Dose- and Time-dependent Effects of Actinomycin D on *Tetrahymena*

With Special Reference to Nucleolar Changes

By JYTTE R. NILSSON

Det Kongelige Danske Videnskabernes Selskab
Biologiske Skrifter **24** : 2

Kommissionær: Munksgaard
København 1985

Det Kongelige Danske Videnskabernes Selskab
udgiver følgende publikationsrækker:

The Royal Danish Academy of Sciences and Letters
issues the following series of publications:

	Bibliographical Abbreviation
Oversigt over Selskabets Virksomhed (8°) *(Annual in Danish)*	Overs. Dan. Vid. Selsk.
Historisk-filosofiske Meddelelser (8°)	Hist. Filos. Medd. Dan. Vid. Selsk.
Historisk-filosofiske Skrifter (4°) *(History, Philology, Philosophy, Archaeology, Art History)*	Hist. Filos. Skr. Dan. Vid. Selsk.
Matematisk-fysiske Meddelelser (8°) *(Mathematics, Physics, Chemistry, Astronomy, Geology)*	Mat. Fys. Medd. Dan. Vid. Selsk.
Biologiske Skrifter (4°) *(Botany, Zoology, General Biology)*	Biol. Skr. Dan. Vid. Selsk.

Selskabets sekretariat og postadresse
The address of the Academy is:

Det Kongelige Danske Videnskabernes Selskab,
H. C. Andersens Boulevard 35
DK-1553 Copenhagen V.
Denmark.

Selskabets kommissionær
The publications are sold by the agent of the Academy:

Munksgaard Eksport- og Tidsskriftsservice/
Munksgaard Export and Subscription Service,
35, Nørre Søgade,
DK-1370 Copenhagen K,
Denmark.

Dose- and Time-dependent Effects of Actinomycin D on *Tetrahymena*

With Special Reference to Nucleolar Changes

By JYTTE R. NILSSON

Det Kongelige Danske Videnskabernes Selskab
Biologiske Skrifter **24**:2

Kommissionær: Munksgaard
København 1985

Contents

Synopsis

Dose- and time-dependent effects of Actinomycin D (AMD), 5–50 μg/ml, were observed on physiological parameters and the fine structure of *Tetrahymena* during a 6-hour exposure.

Dose-dependent effects were seen after a 1-hour exposure as: 1) Changed rate of endocytosis in concentrations above 30 μg AMD/ml. 2) Progressive nucleolar changes involving segregation of the granular and fibrous components, as well as aggregation of the fibrous components apparently through sliding along the nuclear envolope. 3) Presence of 2–3 different nucleolar stages (out of 7 defined stages) in cells at any one dose of AMD. 4) Changed rates of RNA (drastically) and protein (moderately) synthesis (10–40 μg AMD/ml). The number of cells engaged in DNA synthesis and the cell content of ATP remained unaltered on exposure to the drug. *Time-dependent effects* were seen after a 5–6 hour exposure as: A) Cessation of cell proliferation at any one dose of AMD. B) Additional nucleolar aggregation at low AMD doses. C) Formation of large spherical nucleolar bodies of varying electron density especially at the high dose of AMD. D) Appearance of intranuclear rods of a microtubular substructure in some cells. E) Prevention of structural changes in peroxisomes and to some extent in mitochondria. F) Decreased cell content of ATP in concentrations above 10 μg AMD/ml. Prolonged exposures to the drug resulted in spontaneous recovery of cell proliferation in up to 30 μg AMD/ml. The results are discussed in relation to previous reports on the effect of AMD on this ciliate.

JYTTE R. NILSSON
Institute of Cell Biology and Anatomy
Zoological Institutes
University of Copenhagen
Universitetsparken 15
DK-2100 Copenhagen Ø, Denmark

Keywords:
Actinomycin D, nucleolar stages, endocytosis, ATP content, RNA synthesis, protein synthesis, *Tetrahymena pyriformis* GL

Printed in Danmark by Bianco Lunos Bogtrykkeri A/S
ISSN 0366-3612 . ISBN 87-7304-155-6

Introduction

Actinomycin D (AMD) is an antimetabolite inhibiting DNA-dependent RNA synthesis by tight, but reversible, binding to the narrow groove of the DNA molecule (*Hyman* and *Davidson* 1970, *Goldberg* and *Freedman* 1971). Transcription of the various classes of RNA is inhibited in a dose-dependent manner by AMD in mammalian cells with that of ribosomal RNA (rRNA) being most sensitive (*Goldberg* and *Freedman* 1971, *Perry* and *Kelley* 1970), hence low doses of AMD are used routinely in biochemical studies to exclude rRNA synthesis.

The drug also induces fine structural changes in nucleoli in a variety of cell types (*Stevens* 1964, *Simard* and *Bernhard* 1966, *Goldblatt et al.* 1970, *Phillips* and *Phillips* 1971a, 1971b, *Lane* 1969, *Scheer et al.* 1975, *Recher et al.* 1976, *Chaly et al.* 1979, *Jordan* and *McGovern* 1981), including *Tetrahymena* as observed after a 5-hour exposure to either 10 μg AMD/ml (*Satir* and *Dirksen* 1971) or 50 μg AMD/ml (*Eckert* and *Franke* 1975). The most prominent feature of the AMD-induced nucleolar changes is the segregation of the granular and fibrous components, the structural reflection of the inhibited rRNA synthesis.

The purpose of the present investigation has been to study more closely the effects of AMD on the ciliate *Tetrahymena* using a range of doses (5–50 μg AMD/ml) and with special emphasis on a short exposure, corresponding to 1/3 of the generation time, but also to some extent on longer exposures corresponding to 2 normal cell generations. The AMD-treated cells were examined for their capacity to form food vacuoles, their rates of DNA, RNA and protein synthesis, and their content of ATP, in addition to changes in their fine structure, especially those of the nucleolar organization. Both dose- and time-dependent effects of the drug have been found.

Material and methods

Tetrahymena pyriformis GL (*Nanney* and *McCoy* 1976) was grown axenically at 28°C in 2% proteose peptone (Difco) enriched with 0.1% yeast extract (Difco) and inorganic salts (*Plesner et al.* 1964). The 100-ml cultures were shaken and aerated. The cells were used in the exponentially multiplying growth phase where the generation time is 170 min. For determination of the cell density, cell samples were fixed in an equal volume of 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer, and after 10 minutes, the sample was diluted with 0.45% NaCl before counting in a particle counter, Coulter Counter model ZB. In each experiment, 1–2 100-ml cultures were subdivided into equal volumes which received different concentrations of the test compound or served as control samples.

Actinomycin D (AMD; Sigma) was dissolved in a mixture of 87% glycerol and redistilled water (1:1 v/v) because of its low solubility in water; stock solutions of 10 mg AMD/ml were used. The cells were exposed to 5–50 μg AMD/ml, and for each concentration 2 control samples were made, one consisting of untreated control cells and another containing an equivalent volume of the glycerol/water mixture.

For determination of the rate of endocytosis, the cell samples were exposed for 10 minutes to an equal volume of carmine particles (4 mg/10 ml) suspended in culture medium containing the appropriate amount of test compound. After exposure to the carmine suspension, the cells were fixed as described above and the number of labelled food vacuoles was counted in 100 cells per sample, as described previously (*Nilsson* 1972). The capacity of the cells to form food vacuoles was expressed as a percentage of that of the control cells.

The cellular content of ATP was determined by the bioluminescent firefly luciferase assay for ATP

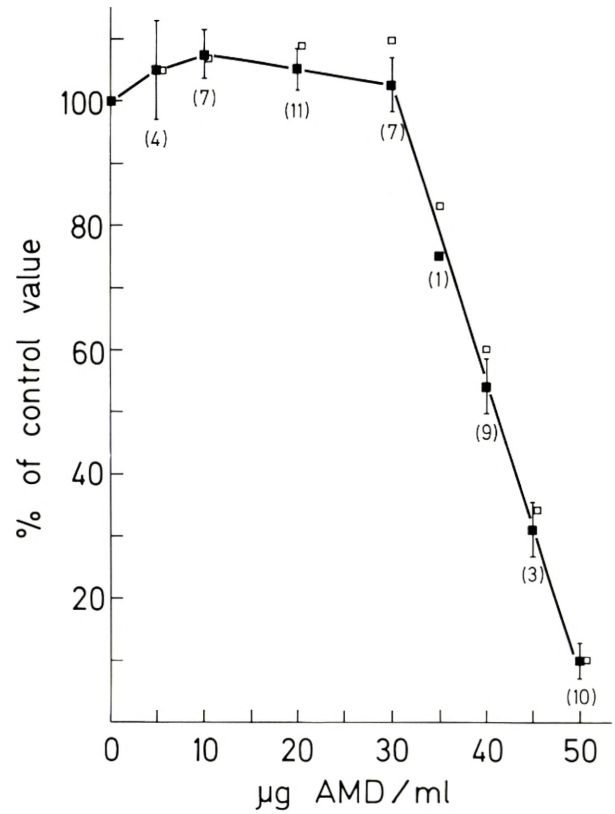


Fig. 1. The effect of actinomycin D (1-hour exposure) on the rate of endocytosis in *Tetrahymena* as determined by a 10-minute uptake of carmine particles. The data are expressed as percent of the corresponding glycerol-treated control cells (drawn curve) and of untreated control cells (open symbols). The number of experiments is indicated in brackets and the standard deviation of the mean is indicated by vertical bars.

