can survive extreme hypoxia for a long time in S phase are inactivated after a few h (18). Little is known about the reason for these differences in response to hypoxia throughout the cell cycle. One would, however, expect that in order to survive extreme hypoxia for a long time the cells would have to acquire a steady state condition with respect to growth and metabolism.

In order to investigate these problems we have constructed an experimental setup which enables us to manipulate our cell cultures under conditions of extreme hypoxia and to study protein metabolism (i.e., protein synthesis and protein degradation) under such conditions. Our aim is to measure protein synthesis and protein degradation as well as the total amount of protein during acute hypoxia and during the first few h after reaeration and relate this to the observed cell cycle progression.

MATERIALS AND METHODS

Cell Culturing Techniques. Cells of the continuous human line NHK 3025 originating from cervical carcinoma in situ (24, 25) were cultivated as monolayers as described previously (19, 25). Under optimal growth conditions these cells meet the requirement set up by Anderson et al. (26) for cells in balanced growth (27). Such growth conditions are obtained by use of medium E2a (28) supplemented with 20% human serum (prepared in the laboratory) and 10% horse serum (Gibco, Paisley, Scotland) and reculturing 3 times/week. Under these conditions the median cell cycle time is 18 h and the median durations of the various phases are: G1 ~ 6.5 h, S ~ 8 h, G2 ~ 2.5 h, and M ~ 1 h.

Maintenance of Hypoxia during Measurement of Protein Synthesis and Protein Degradation. Protein synthesis and degradation are measured as incorporation of radioactive valine into and release from protein, respectively (29). In order to measure these parameters for cells under extremely hypoxic conditions it is necessary to be able to remove and add medium without any introduction of oxygen. For this purpose we have constructed two metal chambers one of which is shown in Fig. 1.

A 5-way Hamilton valve (Bonaduz, Switzerland) was mounted on top of a nickel-plated brass lid. The lid was adjusted to fit a stainless steel chamber which has been described earlier (30) and originally used for maintaining extremely hypoxic conditions during irradiation. Deoxygenation took place by flushing the chamber with a gas mixture of 97% N2 and 3% CO2 containing <4 ppm O2 (31, 32). Four stainless steel hypodermic needles were inserted through the brass lid by use of O-ring sealed boxes. Inside the chamber, but attached to the lid, was a perforated metal tray with indentations adjusted for 3 Anumbra glass dishes (diameter, 8 cm) and 1 glass medium container. The cells were placed around the bottom of the Anumbra dishes covered with only a thin (~0.5 mm) (30) layer of medium. The medium to be added to the cells was kept in the glass container. To assure that this medium became hypoxic it was stirred by use of a glass-covered magnetic stirrer (31, 32). The hypodermic needles were pushed down to the bottom of the dishes, near the wall, all at the same side of the dish, so that the chamber was filled to this side, the dishes could be emptied completely by use of a syringe. After the medium had been removed from a dish new medium was pulled up from the glass container with a glass syringe and, after turning the handle for valve positioning, pushed into the dish. During this procedure 5 liters of N2/CO2 gas mixture were flushed through the chamber per min.

Procedure for Measurement of Protein Synthesis. Cells were grown for 2 or 3 days in normal air atmosphere in medium containing [14C]-valine (Amersham; CFB 75) of constant specific radioactivity (1.0 mm, 0.5 Ci/mol) so that all protein was labeled to saturation. The total
protein content was thus proportional to the amount of incorporated \(^{14}\text{C}\) radioactivity (29). On the day before the experiment the cells were seeded on the Anumbra glass dishes described earlier. Immediately before the start of flushing the medium was changed and 3 ml medium without \(^{14}\text{C}\)valine were added. Medium containing \(^{3}\text{H}\)valine (Amer- 
sham; TRK 533) of known specific radioactivity (1 mm, 20 Ci/mol) 
was added to the glass container.

