EFFECTS OF PUROMYCIN ON THE

NUCLEOPROTEINS OF THE HELA CELL

G. P. STUDZINSKI, Ph.D., and R. LOVE, M.D.

From the Department of Pathology, Jefferson Medical College, Philadelphia

ABSTRACT

The effects of several concentrations of puromycin on the nucleoproteins of HeLa cells grown in monolayers were studied by cytochemical and biochemical techniques. The earliest change at all concentrations of puromycin was a decrease in a granular form of ribonucleoprotein (RNP) that is demonstrable in the normal HeLa cell by the toluidine blue-molybdate (TBM) stain. The other types of RNP revealed by the TBM method were unaltered although the cell volume decreased markedly. Treatment with high concentrations of the antimetabolite resulted in pre-prophase inhibition of mitotic division and led to production of inclusions containing RNP in the cytoplasm; lower concentrations resulted in metaphase arrest. Biochemical analyses confirmed the cytochemical observations and indicated that synthesis of RNA and protein was inhibited to the same extent.

INTRODUCTION

Puromycin inhibits protein synthesis by causing premature release of incomplete polypeptide chains from the ribosomal complex (1, 17, 25, 33). The study of isolated ribosomal systems has shown that this action is direct (19), reversible, and rapid (17). In short term studies, puromycin has no effects other than the inhibition of the formation of protein; no effect on nucleic acid synthesis has been found (25). In growing cells, however, the inhibition of protein synthesis must eventually be followed by inhibition of the synthesis of nucleic acids, since unbalanced macromolecular metabolism cannot continue indefinitely (5). The interrelationship between the primary event of the inhibition of protein synthesis and the cessation of the formation of DNA in mammalian cells has been considered by Powell (24) and Mueller and co-workers (18). More recently, Tamaoki and Mueller (31) and Holland (7) reported the results of their studies on the inhibition of RNA synthesis secondary to puromycin action. It appears that the formation of ribosomal RNA is inhibited, while the production of transfer RNA and of a rapidly labeled fraction of nuclear RNA continues in the presence of puromycin; this fraction may be the "messenger" RNA (7) or a "ribosomal precursor" RNA (31).

In contrast to the wealth of data on the biochemical basis of puromycin action and its frequent use as a specific inhibitor of protein synthesis, cytological observations using this substance have been reported infrequently. Cobb (4) studied the inhibitory action of puromycin on primary explants of human carcinomas and noted cellular disintegration, loss of staining of the cytoplasm, and formation of cytoplasmic vacuoles which enclosed some eosinophilic material. Abnormal mitoses were not noted. Hultin (8) observed that the principal effect of puromycin on fertilized sea urchin eggs was inhibition of mitotic activity; the nucleus swelled but the spindle was not formed and the cell did not divide.

In the present study, an attempt is made to correlate cytological, cytochemical, and biochemi-

cal observations of HeLa cells treated with puromycin and to interpret the results in terms of the underlying fundamental events deduced from the known action of puromycin.

MATERIALS AND METHODS

Tissue Culture

A line of S3 HeLa cells was used in all experiments. Monolayers were grown in Eagle's minimal essential medium supplemented with 8 per cent horse serum, 1 per cent glutamine, and 0.02 per cent kanamycin or 0.005 per cent aureomycin. For most cytochemical and quantitative growth studies, cultures were grown on coverslips sealed to stainless steel rings with paraffin wax (32), and incubated at 37°C in a humid atmosphere of approximately 5 per cent CO_2 in air. When larger numbers of cells were required for chemical analyses, the cells were grown in Petri dishes (60 mm diameter), in some of which coverslips were placed to allow cytochemical examination of the cells in exact parallel with the biochemical analyses. After incubation for 3 days, to establish logarithmic growth, the medium was withdrawn and replaced in some cultures with fresh medium containing the appropriate concentration of puromycin, which was obtained as the dihydrochloride from Nutritional Biochemical Co., Cleveland, Ohio. Other cultures were refed with normal medium and served as controls.