(*Myhrman et al.* 1968) adapted for *Tetrahymena (Nilsson* 1980). The different doses of AMD were tested for inhibition of the output of the bioluminescence using standing amounts of ATP but no interference was observed.

For electron microscopy, the cell samples were mixed with equal volumes of 4% glutaraldehyde in 0.1 M cacodylate buffer. After a 10-minute fixation, the cells were washed in cacodylate buffer and treated for 1 hour with 1% osmium tetroxide in the same buffer. The material was then washed and dehydrated through a graded series of ethanols and finally in propylene oxide before embedding in Epon. The sectioned material was contrasted for 20 minutes in zinc-uranyl acetate (*Weinstein et al.* 1963) and for 3 minutes in lead citrate (*Venable* and *Coggeshall* 1965) before examination in a Zeiss EM9 electron microscope. Micrographs were taken at primary magnification between 1,800 and 20,000 times.

For determination of the rate of DNA, RNA, and protein synthesis, AMD-treated cells (10, 20, 30, and 40 μg AMD/ml), the corresponding glycerol-treated control cells, and an untreated control sample were exposed for 10 minutes (one hour after addition of AMD) to tritiated thymidine or uridine (Amersham Radiochemical Centre, TRA 120 and TRA 178) and for 20 minutes (50 minutes after addition of AMD) to tritiated proline (TRK 323). In all instances, the isotopes were added at a concentration of 20 μCi/ml. After labelling, the cells were fixed for electron microscopy as described above. The sectioned material was mounted on carbon-coated colloidin films, contrasted with zinc-uranyl acetate only, and coated with a thin layer of carbon before applying Ilford L-4 emulsion by the loop method (*Caro* 1969) as described previously (*Nilsson* 1980). After an 8-week exposure, the autoradiograms were developed in Microdol X and contrasted with citrate before examination in the electron microscope. The rate of incorporation of labelled material was determined semi-quantitatively by counting the number of developed silver grains above measured nuclear and cytoplasmic areas (using a planimeter) in micrographs of at least 10 cells per sample; the results were expressed as the number of silver grains per 10 μm^2. The data in Fig. 3 represent the sum of the mean values for the nuclear and cytoplasmic labelling.

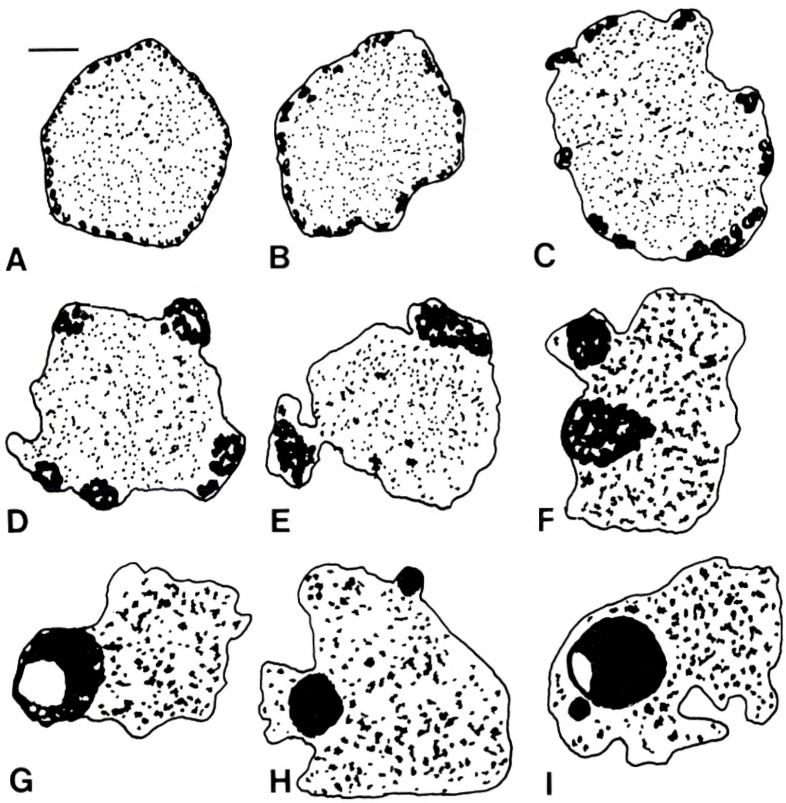


Fig. 2. Diagrams of dose- and time-dependent effects of Actinomycin D (AMD) on the *Tetrahymena* macronucleus after a 1-hour (B–G) and a 5-hour (H,I) exposure, respectively. *A:* Numerous small, unit-sized nucleoli evenly distributed along the nuclear envelope *(STAGE 1)* prior to addition of AMD. *B:* Slightly enlarged nucleoli (aggregation of 2–3 small nucleoli) evenly distributed along nuclear envelope *(STAGE 2);* 10 μg AMD/ml. *C:* Small nucleolar aggregates in uneven distribution along nuclear envelope *(STAGE 3);* 20 μg AMD/ml. *D:* Enlarged nucleolar aggregates in outpockettings of nucleus *(STAGE 4);* 30 μg AMD/ml. *E:* Irregularly shaped entangled nucleolar aggregates *(STAGE 5);* 40 μg AMD/ml. *F:* Enlarged irregular nucleolar aggregates with numerous "islets" of low electron density *(STAGE 6);* 50 μg AMD/ml. *G:* Large spherical nucleolar fusion body with large "nucleolar vacuole" *(STAGE 7);* 50 μg AMD/ml. *H:* Large compact, spherical nucleolar fusion bodies (compare with *C*); 20 μg AMD/ml. *I:* Large compact, spherical nucleolar fusion body with well-defined "nucleolar vacuole" (compare with *F* and *G*); 50 μg AMD/ml. Note the progressive condensation of chromatin from small to large granules during the 1-hour exposure *(A-G)*. For fine structure of nucleolar changes see Plate I, *1–7* and Plate II, *1–4*. Drawn from electron micrographs. Bar indicates 2 μm.

Results

Exponentially multiplying cultures of *Tetrahymena* were exposed to Actinomycin D (AMD) in concentrations ranging from 5–50 μg/ml; for each concentration, 2 control samples (untreated and glycerol-treated cells) were examined. The cells were observed after a 1-hour and a 6-hour exposure to the drug, corresponding to a 1/3 and 2 cell generations, respectively; the two sets of data will be described separately.

Short-time exposure to AMD

The effect of AMD on endocytosis after a 1-hour exposure is shown in Fig. 1. Below 30 μg AMD/ml, the rate of endocytosis is unaffected, whereas a dose-dependent inhibition is seen above this value. The solvent for AMD, glycerol, has a slight stimulatory effect on endocytosis as indicated in the figure by comparison of the data to those of untreated control cells (open symbols). *In vivo* observation of AMD-treated cells revealed the presence of small refractile granules and nucleolar aggregation; these feature were not observed in any of the control cells.

The fine structure of AMD-treated cells was affected by all concentrations of the drug in a dose-dependent manner; most conspicuous were the nucleolar changes. The overall nuclear changes are indicated in Fig. 2, where a control nucleus (Fig. 2, A) is compared to representative nuclei of cells exposed for 1 hour to 10 μg AMD/ml (Fig. 2, B), 20

μg AMD/ml (Fig. 2, C), 30 μg AMD/ml (Fig. 2, D), 40 μg AMD/ml (Fig. 2, E), and 50 μg AMD/ml (Fig. 2, F,G). A progressive aggregation of nucleoli is seen with increasing dose of AMD, although some variation occurs within single samples (Fig. 2, F,G); moreover, a notable chromatin condensation occurs concomitantly. The nucleolar changes involved loss of the granular components and gradual fusion of the remaining fibrous components (Plate I, *2–7*) of the small, unit-sized nucleoli typical of exponentially multiplying *Tetrahymena* (Plate I, *1*). Interestingly, the close association of the small nucleoli with the nuclear envelope (Plate I, *1*) is largely maintained by the nucleolar aggregates in the drug-treated cells (e.g. Plate I, *7*); furthermore, the nucleolar organizers (arrows in Plate I, *1–6*) become distinct in drug-treated cells, apparently due to condensation. At high doses of AMD (> 30 μg/ml), most of the granular nucleolar component was lost during the 1-hour exposure (Plate I, *5–7*), whereas the nucleoli of glycerol-treated control cells remained unchanged (Plate I, *1*).