The experimental timing of protein synthesis measurement during hypoxia is shown in the upper line of Fig. 2. After 2.75 h of flushing, the medium was removed from the 3 Anumbra dishes and 3 ml new 
hypoxic \(^{3}\text{H}\)valine-containing medium were added from the glass con-
tainer. The flushing went on for the whole pulse period of 1 h after 
which the chamber was opened, the medium was quickly removed from the Anumbra dishes, and the dishes were placed on ice. The cells were immediately precipitated with 5 ml cold 10% (w/v) PCA. After 15 min 
the dishes were washed 4 times with 2% PCA (8 ml) before the 
precipitate was dissolved in 3 ml 0.1 M NaOH/0.4% (w/v) sodiumdeoxycholate during 30 min incubation at 37°C. Aliquots (1 ml) were 
taken for measurement of radioactivity by simultaneous liquid scintillation counting. 

At the end of the release period the dishes were also placed on ice 
and the cells were precipitated with 5 ml cold 10% PCA. Total \(^{14}\text{C}\) radioactivity was determined as described above for protein synthesis. 
The rate of protein degradation was calculated as percentage per h as described earlier (33).

Flow Cytometric Measurement of DNA and Protein. Simultaneous 
measurements of the DNA and protein content in single cells were 
performed by two-parametric flow cytometry. The procedure has been 
described elsewhere (33). Briefly, the cells were suspended by trypsin 
treatment and fixed in 50% ethanol before the protein was stained 
overnight at room temperature with fluorescein isothiocyanate (0.1 ¿tg/
ml) in phosphate-buffered saline. Simultaneously the cells were treated 
overnight with RNase (60 ¿g/ml). Thereafter fluorescein isothiocyanate 
and RNase were removed and propidium iodide (17 ¿g/ml in phosph-
ate-buffered saline) was added to stain DNA. The samples were run 
in a laboratory-built flow cytometer of the type described earlier (34). 
The percentage of cells in the different phases of the cell cycle was 
determined by computer fitting of a mathematical model as described 
previously (27).

RESULTS

Protein Synthesis and Protein Degradation. Protein synthesis 
as well as protein degradation was measured in cells that were 
in exponential growth at the onset of hypoxia. Incorporation 
and release of radioactive valine under extremely hypoxic 
conditions were measured during a 1-h period starting 1.75 h after 
the start of flushing (Fig. 2). Similar measurements were also 
performed over a 1-h period starting 2 h after reaeration. Thus, 
in the last case the cells were extremely hypoxic for 3 h and 
then aerated for 2 h before measurement. The results are shown 
in Table 1.

For control cells the values of protein synthesis and protein 
degradation are close to those published previously for these

---

* The abbreviation used is: PCA, perchloric acid.
Table 1  Protein metabolism during and after acute hypoxia

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>During hypoxia*</th>
<th>After reaeration*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein synthesis (%/h)</td>
<td>5.11 ± 0.10</td>
<td>1.83 ± 0.21</td>
<td>5.42 ± 0.05</td>
</tr>
<tr>
<td>Protein degradation (%/h)</td>
<td>1.45 ± 0.10</td>
<td>1.93 ± 0.10</td>
<td>1.44 ± 0.10</td>
</tr>
<tr>
<td>Protein accumulation (%/h)</td>
<td>3.66 ± 0.10</td>
<td>-0.10 ± 0.12</td>
<td>3.98 ± 0.10</td>
</tr>
<tr>
<td>Protein-doubling time (h)</td>
<td>18.9 ± 0.60</td>
<td>17.4 ± 0.50</td>
<td></td>
</tr>
</tbody>
</table>

* Incorporation of [3H]valine and release of [14C]valine was measured over a period of 1 h starting 1.75 h after the start of flushing (see Fig. 2).

The DNA histogram in Fig. 4b has two peaks, the G1 peak and the S phase. In both cases 60,000 cells were analyzed. Protein was stained with fluorescein isothiocyanate and DNA was stained with propidium iodide.