For cell counts, the medium was removed and the cells were dispersed in warm Versene. An electronic counter (Coulter Electronics Co., Chicago) was used to count the cells at thresholds previously standardized by hemocytometer counts. Triplicate counts were performed as routine for each cell number determination and were rejected if the values diverged more than 5 per cent from the mean.

Cytochemistry

The cells were examined on the coverslips on which they were grown. Unfixed cells and smears of nuclei were observed under phase contrast. Cells were fixed for 5 minutes in formol sublimate for Feulgen staining (22). Ribonucleoproteins were stained by the toluidine blue–molybdate (TBM) procedure (13). The specificity of the method for RNP was checked by digestion for 2 hours with ribonuclease (Nutritional Biochemical Co.), 10 mg per cent in tris-(hydroxymethyl) aminomethane buffer. In addition, cells were stained with dilute toluidine blue as described for the demonstration of chromosomal RNA in reference 12.

Chemical Determinations

All operations were performed at 0°C. The cells were harvested by mechanical scraping in 0.14 M

NaCl and washed twice to free the cells of all traces of the medium by repeated centrifugation at 200 g for 10 minutes. The cells were then dispersed by trituration with a pipette, and small aliquots were taken for triplicate cell counts. Other triplicate samples were taken for estimation of protein content by the method of Lowry et al. (15). The remainder of the cell harvest was divided into triplicates for measurements of whole cell nucleic acids and for cell fractionation. Nucleic acids were separated by a modification of the Schmidt-Thannhauser procedure (6), scaled down to require only 0.5 to 1.0 × 10⁶ cells per assay. The DNA was measured by Burton's modification of the diphenylamine reaction (2), while the RNA content was obtained both by the optical density at 260 mμ and by the orcinol reaction (10).

Cell Fractionation

This was performed in two different ways.

(a) CITRIC ACID FRACTIONATION. Cells suspended in ice cold 2.5 per cent citric acid were vigorously shaken for a total of 90 seconds with intervals for cooling. The nuclei were centrifuged at 500 g for 10 minutes, and washed twice with cold 1 per cent citric acid (27).

(b) SUCROSE FRACTIONATION. The cells were homogenized, in a tightly fitting small homogenizer (teflon pestle, clearance 0.002 to 0.004 of an inch), in 0.25 M sucrose-3 mM $MgCl_2$. Ten up and down strokes were given. The nuclei were centrifuged and resuspended in the above medium containing 0.5 per cent Triton-X-100 (Rohm & Haas, Philadelphia). Addition of this detergent has been reported to give cleaner nuclei from rat liver (9). The homogenization was performed as above and repeated on the sediment obtained after centrifugation.

Nuclei obtained by either method were enumerated using the Coulter Counter at a threshold previously obtained by plotting size distribution of the nuclei. The purity of the nuclei and the absence of whole cells were checked visually by phase contrast microscopy and by staining a small drop of the nuclear suspension with 1 per cent toluidine blue. Contamination of nuclei by cytoplasmic fragments did not occur in nuclei prepared by the citric acid method. It was, however, frequent in nuclei separated by the sucrose procedure.

RESULTS

Effects of Puromycin on Cell Division

As the concentration of puromycin was increased above the level of 0.1 μg/ml (2 × 10⁻⁷ M), increasing inhibition of cell multiplication was observed (Fig. 1). With the lower range of concen-

trations (0.1 to 0.5 μg/ml), the inhibitory effect appeared to be more pronounced after the first 24 hours. This may have been due, in part, to the fact that some of the cells came off the glass and were removed with the medium before applying Versene for the cell count. The failure of cells to adhere to the glass was a constant effect of puromycin action and occurred within 24 hours with concen-

