OXYGEN AVAILABILITY AS A CONTROL FACTOR IN THE DENSITY-DEPENDENT REGULATION OF PROTEIN SYNTHESIS IN CELL CULTURE

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SUMMARY

Dilution of a density-inhibited Arachis culture results in a 10-fold increase in capacity for protein synthesis during the first 2 h after dilution. The limitation in the density-inhibited state is not inadequate nutrition, inappropriate pH, or a diffusible inhibitor as the dilution can be carried out in medium obtained by filtration of 14-day cells. The respiratory rate of the culture increases 2-fold immediately after dilution and the ATP level increases 3-fold during the 2-h period subsequent to dilution. These observations suggest that the initial function activated by dilution is an increased availability of oxygen and that this increase in oxygen provides an increased level of ATP, finally resulting in an increased rate of protein synthesis. This idea is further supported by the finding that both the increase in cellular ATP and the acceleration of the rate of protein synthesis can be obtained in dense culture, in the absence of dilution, by maintaining the cells for 2 h under oxygen.

INTRODUCTION

A common property of cells grown in culture is a reduction in the rate of proliferation as the density of the culture increases (Stoker & Rubin, 1967). Although a number of suggestions have been advanced in explanation of this phenomenon (Yeh & Fisher, 1969; Holley & Kierman, 1968; Cecarini & Eagle, 1971), a mechanism of general applicability has as yet not been defined. The approach more generally used in the analysis of control of cell proliferation has been the determination of the conditions required for resumption of cell growth. It appeared to us, however, that an analysis of early molecular events occurring when cells are released from density-dependent inhibition, coupled with an inquiry as to the minimal requirements needed to bring about these early events, would provide a more useful approach to identify the control mechanisms operative in the density-inhibited stationary state. It is possible, indeed probable, that the minimal requirements for changes in early molecular events would be insufficient for resumption of cell growth. Yet the changes in the early events are more likely to be closely related to the controls exerted in the density-inhibited state. Based on this rationale, we have undertaken an analysis of reactions occurring when stationary phase Arachis cells are released from density-dependent growth inhibition. In this communication we report a striking increase in

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the cellular capacity for protein synthesis during the first 2 h after culture dilution. A study of the minimal conditions required for this increase leads us to conclude that the availability of oxygen is a primary molecular factor utilized in the control of growth by cell density.

**MATERIALS AND METHODS**

The *Arachis* cell suspension culture obtained from V-56R peanut (*Arachis hypogaea*, L.) cotyledons (Verma & van Huystee, 1971) was carried as previously described (Verma & Marcus, 1973). Both for maintaining the culture and for experimental analyses, cells were diluted by inoculating 25 ml of a 14-day cell suspension into 225 ml of fresh medium. All other experimental procedures are described in the appropriate legends. The ATP analyses described in Tables 2 and 3 were kindly performed by Dr W. D. Yushok. The data presented are representative of at least 2 experiments giving similar results.

![Graph](image)

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**Fig. 1.** Increased rate of protein synthesis after dilution of the stationary phase culture. Stationary phase cells (containing $1.1 \times 10^9$ cells/ml) were diluted 10-fold in complete medium and incubated at 25 °C. At the times indicated, 10-ml aliquots of the cell suspension were incubated for 5 min with 0.1 µCi of $[^{14}\text{C}]$leucine (specific activity 300 mCi/mM). The cells were harvested on Miracloth, washed with water and homogenized in 5% trichloroacetic acid – 1 mM leucine. An aliquot was removed for analysis of 'uptake' (TCA-soluble radioactivity), and the pellet was rehomogenized in 5% trichloroacetic acid and analysed for hot TCA-insoluble radioactivity (Marcus, Feeley & Volcani, 1966). The 'uptake' data are shown in section A and the TCA-insoluble incorporation is presented in section B.
RESULTS

Growth of the *Arachis* suspension culture maintained on a 2-week cycle (Verma & Marcus, 1973), shows a 3-day lag, a short period of exponential growth (2–3 days), a 4- to 5-day period of sustained growth, and finally a 3- to 4-day stationary phase. In Table 1.