The degree of nucleolar changes varied among cells at any one AMD concentration, as mentioned above, thus not all cells are affected to the same extent by the drug. In order to analyse this feature, 7 stages were defined to characterize the nucleolar organization: *Stage 1:* Small (0.3 μm) unit-sized nucleoli (Plate I, *1*) typical of fast growing cells; they are predominantly granular in substructure and distributed evenly along the inner side of the nuclear envelope (Fig. 2, A). *Stage 2:* Groups of 2–3 unit-sized nucleoli, mainly fibrous in substructure (Plate I, *2*), distributed evenly along the nuclear envelope (Fig. 2, B). *Stage 3:* Small groups of unit-sized nucleoli (Plate I, *3*) separated from one another leaving unoccupied areas along the nuclear envelope (Fig. 2, C). *Stage 4:* Enlarged groups of several small nucleoli (Plate I, *4*) at a few sites on the nuclear envelope (Fig. 2, D). *Stage 5:* Extensive entangled masses of fibrous nucleolar bodies (unit-sized entities recognizable in some parts) with distinct nucleolar organizers (Plate I, *5*); the nucleolar bodies are localized at few sites on the nuclear envelope (Fig. 2, E). *Stage 6:* Large irregularly shaped nucleolar aggregates with numerous irregular "nucleolar vacuoles" (unit-sized entities no longer visible; Plate I, *6*); the nucleolar association with the nuclear envelope is largely maintained (Fig. 2, F). *Stage 7:* Large (up to 4 μm) spherical nucleolar fusion bodies of a uniform fibrous substructure and with a central "nucleolar vacuole" filled with granular material corresponding to dissociated granular nucleolar components (Plate I, *7*); the entire macronucleus contained only 1 or 2 such bodies (Fig. 2, G). Nuclei containing nucleolar stages 3–7 are of an irregular shape (Fig. 2), probably due to the close association of the nucleoli and the nuclear envelope. The distribution of the nucleolar stages was analysed in a large number of drug-treated cells; the data are shown in Table 1. Nucleolar stage 1 was confined to control cells and 2–3 stages were found in each drug-treated sample, which may indicate a certain cell cycle stage dependence. The overall analysis shows a clear dose-related response of the nucleolar organization to the drug. Moreover, the chromatin material condenses in a dose-dependent manner (Fig. 2, B-G).

The cytoplasm of AMD-treated and corresponding glycerol-treated cells was also examined for structural changes, in part to distinguish direct effects of the drug from those possibly induced by the solvent (Table 2). Mitochondria and peroxisomes remained unchanged in all concentrations of AMD, glycogen particles and concentric profiles of the rough endoplasmic reticulum appeared in 10 μg AMD/ml, lipid droplets and autophagic vacuoles appeared in 40 μg AMD/ml, and all drug-treated cells contained small dense granules. In glycerol control cells, glycogen particles, lipid droplets, and dense granules appeared only in the high concentrations; however, enlarged endoplasmic reticulum cisternae, but no concentric profiles, were seen in the high concentrations of glycerol.

The rates of DNA, RNA, and protein synthesis were studied after 1 hour in 10–40 μg AMD/ml, using electron microscope autoradiography. Irrespective of the concentration of AMD, the number

TABLE 1. Distribution of nucleolar stages in *Tetrahymena* treated for 1 hour with Actinomycin D

Actinomycin	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6	Stage 7	Number of Cells
None	100.0	0.0	0.0	0.0	0.0	0.0	0.0	(50)
5 μg/ml	0.0	80.0	20.0	0.0	0.0	0.0	0.0	(10)
10 μg/ml	0.0	44.6	46.4	9.0	0.0	0.0	0.0	(56)
20 μg/ml	0.0	15.9	50.0	29.6	4.5	0.0	0.0	(44)
30 μg/ml	0.0	0.0	31.6	42.1	24.6	1.7	0.0	(57)
40 μg/ml	0.0	0.0	0.0	6.2	18.8	41.7	33.3	(48)
50 μg/ml	0.0	0.0	0.0	0.0	19.0	33.4	47.6	(31)

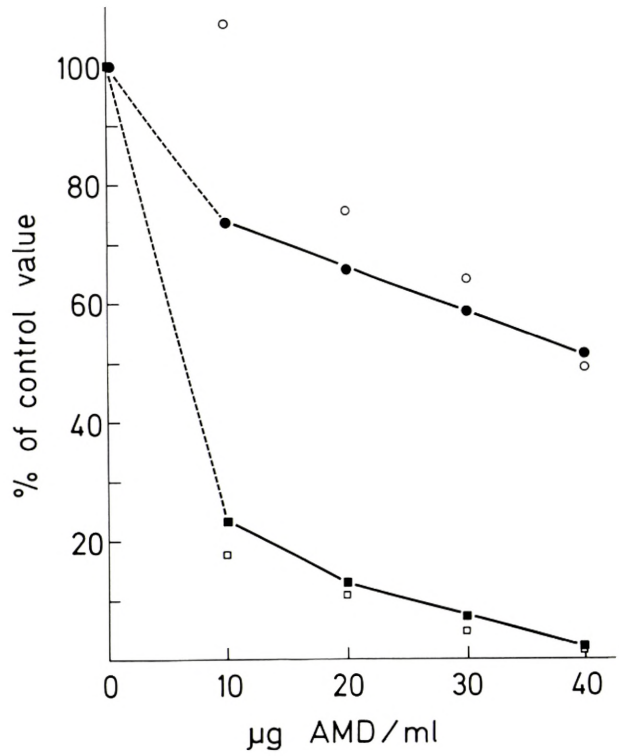
For definition of nucleolar stages see text and Fig. 2, A-G and Plate I.

TABLE 2. Cytoplasmic feature of *Tetrahymena* exposed for 1 hour to Actinomycin D (or solvent: glycerol)

Cell constituent	no AMD	5μg AMD/ml (S = 0.2%)	10μg AMD/ml (S = 0.4%)	20μg AMD/ml (S = 0.8%)	30μg AMD/ml (S = 1.2%)	40μg AMD/ml (S = 1.6%)	50μg AMD/ml (S = 2.0%)
MITOCHONDRIA	light type	unchanged (unchanged)	unchanged (unchanged)	unchanged (unchanged)	unchanged (unchanged)	unchanged (unchanged)	unchanged (unchanged)
PEROXISOMES	light type	unchanged (unchanged)	unchanged (unchanged)	unchanged (unchanged)	unchanged (unchanged)	unchanged (unchanged)	unchanged (unchanged)
GLYCOGEN PARTICLES	absent	absent (absent)	few (absent)	few (absent)	few (absent)	few (absent)	present (few)
LIPID DROPLETS	absent	absent (absent)	absent (absent)	absent (absent)	absent (absent)	few (few)	few (few)
DENSE GRANULES	absent	few (absent)	few (absent)	few (absent)	present (absent)	present (few)	present (few)
AUTOPHAGIC VACUOLES	absent	absent (absent)	absent (absent)	absent (absent)	absent (absent)	few (absent)	present (absent)
ENDOPLASMIC RETICULUM	normal	normal (normal)	normal but concentric profiles (normal)	normal but concentric profiles (normal)	enlarged cisterns, concentric profiles (enlarged cisterns)	enlarged cisterns, concentric profiles (enlarged cisterns)	enlarged cisterns, concentric profiles (enlarged cisterns)

Cells in exponentially multiplying growth phase before exposure.

Fig. 3. The effect of Actinomycin D on the rates of RNA (■) and protein (●) synthesis in *Tetrahymena* as determined after a 1-hour exposure. The data (means of 12–15 cells per sample) are expressed as percent of the values from corresponding glycerol-treated control cells (drawn curves) and from untreated control cells (open symbols). Semi-quantitative determination from electron microscope autoradiography. See text for further explanation.



of cells in DNA synthesis remained unchanged, thus 69% of the untreated control cells had incorporated tritiated thymidine and the figure found for AMD- and glycerol-treated cells was 67% ± 3.7% (analysis of 12 cells per sample); however, whereas the rate of DNA synthesis (number of silver grains per unit nuclear area) was unaltered in 10 µg AMD/ml and the corresponding control, it was reduced to 70% and 64% in 20 and 40 µg AMD/ml, respectively; the corresponding control cells showed similar values. The rates of RNA and protein synthesis are shown in Fig. 3. RNA synthesis was greatly affected by AMD, and not all cells in 30 and 40 µg AMD/ml had incorporated tritiated uridine, whereas all control cells were labelled. Moreover, cytoplasmic RNA labelling was low in all drug-treated cells and it was reduced to 70% in the glycerol-treated cells, thus the nuclear/cytoplasmic ratio was higher than in untreated cells. Protein synthesis was also affected in a dose-dependent manner but to a lesser extent. Labelled material was found above both cytoplasm and nucleus in all cells and, apart from in 40 µg AMD/ml, the nuclear/cytoplasmic ratio was above 1; glycerol-treated control cells showed an increased rate of protein synthesis as compared to untreated control cells (open symbols in Fig. 3). Although the data on tritiated uridine incorporation indicate a low nuclear/cytoplasmic transport as reported by *Eckert et al.* (1975), the data on tritiated proline incorporation show uninhibited transport from cytoplasm to nucleus.

