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Synthesis of Ribosomal Protein during the Cell Cycle of *Escherichia coli* B/r

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Summary. The rate of synthesis of ribosomal protein relative to total protein synthesis (α_r) was found to vary during the cell cycle of *Escherichia coli* B/r. It is greater than the average value in newly arisen daughter cells and below average, when cells near division.

The increase of α_r follows the initiation of chromosome replication and can be understood as a consequence of the duplication of ribosomal protein genes, since many of them are clustered relatively close to the origin of replication. The variations of α_r are being compared with the relative rate of fully induced β-galactosidase synthesis (α_{lac}) over the cell cycle, and possible constitutivity of ribosomal protein genes as proposed by Maaløe (1969) is discussed.

If the ribosomal efficiency is constant during the cell cycle, the observed variation of α_r implies that the increase in total protein deviates from an exponent by 3% over the cell cycle of *E. coli* B/r growing in glycerol medium.

Introduction

Maaløe (1969) proposed that the ribosomal protein "segment" of the bacterial genome is constitutive and that the variation in the fraction of total protein synthesis which is ribosomal (=relative rate of ribosomal protein synthesis, α_r; Schleif, 1967) as a function of growth rate could be due to variation in the overall repression pattern under different growth conditions. Variation in the entire set of repressions should result in different degrees of competition for transcription capacity between actively controlled and constitutive genes, i.e. the variation in α_r should be due to passive control of the ribosomal protein operon(s).

One of the predictions implicit in this model is that α_r varies with cell age under given growth conditions due to the changes in the relative dosage of ribosomal protein genes. Following their replication an abrupt increase and during subsequent growth, when all other genes double (see also Pritchard, 1973) a slow decrease of α_r is to be expected. Furthermore, the extent of variation over the cell cycle should correspond to the relative rate of synthesis from any consitutive gene or of any fully induced enzyme such as β-galactosidase, assuming that secondary controls like catabolite repression are not subject to variations over the cell cycle.

We have measured α_r as function of cell age and of the replication state of the chromosome in synchronous cultures of *Escherichia coli* B/r. α_r was determined in principle as described by Schleif (1967). Schleif had himself measured α_r during the cell cycle of *E. coli* B/r growing in glucose minimal medium without observing significant changes.

From the present analysis it appears that α_r does vary as function of cell age and with the replication state of the chromosome in cultures of *E. coli* B/r growing in glycerol minimal medium. The fluctuations of α_r correlate with the change of relative gene dosage for ribosomal proteins. Also, a comparison is made with the relative rate of fully induced β-galactosidase synthesis (α_{lac}) which we calculated from data which had been obtained in similar synchronous cultures by Pato and Glaser (1968).

Implications of the variation of α_r for the mass and volume growth of individual cells are discussed.

Materials and Methods[1]

a) Bacterial Strains and Growth Conditions

E. coli B/r (CP14, Clark and Maaløe, 1967) and a *thy*$^-$, *drm*$^-$ derivative (CP366, Zeuthen and Pato, 1971) were used in this work. For growth of CP366 all media were supplemented with 20 μg/ml of thymine, which is more than 10 times the concentration needed for its "normal" growth (Zaritsky and Pritchard, 1971; P. Meacock, pers. comm.). For each experiment a colony of the appropriate strain was picked from a glucose minimal medium plate and inoculated into glycerol (0.4%) minimal medium A + B (Clark and Maaløe, 1967). A series of dilutions from this culture was made with the same medium. The cultures were incubated overnight in a reciprocating water bath shaker at 37°C. One of the subcultures with an optical density between 0.02–0.04 was then followed for at least 3 doublings of mass before start of the experiment. Eppendorf spectrophotometer with a 436 nm filter was used.

b) Synchronization Procedure

Synchronous growth was achieved by the membrane selection technique developed by Helmstetter and Cummings (1963, 1964). A culture of the appropriate strain was grown as described above. At $A_{436} = 0.30$–0.35 cells from ca. 100 ml culture were collected on a Millipore membrane filter (142 mm diameter, 0.22 μm pore size) and washed with the same volume of fresh prewarmed medium. The filter holder was inverted and fresh prewarmed medium was pumped through the filter in the opposite direction at a rate of approximately 3.5 ml/min. After a period of 25 min of washing off loosely bound cells the eluate was collected for 70 min into an ice-cooled container. Then the cells were concentrated by filtration and resuspended in 30 ml of fresh medium at 37°C. The moment of resuspension was taken as time zero. The initial A_{436} was between 0.04–0.08, corresponding to 2–5×10^7 cells/ml.

