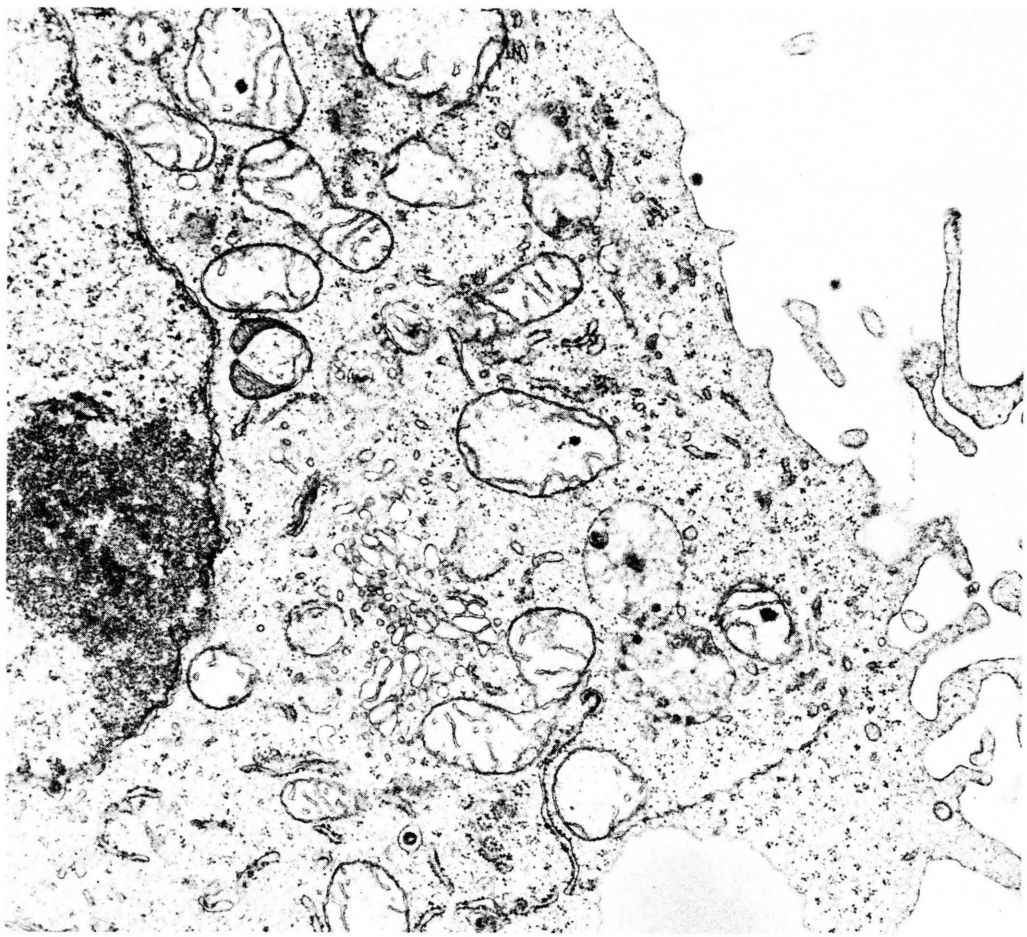
ATP- and ADP levels of controls and of oligomycin treated cells are depicted in Table I. No significant differences between the ATP and ADP content of controls and chloramphenicol treated cells could be detected. Since chemical energy is always a matter of concentration ratios, a more reliable parameter of the energetic state is the ATP/ADP ratio, which is also shown in Table I. These data confirm that a physiological concentration ratio is preserved in the presence of chloramphenicol.

Effects of chloramphenicol on the ultrastructure of EAT-cells

To substantiate further the data obtained, the ultrastructure of controls and chloramphenicol treated cells was examined by electron microscopy. Chloramphenicol is known to bring about alterations in the mitochondrial structure, likely as a

Table I. ATP, ADP concentrations and ATP/ADP ratios of EAT cells grown in the presence of chloramphenicol and of controls.

h	ATP nmol/ 10^6 cells		ADP nmol/ 10^6 cells		ATP/ADP	
	Controls	+ Chloramph.	Controls	+ Chloramph.	Controls	+ Chloramph.
4	7.80	8.14	3.24	3.53	2.41	2.72
8	9.01	10.22	2.60	2.96	3.47	3.48
12	9.16	10.08	2.39	2.41	3.82	4.18
16	8.17	10.03	2.28	2.75	3.80	3.72
20	7.63	8.78	2.29	2.33	3.46	3.60
24	6.37	6.53	1.93	2.04	3.40	3.43



