Energy Parameters, Macromolecular Synthesis and Cell Cycle Progression of *in vitro* Grown Ehrlich Ascites Tumor Cells after Inhibition of Oxydative ATP Synthesis by Oligomycin

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Dedicated to Prof. A. Butenandt on the Occasion of His 80th Birthday

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

1. In order to elucidate the significance of oxidative ATP production for the proliferation of Ehrlich ascites tumor cells, cell cycle progression, energy metabolism and macromolecular synthesis in the presence of oligomycin were studied.

2. In the presence of the inhibitor (20 µg/ml), lactate production and glucose uptake of the cells increased by about 30–35% as compared to controls; oxygen consumption was maximally inhibited by 30–45% and could not further be reduced by higher concentrations of the inhibitor. ATP/ADP ratios of the oligomycin treated cells and control cells were not significantly different.

3. In the first passage in the presence of oligomycin proliferation of the cells is reduced to about 50% that of controls; without severely affecting viability (dye exclusion test). In the second passage with oligomycin cell proliferation completely arrests. As was shown by flow cytometric analysis and BrdU-H33258 technique of flow cytometry, cells accumulate in the early S phase; division of cells which are in the S- and G2M compartment at the beginning of oligomycin treatment accounts for the increase of cell number in the first passage in the presence of oligomycin. On recultivation in the third passage in the absence of the inhibitor cells take up proliferation again; an increase of cell number of about 60% of controls was observed within 24 h.

4. In the presence of oligomycin incorporation of [2-^{14}C]thymidine is reduced to about 20% of the controls within 8 h, incorporation of [U-^{14}C]lysine begins to slow down immediately after treatment with the inhibitor, the same is true for the incorporation of [2-^{14}C]uridine. Transport of α-aminoisobutyric acid into the cells is not severely affected.

5. It is suggested, that not lack of energy by inhibition of oxidative phosphorylation accounts for the arrest of cell cycle progression in the presence of oligomycin but rather the blockade of transport of cytoplasmatic (glycolytic) ATP into mitochondria, which is caused by the high potential built up across the mitochondrial membrane in the presence of this inhibitor.

Growth and proliferation of most normal and malignant animal cells require the presence of oxygen [1–7]. This oxygen dependence of cell cycle progression of animal cells is not well understood. As we have shown in previous experiments, *in vitro* grown Ehrlich ascites tumor cells accumulate in the late G1 phase under exclusion of oxygen and do not enter the S-compartment [5]. An immediate decrease of DNA synthesis as measured by incorporation of [^{14}C]thymidine is observed [4, 8]; replicon initiation of DNA synthesis of the cells is rapidly but reversibly inhibited [9]. The biochemical mechanisms underlying this arrest of proliferation of the cells after deprivation of oxygen are complex. It is frequently assumed that the most important biochemical event of exclusion of oxygen is the inhibition of oxidative ATP-production. However, oxygen deficiency not only interferes with the energy metabolism of cells but also results in the interruption of all oxygen dependent anabolic and catabolic processes.

As an attempt to answer the question wether the transition of G1-phase cells into the S-compartment and initiation of DNA-synthesis depend upon oxidative ATP production, we have studied proliferation, energy metabolism and macromolecular synthesis of EAT cells in the presence of oligomycin. This inhibitor blocks oxidative ATP production without affecting electron transport and without impairing any oxygen dependent metabolic process [10, 11].

The results of our experiments demonstrate that inhibition of oxidative ATP-synthesis by oligomycin does not alter the ATP/ADP ratio of the cells as compared to controls; nevertheless cell proliferation arrests and cells accumulate in the early S-phase. Neither transition of the cells from G1 → S nor mitosis are impaired by inhibition of oxidative phosphorylation.

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Materials and Methods

All chemicals, buffer and media substances were of the purest grade available from Merck (Darmstadt) and Serva (Heidelberg). Biochemicals were from Boehringer (Mannheim) and Sigma (München). Oligomycin was a mixture of Oligomycin A, B and C = 60:25:15 from Serva (Heidelberg). Microcillin was a gift from Bayer (Leverkusen), horse serum was a gift from Behringwerke (Marburg). L-[U-^{14}C]lysine, spec. act. 340 Ci/mol, [2-^{14}C]thymidine, spec. act. 53–61 Ci/mol, [2-^{14}C]-uridin, spec. act. 60 Ci/mol, 2-[^{14}C]-α-Aminoisobutyric acid, spec. act. 51 Ci/mol were purchased from Amersham Buchler (Braunschweig).