The synchrony of chromosome replication was followed by measuring the relative rate of DNA synthesis. Samples of 100 μl of the culture were transferred at various times into small tubes containing ^{3}H-TdR (3.2 μC/0.32 μg/ml) in 100 μl of fresh medium at 37°C. The cells were killed after exactly 2 min by the addition of 3 ml of ice-cold TCA (5%). The precipitated cells were collected on membrane filter (25 mm diameter, 0.45 μm pore size), washed with 6 volumes of cold TCA and dried, and the incorporated radioactivity was determined by scintillation counting (Lindahl and Forchhammer, 1969).

c) The Measurement of α_r

The relative rate of *r*-protein synthesis to total protein synthesis (α_r) was determined by a modification of Schleif's technique (1967) developed by Peter Bennett (see Bennett and Maaløe, 1974). At various times 0.5 ml samples were pulselabelled with ^{3}H-leucine (10 μC/0.025 μg/ml) for one minute and the radioactivity incorporated into polypeptide chains was chased by continued incubation in presence of an excess of cold L-leucine (400 μg/ml) for 100 min into ribosomes and supernatant proteins, respectively. During this chase the RNA was labelled with ^{14}C-uracil (0.1 μC/0.2 μg/ml) for 50 min followed by a 50 min chase [^{12}C-uracil (400 μg/ml)]. The samples were then mixed with crushed ice, 1 ml NaN_3 (1 M) and 25 ml

1 *Abbreviations:* r ribosomal, TdR thymidine, TCA trichloroacetic acid.

of a culture of carrier cells with an $A_{436} = 1.0$–1.5. After centrifugation for 10 min at 10000 rpm the cell pellets were washed twice with TMK buffer (5 mM-TRIS, 10 mM-$MgSO_4$, and 60 mM-KCl, adjusted to pH 7.2 with HCl) and stored at $-70°$C. The pellets were later thawed and resuspended in TMK buffer to a final volume of 1 ml and the cells opened by sonication for 4×2 min intervals (MSE sonicator). 400 µl of sonicate was layered on top of a 10.5 ml D_2O-sucrose gradient [25–65% with a shelf (1.25 ml) of 80% sucrose] prepared in TMK buffer made with D_2O instead of water. The gradients were centrifuged for 15 hrs at 38K rpm (roter type SW41) and fractionated directly into 50 vials in 0.24 ml aliquots. Each aliquot was diluted with 1.5 ml of H_2O and then mixed thoroughly with 15 ml of scintillation fluid (as above) containing 30% (v/v) Triton X-100. The radioactivities were determined by scintillation counting.

By summing up all the ^3H counts over the whole gradient one obtains the ^3H-leucine counts incorporated into total protein (T) within a sample. The ^3H-leucine counts incorporated into the ribosomes (R) were estimated by the use of the $^3H/^{14}C$ ratio in the 70 S peak i.e. the lowest $^3H/^{14}C$ ratio and by multiplying it with the sum of the ^{14}C counts in rRNA over the 70 S peak and the 50 S and 30 S shoulders. Because $\alpha_r = R/T$,

$$\alpha_r = \frac{(^{14}\text{C-counts in rRNA}) \times (^3\text{H}/^{14}\text{C})_{70\,\text{S}}}{T} .$$