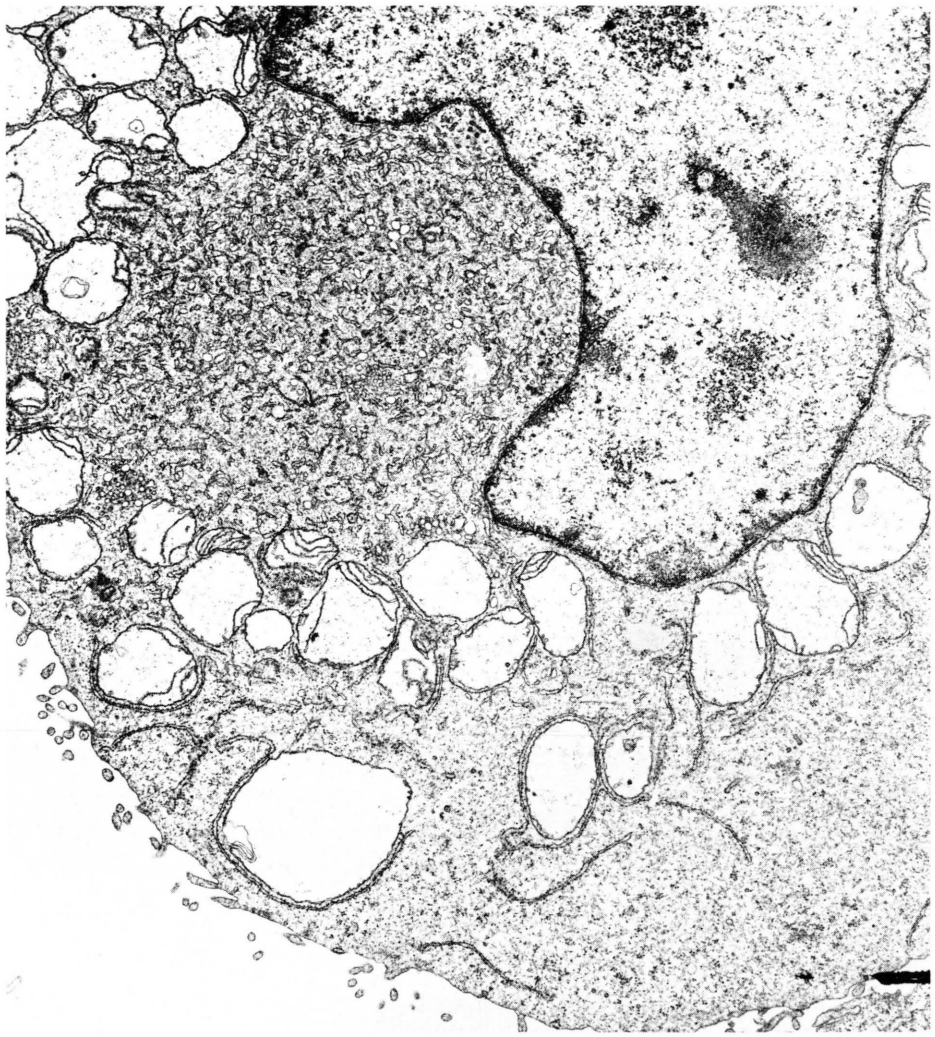
A

Fig. 6. Electron micrographs of the cells. Ⓐ Control cells after 24 h in the second passage *in vitro*, × 8000; Ⓑ cells grown for 24 h in the presence of chloramphenicol, × 8000.

consequence of impairment of the mitochondrial protein synthesizing ability. Electron micrographs of cells grown in the presence of 150 µg/ml chloramphenicol for 12 and 24 h are shown in Fig. 6. These show a high amplitude of swelling of all mitochondria with translucent appearance of the inner compartment, further reduction in the number of cristae within the organelle and loss of cristae orientation. The mitochondrial outer membranes however remain intact. Occasionally mitochondria of near normal appearance were found. An increased number of multivesicular vacuoles are seen in the cytoplasma of chloramphenicol treated cells. The enlarged mitochondrial profiles could result not only from swelling but also from fusion of individual mitochondria as described for other inhibition of mitochondrial functions [18].

Recultivation of cells after treatment with chloramphenicol

Growth of cells cultured for 24 h in the presence of chloramphenicol and transferred thereafter to fresh medium (3. passage *in vitro*) is depicted in Fig. 7. Within 24 h an increase of cell number of chloramphenicol treated cells of about 75% of controls was observed. Increase of DNA and protein of the cultures corresponds to the growth of the cells (not shown). No unbalanced growth of chloramphenicol treated cells could be observed.