Cells and growth techniques

Hyperdiploid Ehrlich ascites tumor cells, strain Karzel, were serially grown in the peritoneal cavity of female NMRI mice; cells for explantation to cultures *in vitro* were usually withdrawn from mice inoculated 5 days previously and transferred to modified Eagle's medium [12] supplemented with 15% horse serum, 25 mg/l streptomycinsulfat, 575 mg/l microcillin to obtain a suspension of $6-7 \times 10^7$ cells/100 ml culture medium; the nutrients of the medium were sufficient for a 24 h culture period; for further details see Karzel and Schmid [13]. No cell growth was observed on glass surfaces using culture flasks cleaned by treatment with a mixture of concentrated H_2SO_4 and HNO_3 (3:1). Growth was estimated by counting of cells in a hemocytometer or by turbidity measurements. Viability of cells was assessed by dye exclusion test with 0.1% nigrosin [14].

After 15 h cultivation at 37° under standard conditions (first passage *in vitro*) the cells were separated by centrifugation ($500 \times g$, 2 min) and transferred to fresh culture medium. The inoculum density was $3.5-4.0 \times 10^5$ cells/ml, in most cases 50 ml cultures were prepared. Oligomycin was added to the cultures in alcoholic solution (20 mg Oligomycin/ml ethanol).

Cell cycle analysis

Cell cycle distribution of the cells was analysed by flow-cytometry. About 10^6 cells were washed twice with cooled Hank's solution, fixed by suspending in 10 ml 96% ethanol and stored at −18 °C. The fixed cells were washed twice with 0.9% NaCl solution, treated with 0.1 ml (10 Kunitz units) RNase for 120 min at 37° (DNase-free, Serva Heidelberg) and after washing again with NaCl solution the nuclei were stained with 3 ml ethidium-bromide (10 mg/l, 50 m M Tris buffer, pH 7.5) and kept in the dark at 4 °C. The fluorescence of DNA bound dye was measured proportionally, stored and graphed with the flow cytometer ICP 11 according to the method of Schumann and Göhde [15]. The quantitative evaluation of the histograms to obtain the fractions of cells in the various compartments is illustrated in reference [16].

Applying the BrdU-H33258 technique [17] 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 lack of deoxycytidine in the cells by BrdU. Cells were stained with the fluorochrome Bisbenzimide H33258 (10 mg/l, 50 mM TRIS/HCl pH 7.0) after treatment with RNase (10 Kunitz units). The fluorescence was measured within 8 h after staining.

The principle of the method is based on the observation that after addition of BrdU the amount of DNA stainable with Bis-benzimid-H33258 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 [17–19].

Electron microscopy

About 10^7 cells were separated by centrifugation in Hank's solution; the pellet was suspended for 30 min at 37 °C in the fixation mixture containing 2.5% glutaraldehyd, 1.2% paraformaldehyd, 0.05% picric acid, 0.075 M sodium cacodylate, pH 7.3. Cells were kept at 4 °C overnight, washed, postfixed with 2% OsO_4 for 60 min at room temperature, dehydrated in an alcohol series and embedded in Epon. Sections were cut on an LKB ultramicrotome and double stained with uranyl magnesium acetate and lead citrate. Electron micrographs were taken with a Zeiss EM 9 microscope at 80 KV.

Measurement of metabolic parameters

L-Lactate production of the cells was assayed as described by Hohorst [20], glucose consumption was measured with the glucose oxidase/perid test from Boehringer (Mannheim); respiration was determined amperometrically with a Clark electrode [21]. The increase in protein content of cell cultures was estimated in aliquots of 1 ml, employing the Folin method [22] with cristallized bovine serum albumin (Behringwerke, Marburg) as standard, after washing the cells three times with Hank's solution.

DNA content of cell cultures was determined fluorometrically in 1 ml cell suspension according to Labarca and Paigen [23]. The cells were incubated in 3 ml buffer (0.05 M phosphate, 2 M NaCl, pH 7.4) containing 1 mg/l of the fluorochrome Bisbenzimide H33258 (Riedel de Haen, Hannover) for 30 min in the dark. Fluorescence was measured at 430 nm, excitation wavelength 360 nm. Calibration curves were obtained with calf thymus DNA (1 – 15 µg/ml).

The relative rates of DNA- RNA- and protein-synthesis were measured by incorporation of [2-^{14}C]-thymidine, [2-^{14}C]uridine and L-[U-^{14}C]lysin into acid insoluble material. Aliquots of 1 ml of cell cultures were withdrawn at appropriate intervals from the cultures and incubated for 15 min at 37° with 0.1 µCi of the labelled precursors. After being washed twice with ice cold Hank's balanced salt solution, the cells were suspended in 5% trichloroacetic acid, collected on a membrane filter (Gelman 0.45 µm), dried and counted in a TRI CARB 300 C using Rotiszint 11.

Rates of uptake of amino acids were measured in the appropriate intervals by incubation of the cells with [2-^{14}C]-α-aminoisobutyric acid and determination of radioactivity in the acid soluble fraction. 0.2 µCi of the amino acid were incubated with 1 ml cell suspension for 10 min at 37 °C. The uptake of radioactivity was measured in 0.5 ml supernatant after precipitation of the cells with trichloro acetic acid.

