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Macromolecular Syntheses and the Course of Cell Cycle Events in the Chlorococcal Alga *Scenedesmus quadricauda* under Nutrient Starvation: Effect of Nitrogen Starvation

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Abstract. Daughter cells of the chlorococcal alga *Scenedesmus quadricauda* incubated under photosynthesizing conditions in a nitrogen-free medium did not make any progress in the cell cycle. Photosynthetic starch formation continued for a period corresponding to a half of the cell cycle and then levelled off. Protein synthesis was very slow and it did not surpass double the initial amount. RNA content decayed from the start of treatment and approached about 2 pg/cell.

When a synchronous population was deprived of nitrogen or of light in the middle of the cell cycle RNA synthesis stopped immediately or very soon afterwards and, in spite of abundant intracellular nitrogen reserves, RNA content slowly declined. This degradation was much extensive in nitrogen starved cells where, eventually, the RNA content attained about half the starting value. In both experimental variants, DNA replications started at the same time as in control culture, but the final amount of DNA attained only half the control value. Protein synthesis stopped immediately in the dark. In the nitrogen-starved cells, it continued for several hours and protein content increased about 70 % of the amount present at the start of starvation. The number of daughter cells formed was proportional to the final protein content in the nitrogen- and light-deprived cells (corresponding division numbers were 6 and 4, respectively). Upon refeeding of daughter cells formed under nitrogen starvation, RNA synthesis started immediately, while protein synthesis displayed a lag of about 5 h. DNA replications were triggered at the time when the ratio of RNA to DNA content attained the same value as in the control culture.

There is little information on the course of the cell cycle events in alga cultures deprived of one of the essential mineral nutrients. In fact the most detailed, and in several respects unique, investigations into this problem are those performed by TAMIYA and his collaborators nearly 30 years ago (HASE *et al.* 1957, TAMIYA 1964). Their results for the chlorococcal alga *Chlorella* have suggested that the course of cell cycle events upon withdrawal

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of nitrogen, sulphur and phosphorus could be used for the analysis of cell cycle controls. It appeared that the lack of a particular element restricts specific basic processes which may arrest the development of the cells at various stages of the cycle.

We were inspired by this possibility and we felt that the most interesting results from TAMIA's school would require interpretation in the framework of the modern concepts of cell cycle controls, in particular, such as the concept of overlapping cell reproductive sequences in chlorococcal algae (ŠETLÍK and ZACHLEDER 1984). To this purpose it was first necessary to supplement the observations on nuclear and cellular divisions with those on macromolecular syntheses.

The cell cycle of chlorococcal algae comprises several (1 to 4, in general n) consecutive cell reproductive sequences. As a result, the mother cell divides at the end of the cell cycle (ŠETLÍK and ZACHLEDER 1984) into 2^n daughter cells. In each of the cell reproductive sequences, the following steps can be distinguished: the growth, DNA replication, nuclear division and cellular division step.

In autotrophically growing algae, photosynthetic energy supply is indispensable for the completion of the growth step. By performing a certain amount of synthetic work and by accumulating energy and material reserves, the cell passes the so called commitment point, after which it becomes committed to divide. The subsequent steps in the cell reproductive sequence can then proceed, and eventually terminate, without an external energy supply, *i.e.* in the dark (ŠETLÍK *et al.* 1972). In cycles which comprise more consecutive cell reproductive sequences, a corresponding number of commitment points must be passed. The replication division sequence which begins at the commitment point, overlaps with the growth step of the next cell reproductive sequence (ŠETLÍK *et al.* 1972, ŠETLÍK and ZACHLEDER 1983, 1984).

The purpose of the experiments reported here and in subsequent papers (ŠETLÍK *et al.* 1988, ZACHLEDER *et al.* 1988) is to investigate the effects of the absence of nitrogen, phosphorus, and sulphur in the medium on the course of cell cycle events in *Scenedesmus quadricauda*. The present paper deals with the response to nitrogen withdrawal in synchronous cultures of this alga.

MATERIAL AND METHODS

Organism

The chlorococcal alga *Scenedesmus quadricauda* (TURP.) BRÉB. strain Greifswald/15 was obtained from the Collection of Cultures of Autotrophic Organisms kept at the Institute of Botany of the Czechoslovak Academy of Sciences, Třeboň, Czechoslovakia.

