Growth Characteristics of *in vitro* Cultured Ehrlich Ascites Tumor Cells under Anaerobic Conditions and after Reaeration

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The effects of deprivation of oxygen on proliferation kinetics of *in vitro* grown Ehrlich ascites tumor cells were studied by means of flow cytometry. The flow cytometric results obtained by applying the BUdR-H 33258 technique showed that 6 – 8 hours after establishment of anaerobiosis cells lose their capacity for proliferation and accumulate in the late G1-phase; during this time late S cells enter the G2 compartment but do not divide while cells, which are in G2+M at the beginning of anaerobiosis divide and enter G1. Cell cycle distribution remained constant up to about 20 hours, thereafter G1-cells enter the S-phase. Up to 24 hours of anaerobiosis cell number does not change and viability of the cells was not severely affected. Recultivation under aerobic conditions of 12 hours anaerobically treated cells revealed, that the cytokinetic properties of G1 cells were not impaired, while S-phase cells after reaeration did not enter G2 but began again to synthesize DNA forming cells with a DNA content greater than 4c. S-phase cells are most sensitive to deprivation of oxygen.

Introduction

Evidence has accumulated that the progress of most mammalian cells through the cell cycle depends on the presence of molecular oxygen [1 – 4]. The biochemical mechanisms underlying the oxygen dependence of cell cycle progression of mammalian cells is unknown although it is clear that molecular oxygen participates in several fundamental processes. In addition to oxygen's performing a central role in respiration, it is substrate of a series of anabolic and catabolic processes.

By systematically investigating the effects of oxygen deprivation on cell growth, cell cycle and metabolism of Ehrlich ascites tumor (EAT) cells, we are attempting to elucidate the various aspects of the function of molecular oxygen in cellular economy of this tumor cell.

In the present report we describe the biological basis of the inhibition of cell growth after exclusion of oxygen by determining whether EAT-cells under anaerobic conditions accumulate at random points or at particular stages of the cell cycle. We further present data on the cytokinetics of anaerobically grown EAT cells after reaeration.

Materials and Methods

Chemicals

All chemicals, buffer and media substances were of the purest grade available from Merck (Darmstadt); Serva (Heidelberg) and Sigma (München). Ethidiumbromide was from Serva, Benzimid H 33258 was from Riedel de Haen (Hannover). [2-^{14}C]thymidine (s. a. 53 – 61 mCi/mM) was purchased from Amersham-Buchler (Braunschweig). Cocktails for scintillation counting were from Roth (Karlsruhe), Argon/CO_2 = 95:5 and air/CO_2 = 95:5 were obtained from Messer-Griesheim (Frankfurt).

Cell culture procedure

Hyperdiploid Ehrlich ascites tumor cells grown serially in the peritoneal cavity of female NMRI mice were used for all experiments after a first shortened *in vitro* passage of 13 – 15 h under standard aerobic conditions in a modified Eagle medium [5] supplemented with 15% horse serum (Behringwerke AG, Marburg), 30 mg/l streptomycin, 575 mg/l microcillin (Bayer, Leverkusen) and 11 mM glucose. This type of ascites tumor cells is made to grow in suspension culture by using glass vessels which are not coated with silicon. Growth of the cells was estimated by enumeration with a hemocytometer.

After 13 – 15 h cultivation under standard conditions the cells were separated by centrifugation

($500 \times g$, 2 min) and transferred to fresh culture medium. The inoculum density was $6-7 \times 10^5$ cells/ml. To obtain anaerobic conditions the flasks were continuously flushed with Argon/CO_2 = 95/5 at a rate of 60 ml/min. The gas passed a heated oxysorb catalyst (R 3-11/M 3610, BASF, Ludwigshafen) and was then humidified by bubbling through a water tower at 37 °C. This procedure provides oxygen free conditions within 30 min. Aerobic cells received air/CO_2 = 95/5. Viability of the cells was assessed by phase contrast microscopy and by dye exclusion tests with 0.1% nigrosine [6]. The increase of protein content of cell cultures was estimated in 1 ml aliquots employing the Lowry method [7], with bovine serum albumin as standard.

For incorporation experiments 1 ml cell suspension was withdrawn at intervals from the cultures and incubated for 15 min at 37 °C with 0.2 µCi [¹⁴C]thymidine. The uptake of label was measured in the TCA insoluble precipitate using Rotiszint 11.

Flow cytometry and BUdR-H 33258 technique

Cell cycle analyses of control cells and anaerobically cultured cells were performed at appropriate time intervals by flow cytometric measurements [17]. A Phywe ICP 11 detector, equipped with a 100 W high pressure mercury lamp, excitation filters BG 38 + BG 3 (Jenaer Glaswerke, Schott Mainz) and barrier filters GG 550 (Leitz, Wetzlar) respectively OG 590 were applied.