Determination of ATP and ADP

ATP and ADP levels of the cells were assayed by applying the luciferin/luciferase system [24] using a Bioluminat 9500 (Bertholt). 0.1 ml cell suspension was added to 0.9 ml of redistilled water at 95° and heated for 3 min in a boiling water bath. ATP was measured in 20 µl aliquots. ADP was estimated by difference after pretreatment of 200 µl aliquots of each sample with pyruvate kinase/phosphoenolpyruvate to convert ADP to ATP.

Results

Viability and cell proliferation in the presence of oligomycin

Increasing concentrations of oligomycin in the range of 0.05 – 50 µg/ml culture medium had no effect on the viability of *in vitro* grown EAT cells over a period of 24 h as assayed by dye exclusion test: the number of nigrosin positive cells did not exceed that of controls and was maximal 2% on an average. For all further experiments a concentration of 20 µg/ml of the inhibitor was chosen. With this concentration a maximal inhibition of respiration was achieved (see below).

Proliferation of the cells in the presence of oligomycin within 24 h is compared with the growth of controls in Fig. 1 A. Growth of the controls within 24 h was 81.8 ± 12.9% (mean of 18 cultures) in the presence of oligomycin proliferation of the cells was reduced to 39.2 ± 8.6% (mean of 52 cultures). In good agreement with the growth of the cultures of 50% of the controls is the increase of DNA as is demonstrated in Fig. 1 B. The corresponding curve for protein is depicted in Fig. 1 C. The protein content of the cells is not significantly affected by treatment with oligomycin: Within 24 h the protein per 10^6 cells increased from 249 ± 35 µg to 306 ± 32 µg (mean of 42 cultures), control values were 249 ± 35 and 315 ± 22.6 µg, respectively. No unbalanced growth could be detected in the presence of the inhibitor.

Cell cycle distribution

Cell cycle distribution of oligomycin treated cells in the second passage is compared to the phase composition of control cultures in the DNA histograms of Fig. 2. The most prominent feature of the histograms of the oligomycin treated cells is the clearance of the G2 compartment and accumulation of the cells in the early S-phase. At the beginning of this typical experiment cell cycle distribution was 33% G1-, 55% S- and 12% G2M-cells; 16 h later the control cultures contained 11% G1-, 58% S- and 31% G2M-cells, while the phase composition of the

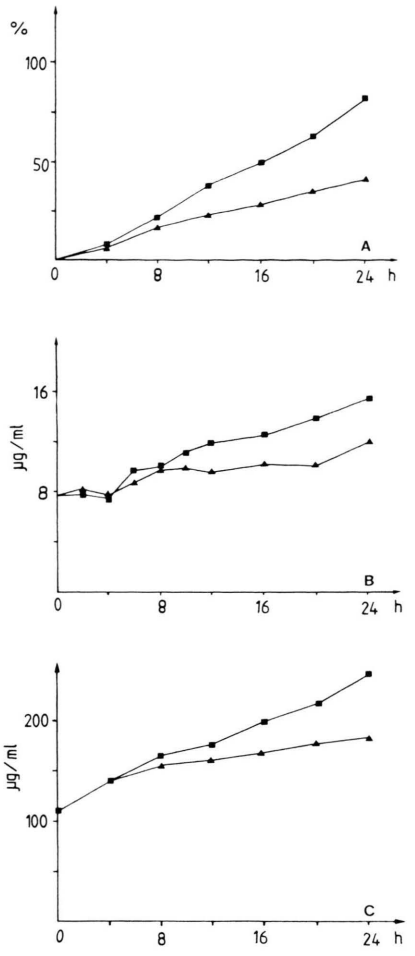

Fig. 1. A, Cell growth $(dN/dt) \cdot (100/N)$; B, increase in DNA; C increase in protein. ▲——▲ in the presence of 20 µg/ml oligomycin, ■——■ of controls.

oligomycin treated cultures was 20% G1-, 70% S- and 10% G2M-cells. In the second passage in the presence of oligomycin (third passage *in vitro*) cell proliferation completely arrests; incorporation of [^{14}C]thymidine dropped to a constant value of 0.82 nmol/10^6 cells incorporation rates of controls after 12 h were 3.41 nmol/10^6 cells.

Further informations on the cycling of the G1-, S- and G2M-cells in the presence of oligomycin were obtained by applying the BrdU-H33258 technique. DNA-histograms grown in the presence of BrdU and in the presence of BrdU plus oligomycin together with histograms of control cells are shown in Fig. 3. From these histograms the following conclusions may be drawn: G1-cells do not divide in the presence of oligomycin within 32 h, because no signals are observed at 1 C = G 1/2 on the left side of the G1 peak in the BrdU histogram series 3C. Under normal conditions the cells pass the S- and G2M compartment within 14 ± 1 h [5], which implies that after this time period fluorescence signals at G 1/2 = 1 C appear. Late S-cells pass the cell cycle in the presence of oligomycin which may be concluded from the appearance of a shoulder on the left side of the G1-peak (48 h, 3C). Cells which are in the G2M-phase at the beginning of the treatment with oligomycin divide and enter G1; the G2M-peak completely disappears within 8 h. Accumulation of the oligomycin treated cells in the early S-phase becomes particularly evident from the DNA histogram 3B, 48 h.