Culture Conditions

The suspensions of synchronous populations of *Scenedesmus* cells were cultivated in plate-parallel vessels (2200 ml) irradiated from one side by incandescent lamps 500 W (Tesla, Czechoslovakia). The inorganic nutrient solution used was that described previously in ZACHLEDER and ŠETLÍK (1982). Carbon dioxide concentration in the aerating gas mixture was 2 % (v/v). The culture vessels were submersed in a water bath at 30 °C (for

more details of the culture equipment see DOUCHA 1979). To be kept under optimal irradiance the cultures were continuously diluted using a dilution rate of 0.10 h^{-1}. The concentration of the culture was routinely assessed in terms of 'optical density' at 750 nm measured with SPEKOL 11 (Carl Zeiss Jena, GDR). The absorbance in the continuous culture at the beginning of the light period was set at 0.4, which corresponded to $4.5 \cdot 10^6$ cells per ml or to 0.3 g dry mass per l. With flow rate of the continuous culture of 0.1 h^{-1}, the absorbance increased in the first half of the light period, and around the 7th h of the cell cycle it attained a value of 0.6, then again slowly decreased. During the dark period the input of fresh nutrient medium was stopped and only samples were withdrawn. At the start of the next light period the culture was again diluted to the starting volume and density. The cultures were synchronized by alternating periods of 14 h light and 10 h darkness. Under this synchronization regime the cells divided into eight-celled daughter coenobia at the end of the cell cycle (DOUCHA 1979).

In nitrogen starved culture, KNO_3 was omitted and ammonia molybdate was replaced by an equimolar concentration of potassium molybdate. The cells were washed twice with N-free medium before nitrogen starvation started.

Assessment of Commitment Curves

To assess the commitment to nuclear and cellular division, samples were taken from synchronous cultures at one- or two-hour intervals and incubated under aerated conditions in the dark at 30 °C. After completion of all the division processes induced during the preceding light period, the percentage of binuclear daughter cells, two-, four- and eight-celled daughter coenobia, and undivided mother cells were estimated. The values obtained were plotted against the time of sampling.

The cell numbers were determined in a Bürker counting chamber. The nuclei were fluorochromed with acridine orange and observed through a fluorescent microscope using the method described by ZACHLEDER *et al.* (1974).

The Measurement of Irradiance

An energy proportional irradiance meter constructed to measure photosynthetically active radiation (PhAR — 400—700 nm) (KUBÍN 1971) was used to measure radiation (Wm^{-2}) at the surface of culture vessels (I_i) as well as radiation transmitted (I_t) through the suspensions. From these two values the mean irradiance was calculated according to the formula:

$$I = (I_i - I_t)/\ln (I_i/I_t).$$

Chemicals

All chemicals used for the analyses were of analytical grade. The DNA, RNA, casein, and albumine for calibration assays were obtained from Serva (Heidelberg, FRG). Other chemicals were supplied by Lachema (Praha, Czechoslovakia).

Biochemical Procedures

Total nucleic acids extraction was performed using a version of WANKA (1962) method as modified by LUKAVSKÝ *et al.* (1973). 10 ml samples of algal

cell suspension were spun down, and then extracted 5 times for 50 min at 20 °C with 0.2 N perchloric acid in 50 % ethanol, 3 times for 10 min at 70 °C by a mixture of ethanol and ether (3 : 1) and finally washed with ethanol (96 %). The pellet was treated with 0.5 N perchloric acid at 60 °C for 5 h to hydrolyze the nucleic acids into soluble fragments. The total concentration of (poly)-nucleotides was measured spectrophotometrically by determining the absorbance at 260 nm. The colorimetric reaction with diphenylamine (BURTON 1956) was used for DNA assay. The RNA content was calculated as a difference between the total nucleic acid content and the DNA content. The sediment after extraction of nucleic acids was used for protein determination after LOWRY *et al.* (1951). Using separate sample the starch content was determined by the method of MCCREADY *et al.* (1950).

RESULTS

The Effect of Nitrogen Withdrawal at the Beginning of the Cell Cycle

A population of daughter cells obtained from a continuous synchronous culture was divided into two parts. One was grown under standard conditions (control culture). The other was washed, transferred to a N-free medium and maintained under conditions similar to the control culture.