About 10^6 cells were washed twice with cooled Hank's solution, fixed by suspending in 10 ml 96% ethanol and stored at − 20 °C if required. The fixed cells were again washed twice with 0.9% NaCl solution, treated with 0.1% RNase (DNase free, Serva, Heidelberg) for 2 h at 37 °C. After, washing with NaCl solution the nuclei were stained with 10 ml ethidium bromide solution (10 mg/l in 50 mM Tris buffer, pH 7.5) and kept in the dark at 4 °C. The quantitative evaluation of the histograms to obtain the fractions of cells in the various compartments is illustrated in reference [9].

Applying the BUdR-H 33258 technique [10] the 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 a lack of deoxycytidine in the cells by BUdR. Cells were stained with the fluorochrome benzimid H 33258 (10 mg/l, 50 mM Tris HCl, pH 7.0) without RNase treatment. The fluorescence was measured immediately after staining using the barrier filter OG 590.

The principle of the method is based on the observation that after addition of BUdR the amount of DNA stainable with Benzimid H 33258 remains constant for each cell even if new DNA is synthesized. Thus, the cells of a certain subcompartment of the cell cycle retain the same fluorescence intensity until they divide. After cell division the two progeny cells appear in the DNA histogram at half the original fluorescence intensity. Cells in G 1 at the time of BUdR addition retain the fluorescence intensity of G 1 cells until they divide, but appear at G 1/2 = 1 c after division. Cells that have been in G 2 + M when treated with BUdR appear at 4 c until they divide and at 2 c after division. The original S-cells appear in the region between 2 c and 4 c and between 1 c and 2 c after division (see Fig. 1). For further details of the method see reference [10, 11].

Results

The generation-time of untreated EAT-cells under our culture conditions was approximately 35 h. In individual experiments it varied from 34−36 h. The mean duration of the G 1-phase was 19 ± 1 h, of the S-phase 9 ± 1 h and of the G 2 + M-phase 7 ± 1 h. As is shown in Fig. 2, cell growth arrests about 8 h after beginning of exclusion of oxygen and reaches a plateau. Protein and cell volume however increase to about 12 respectively 24 h. As was shown by dye exclusion test, viability of the cells is not severely affected by a 24 h period of anaerobiosis.

The distribution through the cell cycle of the ascites cells during a 24 h period of anaerobiosis is demonstrated in Fig. 3. From this figure, which illustrates the results of a typical single experiment, we may draw the following conclusions: The flow cytometric analysis of the cell cycle distribution confirms the arrest of cell growth after 8 h of exclusion of oxygen. Within the first 8 h of anaerobiosis the number of G 1-phase cells increases, while the numbers of S- and G 2 + M cells decrease. During the following 12 h the distribution of the cells through the cell cycle does not change. About 20 h after beginning of deprivation of oxygen, G 1-cells enter the S-phase, while the number of G 2 + M-phase cells and the total cell number remained constant.
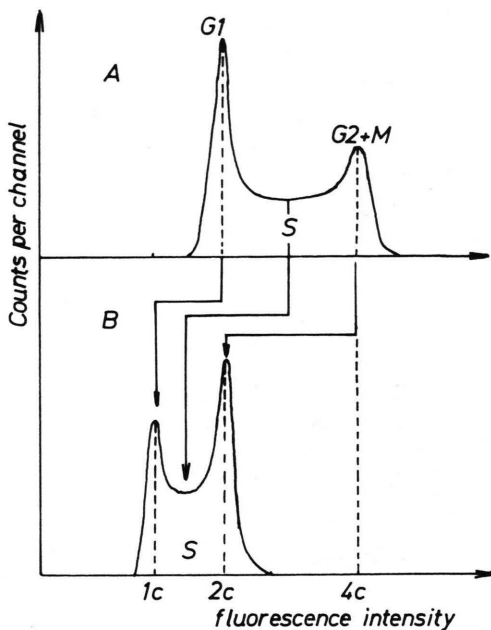
Fig. 1. Illustration of the BUdR-H33258 technique of flow cytometry. A) normal DNA-histogram, B) DNA-histogram after division of a population grown in the presence of BUdR; for further details see text.