In further experiments the growth of oligomycin treated cells in the 3rd passage in the absence of the inhibitor was checked. An increase of cell number of about 80% within 24 h was observed in control cultures, while the cells pretreated with oligomycin in the 2. passage increased by about 55% (see Fig. 4). The protein content of the cells pretreated with oligomycin did not change as compared to the controls. These results demonstrate that the effects of oligomycin on intact cells are fairly reversible.

DNA-, RNA- and proteinsynthesis

The incorporation of [2-^{14}C]thymidine into acid insoluble material of control cells and oligomycin treated cells is shown in Fig. 4A. A typical incorporation pattern with a maximum at about 8 h was obtained under normal conditions. As is illustrated in Fig. 4A in the presence of the inhibitor, the incorporation of [2-^{14}C]thymidine into DNA decreases to about 25% of the controls within 8 h and remains constant up to 24 h. This rate of DNA synthesis of the cells in the presence of oligomycin is compatible with the increase of DNA (see Fig. 1), and the feature of the DNA-histograms (Fig 2B and 3B). The results of incorporation studies with [2-^{14}C]uridine are depicted in Fig. 4B. RNA-synthesis seems to be rapidly and powerful impaired by oligomycin. This is in accordance with results from Pachenko [25] and Stellestkaya [26], who have shown that oligomycin is a strong inhibitor of the r-RNA-synthesis but has no direct effect on RNA-polymerase activity.

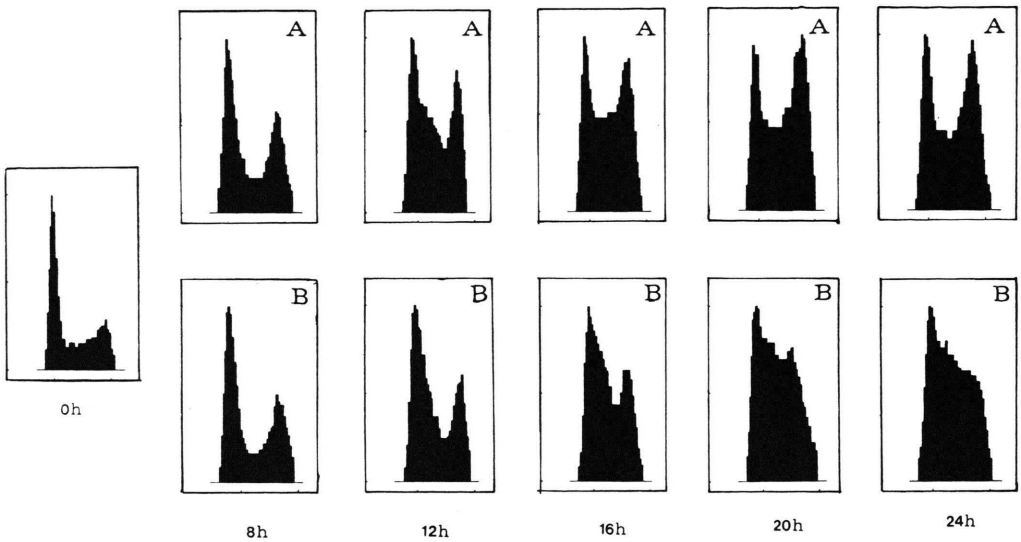

Fig. 2. DNA histograms of EAT cells in the second passage. Cells were stained with Ethidiumbromid. A, control cultures; B, oligomycin treated cells. The first peak: 2C DNA = G1 cells, the second peak: 4C DNA = G2M cells, in between are S cells.