Thus, since there is no increase in total protein content of the cell population during hypoxia (Table 1) while the cell number during hypoxia increases slightly due to cell division (Fig. 3a) the decrease in mean protein content per cell demonstrated in Fig. 3b is well explained.

Fig. 4 shows a two-parametric DNA-protein histogram for cells treated with extreme hypoxia for 3 h and then grown under aerated conditions for 3 h before the cells were fixed. While Fig. 4a is a contour plot representing an array of 64 × 64 channels Figs. 4b and 4d represent the corresponding DNA and protein histograms, respectively. Figs. 4c and 4e represent DNA and protein histograms, respectively, of the subpopulations defined by the numbered slices in Fig. 4a.
Table 2  Fraction of NHIK 3025 cells in various cell cycle phases as measured by analysis of flow cytometric DNA histograms

The control represents a standard cell population in continuous exponential growth. The cells denoted hypoxic were treated with extreme hypoxia for 3 h before they were fixed and stained for flow cytometric recording. The theoretical calculations are based on the supposition that during 3 h of extreme hypoxia no cells enter S from G1, there is no DNA synthesis, and no cells enter G2 from S. The fractions of cells in G1, S, and G2 + M were calculated for various cell cycle progression rates in G2 + M as described in the “Appendix.”

<table>
<thead>
<tr>
<th>Experimental results</th>
<th>G1 (%)</th>
<th>S (%)</th>
<th>G2 + M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerated cells (control)</td>
<td>49.0</td>
<td>28.5</td>
<td>22.5</td>
</tr>
<tr>
<td>Hypoxic cells</td>
<td>55.6</td>
<td>28.0</td>
<td>16.4</td>
</tr>
</tbody>
</table>

Theoretical calculations for various values of \( \alpha \)

<table>
<thead>
<tr>
<th>( \alpha )</th>
<th>G1 (%)</th>
<th>S (%)</th>
<th>G2 + M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>50.7</td>
<td>28.2</td>
<td>21.1</td>
</tr>
<tr>
<td>0.2</td>
<td>52.4</td>
<td>27.9</td>
<td>19.7</td>
</tr>
<tr>
<td>0.3</td>
<td>54.2</td>
<td>27.5</td>
<td>18.3</td>
</tr>
<tr>
<td>0.4</td>
<td>55.8</td>
<td>27.3</td>
<td>16.9</td>
</tr>
<tr>
<td>0.5</td>
<td>57.5</td>
<td>26.9</td>
<td>15.6</td>
</tr>
<tr>
<td>1.0</td>
<td>65.6</td>
<td>23.3</td>
<td>9.1</td>
</tr>
</tbody>
</table>

*Cell cycle progression rate in G2 + M under extremely hypoxic conditions relative to that under aerated conditions.

able to enter S as a synchronous subpopulation immediately after they are reaerated.

In Fig. 4c the DNA histogram of the synchronous subpopulation in S (Fig. 4a, Slice 4) is compared with that of cells in G1 (Fig. 4a, Slice 3). The DNA content of the synchronous cells is about 30% above that of the G1 cells, indicating that the synchronous cells have completed one-third of their total DNA synthesis during the 3-h period after reaeration. Thus, the rate of DNA synthesis of these cells equals that of untreated control cells since S phase lasts for 8 to 9 h for NHIK 3025 cells.

From Fig. 4e it is possible to compare the protein content of the subpopulation in S phase relative to that for cells in G1 since the line denoted by I represents the protein content of the cells in G1 (Fig. 4a, Slice 1) and the squares denoted by 2 represent the protein content of the synchronous subpopulation in S (Fig. 4a, Slice 2). Calculating the mean value of the two histograms in Fig. 4e, we find that subpopulation 2 has a mean protein content per cell about 25% above that of the G1 cells (subpopulation 1). Under normal, aerated growth conditions the amount of protein per cell would increase about 12.5% over a 3-h period (29). Thus, protein accumulation in these particular cells during the first 3 h after reaeration seems to be twice as high as in normal ones. This conclusion is based on the assumption that the protein content of cells in G1 after hypoxia is equal to that of G1 cells in the control. This is verified in Fig. 5 where the line represents Curve 1 in Fig. 4e while the squares represent the protein histogram of the G1 cells in the control. The present data thus indicate that the synchronized subpopulation entering S phase after reaeration not only has a rate of protein accumulation twice the normal rate but that they also have a higher protein content than normal.