<table>
<thead>
<tr>
<th>Dilution medium</th>
<th>Rate of protein synthesis, cpn/10^6 cells, at 0, 30 and 90 min after dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete medium</td>
<td>3951 7300</td>
</tr>
<tr>
<td>Water only</td>
<td>2500 6032</td>
</tr>
<tr>
<td>Water + 2% mannitol</td>
<td>2900 7900</td>
</tr>
<tr>
<td>14-day medium†</td>
<td>2369 7875</td>
</tr>
</tbody>
</table>

* Rates of protein synthesis were measured as described in the legend to Fig. 1 after dilution in the various media.
† 14-day medium was obtained by filtering 14-day-old stationary phase cultures through a sintered glass funnel. The filtrate was used immediately after preparation.

Table 2. Rate of respiration as affected by dilution of stationary phase cultures

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Rate of respiration, µl O_2 consumed/min/10^6 cells</th>
<th>Intracellular ATP level, nmol/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stationary phase (undiluted)</td>
<td>0 6</td>
<td>—</td>
</tr>
<tr>
<td>Immediately after dilution</td>
<td>1 5</td>
<td>7 2</td>
</tr>
<tr>
<td>2 h after dilution</td>
<td>1 6</td>
<td>21 9</td>
</tr>
</tbody>
</table>

Ten millilitres of the indicated cell suspensions were incubated at 25 °C under constant stirring and the rate of oxygen uptake was followed for 5 min with an oxygen probe (Yellow Spring Instrument Co., model 5331). The amount of oxygen consumed per cell was calculated by using the Bunsen coefficient of oxygen in water (Umbreit, Burris & Stauffer, 1964). For measurement of intracellular level of ATP, 1 ml of settled cells (10⁶ cells) were extracted with 0 5 ml of 1 N perchloric acid and the insoluble material was removed by centrifugation. The extract was then neutralized to pH 6-8 with 1 N KOH; the resultant precipitate was again removed by centrifugation and an aliquot of the supernatant was assayed for ATP with hexokinase and glucose-6-P-dehydrogenase (Lamprecht & Trautschold, 1963).

earlier studies we observed that the capacity for synthesis of ribosomal RNA is delayed for about 24 h after dilution from stationary culture (Verma & Marcus, 1973). To ascertain what processes might be more closely related to the release from density-dependent inhibition, capacity for protein synthesis was measured in aliquots of the culture at various times after dilution. The results (Fig. 1) show that almost immediately after dilution there occurs a 10-fold increase in capacity for amino acid incorpora-
tion into protein. Analysis of the uptake of the radioactive amino acid into the soluble pool (Fig. 1A) as well as the levels of endogenous free amino acid (data not shown) indicate that both of these components are unaffected during the two hours in culture. Allowing an additional 2 h (i.e. incubation to 4 h) provides no further increase in capacity for amino acid incorporation. Thus the increase in protein-synthetic capacity appears to be a phenomenon closely related to the dilution of the density-inhibited culture.

Table 3. Effect of increased oxygen tension on protein synthesis capacity of stationary phase culture

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Rate of protein synthesis, cpm/10⁶ cells</th>
<th>Intracellular ATP level, nmol/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stationary phase - air (zero time after dilution)</td>
<td>1675</td>
<td>67</td>
</tr>
<tr>
<td>Stationary phase - air (2 h after dilution)</td>
<td>9937</td>
<td>219</td>
</tr>
<tr>
<td>Stationary phase - 2 h oxygen (zero time after dilution)</td>
<td>7098</td>
<td>151</td>
</tr>
</tbody>
</table>

Rates of protein synthesis were measured as described in the legend to Fig. 1 and ATP content was determined as in Table 2.

Table 1 shows the effect of varying the diluting medium on the increased capacity for protein synthesis. While dilution with water is less effective than that obtained with culture medium, the mannitol experiment (line 3) suggests that the medium requirement is solely for the maintenance of osmotic pressure. The most revealing observation is, however, that the dilution can be carried out in medium obtained by filtration of 14-day cells with full retention of the increase in protein-synthetic capacity. This result rules out inadequate nutrition, inappropriate pH, or a diffusible inhibitor, as controlling factors in the stationary phase. It suggests that the regulation of protein-synthetic capacity in the 14-day culture is a direct consequence of cell density per se.