B

Fig. 6B

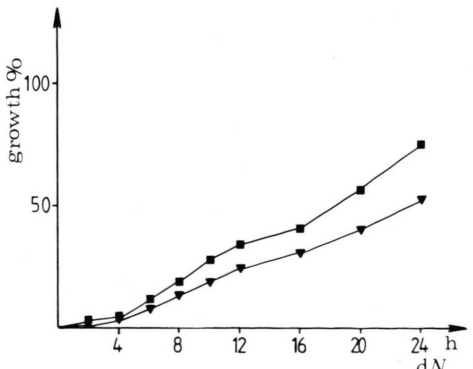


Fig. 7. Relative increae in cell number $\frac{dN}{dt} \cdot \frac{100}{N}$ = percentage growth of control cultures ■—■ and of cells treated with chloramphenicol for 24 h and transferred to fresh medium ▲—▲.

The incorporation pattern of labelled thymidine and uridine of cells shifted to chloramphenicol free medium is compared with that of controls in the 3. passage in Fig. 8A–B. The reduction of DNA and RNA synthesis already found in the presence of the inhibitor in the second passage *in vitro* is also observed in the third passage after transfer of the cells to chloramphenicol free medium. Only moderate differences in the course of DNA- and RNA-synthetic capacity between the cells in the second passage in the presence of chloramphenicol (Fig. 5A–B) and the rate of macromolecular synthesis after removal of the inhibitor are observed. The uptake of α-amino isobutyric acid is not impaired.

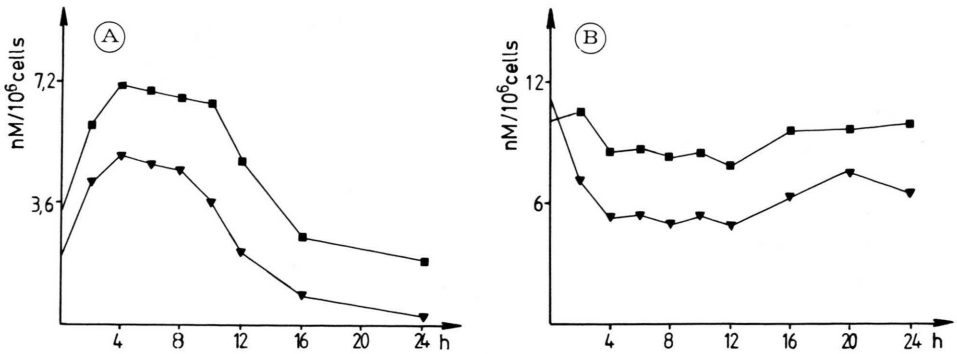


Fig. 8. Incorporation of [^{14}C]thymidine Ⓐ, [^{14}uridine Ⓑ into acid insoluble precipitate of the cells grown for 24 h in the presence of 150 µg/ml chloramphenicol and thereafter shifted to normal medium (third passage *in vitro*). ▲---▲, controls ■—■.