To check the energy level of the cells, their content of ATP was measured. However, this parameter was unaffected by the drug (Table 3).

TABLE 3. Effect of Actinomycin D on content of ATP in *Tetrahymena*

Actinomycin D/ml	1-hour exposure	5-hour exposure
5 µg	102.8 (109.8)	97.8 (112.4)
10 µg	106.4 (103.6)	79.8 (89.5)
20 µg	103.2 (98.2)	86.8 (89.7)
30 µg	98.4 (95.3)	71.8 (74.2)
40 µg	100.7 (91.5)	55.4 (58.6)
50 µg	124.2 (95.4)	53.8 (58.9)

Data expressed as percent of glycerol-treated, and untreated proliferating (brackets), control cells. Means of 3 experiments with a standard deviation of less than 10%.

Prolonged exposure to AMD

After the first hour in up to 30 µg AMD/ml, a 20–30% increase in cell density was found but no

further increase occurred during the following 5 hours; no proliferation was seen at the higher doses of AMD. The glycerol-treated control cells proliferated at normal rate, but only after a lag period of up to 2 hours in the high concentrations; this lag period indicates an adaption of *Tetrahymena* to glycerol and may explain the above-mentioned reduced RNA synthesis and increased protein synthesis of these cells. In all AMD-treated cells, the rate of endocytosis, after a 3-hour exposure, was unaltered from the situation seen after 1 hour (Fig. 1); however, after a 6-hour exposure the rate had decreased in concentrations above 10 μg AMD/ml to 81, 35, 6 and 2% in 20, 30, 40, and 50 μg AMD/ml, respectively (4 experiments). Concomitantly, the cellular content of ATP was affected (Table 3), thus AMD has a time-dependent effect on both endocytosis and the cell content of ATP; furthermore, the number of small, refractive granules increased with time in AMD-treated cells. Extended exposures to the drug resulted in spontaneous recovery of cell proliferation in concentrations of up to 30 μg/ml.

The fine structure of cells exposed to 10–50 μg AMD/ml was examined after a 5-hour exposure during complete arrest of cell proliferation. Typically, the cells contained large glycogen islets, lipid droplets, increased number of dense granules, some autophagic vacuoles, concentric profiles of rough endoplasmic reticulum, and dense type mitochondria but light type peroxisomes; however, cells in 50 μg AMD/ml contained both light and dense type motochondria (Plate II, *5*). Most prominent, however, were the nucleolar structures; all nucleoli were large and appeared as compact, spherical bodies with occasional "nucleolar vacuoles" (Figs. 2 H, I and Plate II, *1–4*), thus additional nucleolar aggregation had occurred in the low concentrations of the drug (compare C and H in Fig. 2). The nucleolar bodies had a uniform fibrous substructure with no trace of the granular component. Moreover, conspicuous small, dense structures had accumulated on the surface of the compact nucleoli or within "nucleolar vacuoles" (Plate II, *3–4*); presumably, they represent condensed nucleolar organizers as they differ distinctly from the condensed chromatin granules (Plate II, *2, 3*). Some cells, irrespective of the concentration of AMD, contained intranuclear rod structures juxtaposing or penetrating the compact nucleoli (Plate II, *1*). These intranuclear rods are composed of microtubules embedded in an amorphous material (Plate II, *2*). Not only did the nucleolar bodies vary in size but also in electron density of their fibrous substructure as seen in separate nucleoli (Plate II, *4*) or within a larger composite nucleolar body (Plate II, *3*) at the high concentration of AMD.

By the end of the 5-hour observation time, the control cells had reached the early stationary phase of growth. Concomitantly their nucleoli had changed in structure (Plate II, *6*) to the typical configuration of decelerating metabolism (growth) involving nucleolar aggregation and segregation of their fibrous and granular components, but unlike the nucleolar changes induced by AMD, the granular components "peel off" in nucleolonema-like configurations (*Nilsson* and *Leick* 1970) (compare Plate I, *2* and Plate II, *6*).

Discussion

In agreement with the known action of actinomycin D (AMD) on rRNA synthesis, the most conspicuous effect of the drug on *Tetrahymena* was the induction of nucleolar changes, as reported previously after a 5-hour exposure to 10 μg AMD/ml (*Satir* and *Dirksen* 1971) and 50 μg AMD/ml (*Eckert* and *Franke* 1975). However, the dose response of progressive nucleolar changes during a 1-hour exposure to AMD has not been demonstrated previously.

Short exposure to AMD

The dose-dependent nucleolar changes indicate a sequential change in the nucleolar organization of *Tetrahymena,* a change which accelerates in the high

concentrations of AMD. This conclusion was reached after classification of the nucleolar configurations in cells treated with 5–50 μg AMD/ml and the finding that all AMD-treated cells had nucleoli differing from those of untreated cells; 7 nucleolar stages were defined. Stage 1 is typical of *Tetrahymena* from exponentially multiplying cultures where 500–1000 small unit-sized nucleoli, predominantly granular in substructure, juxtapose the nuclear envelope (*Nilsson* and *Leick* 1970) to which they are attached by fine filaments (*Satir* and *Dirksen* 1971, *Eckert* and *Franke* 1975). In the presence of AMD, the nucleoli segregated into their granular and fibrous components as described for a variety of cells (*Simard* and *Bernhard* 1966, *Goldblatt et al.* 1970, *Phillips* and *Phillips* 1971a, 1971b, *Snow* 1972, *Lane* 1969, *Simard et al.* 1974, Scheer et al. 1975, *Recher et al.* 1976, *Chaly et al.* 1979, *Jordan* and *McGovern* 1981). However, in *Tetrahymena* this segregation of the nucleolar components is accompanied by aggregation of the fibrous components into entangled masses at low doses, and into huge homogeneous bodies at high doses of the drug. The nucleolar aggregates maintain a close association with the nuclear envelope, a finding which indicates sliding of the small unit-sized nucleoli along the envelope during aggregation. This phenomenon of nucleolar aggregation in *Tetrahymena* may reflect the extrachromosomal nature of the rRNA genes (*Gall* 1974); it occurs in response to numerous factors interfering with growth of this ciliate (e.g. *Nilsson* 1976, *Pyne et al.* 1983), although not to such a dramatic extent as that observed at high doses of AMD. In AMD, the aggregation cannot involve much reduction in nucleolar volume because a compact, spherical nucleolar body of a diameter of 3 μm may in fact accomodate 1000 of the small (0.3 μm) unit-sized nucleoli.

The analysis of the distribution of the nucleolar stages in cells exposed to the different concentrations of AMD, revealed more than one stage at any one AMD concentration. The finding indicates a cell cycle dependent response as that reported (*Cleffmann et al.* 1974) for incorporation of tritiated AMD in *Tetrahymena*. However, considering that the drug enters the cells primarily by food vacuole formation (*Nachtweg* and *Dickinson* 1967), this incorporation of tritiated AMD coincides with the capacity of individual cell stages in the cell cycle to form food vacuoles (*Nilsson* 1976). Hence the differing responses of individual cells may depend on the amount of drug taken up rather than on their

Plate I. Fine structure of nucleolar changes induced by a 1-hour exposure to different doses of Actinomycin D (AMD) and definition of nucleolar stages 1–7. All figures × 50,000. (See Fig. 2 for overall features of the nuclei). Bar indicates 0.5 μm.

1. Stage 1: Small unit-sized nucleoli, predominantly granular in substructure, in close association with the nuclear envelope (e) and with a central nucleolar organizer (arrow). Typical of *Tetrahymena* from fast growing cultures (no AMD).

2. Stage 2: Enlarged nucleoli due to fusion of 2–3 small unit-sized nucleoli, the substructure is predominantly fibrous with a rim (arrow heads) of granular material (nucleolar segregation); note that the association with the nuclear envelope is maintained (e). (10 μg AMD/ml).