Results

E. coli B/r (CP14) grows in glycerol minimal medium with a mean generation time of 55–60 min (Zeuthen and Pato, 1971). The replication time of the chromosome is 45 min (Helmstetter and Cooper, 1968). Thus, chromosome replication occupies most of the division cycle under these conditions. The results of typical experiments in which the relative rate of DNA synthesis in synchronous glycerol cultures was measured are shown in Figs. 1 and 3. The first generation appears to be reasonably synchronous with respect to chromosome replication. The difference in relative rate of DNA synthesis between synchronous and random exponential culture may be seen by comparing Figs. 1 and 3 with Fig. 2, respectively. The synchrony achieved by the membrane selection technique (Helmstetter and Cummings, 1963, 1964) is not perfect, and there is a relatively wide distribution of generation times of individual cells (Powell, 1956, 1958; Kubitschek, 1971). Therefore, the initiation of chromosome replication seems to coincide with the termination of the previous replication cycle, which had been initiated one mass doubling earlier.

α_r, determined in such a synchronous culture (Fig. 1 b) has a maximum value of about 0.13 at the beginning of the cell cycle, falls between 10 and 40 min gradually to 0.10–0.11 and subsequently rises again. At about 70 min, i.e. one doubling time after the maximum at the beginning of the first division cycle α_r again reaches values in excess of 0.115 characteristic of batch cultures in this glycerol medium. The oscillations of α_r are reproducible in pattern and degree.

The following experiment and considerations show that the oscillation of α_r in the synchronous culture is not an artefact induced by the treatment of the cells, which included collection of baby cells in the cold, concentration by filtration and dilution into fresh medium. (a) DNA synthesis and α_r are not affected by subjecting random populations to the same treatment, i.e. the rate of DNA synthesis increases gradually (Fig. 2a), and α_r remains within the range of 0.105–0.115 and even more important, it is initially not increased (Fig. 2b).

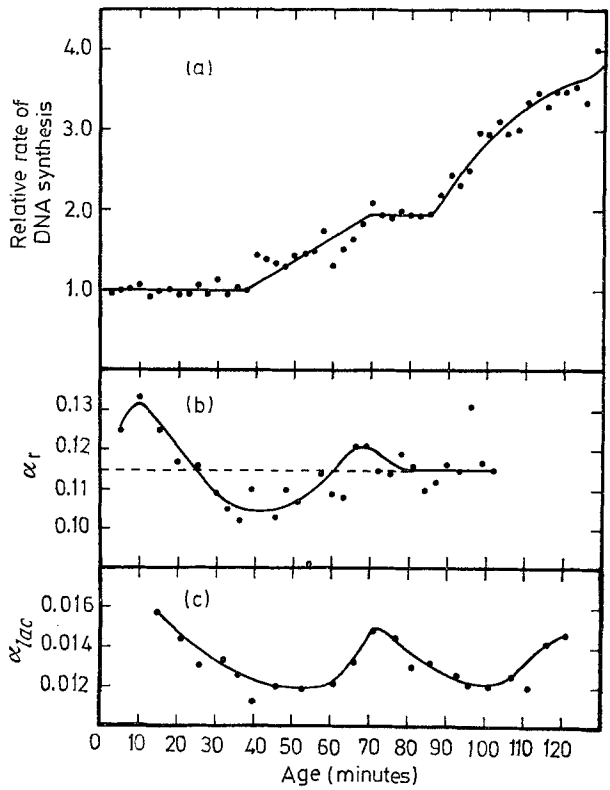

Fig. 1a—c. α_r, α_{lac} and rate of DNA synthesis during the cell cycle of *E. coli* B/r growing in glycerol minimal medium. a) The cts/min/2 min pulse of ^{3}H-TdR are normalized such that their average during the first 38 min gives a value of 1.0. b) The dashed line is the expected average ($\bar{\alpha}_r$) in a steady-state exponentially growing culture, based on the calculation:

$$\bar{\alpha}_r = \ln 2 \cdot \int_0^{60} \alpha_r(t) \cdot 2^{60-t} \cdot dt.$$