trations of more than 0.5 μg/ml, and after a longer delay, with lower doses (Fig. 1). There was, therefore, a considerable drop in the cell count when the puromycin-containing medium was replaced by normal medium 50 hours after the beginning of the experiment (Fig. 1). The cells remaining on the glass began to divide again after a delay of approximately 48 hours (Fig. 1).
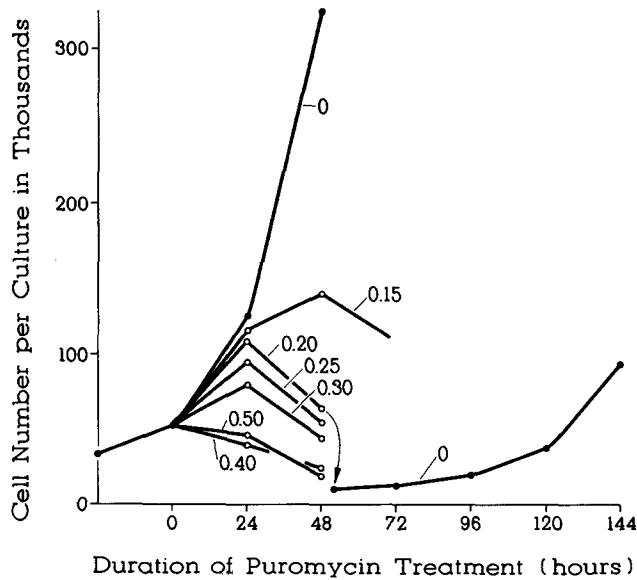

FIGURE 1 Effects of various concentrations of puromycin on multiplication of HeLa cells. The numbers on the graph indicate the puromycin concentration used in μg/ml. Cultures treated with 0.20 μg/ml were washed twice with normal medium at the point indicated by the arrow. Concentrations greater than 0.50 μg/ml had effects similar to those obtained wih 0.50 μg/ml.
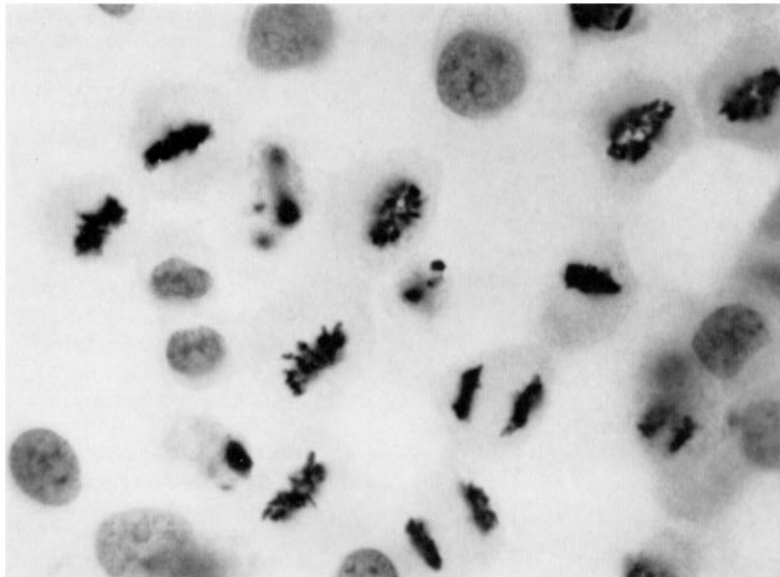

FIGURE 2 Cells treated with 0.15 μg/ml of puromycin for 24 hours. There is an accumulation of mitotic figures at all stages of division. Some metaphases show clumping, and some disintegrating cells are also present. Feulgen stain. × 1150.

Microscopical examination of Feulgen-stained cultures indicated that the early event responsible for retardation of culture growth by minimal concentrations of puromycin (0.1 to 0.2 μg/ml) was a general slowing of the mitotic process. This was followed in time by metaphase arrest. Fig. 2 illustrates a field from a culture treated with a low concentration of puromycin, selected to show the high accumulation of mitotic figures at all stages of the division process. The results of mitotic analyses presented in Fig. 3 showed that this was true of the culture as a whole, the principal effect being metaphase and telophase arrest. At somewhat higher concentrations of puromycin (0.2 to 1.0 μg/ml) no mitotic figures could be seen, i.e., there was complete preprophase block.