In the N-free medium, the reproduction processes were completely inhibited. No DNA replication (Fig. 2C) and no nuclear and cellular division (Fig. 1) took place for a period corresponding to the completion of the cell

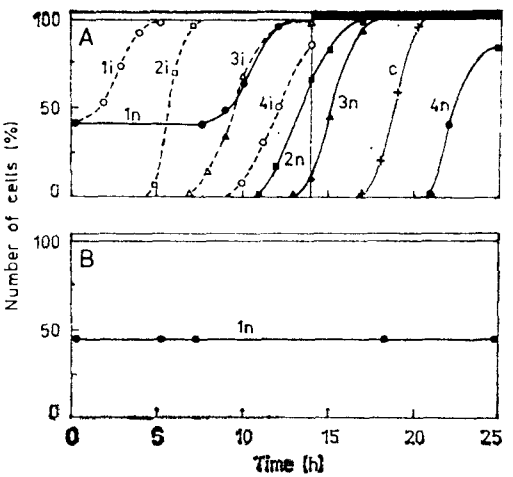

Fig. 1. Time courses of commitments to nuclear and cellular divisions and termination of these division processes during the first cell cycle in control (A) and nitrogen-starved (B) synchronous populations of *Scenedesmus quadricauda*. — Control culture conditions: mean irradiation 70 Wm^{-2} (PhAR), dilution rate 0.1 h^{-1}, nitrogen-starved culture: N-withdrawal at the beginning of the light period, no dilution during starvation. 1i, 2i, 3i, 4i: fraction of the cells attaining commitment to the first, second, third, and fourth nuclear division. 1n, 2n, 3n, 4n: fraction of the cells in which the first, second, third, and fourth nuclear division was terminated. c: fraction of the cells which released daughter coenobia. Light and dark periods are indicated by white and black strips at the top of panels.

cycle in the control culture. The RNA content in the cells declined steadily to about half of the initial level (Fig. 2A). A very slow but significant increase in the protein content was detectable up to the 12th h of the experiment (Fig. 2B). Consequently, the ratio of protein to RNA increased markedly (Fig. 2E). Photosynthetic starch accumulation proceeded at a rate similar to that in the control culture during the first half of the cycle, but then gradually levelled off, attaining about half the amount per cell characteristic of mother cells in the unlimited culture (Fig. 2D). Since there was no growth of the cells, the starch reserves were exceedingly high (the ratio of starch to protein amount was 7 as compared with 1.5 the normal cells).

