OSCILLATIONS IN PROTEIN AND RNA CONTENT DURING SYNCHRONOUS GROWTH OF *ACANTHAMOEBA CASTELLANII*

Evidence for periodic turnover of macromolecules during the cell cycle

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1. Introduction

The generally-accepted concept of cellular growth of microorganisms is that under ideal conditions, metabolic activity is entirely directed towards the biosynthesis of new cellular material and that only during growth at suboptimal conditions does turnover of existing material become significant [1]. Thus, during the growth and division cycles (cell-cycles) of many microorganisms, the synthesis of macromolecules such as protein [2] or RNA [3] have been reported to increase continuously except during certain periods of the cell cycle, e.g., during mitosis where synthesis may decrease or stop [4].

In contrast to these patterns of synthesis of macromolecules, periodic development of respiratory activity has been reported during the cell cycles of many eukaryotic microorganisms [5]. Oxygen uptake rates of synchronously-dividing cultures of the amoeba, *Acanthamoeba castellanii* doubled overall during 1 cell cycle, but rose to reach 7 discrete maximal values during this period [6]. Oscillations in the levels of adenine nucleotides, ATP/ADP ratios and adenylate energy charge values were also observed, and it was concluded that the overall changes in respiration rates reflected in vivo mitochondrial respiratory control, i.e. the control of respiration rate by the availability of ADP [7]. While these oscillations in levels of adenine nucleotides contravene the hypothesis of stabilized adenylate charge [8], such changes predict variations in the balance between energy-utilizing and energy-synthesizing pathways [8]. Also, the recent finding that the half-life of products of mitochondrial protein synthesis in *Saccharomyces cerevisiae* is ~60 min [9] open to question the belief that the sequence of events leading to the accumulation of new cellular material during rapid cell growth is entirely directed towards biosynthesis. This investigation reports the changes in RNA and total cell protein levels during synchronous growth of *A. castellanii* and shows that the cell cycle may be divided into a number of subcycles of biosynthesis and degradation in which energy-utilization is tightly coupled to energy generation.

2. Materials and methods

Acanthamoeba castellanii was grown axenically, with shaking at 30°C, exactly as in [10]. Organisms were counted in a Fuchs-Rosenthal haemocytometer slide (Baird and Tatlock, Chadwell Heath, Romford, Essex) after a suitable dilution with either fresh growth medium or 50 mM $MgCl_2$ (pH 7.4). Synchronous cultures were prepared by centrifugation of exponential cultures in sterile, stoppered tubes at 300 rev/min ($10 \times g$; r_{av} 10.0 cm) for 2 min in an MSE bench centrifuge [11]. After centrifugation the supernatant, containing the most slowly sedimenting population of cells (~10% of the original exponential culture) was decanted and grown as a synchronous culture. The degree of synchrony was assessed by the synchrony index (F) [12] where:

$$F = (N/N_0) - 2^{t/g}$$

F has a maximum value of 1.0 in a culture exhibiting theoretical perfect synchrony and 0 in an exponential

culture. N is the number of organisms at time g (the generation time, taken as that period from time zero to when all the cells had completed division), N_0 is the population before the commencement of cell division and t is the time interval from the initiation to the completion of cell division. Total cell protein was measured by the method in [13] using bovine serum albumin as standard. For RNA and DNA measurements cells were suspended in 0.15 M NaCl and sonicated for 1 min (20 kHz, 500 W MSE sonicator, power setting 5) before precipitation with trichloracetic acid (5% final conc.). Further extraction and estimation of RNA was by the orcinol method [13]. After the final hot trichloracetic acid extraction, the supernatant was assayed for RNA while the insoluble fraction was assayed for DNA by the fluorimetric method in [14]. Yeast RNA and calf thymus DNA, respectively, were used as standards.

Measurements of adenine nucleotides were as in [15] using luciferase assays on extracts from 1 ml culture prepared by chloroform extraction. ATP, ADP and AMP were estimated by the method in [16] using ATP as calibration standard.

Oxygen uptake measurements were made polarographically using a Clark electrode [17] on undiluted suspensions in growth medium.