The metaphase arrest caused by low doses of puromycin resembled in many features that produced by colchicine. However, chromatin clumping with "ball" and "star" metaphases was particularly evident, and the picture was rather similar to the "initial C-mitosis" of Levan (11), generally thought to result from toxic action on the chromosome as well as interference with the spindle mechanism. Examination of the cells under phase contrast showed that the spindle fibrils were absent in the cells at this stage. The clumped metaphases appeared to degenerate and were seen in various stages of disintegration. A small number of cells with multipolar mitoses, chromosome bridges, and lagging chromosomes were observed
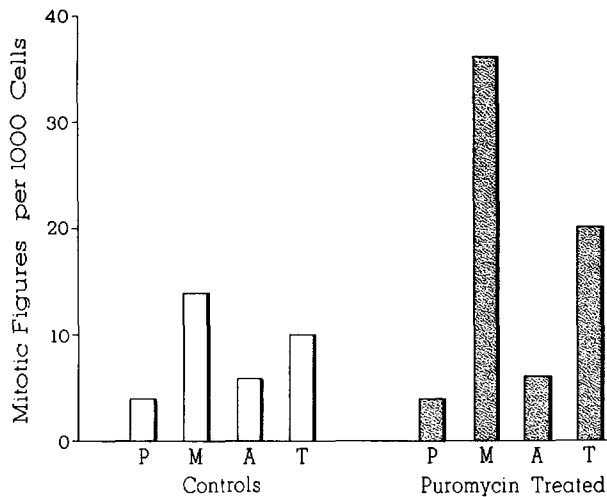

FIGURE 3 Frequency distribution of mitotic figures in an untreated culture and in puromycin-treated culture stained by the Feulgen method (0.15 μg/ml for 24 hours). P = prophase, M = metaphase, A = anaphase, T = telophase.
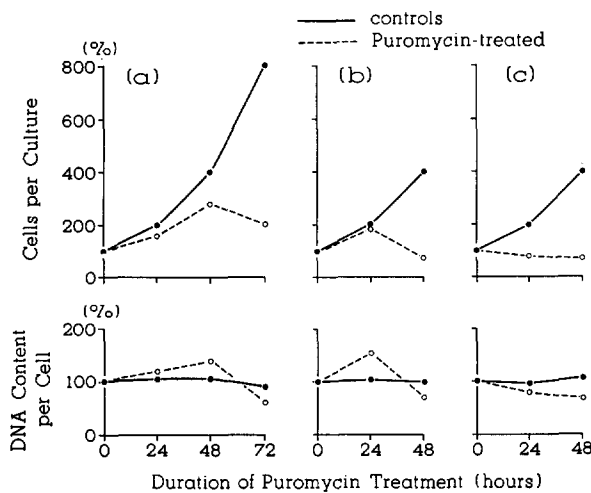

FIGURE 4 Comparison of cell number per culture with mean cellular DNA content. Puromycin was present at the following concentrations (a) 0.15 μg/ml, (b) 0.30 μg/ml, (c) 0.40 μg/ml. The results are expressed as percentage of the initial value which was around 16 μg/10⁶ cells. Statistical tests showed that the values for the cell DNA content differed significantly from the controls ($P < 0.05$) after 48 and 72 hours of treatment in all groups, and also after 24 hours treatment with 0.30 μg/ml ($P < 0.01$). Microscopical examination of puromycin-treated cultures showed that metaphase arrest was most marked at the point of time at which the cell number per culture was maximal.

with equal frequency in treated and control cultures.