Further informations on the cell cycle progression of the cells under exclusion of oxygen were obtained by applying the BUdR technique of flow cytometry. This method proved to be suited to answer the following questions: Do G 1-phase cells pass the cell cycle during anaerobiosis? Do cells from late S-phase enter G 2, do they divide and enter G 1? DNA histograms from anaerobically grown cells in the absence and presence of BUdR are depicted in Fig. 4. From this fig. the following conclusions may
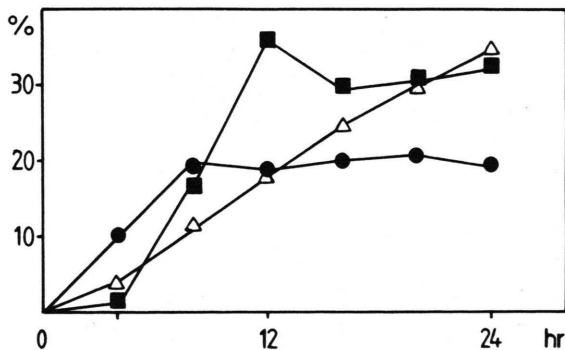
be drawn: Histograms of cell populations after addition of BUdR lack a peak at $G\,1/2 = 1\,c$, which indicates that in the absence of oxygen, G 1-phase cells do not divide within 24 h. Cells of the late S-phase do also not divide because no shoulder at the left side of the G 1-peak of the BUdR histograms appeared. These cells enter however the G2-phase as becomes evident from a comparison of the $G\,2 + M$ peak in the absence and presence of BUdR: Measurements of the population 6–8 h after BUdR addition show the clearance of the $G\,2 + M$ compartment, the cells present in this compartment at the beginning of anaerobiosis have divided and entered G 1; late S-phase cells, which have incorporated BUdR and enter G 2 would remain in their fluorescence compartment. The histograms of cells grown in the absence of BUdR prove, that indeed late S-phase cells have entered G 2 and do not leave this phase within the time of the experiment. These are the G 2-cells of Fig. 3, which do not divide up to 24 h.

Flow cytometric histograms of anaerobically grown EAT-cells after reaeration

In order to characterize further the effects of exclusion of oxygen on the various compartments of the cell cycle, we have studied the proliferation kinetics of anaerobically cultured EAT-cells after reaeration. Flow cytometric analyses of anaerobically pretreated cells recultivated in the presence and absence of BUdR under normal conditions were
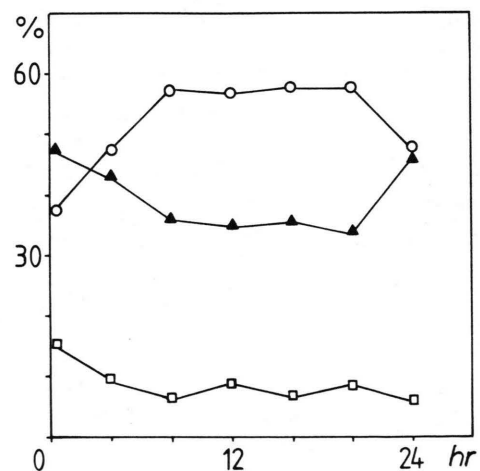

Fig. 2. Cell growth ● — ●, protein content ■ — ■, cell volume △ — △ of anaerobically grown EAT cells.