**DISCUSSION**

Cell Cycle Progression during Acute Hypoxia. In our earlier studies (18, 19) we showed that cell cycle progression under extremely hypoxic conditions is zero for cells in S phase while cells in G1 to some extent progress to the G1-S border, where they are arrested until they become reaerated. These findings were in good agreement with other reports on different cell systems (12, 14) indicating that these effects are not confined only to a special cell type but may be common for mammalian cells.

From the present data in Table 2 the fraction of cells in G2 + M has, during extreme hypoxia, been reduced from 22.5% to 16.4%. This reduction is not fully explained by suggesting that...
only mitotic cells are able to progress during extreme hypoxia since NHIK 3025 cells in exponential growth (which is the situation at the onset of hypoxia) have a mitotic index of not more than 3% (27). Thus, the present results indicate that mitotic as well as G2 cells are able to progress under extremely hypoxic conditions. In a recent paper by Shrieve and Begg (21) on Chinese hamster V79-379A cells, a slight progression of late S-phase cells into G2 was observed after 6 and 12 h of extreme hypoxia. Whether any cells were able to divide during the first 3 h of extreme hypoxia was not clear from their data. However, their observation is interesting on the basis of our present results and may indicate that cells having completed a certain fraction of their cell cycle before the onset of hypoxia will be able to proceed slowly even during extreme hypoxia.

**Protein Metabolism during Acute Hypoxia.** Under extremely low concentrations of oxygen (<4 ppm O2) net protein accumulation as measured in the asynchronous cell population is zero (Table 1). As was explained in \("\text{Results}\) the flow cytometric recording of the protein content per cell after 3 h of extreme hypoxia supports this conclusion. One may notice, however, that we cannot from the present results exclude the possibility that a reduction in the protein content and thus a negative protein balance may take place under extremely hypoxic conditions for some of the cells, e.g., for cells in some specific part of the cell cycle.

Even under extremely hypoxic conditions there is a significant protein synthesis of slightly less than 2%/h as compared with about 5%/h under normal, well-aerated conditions (Table 1). This finding accords with that reported by Shrieve et al. (17) on EMT6/SF mouse mammary tumor cells, which showed that incorporation of radioactive amino acids under extremely hypoxic conditions was reduced to about 40% of the rate under normal well-oxygenated conditions.

The present results show, however, that under extremely hypoxic conditions the protein synthesis is balanced by an energy-consuming process the amount of energy used for this activity must be increased under hypoxic as compared with aerobic conditions. In a situation of acute energy shortage this would hardly occur unless protein degradation was of importance to cellular function or cell survival. From our previous studies showing that cells in S phase are readily inactivated under extremely hypoxic conditions (18) it is probable that cells during such hypoxia are protected by the arrest at the G1-S border. Therefore it is tempting to suggest that the increased protein degradation seen under extremely hypoxic conditions is afforded in order to induce this arrest, perhaps by specific degradation of factors initiating DNA synthesis (initiation factors) as well as other growth-related proteins.

**Protein Metabolism after Reaeration.** In our previous studies (18, 19) we showed that NHIK 3025 cells arrested at the G1-S border during 3 or 12 h of extremely hypoxic conditions will enter S phase immediately and proceed with a normal rate of DNA accumulation after reaeration. The present results as shown in Fig. 4c is in good agreement with these findings. Obviously our cells differ from the V79-379A cells studied by Shrieve and Begg (21) in this respect since in their experiment few G1 cells were able to enter S phase 4 h after reaeration following 12 h of extreme hypoxia.