An attempt was made to supplement the microscopical observations of metaphase arrest, followed by prophase block, with analysis of changes in the cell DNA content as measured by chemical methods. Fig. 4 shows that the mean cell DNA content varied with the stage of inhibition of culture growth. Low concentrations of puromycin (0.1 to 0.3 μg/ml), which allowed suboptimal culture growth, initially produced an increase in content of DNA per cell reaching a maximum of about 140 per cent of the mean DNA content of logarithmic cultures (Fig. 4 a and b). Microscopical examination at this stage revealed a striking increase in the number of metaphases (Fig. 2). At later stages of inhibition with low concentrations of puromycin (Fig. 4 a and b), or after shorter exposure to higher doses of the inhibitor (Fig. 4 c), the decrease in the cell number per culture was accompanied by a decrease in the cell DNA content (approximately 70 per cent of the original value). The magnitude of these changes implies that at first the cells were arrested in the G2 period as well as in metaphase, and that subsequently such arrested cells disintegrated or came off the glass, leaving behind a population of cells which had not completed DNA synthesis.

Effects of Puromycin on the HeLa Cell in Interphase

(a) The Cytoplasm

The earliest cytochemical effect of puromycin was a decrease in the staining of the discrete cytoplasmic RNP granules which could be visualized in the cytoplasm by TBM (method D) (cf. Figs. 5 and 6). This decrease was frequently marked (Fig. 6), and, when the dose was high or the treatment prolonged, the granules disappeared completely (Fig. 7). The intensity of staining of the diffuse RNP of the cytoplasm stained by the other methods of the TBM procedure was not reduced (Figs. 8 to 10). It may be noted, however, that the actual amount of stained material in each treated cell appeared to be less than in control cells, since the cytoplasm was rounder and decidedly smaller. The fact that puromycin treatment did, indeed, lead to a gradual decrease of cell volume is illustrated in Fig. 11. Cytoplasmic fragmentation and "blebbing" was seen frequently in puromycin-treated cultures.

Loss of cytoplasmic fragments would have the effect of reducing the cytoplasmic volume. In addition, an increased proportion of cells in G1 period would tend to contribute to a reduction in over-all cell size.

Cytoplasmic "inclusions" were seen only after treatment with concentrations of puromycin greater than 5 μg/ml (Fig. 12). Even prolonged treatment with smaller doses did not produce such formations. These cytoplasmic particles were composed principally of RNP since they were not stained by the TBM method after digestion with ribonuclease.

(b) The Nucleus

The nucleoproteins of the interphase nucleus appeared to be structurally more resistant to puromycin than the cytoplasm of the HeLa cell. All forms of RNP demonstrable by the TBM method, in the nucleus were unaltered despite the presence of marked cytoplasmic changes. In particular, the nucleolus retained its structural detail up to the point of total nuclear disintegration. Nucleolini were visualized in the nucleoli even after treatment for 48 hours (Figs. 9 and 10). In contrast to the results of treatment with other inhibitors (3, 14), these nucleolini showed no departure from normality, appearing discrete and showing undiminished metachromasia. Staining by TBM (method C), which most clearly demonstrates the pars amorpha of the nucleolus, showed that this type of nucleolar RNP also appears unaffected by puromycin treatment (Fig. 13).

(c) Biochemical Measurements of the Effects of Puromycin on RNA and Protein Content of the Cell

Biochemical analyses for DNA, RNA, and protein were performed at the same times at which cells were also taken for morphological observations of the effects of puromycin. Such analyses confirmed the inhibitory effect of puromycin on protein synthesis and allowed a correlation to be made between RNA content and TBM staining. The results of DNA measurement have been described above. The inhibition of RNA formation in cultures treated with 0.40 μg/ml of puromycin was proportional to the inhibition of protein synthesis (Fig. 14). When RNA and protein values were expressed as mean cell content, as seen from Table I, the amount of RNA and protein per cell decreased markedly during puromycin action, the

magnitude of the decrease depending on the length of treatment. It was also observed that the nuclei of puromycin-treated cells were more fragile during cell fractionation procedures; in Table I, nuclear content is, therefore, expressed in terms of counted nuclei after fractionation. As indicated, the nuclear content of RNA and protein was less affected by puromycin treatment than the cytoplasmic components. Since preparation of nuclei in citric acid leads to a loss of nuclear protein, these experiments were repeated isolating the nuclei in sucrose; the results were analogous to those shown in Table I.