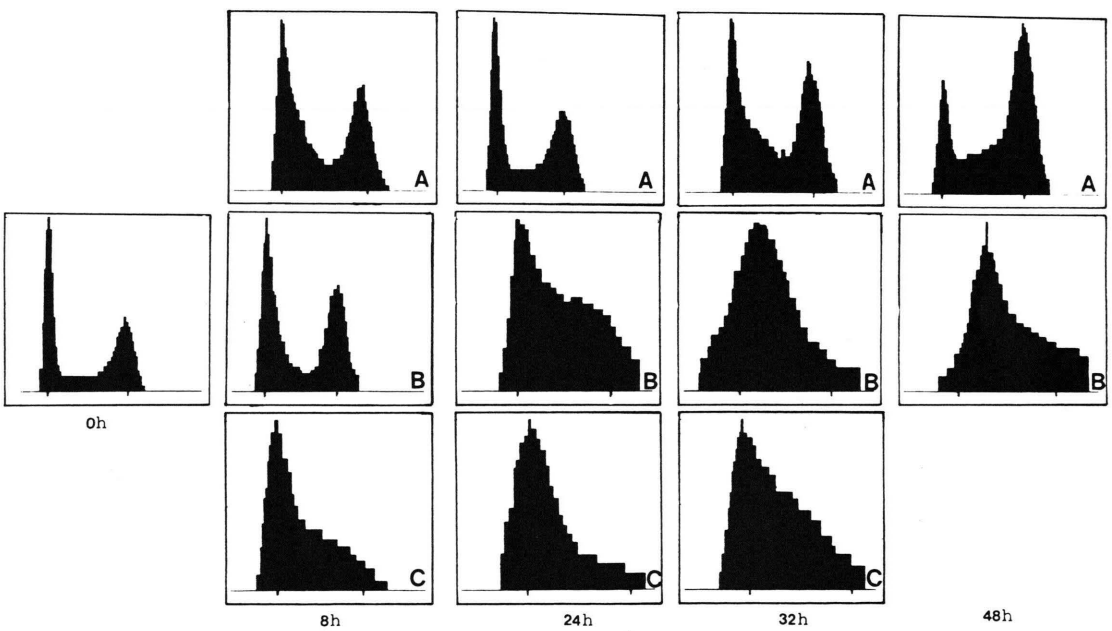

Fig. 3. DNA histograms of EAT cells stained with Bisbenzimide-H33258; A, controls; B, cells grown in the presence of oligomycin; C, cells grown in the presence of oligomycin plus BrdU; for further illustrations see text.

A close correlation between cell growth and protein synthesis was found in incorporation experiments with [^{14}C]lysine. Incorporation of radioactivity from [^{14}C]lysine into the acid insoluble precipitate slows down continuously on treatment of the cells with oligomycin (Fig. 4C). The transport of α-aminoisobutyric acid was not significantly affected by the inhibitor (data not shown). An inhibition of the uptake of the amino acid in the presence of oligomycin was observed by Hempling [27], however, with nongrowing cells in buffer solution.

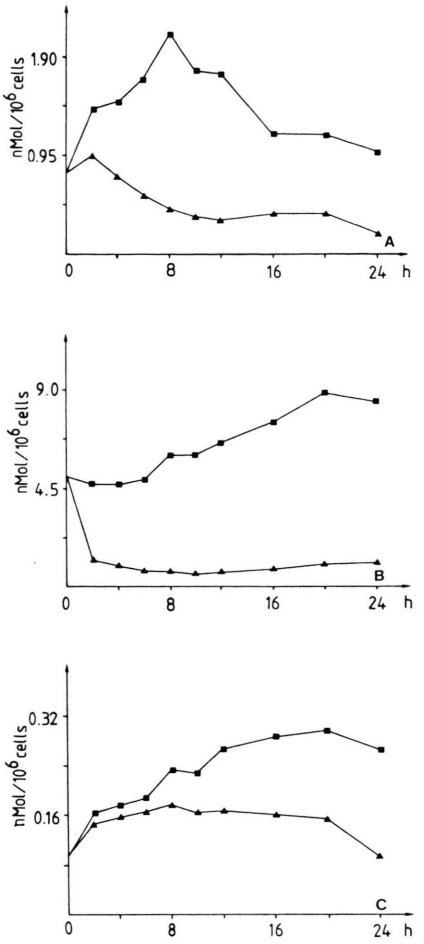

Fig. 4. Incorporation of A, [2-^{14}C]thymidine; B, [2-^{14}C]uridine and C, [U-^{14}C]lysine into acid insoluble precipitate of EAT-cells. ▲——▲ in the presence of oligomycin, ■——■ controls.

Energy metabolism

Inhibition of oxidative ATP production by oligomycin causes an increase of lactate production by about 35% (Controls 0.744 µmol/10^6 cells × h, oligomycin treated cells 0.992 µmol/10^6 cells × h means over a period of 24 h). Under the same conditions the glucose consumption of the controls within 24 h was found to be 0.545 µmol/10^6 cells × h, in the presence of oligomycin it was 0.673 µmol/10^6 cells × h, that is about 136% of the controls.

At oligomycin concentrations of 5 µg/ml a maximal reduction of oxygen consumption of the cells of about 40% was observed; this inhibition of respiration could not be significantly enhanced by higher concentrations (see Fig. 5). Since oligomycin easily passes the plasma membrane of mammalian cells [28] and complete inhibition of the F_1-ATPase of isolated mitochondria was obtained with 2–5 µg/ml of the inhibitor [10], it is suggested that in the presence of 20 µg/ml no ATP is synthesized via oxidative phosphorylation. This is confirmed by experiments with glucose free cultures (see below). Between 4–20 h the oxygen uptake of the control cells was 216 ± 15 nmol O_2/10^6 cells × h; it was reduced maximally to 120 ± 9 nmol/10^6 cells × h. The oxygen consumption of controls and of cells in the presence of the inhibitor over a period of 24 h is summarized in Table I. In contrast to uncouplers of oxidative phosphorylation like DNP, which stimulates respiration, inhibition of ATP synthesis by oligomycin reduces the oxygen consumption. The remaining respiration first of all sustains ATP independent transport processes across the mitochondrial membrane.