Fig. 3. Cell cycle distribution of anaerobically grown EAT-cells. G 1-cells ○ v — v ○, S-cells ▲ —— ▲, G 2 + M-cells □ — □.
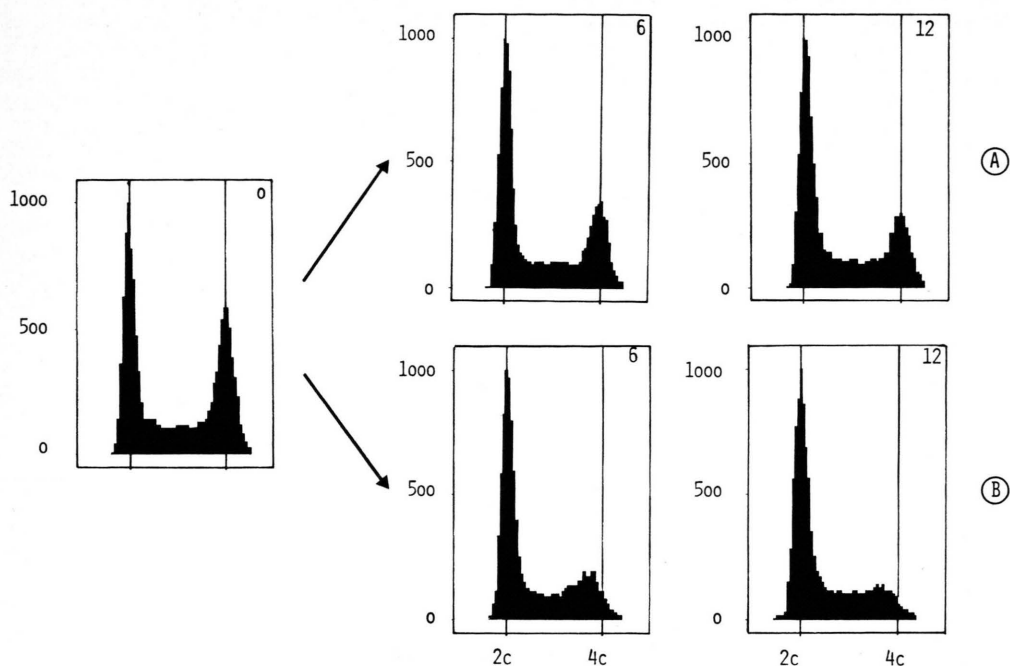
Fig. 4. DNA-histograms of anaerobically grown EAT-cells Ⓐ without BUdR Ⓑ in the presence of BUdR; further illustrations see text.
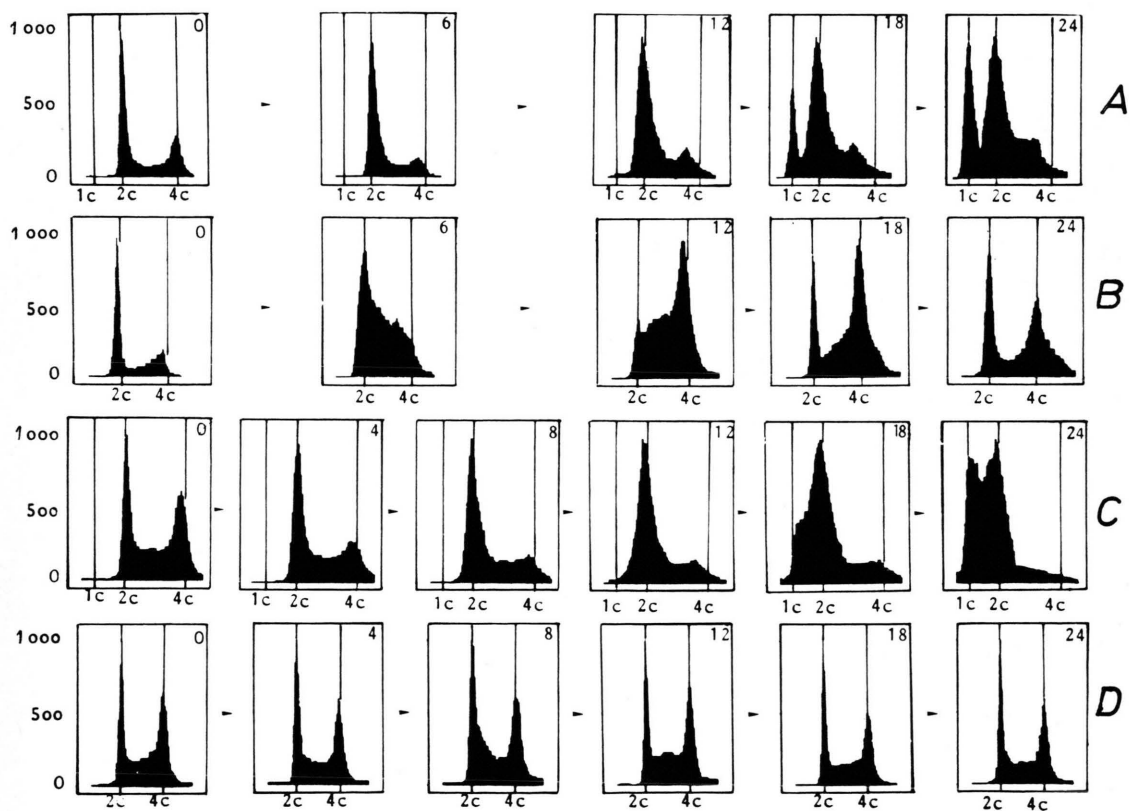

Fig. 5. DNA-histograms. A) of 12 h anaerobically treated cells, recultivated in the presence of BUdR; B) recultivated in the absence of BUdR; C) of aerobically grown cells recultivated in the presence of BUdR; D) recultivated in the absence of BUdR.