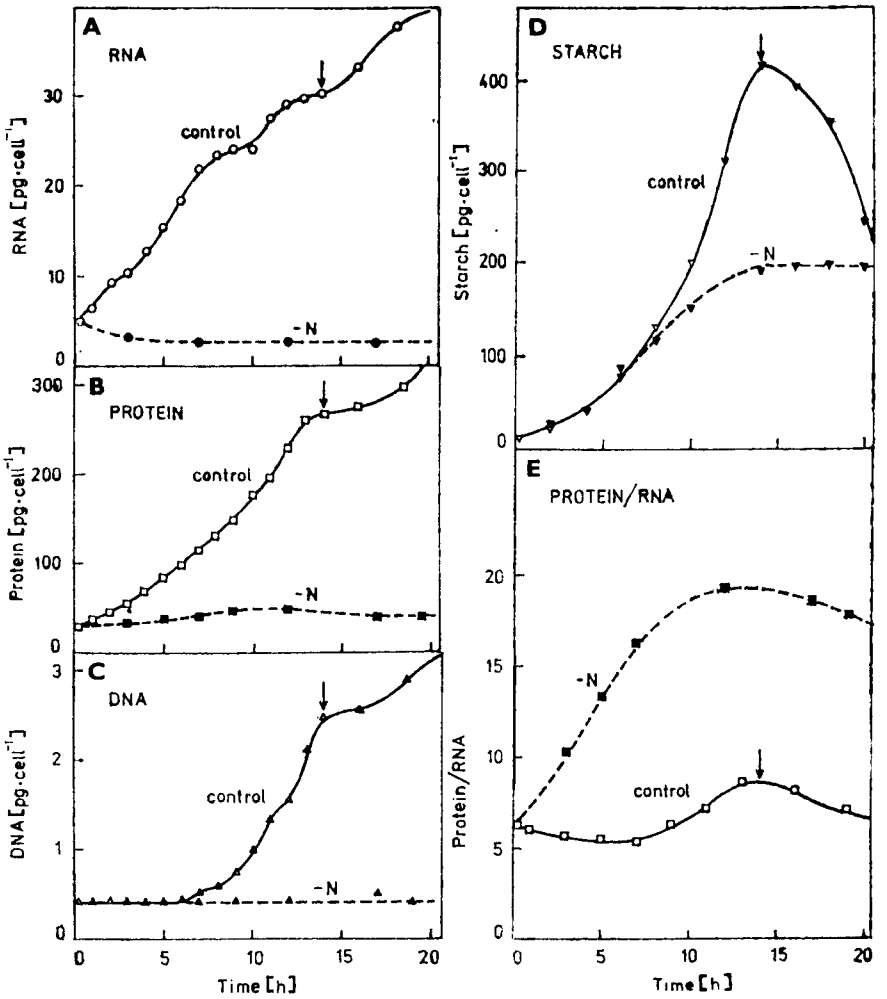

Fig. 2. Variation in RNA (A), protein (B), DNA (C), starch (D) content and in protein to RNA ratio (E) during the cell cycle in control (open symbols) and nitrogen-starved (closed symbols) synchronous populations of *Scenedesmus quadricauda*. — Culture conditions: see Fig. 1. The beginning of the dark period in the control culture is indicated by an arrow. The N-starved culture was kept in continuous light.

The Effect of Nitrogen Withdrawal at the Postcommitment Period of the Cell Cycle

The synchronous population was divided into three subpopulations at the point when most of the cells had reached the commitment point for division into four daughter cells (at the 6th hour of the cell cycle). One subpopulation was washed and its growth continued in N-free medium, the other was deprived of light. The third subpopulation served as a control. In the control

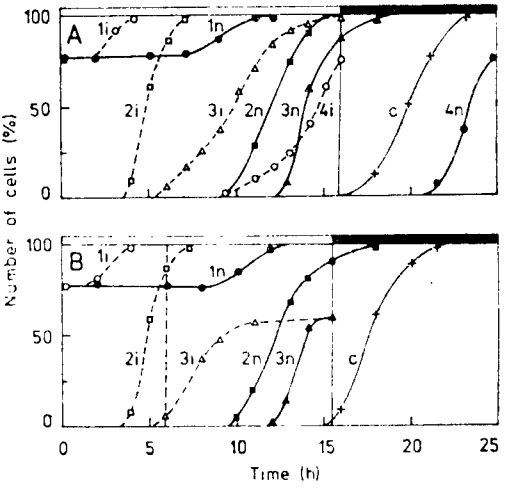

Fig. 3. Time courses of commitment to nuclear and cellular division and termination of division processes during the first cell cycle in control (A) and nitrogen-starved (B) synchronous populations of *Scenedesmus quadricauda*. — Culture conditions: mean irradiance 80 Wm^{-2} (PhAR), dilution rate 0.1 h^{-1}. Nitrogen was withdrawn at the 6th hour of light period (marked by a vertical dashed line). Symbols and labelling of curves: see Fig. 1.

culture, each cell yielded eight daughter cells and about 75 % of these became binuclear during the dark period (Fig. 3A). In the nitrogen-starved culture, all cells divided, but only about 50 % of them reached the third commitment point for division into 8 cells (Fig. 3B, curve 3i). The light-deprived cells divided exclusively into 4 daughter cells.

The synthesis of RNA stopped immediately or shortly after the cells had been deprived of nitrogen or light, but in nitrogen starved cells marked decline of RNA content per cell occurred (Fig. 4A). After nitrogen-deprivation, protein synthesis was unaffected for about 1 h, then it slowed down and eight hours later it stopped (Fig. 4B). In darkened populations the increase in protein stopped immediately. In both nitrogen-starved cells and light-deprived ones, DNA amount started to increase at the same time as in the control culture. In both treated subpopulations the number of DNA replications was reduced so that the final DNA content was proportional to the RNA content at the time of the initiation of DNA replication. There was no difference between the course of accumulation of starch content per cell in the control and nitrogen-deprived cultures (not shown in Figure).