ATP- and ADP-levels of controls and of oligomycin treated cells are also depicted in Table I. 12 h after starting the experiments no differences between the ATP content of controls and oligomycin treated cells could be observed. Since chemical energy is always a matter of concentration ratios, a more reliable parameter of the energetic status is the ATP/ADP ratio (see Table I). Between 4 and 24 h an average ratio of about 4.84 ± 1.3 was found in the absence and presence of oligomycin. The impairment of proliferation activity of the cells by oligomycin seems therefore not to be a consequence of a general lack of energy; other mechanisms must be taken into consideration.

Proliferation activity of the cells in oligomycin containing, glucose deprived medium is illustrated

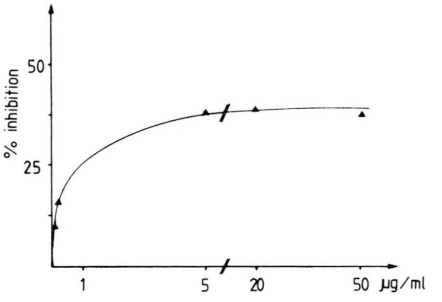

Fig. 5. Relation between oligomycin concentration in the culture medium and inhibition of oxygen consumption of the cells in percent.

Table I. ATP-, ADP-levels, ATP/ADP-ratios and oxygen consumption of *in vitro* grown EAT-cells in the absence and presence of oligomycin.

Time [h]	ATP nmol/10^6 cells		ADP nmol/10^6 cells		ATP/ADP		O_2-uptake nmol/10^6 cell s×h	
	controls	oligomycin	controls	oligomycin	controls	oligomycin	controls	oligomycin
0	8.75 ± 1.35	8.75 ± 1.35	2.05 ± 0.36	2.05 ± 0.36	3.65 ± 1.40	3.65 ± 1.40	173 ± 19	173 ± 19
4	14.12 ± 2.71	11.48 ± 1.47	2.69 ± 0.51	2.23 ± 0.57	5.37 ± 1.19	5.76 ± 1.98	197 ± 23	127 ± 15
8	15.57 ± 2.38	10.38 ± 2.03	4.39 ± 0.28	3.07 ± 0.29	5.64 ± 0.77	4.82 ± 0.98	200 ± 14	149 ± 11
12	11.00 ± 1.78	11.35 ± 1.93	5.60 ± 0.67	3.18 ± 0.61	4.16 ± 0.55	4.45 ± 1.29	218 ± 17	170 ± 13
16	12.34 ± 1.43	12.31 ± 2.57	4.92 ± 0.63	3.26 ± 0.52	4.41 ± 0.61	4.90 ± 1.39	215 ± 17	133 ± 19
20	12.02 ± 2.53	12.19 ± 2.61	4.07 ± 0.38	3.32 ± 0.31	4.48 ± 0.90	4.84 ± 1.13	165 ± 11	117 ± 9
24	10.49 ± 1.81	10.20 ± 1.71	2.23 ± 0.21	2.57 ± 0.19	5.60 ± 1.25	5.45 ± 0.96	169 ± 19	97 ± 12

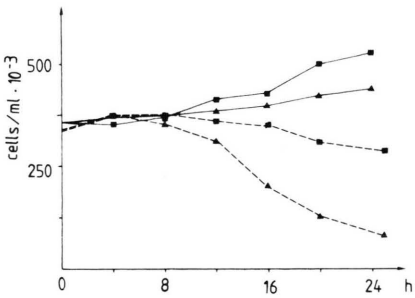

Fig. 6. Cell growth of EAT cells in normal medium ■—■; in the presence of 20 µg/ml oligomycin ▲—▲; in glucose deprived medium ■----■; in glucose deprived medium + oligomycin ▲----▲.

in Fig. 6. 4 h after starting the experiment cell number begins to slow down and 20 h later a decrease of cells of about 75% was measured; most of these cells had lost their viability as a result of complete blockade of energy metabolism.

Electron microscopic studies

Electron microscopic photographs of cells treated with 20 µg/ml oligomycin show a high amplitude swelling of all mitochondria with a translucent appearance of the inner compartment, however, the mitochondrial membranes remain completely intact; even after treatment with oligomycin for 24 h no damage could be observed Fig. 7. The enlarged mitochondrial profiles could result not only from swelling but also from fusion of individual mitochondria as described for other inhibitors of mitochondrial functions [29].

Concluding remarks

In the present experiments we have investigated the role of oxidative ATP synthesis for a regular cell cycle progression of *in vitro* grown Ehrlich ascites tumor cells using oligomycin as a tool to block oxidative phosphorylation. Oligomycin specifically inhibits proton-transport into mitochondria [11], which is coupled to the synthesis of ATP. In contrast to uncouplers of oxidative phosphorylation like DNP, which dissipates the proton gradient, 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} [30]. ATP independent transport processes between cytosol and mitochondrial matrix are however maintained by the high chemi-osmotic potential across the membrane. The oxygen consumption of the cells reveals the bulk of energy required for these processes in the presence of oligomycin. The impairment of energy supply of intramitochondrial ATP dependent processes for the growth and metabolism of the cells seems to be most important.