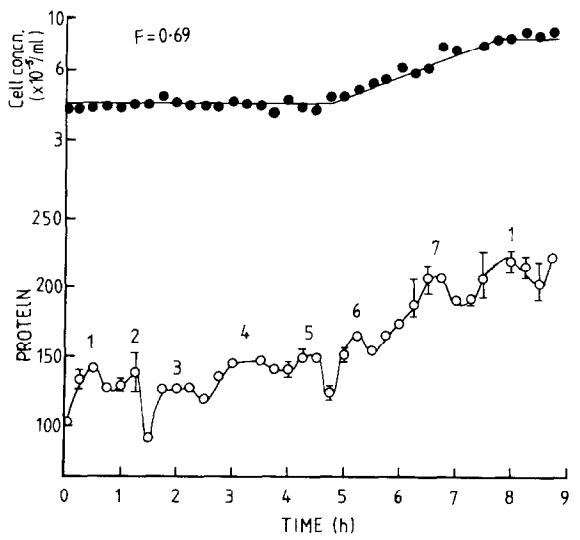

Fig.1. Changes in total protein during synchronous growth. The synchronous culture contained 6% of the exponential culture and shows (•) cell numbers with synchrony index F and (○) changes in total protein levels as μg/ml culture. Cell suspensions were washed twice with 10 vol. 50 mM $MgCl_2$ (pH 7.4) before estimation of total protein as in section 2. Each point is the mean value of 3 separate protein estimations on each sample and the range of values obtained are indicated where these are greater than the diameter of the symbol.

3. Results

3.1. *Changes in total cell protein, RNA and DNA during synchronous growth*

The changes in levels of total protein of a synchronously-dividing culture are shown in fig.1. While levels doubled overall during 1 cell cycle, the pattern of accumulation was discontinuous and levels oscillated to give 7 maxima/cell cycle. Maximal amplitudes (peak–trough, % minimal values) were 54% (fig.1). Similar changes in levels of RNA were also observed during synchronous growth (fig.2). RNA content/ml culture again doubled overall during 1 cell cycle but

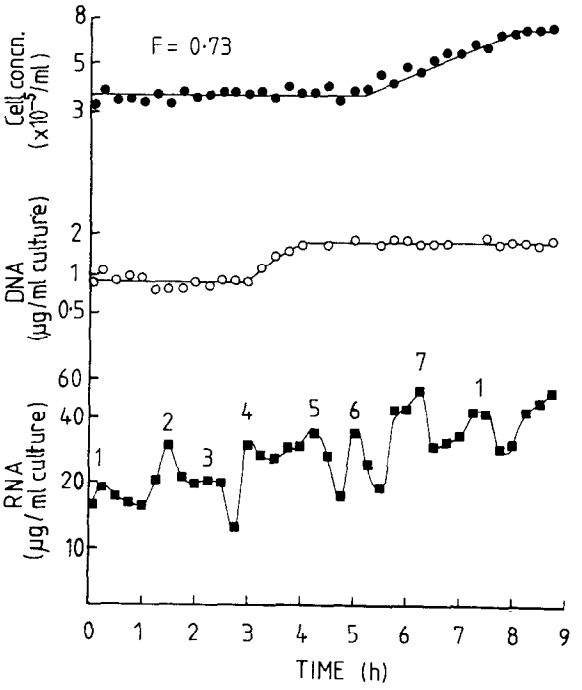

Fig.2. Changes in RNA and DNA levels during synchronous growth. The synchronous culture contained 12% of the exponential culture. (•) Cell numbers, with synchrony index F, (○) DNA and (▪) RNA. DNA and RNA were estimated as in section 2. After the final hot trichloracetic acid extraction the supernatant was assayed for RNA, while the insoluble fraction was assayed for DNA.

rose to reach 7 distinct maximal values during this period. The maximal amplitude (peak–trough, % minimal value) was 95%. After the final extraction of RNA by hot trichloracetic acid, the insoluble fraction was assayed for DNA. No oscillatory changes in this macromolecule were observed (fig.2); levels remained constant until 3 h growth and then doubled in amount by 4 h. The period of DNA synthesis occupied from 0.37–0.5 of the cell cycle time. In a similar experiment, identical changes in the levels of RNA were observed and the maximal amplitude (peak–trough, % minimal value) was 110%.

The values obtained for total protein and RNA amounts during synchronous growth, compare favourably with those obtained for exponential cultures (300–400 and 45 pg/cell, respectively) and to those obtained in [18]. However, direct comparison of values obtained is impossible due to variations of these 2 macromolecules during exponential growth [18,19].