In view of the fact that a striking increase in acid-soluble nucleosides and nucleotides was found when nucleic acid synthesis was inhibited by 5-fluorodeoxyuridine (30), the acid-soluble pool was measured in the puromycin-treated cells. The results presented in Fig. 15 indicate that this did not take place when the inhibition was due to puromycin. On the contrary, there was a decrease in the amount of these substances, probably as a result of the diminished volume of the treated cells.

DISCUSSION

Study of the cell's reaction to noxious agents may provide answers to some of the basic questions in biology. Since puromycin has a clearly defined action on protein metabolism, it is possible to attempt an interpretation of the observed morpho-

logical changes in terms of molecular events. Thus, the dramatic decrease in cytoplasmic granular RNP may be viewed in the light of the observation of Noll and colleagues that puromycin action is accompanied by accelerated breakdown of poly-ribosomes to their constituent monomers (20). Furthermore, Zimmerman found that puromycin can prevent formation of ribosome clusters in the HeLa cell (34). Rather similar is the finding of Marks *et al.* (16) that the decrease in ability to synthesize protein in the maturing reticulocyte is associated with a diminished content of polyribo-somes. Polyribosomes have been found biochemically in the HeLa cell (23, 34). The present finding of the decrease in cytoplasmic granular RNP after treatment with puromycin can, therefore, be interpreted as indicating breakdown of the poly-ribosomes to the monomers. However, the above interpretation is not the only possible one; it may be that the decrease in the total RNA per cell (Table I) creates conditions unfavorable for the formation of cytoplasmic granules during the staining procedure; *e.g.*, a certain minimum number of ribosomes per cell may be necessary to produce the granular type of staining.

Puromycin treatment causes less morphological change in the interphase nucleus than in the cytoplasm, and analyses show that the nuclear content of RNA and protein decreases less rapidly than the cytoplasmic RNA and protein (Table I). These

FIGURE 5   Untreated HeLa S3 cells stained by TBM method D. Note prominent cytoplasmic granules of ribonucleoprotein. × 1150.

FIGURE 6   Effect on the cytoplasmic granular RNP of 0.20 µg/ml of puromycin acting for 24 hours. TBM method D. × 1150.

FIGURE 7   0.40 µg/ml of puromycin for 24 hours. Note the complete absence of cytoplasmic granular RNP. TBM method D. × 1150.

FIGURE 8   Untreated HeLa cells stained by TBM method B, showing nucleolini (arrows) within the pars amorpha of the nucleolus. Cytoplasmic ribonucleoproteins stain diffusely in this method. × 1150.

FIGURE 9   Cells treated with 0.30 µg/ml of puromycin for 24 hours. Cells appear to be smaller and rounder but the intensity of cytoplasmic staining is not diminished. The appearance of the nucleolus is unchanged and the nucleolini can be clearly distinguished (arrows). TBM method B. × 1150.