c) α_{lac}, the relative rate of β-galactosidase synthesis (as fraction of rate of total protefn synthesis) was *calculated* on basis of the data in Fig. 2 in Pato and Glaser (1968). The induction rates determined by them are taken as a measure for the rate of fully induced synthesis oi β-galactosidase (dE/dt). Total protein is assumed to increase exponentially over the cell cycle: $P(t) = P(o)e^{kt}$, ($k = \ln 2/\tau$), i.e. $dP/dt = P(o)ke^{kt}$. Division of dE/dt by the rate of total protein synthesis, dP/dt, yields dE/dP which is proportional to α_{lac}. Since 1–2% of total protein synthesis is synthesis of β-galactosidase in a fully induced glycerol culture we choose—to get approximate α_{lac} values—the first dE/dP value at 12 min to be 0.015 and adjusted the subsequent ratios accordingly

(b) Using the α_r values of the synchronous cultures (Fig. 1b) and the age distribution of the cells in a random population (Powell, 1956), an α_r value of 0.115 was calculated for a randomised populatíon. This is indistinguishable from the experimentally determined value for α_r in glycerol cultures (Fig. 2b; see also Bennett and Maaløe, 1974). This computation (legend to Fig. 1b) gives weight to each result according to the proportion of cells in a random culture that are in the corresponding age group.

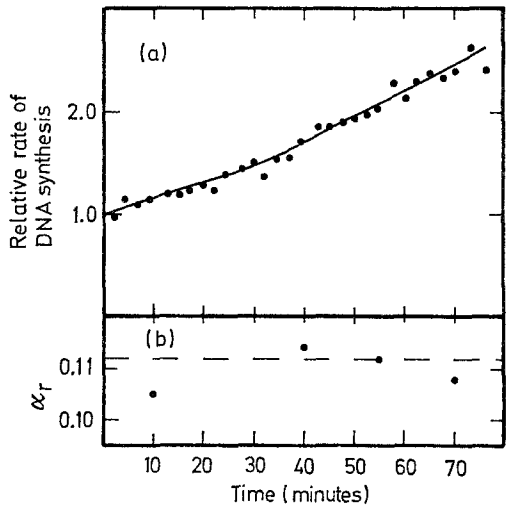

Fig. 2. α_r (b) and rate of DNA synthesis (a) in a steady-state culture of *E. coli* growing exponentially in glycerol minimal medium. The culture was treated in an analogous way as for synchrony, except that instead of collecting the cells on a membrane filter (see Materials and Methods) they were diluted 1:100 directly into ice-cold fresh medium

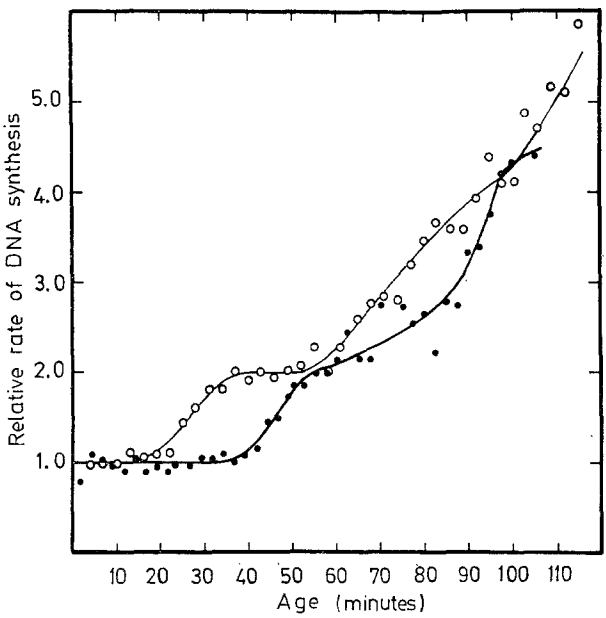

Fig. 3. The relative rate of DNA synthesis in synchronous cultures of *E. coli* B/r (CP14; closed circles) and its *thy*-derivative (CP366; open circles)