In order to determine whether the above oscillations in protein and RNA levels were the result of adverse metabolic effects of the centrifugation conditions employed to produce the synchronous cultures, the control experiment shown in fig.3 was performed. An exponentially-growing culture was centrifuged under conditions identical with those employed to produce synchronous cultures, but after centrifugation the pellet and supernatant were remixed and the entire contents returned to the growth vessel. Cell numbers increased exponentially (mean generation time of 8 h) from time zero (fig.3) as did total cell protein and RNA levels. The maximal variations from the exponential increases for protein and RNA were 5% and 12%, respectively.

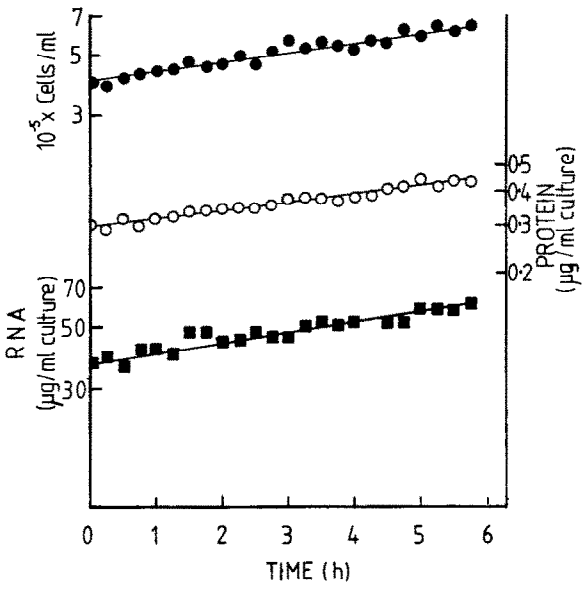

Fig.3. Changes in cell numbers, RNA and protein levels after centrifugation and resuspension of exponentially-growing cultures. An exponential culture was transferred to 4 sterile 50 ml stoppered tubes and centrifuged at 300 rev./min for 2 min in the 4 X 50 ml head of an MSE bench centrifuge. After centrifugation the pellet and supernatant were resuspended and returned to the growth vessel. At 15 min intervals samples were removed for cell counts (•) and measurements of RNA (▪) and protein (○) as in section 2.

3.2. *Timings of maxima of protein and RNA levels in relation to the cell cycle*

Oxygen uptake rates and levels of adenine nucleotides have been shown to oscillate during the cell cycle of *A. castellanii* [6] with periodicities similar to those reported here for RNA and protein amounts. The

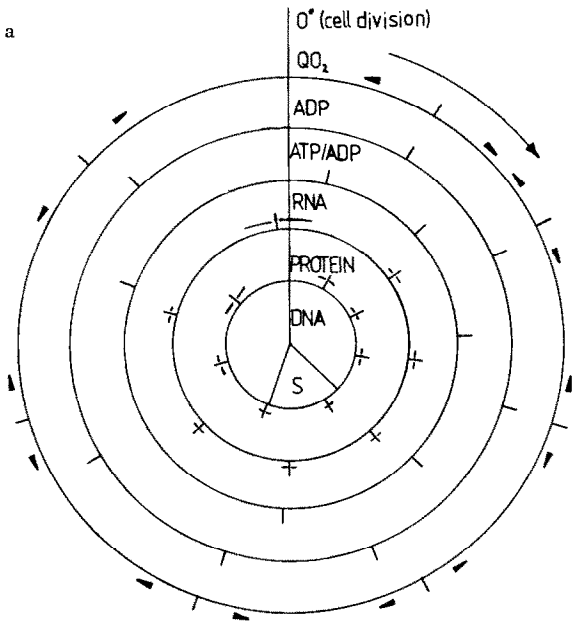

Fig.4a. Timings of cell cycle events in *A. castellanii*. The cell cycle is represented as a circle from 0° to 360°. 0° represents time zero in a synchronous culture and 360° the time when all the cells have completed division. Maxima of cell cycle events are thus shown as phase angles with respect to their timings in synchronous cultures. Data is included from [6] and the present report. Maxima of total protein (3 expt) and RNA (2 expt) are shown as means plus range of values obtained.