FIGURE 10   Cells treated with 0.30 µg/ml of puromycin for 48 hours. Despite marked degenerative changes present in many cells at this stage of treatment, the few cells in structurally normal interphase still have apparently normal nucleoli with nucleolini (arrow). The cytoplasm stains with normal intensity. TBM method B. × 1150.
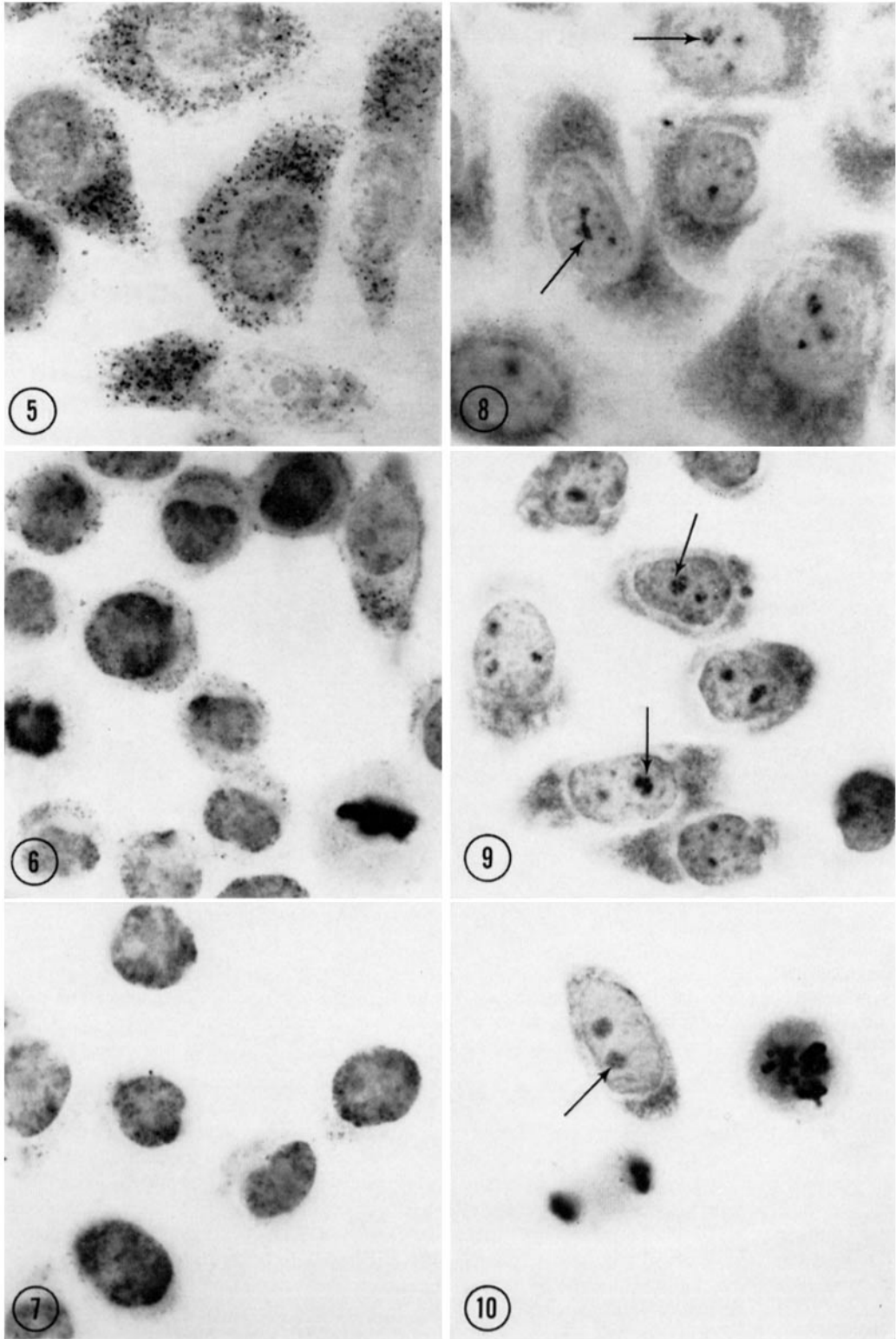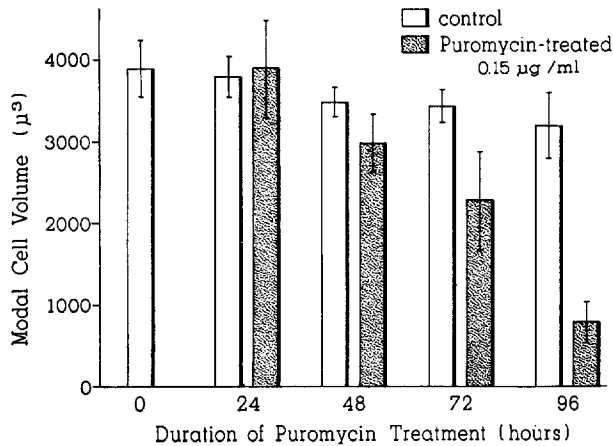
FIGURE 11 Effect of puromycin treatment on cell volume. Modal values at each time of treatment are expressed as the mean and standard deviation of 3 experiments.
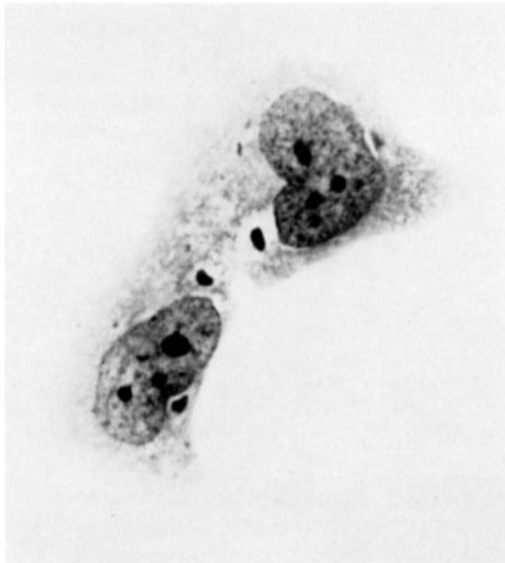