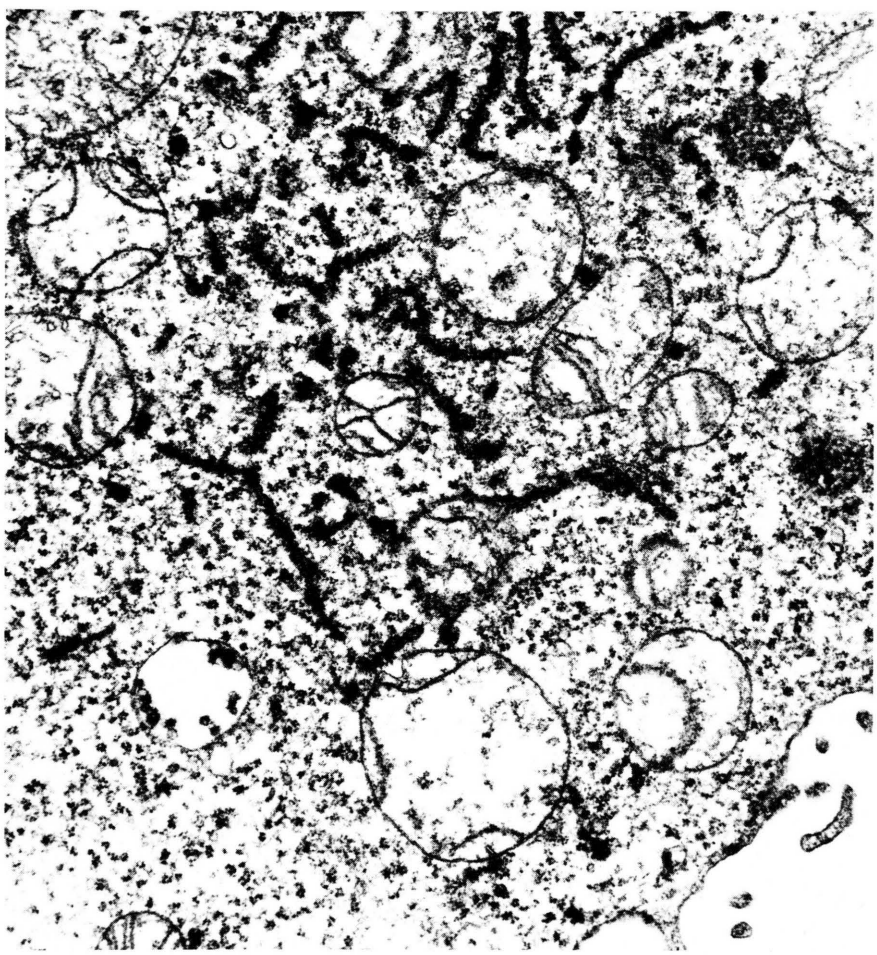


Fig. 9. Electron micrograph of cells grown for 24 h in the presence of chloramphenicol and thereafter for 12 h in normal medium (third passage) × 8000 (controls see Fig. 6A).

Electron micrographs of cells grown in the presence of chloramphenicol for 24 h and shifted to normal medium are depicted in Fig. 9. 12 h after recultivation, recovery of the structure of the inner mitochondrial membranes is going on. Since growth of eukaryotic cells in the presence of chloramphenicol usually does not lead to a loss of mitochondrial nucleic acids [19] the organelles take up translation activity after removal of the inhibitor.

Discussion

The results presented in this paper extend our knowledge on the effects of chloramphenicol on cell cycle kinetics and metabolism of *in vitro* grown Ehrlich ascites tumor cells. Concerning the concentration of the inhibitor a substantial inhibition of the mitochondrial protein synthesizing system should be achieved without exerting nonspecific side effects in other areas of cell metabolism. A concentration of about 100–150 µg/ml meets this conditions fairly well as may be concluded from many experiments described in the literature [20]. No complete inhibition of mitochondrial protein synthesis, which is only 2% of the total protein synthetic activity of the whole cell [21], may however be achieved by this concentration.

The electron micrographs presented demonstrate that the formation of cristae is severely affected in the presence of chloramphenicol while the structure of the outer membrane is apparently unaffected; these are well known effects of chloramphenicol on the mitochondria of eukaryotic cells [22].

In proliferative cell populations, the inhibitory effect of drugs on mt-DNA transcription and translation may be assessed by measuring the decrease in oxyen consumption. As is described in [23], chloramphenicol reduces oxygen consumption of chick-embryo cells by 15% at 100 µg/ml; at 350 µg/ml respiration was inhibited by about 50%, a value close to that reported for other cultured cell lines. In the present experiments we found a reduction of oxygen consumption after 24 h of incubation with chloramphenicol by about 40% of controls.

If mitochondrial translation is substantially inhibited, cellular cytochrome oxidase activity should be affected. Such is indeed demonstrated in different animal cell lines treated with chloramphenicol [21, 24, 25]. After one population doubling = 100% growth, cytochrome oxidase activity should therefore be reduced by 50%, after two by 25% and so on [26]. Since in our experiments in the presence of chloramphenicol an increase of cell number of about 50% is observed within 24 h, the reduction of oxygen consumption between 30–40% is compatible with a nearly complete inhibition of cytochrome oxidase synthesis.

The most significant effect of chloramphenicol on cell cycle kinetics is the retardation of cycle progression within short time after treatment of the cultures with the antibiotic. Over two passages *in vitro* an arrest of cell growth could not be detected. These results do not support our suggestion [1], that accumulation of EAT cells in the presence of oligomycin in the early S-phase is caused by inhibition of mitochondrial protein synthetic activity; however it cannot be excluded, that arrest of cell growth may occur after a longer time period of cultivation in the presence of chloramphenicol.

An increase of cell population doubling time in the presence of chloramphenicol was observed also with other animal cells [20] but a more subtile analysis of cell cycle kinetics has not been published.

It seems quite clear, that mitochondria of animal cells perform indispensable functions for cell growth, which are not related to energy metabolism. Mitochondria are involved in many anabolic and catabolic pathways and it is conceivable that disorganization of the inner membrane by chloramphenicol might well be sufficient to impair their functions. It is further well established, that the mitochondrial and the cytoplasmic protein synthesizing system are tightly coordinated. This coordination probably occurs at several different levels. The impairment of cell cycle progression by chloramphenicol is therefore not surprising.

Acknowledgements

This work was supported by DFG (SFB103), Fond der biologischen Chemie and Kuleman Stiftung, Marburg.

The technical assistance of Thomas Stein is gratefully acknowledged. We thank U. Lehr, Anatomisches Institut Marburg for performing electron microscopy.

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