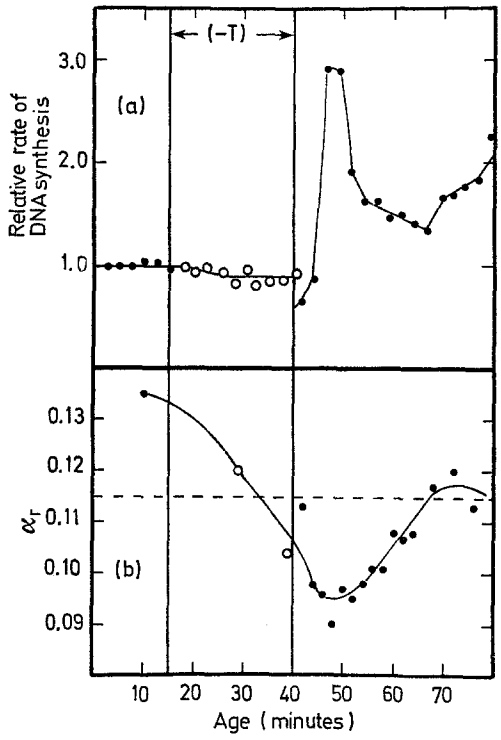

Fig. 4. α_r (b) and rate of DNA synthesis (a) in a synchronous culture of glycerol grown *E. coli* B/r (CP366) during thymine starvation between age 15 to 40 min and following the readdition of thymine at 40 min

To improve synchrony of chromosomal replication, a synchronous culture of a thymineless derivative (CP366) of *E. coli* B/r was thymine-starved just prior to initiation of chromosome replication. Since the replication time in a *thy*$^-$ strain is longer than in its *thy*$^+$ counterpart (Zaritsky and Pritchard, 1971), although the mean generation time is the same, it was necessary to establish the replication cycle in this particular strain. Fig. 3 shows that in strain CP366 initiation occurs ca. 20 min earlier than in the parent strain CP14. This indicates that the period from initiation of chromosome replication until the subsequent cell division [(C+D) Helmstetter *et al.*, 1968] is 20 min longer in CP366 than in CP14. Improved synchrony of chromosomal initiation was therefore achieved by depriving a synchronous population of CP366 of thymine at an age of 15 min restoring thymine 25 min later. During and following such a treatment of random, exponentially growing cultures of CP366 there was no significant change in polysome profile or in the rates of RNA and protein synthesis (A. Z., unpublished results).

Shortly after the readdition of thymine, the rate of DNA synthesis (2 min ^{3}H-TdR pulses) abruptly increased three fold, then dropped to twice the prestarvation value, indicating a synchronous initiation followed by the termination of the previous replication cycle (Fig. 4a). Using this method (synchronized replication after selection synchrony) the variation of α_r with age is greater (Fig. 4b).

From 50 to 70 min a well defined increase of α_r is observed from 0.09 to 0.12. The greater variation of α_r compares well to the very high degree of synchrony of initiation.

Discussion

a) Control of Ribosomal Protein Synthesis

It was recently shown that the replication of the bacterial chromosome is bidirectional (Masters and Broda, 1971; Bird *et al.*, 1972) and that the origin of replication is situated at about 74 min (Bird *et al.*, 1972) on the genetic map of *E. coli* (Taylor, 1970). Many of the *r*-protein genes have been mapped in the 62–64 min region (Takata, 1972). Assuming that most of the *r*-protein genes map close by and accepting bidirectional chromosome replication the dosage of *r*-protein genes, relative to all other genes, is expected to double ca. 10 min after replication is initiated.