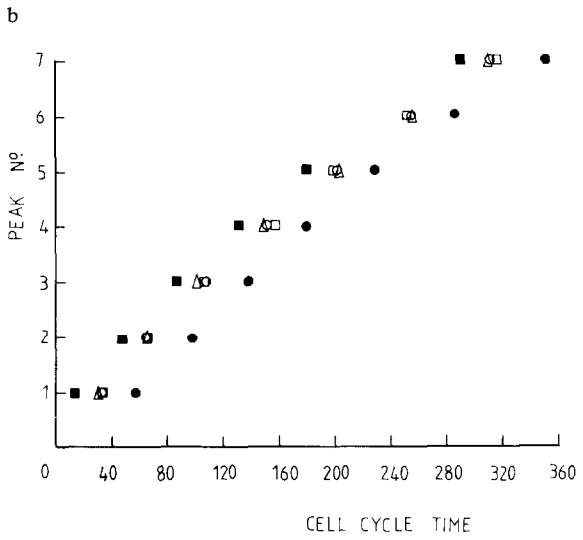

Fig.4b. Maxima of events occurring in synchronously dividing cultures as a function of normalized cell cycle time. The cell cycle is represented linearly from 0° (time zero in a synchronous culture) to 360° (the time when all the cells have completed division). Data is shown for respiration rate (•), ADP (□), ATP/ADP ratio (▪), RNA (○) and protein (△).

phase relationships between maxima of oxygen uptake rates, ADP and ATP/ADP ratios, together with the differential effects of uncouplers or inhibitors of respiration at respiratory maxima and minima, suggest that the overall changes in respiratory activity represent in vivo mitochondrial respiratory control. It was therefore of interest to compare the timings of maxima of RNA and protein levels to the reported respiratory oscillations [6]. Figure 4a shows that maximum levels of total protein correspond to those points in the cell cycle where respiration rates and ADP levels are highest whereas maxima of RNA amounts are out of phase with these but in phase with maximal ATP/ADP ratios.

A plot of peak number as a function of normalized cell cycle time (fig.4b) clarifies the regularity of the periodicities and emphasizes the similarities and differences in phase inter-dependencies.

In order to eliminate any errors resulting from possible variations in the timings of cell events between different experiments, all these parameters were measured during a portion of growth of a single synchronous culture (fig 5). This experiment clearly confirms the data presented in fig.4a.