During the first 3 h after reaeration the cells which were arrested at the G1-S border during extreme hypoxia accumulate twice as much protein as aerated control cells in exponential growth. The reason for the increased protein accumulation is not clear. However, we can exclude that these cells have a need to restore their overall protein content since it was shown that in these particular cells the total protein content 3 h after reaeration was above normal (comparison between Fig. 4c and Fig. 5). More likely, the cells have a need for specific proteins during the first hours after reaeration. What kind of proteins and for what purpose is at this stage a matter of speculation. An obvious possibility is, however, that these cells after reaeration would need initiation factors as well as other proteins of importance to cell growth. Due to the early initiation of DNA synthesis after reaeration one may in fact speculate whether these specific proteins are synthesized also during hypoxia. This would decrease the time needed to initiate DNA synthesis after reaeration but would imply that it would be necessary to degrade these proteins efficiently during hypoxia in order to stop initiation of DNA synthesis and thereby protect the cells against damage caused by the lack of oxygen. Thus, while specific degradation of initiation factors and other growth-related proteins might be the reason for the increased protein degradation observed under extremely hypoxic conditions, synthesis of the same proteins could be the reason for the abnormally high protein synthesis observed after reaeration.

**ACKNOWLEDGMENTS**

The skillful technical assistance of Charlotte Borka and Ursula Prehn Hansen is gratefully acknowledged.

**APPENDIX**

**Calculation of the Number of Cells That Have Completed G2 + M during 3 h of Extreme Hypoxia**

At the onset of extreme hypoxia our cells are distributed throughout the cell cycle according to the age-distribution function, \( f(t) \), valid for cells in exponential growth

\[
 f(t) = \frac{2N_r \ln 2}{\tau_c} \cdot e^{-\frac{\ln 2}{\tau_c} t} \tag{A}
\]

in which \( N \) is the total cell number, \( \tau_c \) is the cell cycle duration, and \( t \) is the time interval of hypoxia.

\[
 f(t) = \frac{2N_r \ln 2}{\tau_c} \cdot e^{-\frac{\ln 2}{\tau_c} t} \tag{A}
\]

for \( t \) in which \( N \) is the total cell number, \( \tau_c \) is the cell cycle duration, and \( t \) is the time interval of hypoxia.
is the time after start of cell cycle (i.e., start of $G_1$). During a time period of 3 h the number of cells that are able to divide in an aerated control ($N_3$) is

$$N_3 = \int_{t = 0}^{T_c} f(t) \, dt \quad (B)$$

If the cell cycle progression rate is a fraction $\alpha$ of that in an aerated control, the number of cells able to divide over a 3-h period is

$$N_{3\alpha} = \int_{t = 0}^{T_c} \alpha f(t) \, dt$$

Inserting Equation A for $f(t)$ gives

$$N_{3\alpha} = N_T \left( e^{-\frac{T_c}{2}} - 1 \right) \quad (C)$$

Since each of these cells form 2 new cells the new cell number ($N$) will be

$$N = N_T + N_{3\alpha} \quad (D)$$

Under extremely hypoxic conditions no recruitment takes place from $G_1$ to $S$ or from $S$ to $G_2$. For NHIK 3025 cells $G_2 + M$ lasts for more than 3 h. Thus the $N$ cells that divide come from the $G_2 + M$ fraction only, and therefore the number of cells in $G_2 + M$ is reduced with $N$. The number of cells in $G_1$ increase with $2 \times N$ and the number of cells in $S$ is the same as before hypoxia. The fraction of cells in $G_1$, $S$, and $G_2 + M$ after 3 h of extreme hypoxia is thus calculated for various values of $\alpha$ by using Equations C and D and using the fractions for aerated cells (Table 2, Line 1) as starting values.

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Regulation of Protein Metabolism of Human Cells during and after Acute Hypoxia

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