As can be seen in Figs. 1a and 3, chromosome replication in *E. coli* B/r growing in glycerol minimal medium, is initiated at 45–55 min. Since the doubling time is 60 min, initiation of chromosome replication takes plac at about 10 min before physical division. Thus, in cells of age 0 (i.e. those collected by elution from the membrane filter) chromosome replication has proceeded about 20% (= 10 min). Thus, genes coding for *r*-proteins being 25% of a replication time away from the origin are just being replicated in these cells. At age 10 min α_r values are exceeding the average value considerably, then decrease and rise again one doubling time later (Fig. 1b); this finding *per se* is compatible with the prediction from Maaløe's proposal (1969) that *r*-protein genes are constitutive, namely that doubling of the genes will be expressed immediately and only later balanced out by the increase of the dose of other genes and changing repression pattern. A time delay of 5 to 10 min between the calculated replication time of *r*-protein genes and the maximum of α_r can be accounted for by transcription time and the time for effective increase of the corresponding messenger RNA concentration. The more diffuse initiation at age 50–55 min (Fig. 1a) is followed by a smaller peak of α_r at age 65–70 min, i.e. when the daughter cells are again 5–10 min of age. The lesser variation of α_r in the second generation most likely reflects loss of synchrony due to the relatively high coefficient of variation of inter-division times (Powell, 1956, 1958; Kubitschek, 1971).

A comparison can be made with the variations of the relative rate of synthesis of β-galactosidase (α_{lac}, Fig. 1c) which was calculated from the data of Pato and Glaser (1968) on the inducibility of β-galactosidase as function of cell age in glycerol cultures of *E. coli* B/r. The calculation was performed as described in the legend to Fig. 1c assuming that the rate of total protein synthesis is increasing truly exponentially over the cell cycle (see Discussion below). The oscillations of α_{lac} have a somewhat bigger amplitude and do apparently not damp out as rapidly (different degrees of synchrony?). The increase of α_{lac} at 65 min which reflects the doubling of the *lac* operon (Pato and Glaser, 1968) is about 5–10 min later than the one of α_r. From the map position of the *lac*-operon one should expect a 10–15 min time difference. This is, probably, only an apparent discrepancy since the rates of β-galactosidase synthesis were measured over 10 min periods (starting at the indicated times; Pato and Glaser, 1968) such that the actual values should

be delayed by about 5 min. The apparent lesser variation of α_r might indicate that a considerable fraction of the ribosomal protein genes are located outside the *strA* region or that there is one or another active control mechanism moderating the gene dosage response of α_r. Pattern and extent of the α_r variation are expected to be identical to the one of α_{lac} if the ribosomal genes were truly constitutive (Maaløe, 1969).

We would also like to indicate that Schleif's data on a culture in glucose minimal medium actually show a variation, though less expressed, of α_r over the cell cycle (Fig. 10, Schleif, 1967). α_r appears to increase at the time of division in two consecutive cell cycles. DNA replication in cells growing in glucose minimal medium (with a doubling time of 45–50 min) is initiated about 20 min before division (Clark and Maaløe, 1967; Helmstetter *et al.*, 1968). Thus, the increase of α_r seems to follow the expected doubling of the ribosomal protein genes also at the somewhat faster growth rate in glucose minimal medium.

The degree of synchrony achieved by thymine-starving the synchronous culture is such that all chromosomes initiate a round of replication during a short period of about 5 min (43–48 min of age, Fig. 4a). We find that α_r starts to increase 5 to 10 min later, and increases maximally at about 60 min, exceeding subsequently the mean value for a random culture (0.115, broken line in Fig. 4b). Its well defined increase from 0.09 to 0.12 between 10 and 30 min after the highly synchronous initiation, i.e. shortly after the presumptive doubling of the ribosomal protein genes, can be taken to reflect a gene dosage response due to constitutivity (Maaløe, 1969).

However, it should be noted that expression of gene dosage changes is one but not the only expectation for the behaviour of constitutive genes. It is certainly indicative of a relatively loose active control circuit, if any. Contrary to the variations of α_r demonstrated here, Matzura *et al.* (1973) could not detect any significant variation of the relative rate of RNA polymerase synthesis (α_P) over the cell cycle of *E. coli* B/r (in glucose medium) which suggests a tight control at the RNA polymerase "operon".