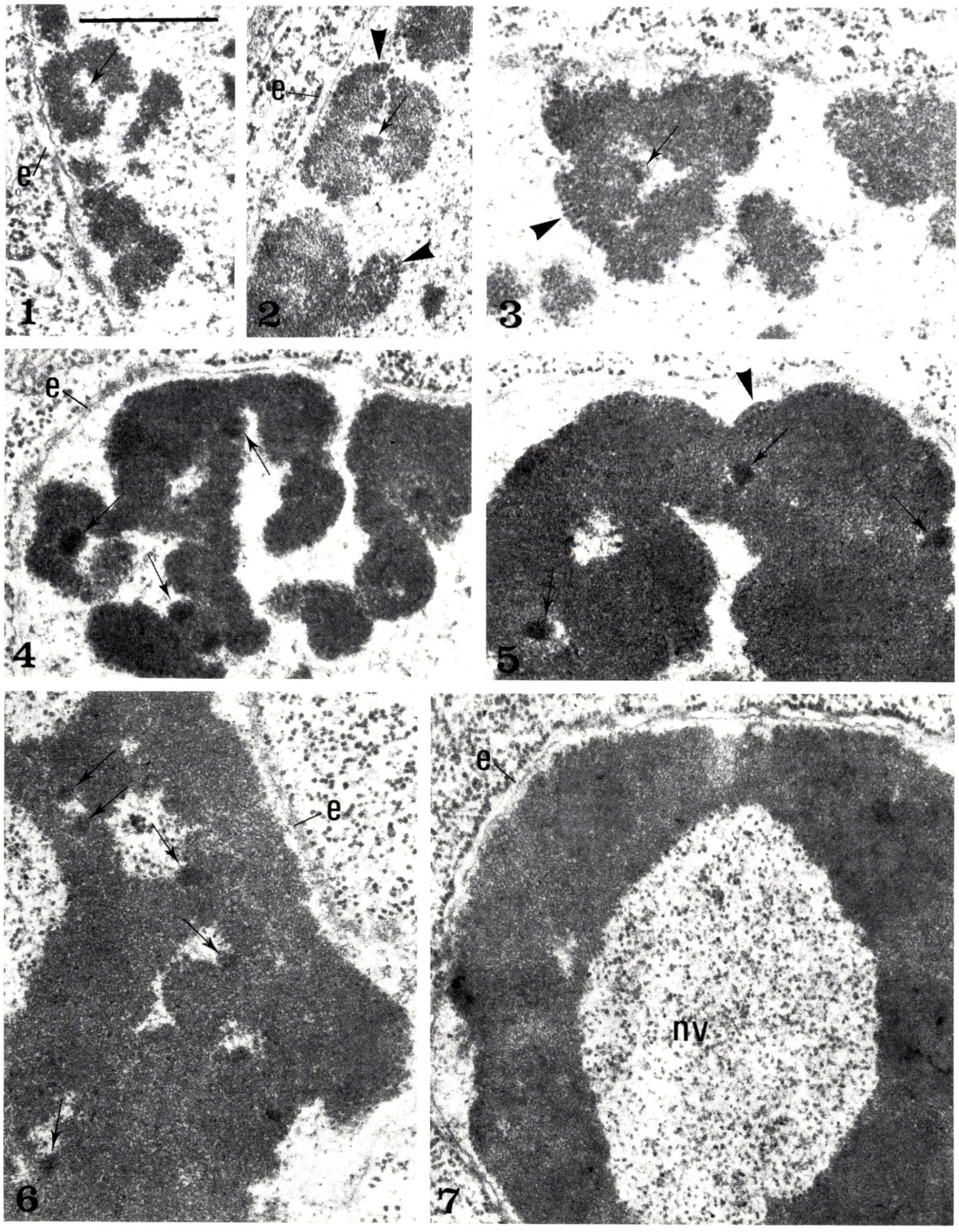
3. Stage 3: Further aggregation of the small nucleoli (unit entities recognizable); part of the fibrous aggregate maintains the position on the nuclear envelope and little granular nucleolar component (arrow head) is visible. Nucleolar organizer (arrow). (5 μg AMD/ml).

4. Stage 4: Enlarged aggregates in which unit-sized nucleoli may be recognized. Several condensed nucleolar organizers may be seen (arrows). (30 μg AMD/ml).

5. Stage 5: Entangled mass (only partly shown) of fibrous nucleolar material (entities not clearly identifiable) with distinct nucleolar organizers (arrows) and little granular component (arrow head). (50 μg AMD/ml).

6. Stage 6: Large irregularly shaped nucleolar aggregate (only small section shown), the unit-sized entities no longer visible in the entangled fibrous mass. The nucleolar organizers (arrows) are confined to irregularly shaped cavities ("nucleolar vacuoles") in which free granular material corresponding to the granular nucleolar component is seen. An extensive portion of the nucleolar aggregate is associated with the nuclear envelope (e). (40 μg AMD/ml).

7. Stage 7: Large spherical nucleolar body (minor portion shown but see Fig. 2, G) of a compact fibrous substructure and with a large "nucleolar vacuole" (nv) filled with granular material corresponding to dissociated granular nucleolar component and resembling ribosomes outside nuclear envelope (e). An extensive portion of the nucleolar body (4 μm in diameter) is associated with the nuclear envelope (e). (50 μg AMD/ml).


e
1
e
2
3
e
4
5
e
6
e
nv
7

cell cycle stage. One hour corresponds to 1/3 of a cell generation of *Tetrahymena* and the 20–30% increase in cell density seen after this time in up to 30 μg AMD/ml indicates that the most advanced cell stages complete the cycle, whereas cell division is abolished at higher concentrations. These findings are in accordance with those found using heat synchronized *Tetrahymena* populations, where inhibition of cell division depends on the time of addition (about 1 hour before synchronous division) and the concentration of the drug (*Lazarus et al.* 1964, *Whitson* and *Padilla* 1964, *Moner* 1965, *Frankel* 1965, *Dugaiczyk* and *Eiler* 1968, *Fink* and *Zeuthen* 1980). Although cell division may be inhibited after a 1-hour exposure to AMD, the number of cells in DNA synthesis remained constant, but the rate of thymidine incorporation decreased with increasing dose of the drug, presumably due to the known binding of AMD to DNA (*Goldberg* and *Friedman* 1971, *Kersten* 1971) possibly reflected by the observed chromatin condensation.

At low doses of AMD (below 1 μg/ml) rRNA synthesis is selectively inhibited in mammalian cells and total RNA synthesis is abolished above 5 μg AMD/ml (*Perry* and *Kelly* 1970). Higher doses of the drug are needed to affect RNA synthesis in *Tetrahymena,* thus after a 3-hour exposure to 5 μg AMD/ml, rRNA synthesis is inhibited by only 50% and concomitantly tRNA synthesis was lowered by 30% (*Leick* 1969). The question of whether AMD may be a non-selective inhibitor of rRNA synthesis in *Tetrahymena* was investigated by *Ernst* and *Oleinick* (1977); RNA synthesis was effected in a dose- and time-dependent manner in 0.5–10 μg AMD/ml with almost equal inhibition of Poly (–) and Poly (+) RNAs, quite unlike the situation in mammalian cells. The low rates of RNA synthesis, found by electron microscope autoradiography in the present study, are in good agreement with the biochemical findings within the same range of AMD doses (*Ernst* and *Oleinick* 1977, *Eckert* and *Franke* 1975). In addition, protein synthesis in *Tetrahymena* is affected by the drug, thus a 23% dissociation of polysomes occurs within 1 hour in 10 μg AMD/ml (*Ernst* and *Oleinick* 1977), a figure which equals the 74% value for protein synthesis in the present study. Only a slight additional effect is seen on protein synthesis with increasing doses of AMD (*Fink* and *Zeuthen* 1980, present study).

Plate II. Fine structure of cells exposed for 5 hours to actinomycin D (AMD) and of a control cell (*6*). Time-dependent effects of AMD.

1. Aggregation of two large spherical nucleolar bodies of a compact fibrous substructure. Conspicuous intranuclear rods (arrows) juxtapose or penetrate the nucleoli. Note the compact condensed chromatin granules in the nucleoplasm (n). (10 μg AMD/ml). × 1,800. Bar indicates 0.5 μm.

2. Enlargement of lower part of *1* showing portion of the intranuclear rod juxtaposing the nucleolar body. The substructure of the rods (arrows) is clearly microtubules embedded in amorphous material. Condensed chromatin granules (ch). (10 μg AMD/ml). × 60,000. Bar indicates 0.5 μm.

3. Part of 3.5 μm large composite nucleolar body revealing 3 spherical portions (1, 2, 3) of different electron density. Nucleolar organizers (arrows) are seen in the "nucleolar vacuole" in the body labelled 2; they differ from the condensed chromatin granules (ch) on the surface of the nucleolar body. (50 μg AMD/ml). Magnification as in *2.*