It was desirable to know whether those reproduction processes to which the cells became committed in the complete medium would take place in the absence of nitrogen supply. The samples for the commitment assay were

therefore incubated in the dark both in a complete and a nitrogen-free medium. There was no difference between the two in the final result (Fig. 5).

Recovery from the Nitrogen-Starvation

The refeeding experiment was performed with daughter cells released from mother cells starved in the second third of the cell cycle (see Fig. 3). These

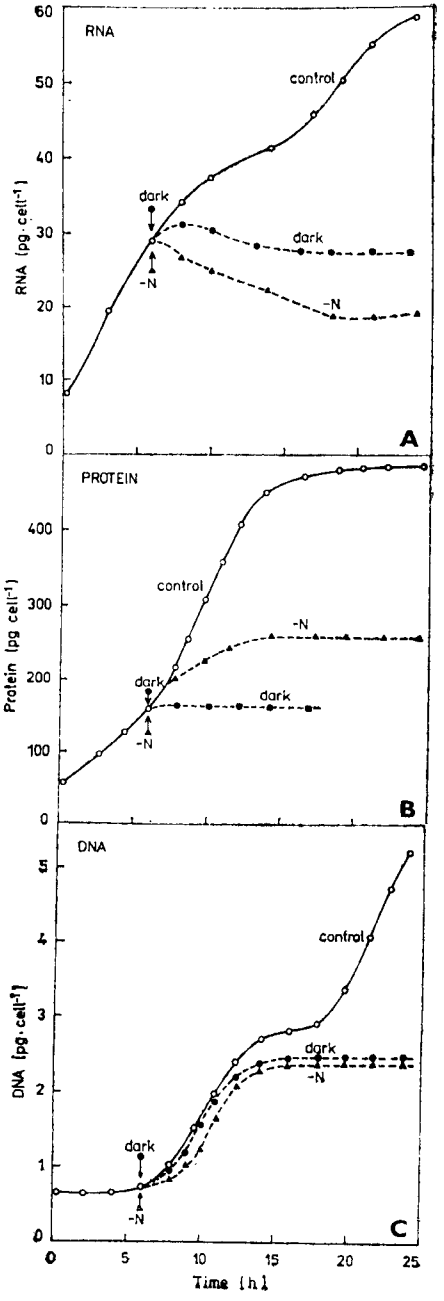

Fig. 4. Variation in RNA (A), protein (B) and DNA (C) content during the first cell cycle in control (open symbols) or light-deprived and nitrogen-starved (closed symbols) synchronous populations of *Scenedesmus quadricauda*. — Culture conditions: see Fig. 3. The times of nitrogen withdrawal and light deprivation in the nitrogen-starved culture and in light-deprived one and the beginning of the dark period in the control culture are indicated by arrows.

daughter cells were born uninuclear, unigenomic and with a very low RNA content per genome (2 pg per cell) while control daughter cells were mostly binuclear, bigenomic or tetragenomic, and their RNA content was 10 pg per cell.

When these daughter cells were exposed to light in a complete medium, only the RNA content increased during the first 5 hours (Fig. 6). After the

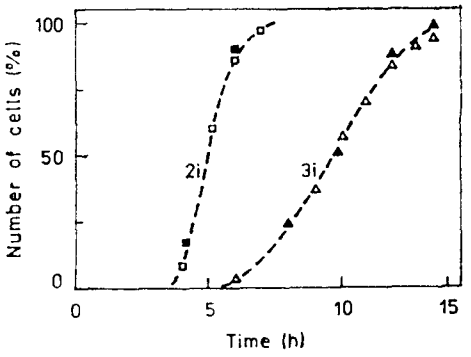

Fig. 5. Time courses of commitment to nuclear and cellular divisions in subcultures derived from a control synchronous population of *Scenedesmus quadricauda* and incubated in the dark in the complete medium (open symbols) and in the nitrogen-free medium (closed symbols). — Culture conditions and labelling of the curves: see Fig. 1.

normal ratio of protein to RNA content was restored in this way, the protein synthesis started. DNA replication was initiated as soon as the proper value of the RNA/DNA ratio was attained (the 8th h of the cell cycle) (Fig. 6). The division processes and their commitments were also triggered with delay (about 5 h) and only 50 % of cells succeeded in dividing into 8 daughter cells (Fig. 7).