An important aspect of ribosomal biogenesis is the coordination between rRNA and *r*-protein synthesis. Dennis (1971, 1972) has indicated that the rate of RNA synthesis increases exponentially during the cell cycle in glucose as well as in succinate-grown cultures. If so, this suggests in conjunction with the present finding of an oscillating α_r in glycerol culture as well as in succinate-grown cells (A. Z., unpublished results) over the cell cycle that the coordination between rRNA and *r*-protein synthesis is not strict.

b) Rate of Protein Synthesis during the Cell Cycle

The ribosomal efficiency (polypeptide chain growth rate and fraction of ribosomes engaged in polysomes) appears to be nearly constant under various growth conditions which give widely different growth rates (e.g. Kjeldgaard and Kurland, 1963; Lindahl and Forchhammer, 1969; Forchhammer and Lindahl, 1971; and see for discussions: Maaløe and Kjeldgaard, 1966; Maaløe, 1969; Koch, 1970, 1971). If we assume that over the cell cycle (a) the ribosomal efficiency is also constant, (b) the ribosomal protein pool is small (as in random populations:

Schleif, 1967; Gupta and Singh, 1972; K. Gausing, 1974) and (c) thematuration time for ribosomal subunits is short (1–2 min in exponential cultures: Lindahl, 1973), then the results presented here imply that the rate of total cellular protein synthesis, dP/dt does not increase in a strict exponential fashion over the cell cycle as would be expected if α_r were constant.

Thus,

$$\frac{dP}{dt} = \gamma r(t) \tag{1}$$

where $r(t)$ is the amount of r-protein at time (t) and γ is a constant. The rate of r-protein synthesis,

$$\frac{dr}{dt} = \frac{dP}{dt} \cdot \frac{dr}{dP} = \frac{dP}{dt} \cdot \alpha_r \tag{2}$$

since α_r was defined (see Introduction) as $\frac{dr}{dP}$.

Substituting Eq. (1) in (2) gives

$$\frac{dr}{dt} = \gamma r(t)\, \alpha_r \tag{3}$$

$$\frac{dr}{r(t)} = \gamma \alpha_r\, dt. \tag{4}$$

On integration this gives

$$r(t) = r(0)\, e^{\gamma A(t)} \tag{5}$$

where

$$A(t) = \int_0^t \alpha_r(t)\, dt, \text{ and } r(0) \text{ is the value of } r(t) \text{ at } t = 0.$$

But

$$\frac{dP}{dt} = \gamma r(t) = \gamma r(0)\, e^{\gamma A(t)}.$$

Therefore,

$$P(t) = P(0) + \gamma r(0) \int_0^t e^{\gamma A(t)}\, dt. \tag{6}$$

Using Fourier analysis the data of Fig. 1 b have been fitted by a function with two harmonics to represent the variation of α_r with time. From this function $A(t)$ was computed[2].

The relative values of $P(t)$ for every $t = 1, 2, \ldots, 60$ min were computed substituting the numerical equivalents of the above parameters in Eq. (6). If these calculated values are compared with the values expected on the basis of exponential increase (α_r constant) a maximum difference in total protein per cell is exhibited at age 26–30 min in glycerol-grown cells such that the calculated

2 Fourier's analysis resolves every function into a series of oscillating sinusoidal functions of the form:

$$A_0 + A_1 \cdot \cos\left(\frac{2\pi t}{\tau} + \phi_1\right) + A_2 \cdot \cos\left(\frac{4\pi t}{\tau} + \phi_2\right) + \cdots.$$

In this case the best fit for the data of Fig. 1 is given by the following parameters:
$A_0 = 0.11468$, $A_1 = -0.01188$, $\phi_1 = -62.89$;
$A_2 = 0.00206$, $\phi_2 = -125.10$;
$A_0 = 0.11468$ is the average value of α_r taken over one generation time ($\tau = 60$ min).

value exceeds the value for exponential increase by 3%. A comparison between linear and exponential increase in total protein gives a maximum difference of 9%.

As previously stated (e.g. Kubitschek, 1968) it is not presently possible to distinguish experimentally between linear and exponential increase of the amount of cellular proteins. The results of the present analysis indicate that the difference that can be anticipated on basis of the observed variation of α_r is even smaller.

A question of particular interest is whether the density of the cell varies with age. Since the density depends on the cellular volume and mainly on the amount of protein and RNA present, prior knowledge of the rates of increase of these three variables during the cell cycle is needed.

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