Insight into the nature of the control in dense culture is provided by the data of Table 2. Immediately after dilution there occurs a greater than 2-fold increase in the respiratory rate. During the subsequent 2 h there is little further change. Analysis of the endogenous levels of ATP (Table 2) reveals a 3-fold increase subsequent to dilution, i.e. during the 2-h incubation. These data suggest that the initial result of dilution is an increased respiratory rate, presumably as a consequence of increased oxygen availability. The increase in respiration in turn provides an increased level of ATP, finally resulting in the increased rate of protein synthesis.

Further support for this contention is presented in Table 3. In this experiment, a stationary phase culture was maintained for 2 h under oxygen. The rationale of the experiment is that if oxygen availability is the component limiting the rate of protein synthesis in the stationary culture, this process should be accelerated by providing an oxygen atmosphere. The data show that the oxygen atmosphere is effective in increasing capacity for protein synthesis in dense culture. Just as in the dilution experiments, the cellular levels of ATP are increased concomitantly with the utilization of the oxygen atmosphere.
The studies presented attempt to distinguish processes involved in the initial release of the density-imposed stationary state from processes needed for subsequent cellular growth. The two most significant observations are the increase in ATP and the critical role of oxygen availability. The increase in ATP is a secondary consequence and its significance to growth control is in its relation to the increased protein-synthetic capacity. In further studies, we have examined in detail some of the molecular aspects of the 10-fold increase in protein-synthetic capacity (Verma & Marcus, 1974). There is a 3- to 4-fold increase in mRNA synthesis and in the appearance of this mRNA in polyribosomes, as well as a 2.5-fold decrease in 'transit time', i.e. the time required for the particular polyribosome population to complete a protein chain. This latter change is presumably due to an increased rate of both chain initiation and elongation. It appears possible that both of these processes, mRNA synthesis and mRNA translation, could be directly affected by the cellular levels of ATP. In the case of mRNA biosynthesis ATP is a precursor, thereby allowing direct substrate regulation and indeed, ATP has been shown to be a regulator of RNA synthesis in E. coli under conditions of phosphate starvation and recovery (Nazar, Tyfield & Wong, 1972). With regard to regulation at the level of translation, ATP functions in providing both aminoacyl tRNA and GTP, and in wheat embryo, ATP has been shown to be obligatory for chain initiation (Marcus, 1970; Seal, Bewley & Marcus, 1972).

The relationship between density inhibition, ATP level, and protein-synthetic capacity has also been noted in mammalian systems. In studies with 3T3 fibroblasts it has been reported that as the cells reach confluency there is a drop in amino acid incorporation and in polyribosome content to about one third of that in subconfluent cells (Hodgson & Fisher, 1971). A similar result has been reported for hepatoma cell cultures in stationary phase (Ward & Plagemann, 1969). Other studies (Freudenberg & Mager, 1971; Van Venrooij, Henshaw & Hirsch, 1972) have shown a strong correlation between cellular ATP content and capacity for protein synthesis. Thus the possible link between the cellular level of ATP, capacity for protein synthesis, and density-dependent growth inhibition, is not a restricted one. On the other hand, direct analysis of monolayer cultures of chick embryo and 3T3 cells (Colby & Edlin, 1970; Weber & Edlin, 1971), has shown that the ATP levels of density-inhibited cells and cells growing exponentially are similar. These results would suggest that the significance of the ATP level to growth control might apply only to cells growing in suspension culture. Alternatively, the control exerted by the level of cellular ATP might be related primarily not to the decreased rate of cell proliferation but rather to the maintenance of the stationary state. The decrease in ATP would then be seen only in cultures where a stable stationary state could be maintained.

The critical role of oxygen availability to growth of the Arachis culture is of primary significance. It seems not unreasonable that a decreased oxygen availability would occur with all cells growing in close proximity, independent of the cell type. Thus the observation that oxygen availability is a primary factor in density control may be of
general significance. The specific mechanism exerted by the decreased level of oxygen in maintaining the density-inhibited control may be a reduced respiratory capacity (see Table 2). Alternatively, a more complex process could be involved, e.g. the accumulation of lactic acid or the conversion of an enzymic component to a less active form under conditions of reduced oxygen (see Glinos, Werrlein & Papadopoulos, 1965). An understanding of the specific mechanism is of particular importance since it could provide information pertinent to the relative insensitivity of transformed mammalian cells to density-dependent inhibition. The Arachis cell culture might be a useful system for obtaining this information.

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