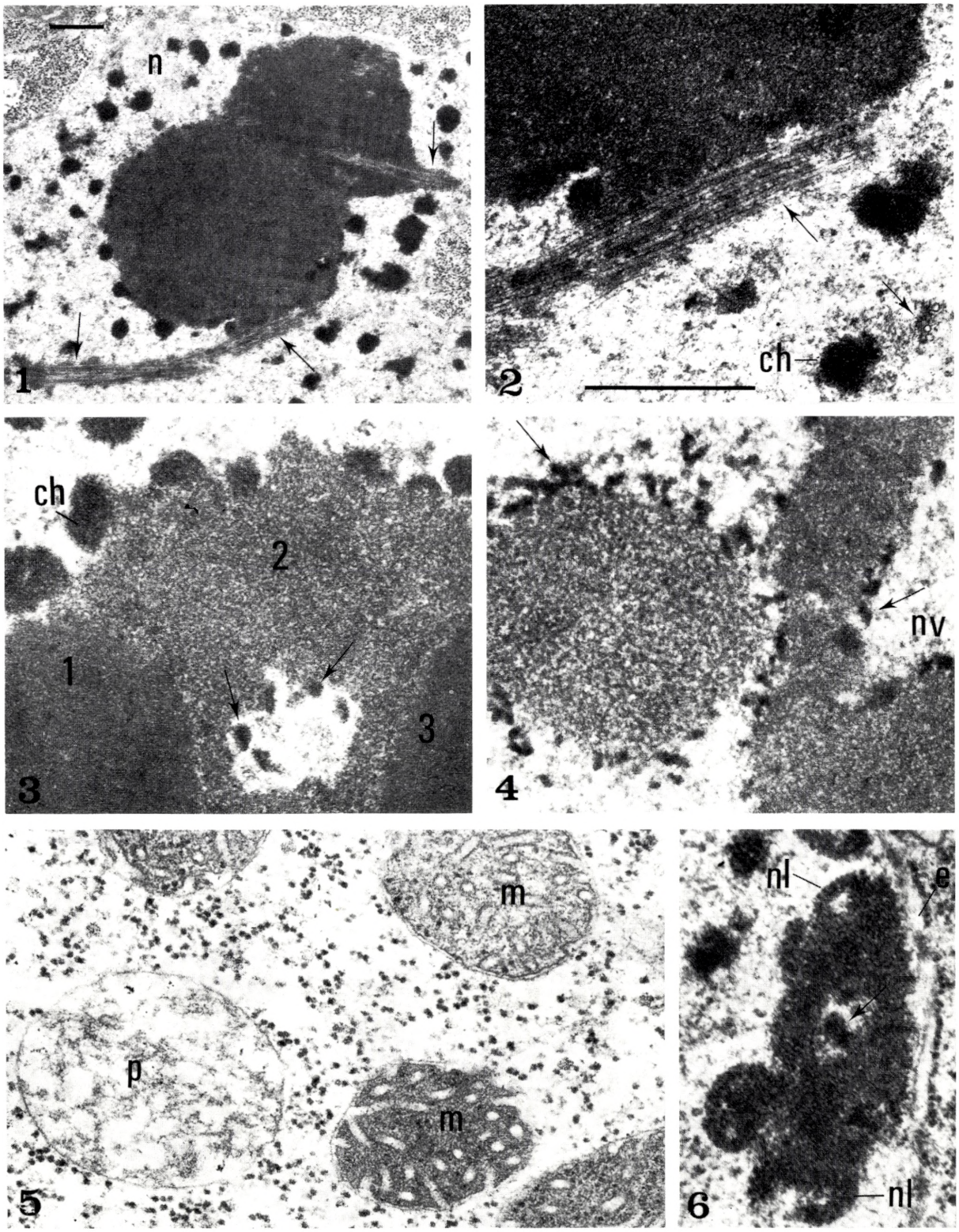
4. A small spherical nucleolar body surrounded by several nucleolar organizers (arrow) resembling those found within the "nucleolar vacuole" (nv) of the adjacent large nucleolar body (see full outline of the two nucleoli in Fig. 2, I). Note the different electron density of the nucleolar bodies. (50 μg AMD/ml). Magnification as in *2.*

5. Portion of cytoplasm of cell in 50 μg AMD/ml. Note the light type peroxisomes (p) and light and dense type mitochondria (m). Magnification as in *2.*

6. Nucleolus of control cells at early stationary growth phase. Nucleolar aggregation has occurred (note association with nuclear envelope (e)) as well as segregation of the granular and fibrous component; however, the granular component typically "peel off" in nucleolonema-like structures (nl) unlike the situation in AMD (compare to Plate I, *2*). (Glycerol-treated control cell). Magnification as in *2.*

Extended exposure to AMD

The time-dependent effects of AMD on the *Tetrahymena* nucleoli were evident after the 5–6 hour exposure. All nucleoli, irrespective of the concentration of the drug, were spherical, compact


n
1
ch
2
ch
2
1
3
3
nv
4
m
p
m
5
nl
e
nl
6

fibrous bodies with no trace of the granular component; they were few in number at any one concentration of AMD which means that additional aggregation has occurred in cells at the low doses of the drug. Thus the nucleolar bodies found in 10 μg AMD/ml differ from those reported by *Satir* and *Dirksen* (1971) for which no explanation can be offered; however, the nucleolar bodies observed in 50 μg AMD/ml resemble those reported by *Eckert* and *Franke* (1975). An additional observation is that the electron density of the large fusion bodies may vary; such a segregation of the fibrous nucleolar component into types of varying electron density has been observed in other AMD-treated cells (*Stevens* 1964, *Phillips* and *Phillips* 1971a, 1971b, *Simard et al.* 1974, *Jordan* and *McGovern* 1981). The phenomenon could be ascribed to the random release of immature pre-rRNA from the template as observed in AMD-treated oocytes (*Scheer et al.* 1975). In *Tetrahymena*, pre-rRNA becomes stably bound to the nucleoli (fibrous component) after synthesis, whereas the precursors of cytoplasmic ribosomes, containing 26S and 17S rRNAs, are bound more loosely (granular component) (*Herlan et al.* 1979). Although no considerable processing or degradation of pre-rRNA could be found during a 90-minute exposure to 50 μg AMD/ml (*Eckert et al.* 1975), a slow processing and/or release of pre-rRNA could occur during the additional 4-hour exposure, thus leaving some nucleoli with reduced mass. The less dense nucleolar structures could therefore represent a nucleolar matrix as found in cultured human fibroblasts (*Jordan* and *McGovern* 1981). Moreover, the time-dependent loss of the granular nucleolar component from the *Tetrahymena* macronucleus also indicates degradation or transport of rRNA to the cytoplasm; although AMD has been reported to inhibit nuclear/cytoplasmic transport (*Eckert et al.* 1975), the present study demonstrates that cytoplasmic/nuclear transport takes place in the presence of the drug as newly synthesized protein enters the nucleus.

Intranuclear rod structures were found in some AMD-treated cells. The structures are composed of microtubules embedded in an amorphous material and they appear under conditions of arrested cell division; they could represent remnants of division-associated microtubules in cells with abolished division. AMD also induces intranuclear structures in oocytes (*Lane* 1969, *Snow* 1972, *Eckert et al.* 1972); however, these structures differ from the intranuclear rods in *Tetrahymena*, but they resemble the actin bundles induced by dimethyl sulphoxide in this ciliate (*Nilsson* 1980, *Katsumara* and *Fukui* 1982).

The cytoplasmic changes induced by AMD in *Tetrahymena* primarily reflect a general cessation of cell proliferation as seen under stationary phase conditions (e.g. *Nilsson* 1976). However, peroxisomes and mitochondria did not appear in their expected dense type (*Nilsson* 1976, 1981), which indicates interference with the biochemical events responsible for the transformation of the light to the dense type. In fact, AMD inhibits the increase in the specific activity of the peroxisomal enzyme, isocitrate lyase, normally seen in *Tetrahymena* at cessation of growth (*Levy* 1967), moreover the drug affects mitochondrial transformation and mitochondrial RNA synthesis in other cells (*Kahri* 1968, *Young* and *Zimmerman* 1974).

Although AMD prevents cell proliferation of *Tetrahymena* for several hours, this inhibition is abolished during prolonged exposures to up to 30 μg AMD/ml (*Satir* 1967 and present study). The study by *Satir* and *Dirksen* (1971) is a description of such growth during a 45-hour exposure to 10 μg AMD/ml. Whether this recovery of growth after a long lag period in AMD is due to the drug being metabolized or to adaption of the cells is not known.

Acknowledgements

Dr. Cicily Chapman-Andresen and Professor Christian Overgaard Nielsen are thanked for critical reading of the manuscript. Mrs. Karen P. Meilvang is thanked for excellent technical assistance. The financial support from the Carlsberg Foundation and the Novo Foundation is gratefully acknowledged.

References

Caro, L. G. 1969: A common source of difficulty in high resolution autoradiography. J. Cell Sci. *41*, 918–919.

Chaly, N., Lord, A. & Lafontaine, J. G. 1979: Nucleolar organization in the euglenoid *Astasia longa* as disclosed by selective staining, actinomycin D treatment, and cold shock. Can. J. Bot. *57*, 2031–2043.

Cleffmann, G., Fehrendt, I. & Behrendt, W. 1974: The rate of uptake of H^3-actinomycin during the cell cycle of *Tetrahymena*. Exp. Cell Res. *87*, 139–142.

Dugaiczyk, A. & Eiler, J. J. 1968: The inhibition of synchronous division of *Tetrahymena pyriformis* by actinomycin D. Curr. Mod. Biol. *1*, 333–336.

Eckert, W. A. & Franke, W. W. 1975: Changes in fine structure and composition of macronuclei of *Tetrahymena pyriformis* induced by drugs interfering with RNA synthesis and processing. Cytobiologie *11*, 392–418.

Eckert, W. A., Franke, W. W. & Scheer, U. 1972: Actinomycin D and the central granules in the nuclear pore complex: Thin sectioning versus negative staining. Z. Zellforsch. *127*, 230–239.