performed over a period of 24 h. Typical DNA histograms of these cells are compared with histograms of untreated cells in Fig. 5. The following effects fo a 12 h period of exclusion of oxygen on the cytokinetic properties of the various compartments of the cell cycle may be inferred from the histograms: 18 h after addition of BUdR to the recultivated cells, a distinct G 1/2 peak has appeared suggesting that cells being in G 1 at the beginning of the experiment start to divide after about 14−16 h (series A). This period corresponds to the duration of S + G 2 + M. A fraction of cells being in the S-compartment at the start of recultivation has not moved into G 2 after 24 h. They are passed by original G 1 cells. This may be concluded from a remaining S-plateau in the histogram series A and from the few signals between G 1/2 and G 1, while in the control series C an undisturbed clearance of the G 2- and S-compartments is observed. The 24 h histogram of series B shows a distinct shoulder at the right of the G 2 + M peak indicating that part of the S-cells have lost the control of mitosis forming cells with a DNA content greater than 4c. This phenomenon is not observed in the corresponding histograms of the untreated cells (series D). Between 6−8 h the G 2 + M peaks of both BUdR histograms (series A and C) disap-

peared. In further experiments, the influence of different periods of anaerobiosis on the proliferation kinetics of the cells after reaeration was studied. Cell cycle distribution of cells anaerobically grown for 8, 12 and 24 h are depicted in Fig. 6 a−c. As can be inferred from Fig. 6 a, after a 12 h period of anaerobiosis the cells have accumulated in a smaller range of the G 1 compartment than after 8 or 24 h exclusion of oxygen. It may be possible that most cells have arrived a "control" point in late G 1. This question will be discussed later. After 8 h of anaerobiosis, cells are less homogeneous with respect to the G 1-compartment. A large part of these G 1-cells has not arrived the late G 1-compartmenrts and is distributed over the whole phase. Towards the end of a 24 h exclusion of oxygen, cells begin to leave the G 1-phase (see Fig. 3) and enter the S-phase; they have already passed the maximum of "synchronization". This interpretation of the kinetics of the G 1-phase cells of anaerobically treated cultures is confirmed by the analysis of the proliferation of the S- and G 2 + M-phase cells (Fig. 6 b and 6 c). S-phase cells of 12 h anaerobically pretreated cultures traverse a maximum between 6−8 h after reaeration. This maximum coincides with the rise of the G 2-phase cells, which in turn pass a maximum at the same time where the S-phase cells go through
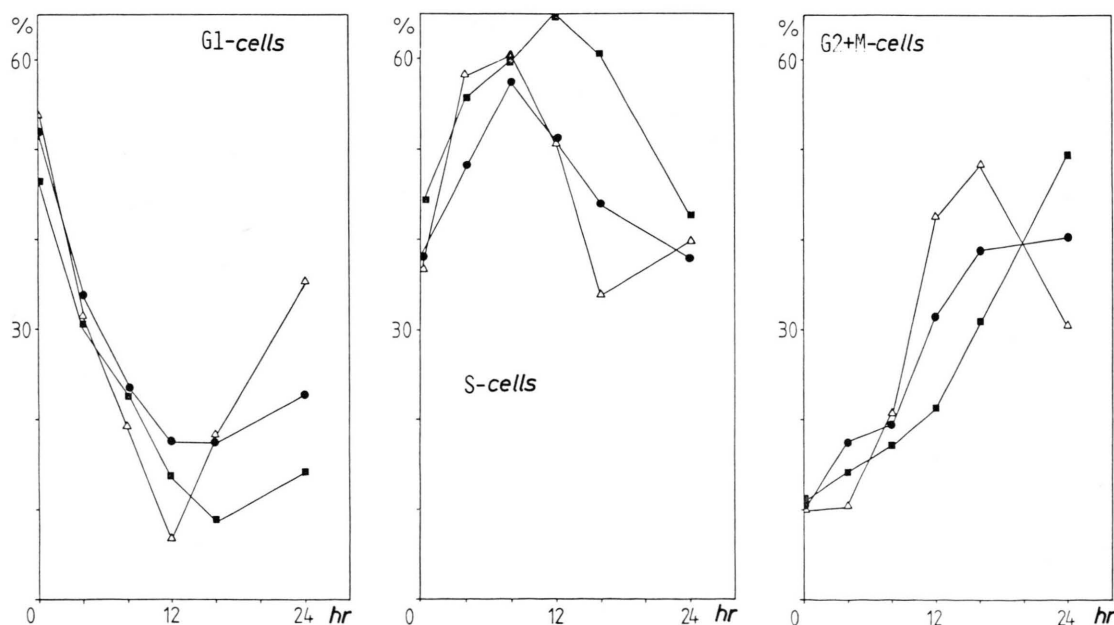