Of particular interest is the inhibition by oligomycin of cell cycle progression and accumulation of the cells in the early S-phase. Since the ATP/ADP ratio is not severely affected and oligomycin has – as far as we known – no other effects on cellular metabolism than inhibition of oxidative phosphorylation (only a hundredfold of the concentration chosen in the present experiments causes inhibition of Na^+, K^+-ATPase of the plasma membrane [31, 32]) we suggest, that impairment of mitochondrial macromolecular synthesis as a result of lack of energy supply is the main reason for the arrest of cell growth. First of all we think of protein synthesis, because it was recently shown [33] that ATP synthesized via oxidative phosphorylation, not GTP synthesized from substrate phosphorylation, limits the rate of mitochondrial protein synthesis. The

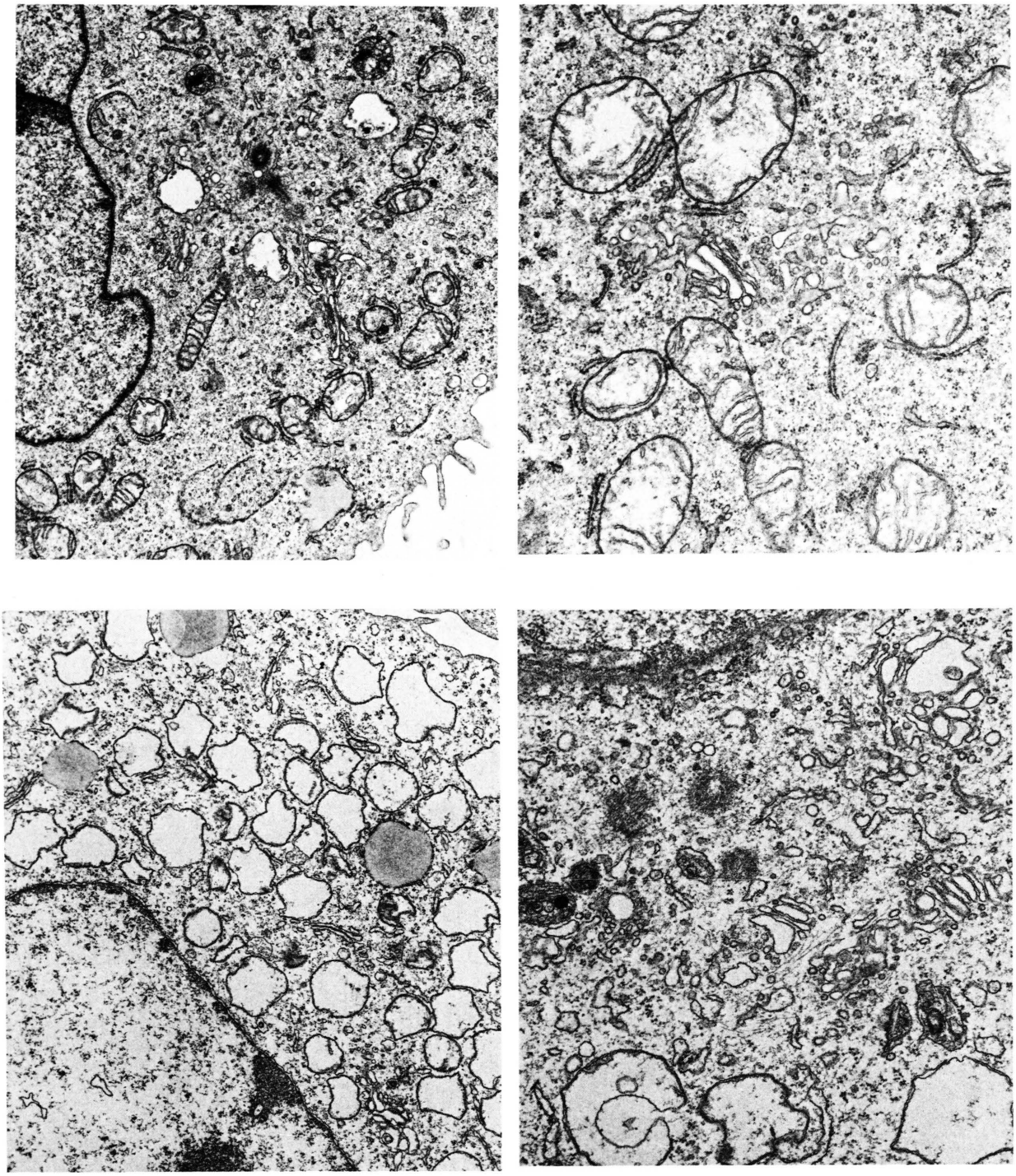

Fig. 7. Electron micrographs of EAT cells. A, control cells after 15 h in the second passage *in vitro* × 4400 (left) × 8000 (right); B, cells treated for 15 h with 20 µg/ml of oligomycin × 4400 (left) × 8000 right.

significance of mitochondrial protein synthesis for regular cell cycle progression can be tested by applying a specific inhibitor of mitochondrial protein synthesis *e.g.* chloramphenicol, which is presently under investigation.