– 1975: Nucleocytoplasmic translocation of RNA in *Tetrahymena pyriformis* and its inhibition by actinomycin D and cycloheximide. Exp. Cell Res. *94*, 31–46.

Ernst, S. G. & Oleinick, N. L. 1977: Actinomycin D in *Tetrahymena*. Non-specific inhibition of RNA synthesis and primary and secondary effects on protein synthesis. Exp. Cell Res. *110*, 363–373.

Fink, K. & Zeuthen, E. 1980: Heat shock proteins in *Tetrahymena* studied under growth conditions. Exp. Cell Res. *128*, 23–30.

Frankel, J. 1965: The effect of nucleic acid antagonists on cell division and oral organelle development in *Tetrahymena pyriformis*. J. Exp. Zool. *159*, 113–148.

Gall, J. G. 1974: Free ribosomal RNA genes in the macronucleus of *Tetrahymena*. Proc. Nat. Acad. Sci. USA *71*, 3078–3081.

Goldberg, I. H. & Friedman, P. A. 1971: Antibiotics and nucleic acid synthesis and function. In: Drugs and cell regulation (Mihich, E. ed.), pp. 99–119. New York: Academic Press.

Goldblatt, P. J., Verbin, R. S. & Sullivan, R. J. 1970: Induction of nucleolar segregation by actinomycin D following inhibition of protein synthesis with cycloheximide. Exp. Cell Res. *63*, 117–123.

Herlan, G., Eckert, W. A., Kaffenberger, W. & Wunderlich, F. 1979: Isolation and characterization of an RNA-containing nuclear matrix from *Tetrahymena* macronuclei. Biochemistry, *18*, 1782–1788.

Hyman, R. W. & Davidson, N. 1970: Kinetics of the in *vitro* inhibition of transcription by actinomycin. J. Mol. Biol. *50*, 421–438.

Jordan, E. G. & McGovern, J. H. 1981: The quantitative relationship of the fibrillar centres and other nucleolar components to changes in growth conditions, serum deprivation and low doses of actinomycin D in cultured diploid human fibroblasts (strain MRC-5). J. Cell Sci. *52*, 373–389.

Kahri, A. I. 1968: Effects of actinomycin D and puromycin on the ACTH-induced ultrastructural transformation of mitochondria of cortical cells of rat adrenals in tissue culture. J. Cell Biol. *36*, 181–195.

Katsumara, H. & Fukui, Y. 1982: In vivo identification of *Tetrahymena* actin probed by DMSO induction of nuclear bundles. Exp. Cell. Res. *137*, 353–363.

Kersten, H. 1971: Use of inhibitors to study the structure and function of nucleic acids and ribosomes. FEBS Letters *15*, 261–268.

Lane, N. J. 1969: Intranuclear fibrillar bodies in actinomycin D-treated oocytes. J. Cell Biol. *40*, 286–291.

Lazarus, L. H., Levy, M. R. & Scherbaum, O. H. 1964: Inhibition of synchronized cell division in *Tetrahymena* by actinomycin D. Exp. Cell Res. *36*, 672–676.

Leick, V. 1969: Effect of actinomycin D and DL-p-fluorophenyalanine on ribosome formation in *Tetrahymena pyriformis*. European J. Biochem. *8*, 215–220.

Levy, M. R. 1967: Effects of inhibitors of RNA and protein synthesis on activation of glyconeogenesis in *Tetrahymena*. J. Cell Physiol. *69*, 247-252.

Moner, J. G. 1965: RNA synthesis and cell division in heat-synchronized populations of *Tetrahymena pyriformis*. J. Protozool. *12*, 505–509.

Myhrman, A., Lundin, A. & Thore, A. 1978: The analytical application of ATP monitoring using firefly bioluminescence. LKB Application Note 314.

Nachtweg, D. S. & Dickinson, W. J. 1967: Actinomycin D: Blockage of cell division of synchronized *Tetrahymena pyriformis*. Exp. Cell Res. *47*, 581–595.

Nanney, D. L. & McCoy, J. W. 1976: Characterization of the species of the *Tetrahymena pyriformis* complex. Trans. Amer. microsc. Soc. *95*, 664–682.

Nilsson, J. R. 1972: Further studies on vacuole formation in *Tetrahymena pyriformis* GL. C. R. Trav. Lab. Carlsberg *39*, 83–110.

– 1976: Physiological and structural studies on *Tetrahymena pyriformis* GL. With special ref erence to endocytosis and to structural changes during the cell cycle and in response to alterations in the cellular environment. C. R. Trav. Lab. Carlsberg *40*, 215–355.

– 1980: Effects of dimethyl sulphoxide on ATP content and protein synthesis in *Tetrahymena*. Protoplasma *103*, 189–200.

– 1981: On cell organelles in *Tetrahymena*. With special reference to mitochondria and peroxisomes. Carlsberg Res. Commun. *46*, 279–304.

Nilsson, J. R. & Leick, V. 1970: Nucleolar organization and ribosome formation in *Tetrahymena pyriformis* GL. Exp. Cell Res. *60*, 361–372.

Perry, R. P. & Kelley, D. E. 1970: Inhibition of RNA synthesis by actinomycin D: Characteristic dose-response of different RNA species. J. Cell Physiol. *76*, 127–140.

Phillips, S. G. & Phillips, D. M. 1971a: Nucleoli of diploid cell strains. Their normal ultrastructure and the effects of toyocamycin and actinomycin D. J. Cell Biol. *49*, 785–802.

– 1971b: Distinctive characteristics of nucleoli of two established cell lines. J. Cell Biol. *49*, 803–815.

Plesner, P., Rasmussen, L. & Zeuthen, E. 1964: Techniques used in the study of synchronous *Tetrahymena*. In: Synchrony in cell division and growth (Zeuthen, E. ed.), pp. 543–563. New York: Interscience.

Pyne, C. K., Iftode, F. & Curgy, J.-J. 1983: The effects of cadmium on the growth pattern and ultrastructure of the ciliate *Tetrahymena pyriformis*, and the antagonistic effect of calcium. Biol. Cell *48*, 121–132.

Recher, L., Sykes, J. A. & Chan, H. 1976: Further studies on the mammalian cell nucleolus. J. ultrastr. Res. *56*, 152–163.

Satir, B. 1967: Effect of actinomycin D on cultural growth phases and on the pattern of total RNA synthesis in *Tetrahymena*. Exp. Cell Res. *48*, 253–262.

Satir, B. & Dirksen, E. R. 1971: Nucleolar aging in *Tetrahymena* during the cultural growth cycle. J. Cell Biol. *48*, 143–154.

Scheer, U., Trendelenburg, M. F. & Franke, W. W. 1975: Effects of actinomycin D on the association of newly formed ribonucleoproteins with the cistron of ribosomal RNA in *Triturus* oocytes. J. Cell Biol. *65*, 163–179.

Simard, R. & Bernhard, W. 1966: Le phénomène de la ségrégation nucléolaire: Spécificité d'action de certains antimétabolites. Int. J. Cancer *1*, 463–479.

Simard, R., Langelier, Y., Mandeville, R., Maestracci, N. & Royal, A. 1974: Inhibitors as tools in elucidating the structure and function of the nucleus. In: The cell nucleus Vol. III (Busch, H., ed.), pp. 447–487. New York: Academic Press.

Snow, M. H. L. 1972: The effect of actinomycin D *in vivo* upon peripheral nucleoli and other nuclear organelles in oocytes of *Triturus cristatus*. J. Cell Sci. *10*, 833–855.

Stevens, B. J. 1964: The effect of actinomycin D on nucleolar and nuclear fine structure in the salivary cell of *Chironomus thummi*. J. ultrastr. Res. *11*, 329–353.

Venable, J. H. & Coggeshall, R. 1965: A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. *25*, 407–408.

Weinstein, R., Abbiss, T. & Bullivant, S. 1963: The use of double and triple uranyl salts as electron stains. J. Cell Biol. *19*, 74A.

Whitson, G. L. & Padilla, G. M. 1964: The effects of actinomycin D on stomatogenesis and cell division in temperature-synchronized *Tetrahymena pyriformis*. Exp. Cell Res. *36*, 667–671.

Young, P. G. & Zimmerman, A. M. 1974: The decay of *Xenopus laevis* mitochondrial rRNA in the presence of ethidium bromide or actinomycin D. Can. J. Biochem. *52*, 911–915.

Indleveret til Selskabet november 1984.
Færdig fra trykkeriet januar 1985.