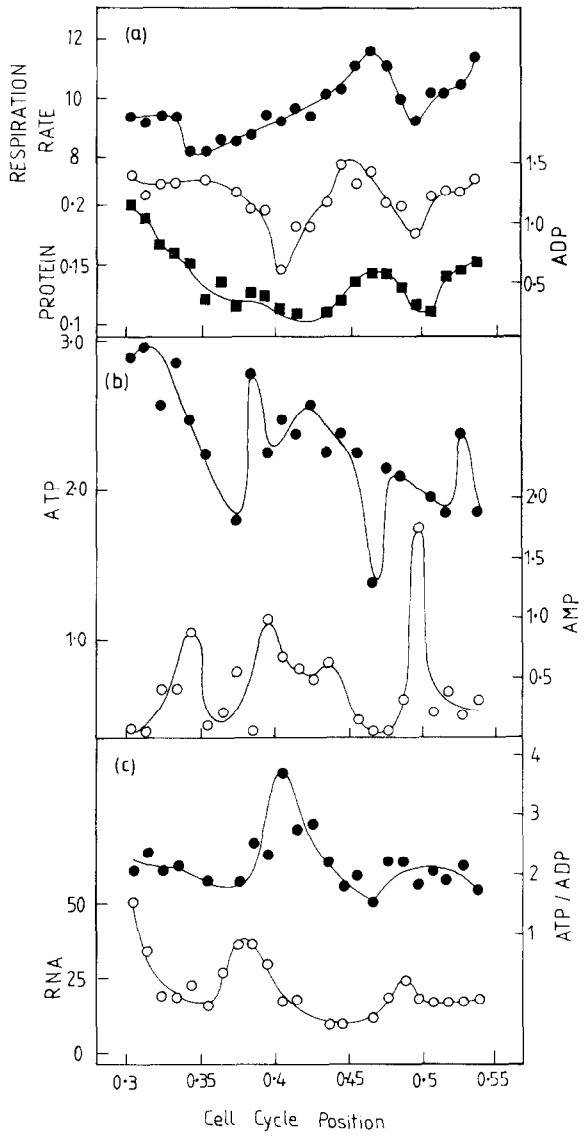

Fig.5. Changes in oxygen uptake rates, adenine nucleotide levels, RNA and protein levels during the cell-cycle of *A. castellanii*. A synchronous culture containing 13% of the exponential culture was prepared and growth followed for 9 h. After ~2.5 h growth, samples were removed at intervals for 2 h for measurements of oxygen uptake rates, adenine nucleotides, RNA and protein levels as in section 2. (a) Shows (•) respiration rates (nmol O_2/min/ml culture). (○) ADP concentration (nmol/ml culture) and (▪) total protein (mg/ml culture). (b) Shows (•) ATP levels and (○) AMP levels, both as nmol/ml culture. (c) Shows (•) ATP/ADP ratio and (○) RNA levels, (μg/ml culture). Data are presented in relation to the proportion of the cell cycle traversed at the time of sampling, assuming a linear representation of the cell cycle from 0–1.0. 0.1 of the cell cycle time represents 0.825 h.

4. Discussion

The patterns of synthesis of total protein and RNA during synchronous growth of *A. castellanii* described here are in contrast to the frequently reported descriptions of synthesis of macromolecules during the cell cycle (see section 1). Our observations suggest that under ideal growth conditions, turnover of cellular components is an important and necessary feature of the sequence of events occurring during growth and division of this organism. That the cell cycle of *A. castellanii* may be divided into a number of subcycles of biosynthesis and degradation opposes the idea that turnover is minimal in cells growing at their maximal growth rate [1]. Oscillations in mean cell volume and total protein, which were in phase with oscillations in activities of lactate dehydrogenase and glucose 6-phosphate dehydrogenase (3/cell cycle) have been reported during the cell cycle of colcemid-treated Chinese hamster cells [20]. These authors suggested that enzyme synthesis at discrete stages of the cell cycle was part of a more generalized phenomena affecting the whole population of proteins possibly initiated by increased synthesis of RNA. It would appear that a similar process may be present during the cell cycle of *A. castellanii.*

Any cell cycle-dependent variations in rate of synthesis or degradation will be time-averaged in an exponentially-growing population; rates of turnover would be underestimated using such a system, unless post-degradative reincorporation can be monitored, and the temporal organization can never be resolved. However calculations of turnover rates allowing for post-incorporation and recycling of radioactive label has led to the finding that in *S. cerevisiae*, the half-life of products of mitochondrial protein synthesis is much shorter than thought in [9], ~1 h. In many cases of reports of synthesis of RNA and total protein during the cell cycle, infrequent sampling may have resulted in over-simplified interpretation of observed patterns. In the present investigation, the generation time of 8 h (compared with ~1–3 h for many bacteria and yeasts) has facilitated fairly high resolution of the events occurring during the period of growth and division of *A. castellanii*. Also, processes of exocytosis [21] may have assisted in the detection of changes in subcellular organization resulting from alterations in metabolic activity. Alternatively, it may be that the periodic synthesis and degradation is the exception rather than the rule.

That the timings of maxima of total protein levels coincide with respiratory maxima (fig.4,5) is keeping with the idea that the changes in overall respiration rates result from in vivo respiratory control [6]. The periodicities of these oscillations suggest that they involve 'epigenetic' feedback loops [22], i.e., result from temporal changes in the control of gene expression and are thus distinct from oscillations of the 'metabolic' type which usually have periods of the order of minutes [23,24]. It can be concluded that the cell cycle-dependent oscillations are associated with the normal sequence of events during the cell cycle since they are not observed in synchronous cultures after exposure to identical centrifugation conditions; the maximal variations from ideal exponential increases for RNA and total protein are an order of magnitude less than the oscillations observed in synchronous cultures. Also, that RNA values oscillate in synchronous cultures where DNA levels show no oscillatory behaviour, but remain constant before and after the S-phase of DNA synthesis, eliminates the possibility that the susceptibility of these macromolecules to the extraction procedures varies during the cell cycle. It is also of interest to note that in cultures where cell division apparently takes ~2.5 h from initiation to completion, events with a periodicity of ~1.0 h^{-1} are observed. However, as reported [6] and as shown in fig.2, the period of DNA synthesis in these synchronous cultures is ~1 h. This clearly indicates that in this organism the degree of synchrony assessed by the direct observation of cell numbers, leads to synchrony indices that are grossly underestimated. The period of DNA synthesis therefore gives a more accurate representation of the degree of synchrony of these cultures than cell counts which involve a degree of indeterminacy. It is often assumed that the availability of energy (in the form of ATP) determines the direction of metabolism to either ATP-generating or ATP-utilising reactions [8,25,26] i.e., when ATP/ADP ratios are low, ATP-generating reactions predominate while when ATP/ADP ratios are high biosynthetic reactions are initiated. Thus, one would expect maximum rates of biosynthesis when ATP/ADP ratios are high and vice versa. But, during the cell cycle of *A. castellanii* maximal rates of protein synthesis occur when ATP/ADP ratios are low (fig.4,5). When protein levels are decreasing, by processes of degradation, the ATP/ADP ratios are high. This suggests that, in *A. castellanii,* the availability of energy does not dictate the rate of bio-