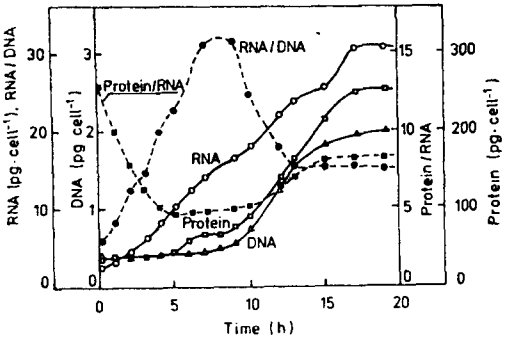

Fig. 6. Variations in RNA, protein, DNA content, RNA/DNA ratio and protein/RNA ratio in a nitrogen-refed synchronous population of *Scenedesmus quadricauda*. The nitrogen-starved daughter cells were transferred to the complete medium after a light period of 24 hours. Culture conditions: see Fig. 1.

DISCUSSION

The present findings showed that RNA was severely restricted as soon as the nitrogen was withdrawn from the nutrient medium, and a not negligible net protein increase occurred in nitrogen-starved algal cells. An amount equal to 80 % of the nitrogen present in the cells as protein is at the same time present in some other form utilizable for protein synthesis.

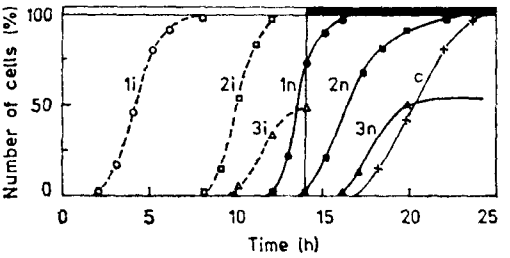

Fig. 7. Time courses of commitments to nuclear and cellular division and termination of division processes upon refeeding of a nitrogen-starved population of *Scenedesmus quadricauda*. The nitrogen-starved population was transferred to the complete medium after the dark period shown in Fig. 3. — Culture conditions: see Fig. 3. Symbols and labelling of the curves see Fig. 1.

We assume that the response of RNA must be controlled by a mechanism which ignores the total nitrogen reserves in the cells because the latter still permits protein synthesis. An indirect confirmation of our results can be found in the experiments of HASE *et al.* (1957), who also found that cells of *Chlorella* deprived of nitrogen from the start of the cycle were still able to double their volume and even to divide into two daughter cells.

Our results showed that all processes following the commitment point were independent not only of light energy input but oals of the external supply of nitrogen. If the hypothesis on the light control over RNA synthesis in *Scenedesmus* (ZACHLEDER and ŠETLÍK 1982) is correct, the same mechanism may be proposed for nitrogen control by nitrogen source withdrawal. In both cases, the mechanism controlling RNA synthesis must include monitoring presence of external sources of energy and nutrients since the deprivation neither of light nor of nitrogen in the medium immediately affect the energy and nitrogen reserves in the cell. Upon deprivation of both light and nitrogen, the RNA increase in the cell always stops much earlier than protein synthesis.

We found that in the population deprived of nitrogen, the decline in RNA content per cell is more pronounced than in the population kept in the dark. Nitrogen withdrawal in the light probably not only stops the synthesis of new RNA but also starts its degradation. An alternative possibility would be that only new synthesis is stopped and that the observed decline results from a rapid turnover of RNA occurring under all conditions. This is, however, rather improbable: a substantial part of the RNA degraded in our case is rRNA and SIERSMA and CHIANG (1971) have demonstrated by radioactive labelling that ribosomes as well as their RNA are conserved from generation to generation in the cells of *Chlamydomonas*.

RNA degradation accompanies the induction of gametogenesis in *Chlamydomonas* by nitrogen starvation (MARTIN *et al.* 1976, SIERSMA and CHIANG 1971). The mechanism by which this RNA degradation is triggered is probably very similar to that operating in *Scenedesmus*, although in the latter the formation of gametes is not induced.