From our experiments we may further conclude that cells which are in the S- and G2-compartment at the beginning of cultivation in the presence of oligomycin divide and are responsible for the increase of cell number in the first 24 h. The transition from S → G2 and mitosis obviously do not depend on the ATP from respiratory chain.

Acknowledgements

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[1] A. K. Balin, D. B. Goodman, H. Rasmussen, and V. J. Christophalo, J. Cell Physiol. **89,** 235–250 (1976).
[2] R. Born, O. Hug, and K. R. Trott, Int. J. Radiation Oncology Biol. Phys. **1,** 687–697 (1976).
[3] W. S. Bullough and M. Johnson, Proc. Roy. Soc. London B, **138,** 562–572 (1951).
[4] H. P. Krause and Fr. Schneider, Hoppe-Seyler's Z. Physiol. Chem. **355,** 1335–1340 (1974).
[5] R. Merz and Fr. Schneider, Z. Naturforsch. **37 c,** 326–334 (1982).
[6] R. Merz and Fr. Schneider, Z. Naturforsch. **38 c,** 313–318 (1983).
[7] R. W. Brosemer and W. J. Rutter, Exp. Cell Res. **25,** 101–113 (1961)
[8] M. Löffler, Eur. J. Biochem. **107,** 207–215 (1980).
[9] H. Probst and V. Gekeler, Biochem. Biophys. Res. Comm. **94,** 55–60 (1980).
[10] H. A. Lardy, D. Johnson, and W. C. McMurray, Arch. Biochem. Biophys. **78,** 587–597 (1958).
[11] E. C. Slater and H. F. Ter Welle in "Inhibitors Tools in Cell Research". 20. Colloquium d. Ges. Biol. Chem. 1969, Mosbach Baden pp. 258–278, Springer Verlag, Berlin 1969.
[12] R. Schindler, M. Day, and G. A. Fischer, Cancer Res. **19,** 47–51 (1959).
[13] K. Karzel and G. Schmid, Drug Res. **18,** 1500–1504 (1968).
[14] J. P. Kaltenbach, M. H. Kaltenbach, and W. B. Lyons, Exp. Cell Res. **15,** 112–117 (1958).
[15] J. Schuman and W. Göhde, Strahlentherapie **147,** 298–307 (1974).
[16] R. Merz, Thesis Marburg 1980.
[17] R. M. Böhmer, Cell Tissue Kinetics **12,** 101–110 (1979).
[18] H. P. Beck, Cell Tissue Kinetics **14,** 163–177 (1981).
[19] R. M. Böhmer and J. Ellwart, Cytometry **2,** 31–34 (1981).
[20] H. J. Hohorst, Methoden der enzymatischen Analyse (U. Bergmeyer, ed.), pp. 1425–1429 Verlag Chemie, Weinheim 1970.
[21] L. V. Clark, R. Wolf, D. Granger, and Z. Taylor, J. Appl. Physiol. **6,** 189–193 (1953).
[22] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. **193,** 265–275 (1951).
[23] C. Labarca and K. Paigen, Anal. Biochem. **102,** 344–352 (1980).
[24] M. DeLuca, Advances Enzymol. **44,** 37–68 (1959).
[25] L. F. Panchenko, Biochim. Biophys. Acta **299,** 103–113 (1973).
[26] N. V. Stellenstkaya, T. V. Sirota, L. F. Pachenko, and N. G. Shuppe, Biokhimiya **38,** 1065–1072 (1973).
[27] H. G. Hempling, Biochim. Biophys. Acta **112,** 503–518 (1966).
[28] R. Johnston, S. Scharf, and R. S. Criddle, Biochem. Biophys. Res. Comm. **77,** 1361–1368 (1977).
[29] M. Albring, K. Radsak, and W. Thoenes, Virchow's Arch. B. Cell Path. **14,** 373–377 (1974).
[30] R. Krämer and M. Klingenberg, in "Function and Molecular Aspects of Biomembrane Transport" ed. E. Quagliariello, pp. 515–518, Elsevier 1979.
[31] F. F. Jobis and M. J. Vreman, Biochim. Biophys. Acta **73,** 346–348 (1963).
[32] H. E. van Groningen and E. C. Slater, Biochim. Biophys. Acta **73,** 527–530 (1963).
[33] A. Mutvei and B. D. Nelson, Biochem. Biophys. Res. Comm. **105,** 916–921 (1982).