Fig. 6. Proliferation kinetics of 8 h ●−●, 12 h △−△ and 24 h ■−■ anaerobically grown cultures after reaeration.
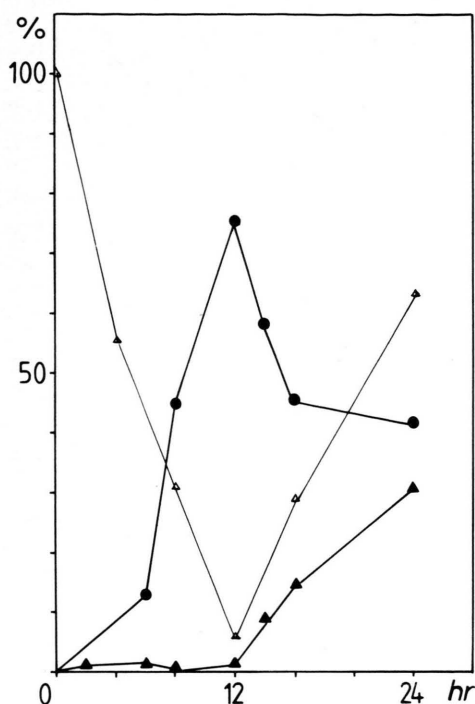
Fig. 7. Increase of protein $(d(P)/dt) \cdot (1/(P) \cdot 100)/10^6$ cells ● — ●, cell growth $(dN/dt \cdot (1/N) \cdot 100)$ ▲ — ▲ and kinetics of G1-phase cells △ — △ of 12 h anaerobically treated cultures after reaeration.

per 10^6 cells decreases, because the cells divide. In Fig. 8 the coincidence between the [^{14}C]thymidine incorporation and the S-phase profile of the cultures is demonstrated. These experiments prove the reliability of the flow cytometric data and the reality of their interpretation.

Morphology of the cells deprived of oxygen and after reaeration

Electron microscopic studies of the cells incubated for 12 h under exclusion of oxygen showed dilated endoplasmic reticulum and significantly reduced density of ribosomes in a translucent cytoplasm. A high amplitude swelling of all mitochondria with a translucent appearance of the inner compartment is further observed (Fig. 9b). As is shown in Fig. 9c, these changes in the intracellular space of the cells are completely reversible after reaeration.
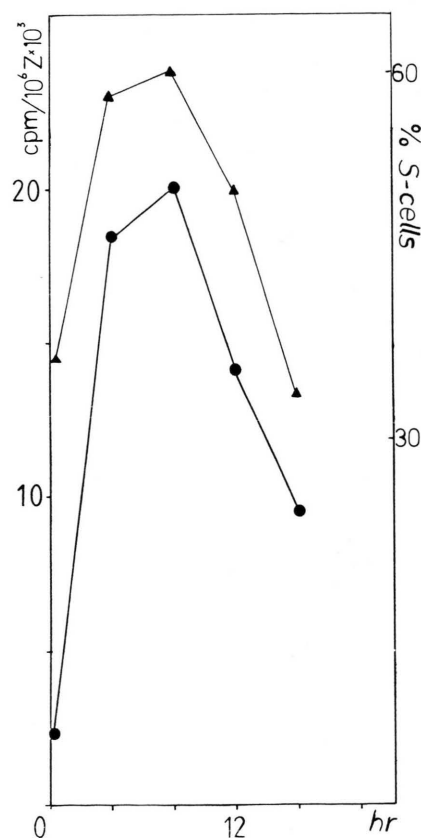
a minimum. The partial synchronization of 12 h anaerobically pretreated cells is retained up to about 16 h. Cells which were grown 8 and 24 h under exclusion of oxygen are completely desynchronized 16 h after reaeration: the G1- and G2 + M cells of these populations increase simultaneously (Fig. 6a and 6c) and the G2 + M-cohorts do not run through a maximum. The kinetics of the G1-, S- and G2 + M-phase cells of cultures which were grown 24 h under exclusion of oxygen, suggest that the progress through the cell cycle is delayed and that the G1-cells need an extended period for recovery.

A further source of information about the proliferation of 12 h oxygen free cultured cells after reaeration was the measurement of cell growth, of protein increase and of [^{14}C]thymidine incorporation. A comparison of these parameters with the flow cytometric data is shown in Fig. 7. Cell growth of the cultures begins 12 h after reaeration, exactly at the time, when the cells of the G1-compartment increase again; at the same time the protein content