Our results showed that much progress in the replication division sequence was attained by cells deprived of nitrogen and further exposed to light than in those that were deprived of light (samples for commitment assay). This can be interpreted (ZACHLEDER 1983) as due to that the protein level determines commitment to nuclear (and cellular) division. Protein synthesis continued after nitrogen withdrawal in the sixth hour of the cycle. Thus, the level of protein in cells gradually attained the values required for commitment to the last nuclear division.

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BOOK REVIEWS

IARC MONOGRAPHS ON THE EVALUATION OF THE CARCINOGENIC RISK OF CHEMICALS TO HUMANS. ALLYL COMPOUNDS, ALDEHYDES, EPOXIDES AND PEROXIDES. — International Agency for Research on Cancer, Lyon 1985. Volume 36. 369 pp. US. $. 25.00; Sw. fr. 70.—.

The 36th volume of Monographs published by IARC contains the evaluation of the genotoxic risk of four groups of chemicals: (i) Allyl compounds, (ii) Aldehydes, (iii) Epoxides and (iv) Peroxides. Several of the evaluated compounds occur naturally, *e.g.* allyl isothiocyanate and eugenol in plants, some of them in the polluted environment (acrolein, acetaldehyde), or are used in large quantities in the industry (allyl chloride, ethylene oxide, propylene oxide, styrene oxide, acetaldehyde, hydrogen peroxide). In addition, several of the evaluated compounds are present in the environment as metabolic products or as endogenous agents, *e.g.* acetaldehyde, hydrogene peroxide and malonaldehyde. Physical and chemical data, further data on production, use, occurrence, methods of analysis and biological data relevant to the evaluation of the genotoxic risks are presented for each of the compounds evaluated. The publication is complemented by a graphical representation of data issued from short-term tests. The tests are classified according to the end-point detected (DNA damage, mutation, chromosome aberration, cell transformation) and the test organism (prokaryots, fungi, green plants, mammalian cells *in vitro*, mammals and humans). Either the minimal effective dose or the maximal dose applied that produced genotoxic effects has been recorded. Readers will finds this new section of IARC Monographs called "Activity profiles for short term tests" very valuable.

T. GICHNER (*Praha*)

IARC MONOGRAPHS ON THE EVALUATION OF THE CARCINOGENIC RISK OF CHEMICALS TO HUMANS. SOME NATURALLY OCCURRING AND SYNTHETIC FOOD COMPONENTS, FUROCOUMARINS AND ULTRAVIOLET RADIATION. VOLUME 40. — International Agency for Research on Cancer, Lyon 1986. 444, pp. Sw. fr. 65.—.

The broad goal of the IARC publication is to collect data on the carcinogenic, mutagenic and teratogenic effects of environmental chemicals. The 40th volume of the IARC Monographs, published jointly with WHO, represents the views of a IARC Working group (Lyon 1985) on the carcinogenic and mutagenic risks of 4 groups of chemicals: 1. Naturally occurring toxins (Bracken fern, citrinin, patulin, rugulosin), 2. Food additives (benzyl acetate, butylated hydroxyanisole, butylated hydroxytoluene, potassium bromate), 3. Amino acid pyrolysis products in food, and 4. Various furocoumarins (*e.g.* angelicins and psoralens). For each compound reported, data on production, use, occurrence, further chemical and physical data and data relevant to evaluation of carcinogenic and mutagenic risks, are covered. Some of the compounds were reviewed in previous IARC Monographs, *e.g.* patulin was evaluated in Volume 10, pyrolysis products of tryptophane in Volume 31, and 8-methoxypsoralen in Volume 24. Most furocoumarins (*e.g.* psoralens) have limited genotoxicity in the dark. However, in the presence of ultraviolet A radiation (UVA) they are activated and can induce mono- and bi-adducts with DNA. Some of these compounds, *e.g.* 8-methoxypsoralen in conjuction with UVA, are used clinically. In the appendix, the reader can find brief information on various types of UV radiation and on the genotoxic effects of UV light alone.

The IARC Monographs should be available in all biological libraries providing information on the genotoxic and carcinogenic effects of environmental pollutants.

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