synthesis, but that the changing biosynthetic requirements of the cell result in the observed changes in ATP/ADP ratios.

Further work is necessary to determine:

1. The activities of individual enzymes during the cell cycle.
2. The amplitude of the oscillations that would be observed in a theoretically perfect synchronous culture, *i.e.* the amplitude observed in a single cell.
3. Whether the phenomenon of periodic turnover is restricted to *Acanthamoeba* or is of more widespread occurrence.

We may conclude, as in [27], that the basis of temporal control in the cell cycle of eukaryotes remains to be explained.

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References

[1] Bartley, W. and Birt, L. M. (1970) in: Essays in Cell Metabolism (Bartley, W. et al. eds) pp. 1–44, Wiley-Interscience, London.
[2] Prescott, D. M. (1955) Exp. Cell Res 9, 328–337.
[3] Keiding, J. and Andersen, H. A. (1978) J. Cell Sci. 31, 13–23.
[4] Kessler, D. (1967) Exp. Cell Res. 45, 676–680.
[5] Lloyd, D. (1974) The Mitochondria of Microorganisms, Academic Press, London, New York.
[6] Edwards, S. W. and Lloyd, D. (1978) J. Gen. Microbiol. 108, 197–204.
[7] Chance, B. and Williams, G. R. (1956) Adv. Enzymol. 17, 65–134.
[8] Chapman, A. G. and Atkinson, D. E. (1977) Adv. Microb. Physiol. 15, 253–306.
[9] Bakalkin, G. Y., Kalnov, S. L., Galkin, A. V., Zubatov, A. S. and Luzikov, V. N. (1978) Biochem. J. 170, 569–576.
[10] Edwards, S. W., Chagla, A. H., Griffiths, A. J. and Lloyd, D. (1977) Biochem. J. 168, 113–121.
[11] Chagla, A. H. and Griffiths, A. J. (1978) J. Gen. Microbiol. 108, 39–43.
[12] Blumenthal, A. K. and Zahler, S. A. (1962) Science 135, 724.
[13] Herbert, D., Phipps, P. J. and Strange, R. E. (1971) Methods Microbiol. 53, 209–344.
[14] Kissane, J. M. and Robins, E., (1958) J. Biol. Chem. 233, 184–188.
[15] Edwards, C., Statham, M. and Lloyd, D. (1975) J. Gen. Microbiol. 88, 141–152.
[16] Chapman, A. G., Fall, L. and Atkinson, D. E. (1971) J. Bacteriol. 108, 1072–1086.
[17] Lloyd, D. and Brookman, J. S. G. (1867) Biotechnol. Bioeng. 9, 271–272.
[18] Byers, T. J., Rudick, V. L. and Rudick, M. J. (1969) J. Protozool. 16, 693–699.
[19] Edwards, S. W. and Lloyd, D. (1977) J. Gen. Microbiol. 102, 135–144.
[20] Klevecz, R. R. and Ruddle, F. H. (1968) Science 159, 634–636.
[21] Pigon, A. (1976) Cytobiology 13, 107–117.
[22] Goodwin, B. C. (1976) Analytical Physiology of Cells and Developing Organisms, Academic Press, London, New York.
[23] Pye, K. and Chance, G. (1966) Proc. Natl. Acad. Sci. USA 55, 888–894.
[24] Hess, B. and Chance, B. (1978) Theor. Chem. 4, 159–179.
[25] Goldbeter, A. (1974) FEBS Lett. 43, 327–330.
[26] Sel'Kov, E. E. (1975) Eur. J. Biochem. 59, 151–157.
[27] Halvorson, H. O. (1977) in: Cell Differentiation in Microorganisms, Plants and Animals (Nover, L. and Mothes, K. eds) pp. 377–401, Gustav Fischer Verlag, Jena.