Fig. 8. Incorporation of [^{14}C]thymidine ● — ● and kinetics of the S-phase cells ▲ — ▲ of 12 h anaerobically treated cultures after recultivation.
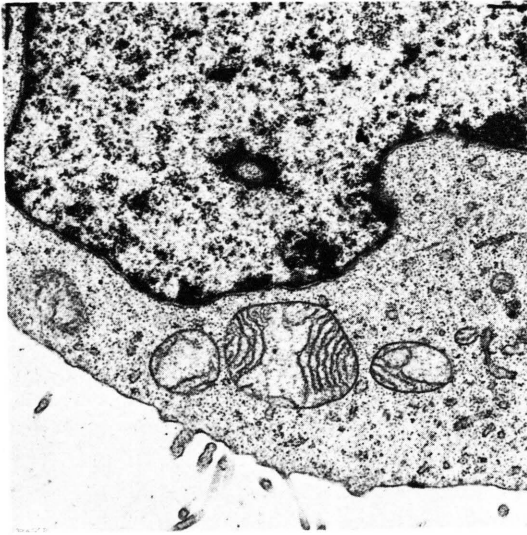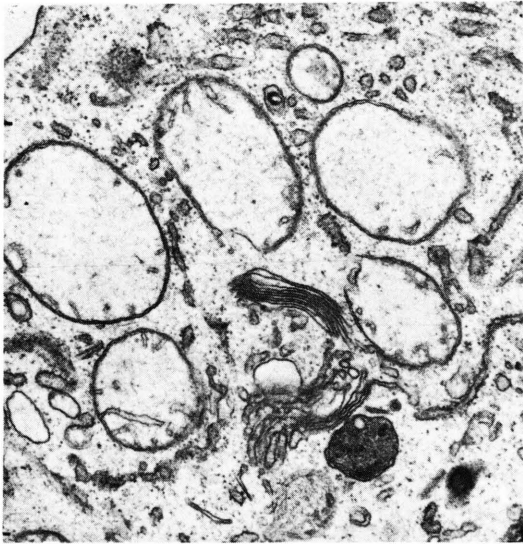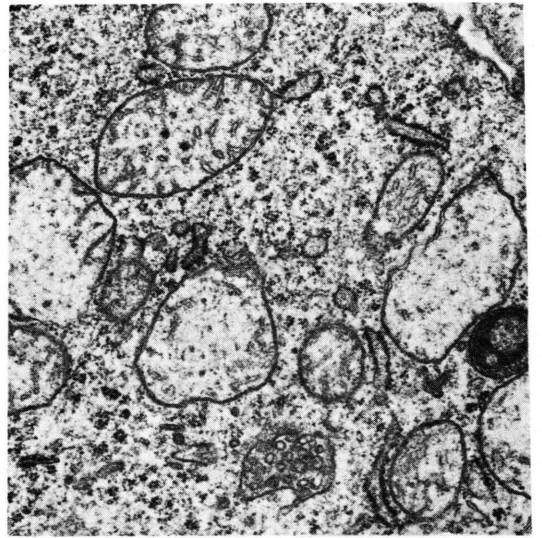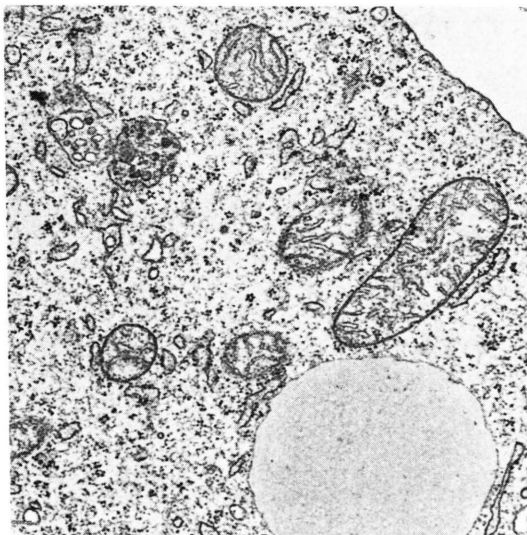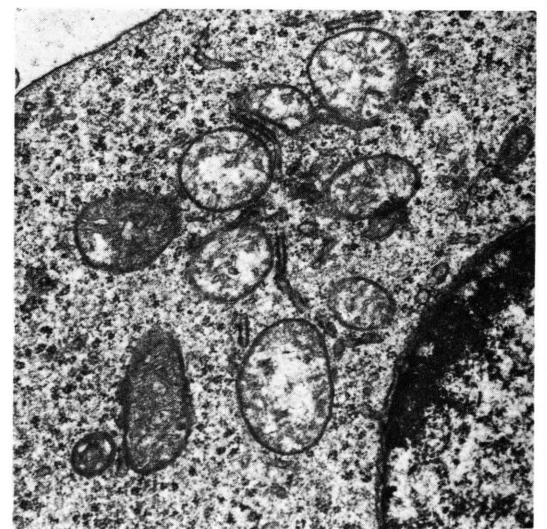
Fig. 9. Electron micrographs of 1) controls, 2a) 12 h anaerobically treated cells, 2b) 12 h aerobically grown control cells, 3a) anaerobically treated cells 12 h after reaeration; 3b) controls. × 4400.