Biologiske Skrifter

Biol. Skr. Dan. Vid. Selsk.
Priser excl. moms

Vol. 17 (DKr. 330.–)

1. Degerbøl, Magnus, and Fredskild, Bent: The Urus (*Bos primigenius* Bojanus) and Neolithic Domesticated Cattle (*Bos taurus domesticus* Linné) in Denmark. With a Revision of *Bos*-Remains from the Kitchen Middens. Zoological and Palynological Investigations. 1970 ... 200.–
2. Hansen, Hans Jørgen: Electron-Microscopical Studies on the Ultrastructures of some Perforate Calcitic Radiate and Granulate Foraminifera. 1970 ..... 50.–
3. Mathiesen, Fr.J.: Palaeobotanical Investigations into some Cormophytic Macrofossils from the Neogene Tertiary Lignites of Central Jutland. Part II: *Gymnosperms*. 1970 ..... 80.–

Vol. 18 (DKr. 450.–)

1. Hansen, Georg, Nørgaard: On the Structure and Vascularization of the Pituitary Gland in some Primitive Actinopterygians (*Acipenser, Polyodon, Calamoichthys, Polypterus, Lepisosteus* and *Amia*). 1971 ..... 70.–
2. Dyck, Jan: Structure and Spectral Reflectance of Green and Blue Feathers of the Rose-faced Lovebird (*Agapornis roseicollis*). 1971 ..... 60.–
3. Perch-Nielsen, Katharina: Elektronenmikroskopische Untersuchungen an Coccolithen und verwandten Formen aus dem Eozän von Dänemark. 1971.... 160.–
4. Böcher, Tyge W., and Lyshede, Ole B.: Anatomical Studies in Xerophytic Apophyllous Plants. II. Additional Species from South American Shrub-Steppes. 1972 ..... 160.–

Vol. 19 (DKr 410.–)

1. Humphreys, William F., and Lützen, Jørgen: Studies on Parasitic Gastropods from Echinoderms. I. On the Structure of the Parasitic Gastropod Megadenus cantharelloides n.sp., with Comparisons on Paramegadenus n.g. 1972 ..... 30.–
2. Surlyk, Finn: Morphological Adaptations and Population Structures of the Danish Chalk Brachiopods (Maastrichtian, Upper Cretaceous). 1972 ..... 60.–
3. Hammer, Marie: Tahiti. Investigation on the Oribatid Fauna of Tahiti, and on some Oribatids found on the Atoll Rangiroa. 1972 ..... 80.–
4. Wingstrand, Karl Georg: Comparative Spermatology of a Pentastomid, *Raillietiella Hemidactyli*, and a Branchiuran Crustacean, *Argulus Foliaceus*, with a Discussion of Pentastomid Relationships. 1972 ..... 80.–
5. Böcher, Tyge W., and Jørgensen, C.A.: Jyske dværgbuskenheder. Eksperimentelle undersøgelser af forskellige kulturindgrebs indflydelse på vegetationen. With an English Summary. 1972 ..... 50.–
6. Lützen, Jørgen: Studies on Parasitic Gastropods from Echinoderms. II. On *Stilifer* Broderip, with Special Reference to the Structure of the Sexual Apparatus and the Reproduction. 1972 ..... 30.–
7. Rasmussen, H. Wienberg: Lower Tertiary Crinoidea, Asteroidea and Ophiuroidea from Northern Europe and Greenland. 1972 ..... 80.–

Vol. 20 (DKr. 490.–)

1. Blom, Lars: Ridge Pattern and Surface Ultrastructure of the Oviducal Mucosa of the Hen (*Gallus domesticus*). 1973 ..... 30.–
2. Jensen, Poul Vagn: Structure and Matemorphosis of the Larval Heart of *Calliphora erythrocephala*. 1973 .... 30.–
3. Hammer, Marie: Oribatids from Tongatapu and Eua, the Tonga Islands, and from Upolu, Western Samoa. 1973 ..... 80.–
4. Gooding, Richard U., and Lützen, Jørgen: Studies on Parasitic Gastropods from Echinoderms. III. A Description of *Robillardia Cernica* Smith 1889, Parasitic in the Sea Urchin *Echinometra* Meuschen, with Notes on its Biology. 1973 ..... 30.–
5. Manton, I., and Leadbeater, B. S. C.: Fine-structural Observations on six Species of *Chrysochromulina* from Wild Danish Marine Nanoplankton, including a Description of *C. campanulifera* sp. nov. and a Preliminary Summary of the Nanoplanktion as a Whole. 1974 ..... 40.–

fortsættes side 4

6. Birkelund, Tove, and Hansen, Hans Jørgen: Shell Ultrastructures of some Maastrichtian Ammonoidea and Coleoidea and their Taxonomic Implications. 1974 50.–
7. Poulsen, Chr.: Silurian Pelecypoda, Monoplacophora, and Gastropoda from the Reefy Facies of the Offley Island Formation of Washington Land and Offley Island (Northwest Greenland). 1974 ..... 30.–
8. Böcher, Tyge W.: Structure of the Multinodal Photosynthetic Thorns in *Prosopis Kuntzei* Harms. 1975 .... 60.–
9. Mathiesen, Fr. J.: Palaeobotanical Investigations into some Cormophytic Macrofossils from the Neogene Tertiary Lignites of Central Jutland, Part III: Angiosperms. 1975 ..... 80.–
10. Bromley, R. G., Schultz, M.-G., and Peake, N. B.: Paramoudras: Giant Flints, Long Burrows and the Early Diagenesis of Chalks. 1975 ..... 60.–

Vol. 21 (DKr. 650.–)

1. Nygaard, Gunnar: New or Interesting Plankton Algae. 1977 ..... 150.–
2. Peel, John S.: Systematics and Palaeoecology of the Silurian Gastropods of the Arisaig Group, Nova Scotia. 1977 ..... 150.–
3. van der Land, Jacob, and Nørrevang, Arne: Structure and Relationship of Lamellibrachia (Annelida, Vestimentifera). 1977 ..... 200.–
4. Hammer, Marie: Investigations on the Oribatid Fauna of the North West Pakistan. 1977 ..... 150.–

Vol. 22 (DKr. 710.–)

1. Wingstrand, K. G.: Comparative Spermatology of the Crustacea Entomostraca 1. Subclass Branchiopoda. 1978 ..... 150.–
2. Alexandersen, Verner: Sūkās V. A. Study of Teeth and Jaws from a Middle Bronze Age Collective Grave on Tall Sūkās 1978. (Publications of the Carlsberg Expedition to Phoenicia 6. The other reports from this expedition are printed in the *Historisk-filosofiske Skrifter*, including no. 5, *Hist.-fil. Skr.* 10:1, with the report of the excavation in question: Henrik Thrane: Sūkās IV, 1978, 100 DKr.) 80.–
3. Böcher, Tyge W., and Olesen, Peter: Structural and Ecophysiological Pattern in the Xero-Halophytic C_4 Grass, *Sporobolus rigens* (Tr.) Desv. 1978 ..... 100.–
4. Hammer, Marie, and Wallwork, John A.: A Review of the World Distribution of Oribatid Mites (*Acari: Cryptostigmata*) in Relation to Continental Drift. 1979. 50.–
5. Jørgensen, C. Barker; Larsen, Lis Olesen, and Lofts, Brian: Annual Cycles of Fat Bodies and Gonads in the Toad *Bufo Bufo Bufo (L)*, Compared with Cycles in Other Temperate Zone Anurans. 1979 .... 40.–
6. van der Hammen, Thomas: Changes in Life Conditions on Earth during the past One Million Years. 1979 40.–
7. Thom, René: Théorie des catastrophes et biologie: Plaidoyer pour une biologie théorique. 1979 ..... 30.–
8. Böcher, Tyge W.: Xeromorphic Leaf Types. Evolutionary Strategies and Tentative Semophyletic Sequences. 1979 ..... 100.–
9. Hammer, Marie: Investigations on the Oribatid Fauna of Java. 1979 ..... 120.–

Vol. 23 (DKr. 700.–)

1. Nielsen, Anker: A Comparative Study of the Genital Chamber in Female *Trichoptera*. 1980 ..... 200.–
2. Böcher, Tyge W.: Evolutionary Trends in Ericalean Leaf Structure. 1981 ..... 90.–
3. Larsen, Torben B.: The Butterflies of Yemen Arab Republic. With a Review of Species in the *Charaxes viola*-Group from Arabia and East Africa by A. H. B. Rydon. 1982 ..... 120.–
4. Böcher, Tyge W.: A Developmental Analysis of the Photosynthesizing Organs in *Prosopis Kuntzei*. 1982... 90.–
5. Salomonsen, Finn: Revision of the Melanesian Swiftlets *(Apodes, Aves)* and their Conspecific Forms in the Indo-Australien and Polynesian Region. 1983 ..... 200.–

Vol. 24

1. Andersen, Svend Th.: Forests at Løvenholm, Djursland, Denmark, at present and in the past. 1984 .... 200.–
2. Nilsson, Jytte R.: Dose- and Time-dependent Effects of Actinomycin D on *Tetrahymena* with Special Reference to Nucleolar Changes. 1985 ..... 40.–
3. Friis, Else Marie: Angiosperm Fruits and Seeds from the Middle Miocene of Jutland (Denmark).

Printed in Denmark by Bianco Lunos Bogtrykkeri A/S. ISSN 0106-0481. ISBN 87-7304-155-6