Discussion

As was already demonstrated by Warburg [12] exclusion of oxygen reversibly inhibits the growth of *in vitro* cultured hyperdiploid Ehrlich ascites tumor cells [4]. In the present report we tried to determine whether cells inhibited by deprivation of oxygen occur at random points or accumulate at particular stages of the cell cycle. We further attempted to elucidate the effects of different periods of exclusion of oxygen on the various compartments of the cell cycle. In our experiments we have applied the BUdR-H 33258 technique of flow cytometry; this method allows to follow the progression of all cells of a population through the compartments of the cell cycle.

The data we obtained show that the cells cultured 12 h under anaerobic conditions accumulate in the late G1-phase of the cell cycle. This may be concluded from the observation that up to 12−14 h after deprivation of oxygen the protein content of the cultures and the cell volume increase (Fig. 2) while the increase in cell number has already ceased after 6−8 h. It may be further inferred from Fig. 6 a: A steep drop, a distinct minimum and a steep increase characterize the growth curve of the G1-cells of a 12 h anaerobically treated culture. A progressive accumulation of *in vivo* grown Ehrlich ascites cells in G1 was described by Patt *et al.* [13]. These authors suggested that the accumulation reflects the progressively larger proportion of anoxic cells in the ascites tumor of mice. It may be possible to use a 12 h period of anaerobiosis to obtain a reversibly inhibited G1-enriched population.

It has been proposed that cells could make the commitment to continue to proliferate or to enter a quiescent state at a single point in the G1-phase called the restriction point [14]. Since about 20 h after establishment of hypoxia G1 cells begin to enter the S-phase again we suppose, that cells under anaerobic conditions do not withdraw from the cell cycle into a qualitatively distinct quiescent state (Go-state) but that the "arrested" cells slowly traverse the cell cycle with a significantly extended G1-period. It should be noted that the stage of the cell cycle at which anaerobically grown cells accumulate is not necessarily the stage in which the most sensitive site to deprivation of oxygen is located.

As we have shown in previous experiments, DNA synthesis seems to be one of the most sensitive sites to exclusion of oxygen [4, 15]. Incorporation of [^{14}C]thymidine and replicon initiation of DNA is reversibly blocked by anaerobiosis. When oxygen was resupplied initiation reappeared subsequently. The kinetics of restoration of a normal length distribution of the daughter chains of operating units indicated that the initiation pattern in the reaerated cells was normal [15]. However, from the present studies we must conclude, that S-phase cells are changed by extended exclusion of oxygen. This may be concluded from our recultivation experiments. S-phase cells of anaerobically pretreated cultures leave the cell cycle and form cells with a DNA content greater than 4c. The mechanisms which might govern the influence of hypoxia on the initiation of DNA-synthesis are still unclear. As was shown in previous experiments from our laboratory inhibition by 5-aza-dihydro-orotate of the pyrimidine synthesis has the same effects on DNA-, RNA-, protein synthesis and cell cycle of EAT-cells as exclusion of oxygen. This inhibitor blocks the oxygen dependent step of the UMP-synthesis, namely the dihydro-orotate-dehydrogenase. Substitution of cell cultures with 0.1 mM uridine added together with the inhibitor restores proliferation completely but is ineffective in the case of anaerobically cultured cells [16]. In the absence of oxygen, cell proliferation may be arrested by inhibition of the dihydro-orotate-dehydrogenase alone but additional impairments of cell metabolism must play an important role.

In a recent paper, Paoletti *et al.* [17] have emphasized that initiation of DNA synthesis depends upon the electron transport through the respiratory chain but not on the respiratory ATP. The authors suggest, that the shift of the cytosolic redox state by blocking of the respiratory chain might impair important biosynthetic processes which occur in the cytosol. It is assumed that the transfer of reducing equivalents from the cytosol into the mitochondria and their oxidation via the respiratory chain regulates the redox state of the cytoplasma.

Comparison of the effects of deprivation of oxygen and of different inhibitors of the respiratory chain on the proliferation kinetics of EAT cells, performed in our laboratory, revealed that growth cessation of the cells is not only caused by interference of the inhibitors with the respiratory chain but may be the result of a multitarget action [18]. We suspect that most inhibitors of respiration because of a lack of specificity are not well suited to

study relations between electron transport and cell proliferation. The elucidation of the complex effects of deprivation of oxygen on the proliferation and cellular economy of animal cells requires further investigations.

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