FIGURE 12 HeLa cells treated with 50 μg/ml of puromycin for 20 hours, showing large cytoplasmic inclusions of RNP. Cells containing cytoplasmic inclusions generally appear larger than other cells. Dilute toluidine blue. × 1150.

FIGURE 13 TBM method C demonstrating normal pars amorpha of the nucleoli in puromycin-treated cells (0.40 μg/ml for 24 hours). × 1150.

observations can be explained by slower turnover of nuclear protein, but it is possible also that protein synthesis in the nucleus proceeds, at least in part, by a pathway insensitive to puromycin, perhaps of the type suggested by Stone and Joshi (29). In either case, the unaltered nucleolar substructure shows that puromycin-sensitive protein synthesis is not essential for the preservation of nucleolar integrity. This is in sharp contrast to the
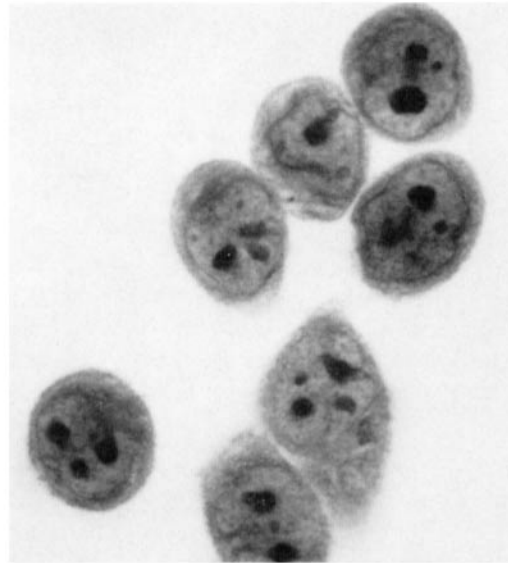
effects of inhibitors of nucleic acid metabolism; inhibition of DNA synthesis by fluorodeoxyuridine results in progressive enlargement and vacuolization of nucleolini leading to their disappearance (14), while interference with DNA-primed RNA synthesis causes total disappearance of the nucleolar RNA (3). Thus, the finding of entirely normal nucleoli in cells treated with all doses of puromycin seems rather remarkable, since, as already described, puromycin action leads eventually to the inhibition of DNA and RNA synthesis. It seems,
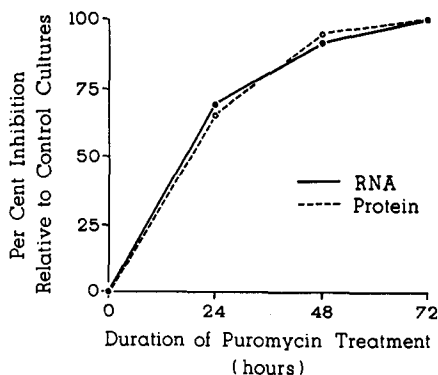
FIGURE 14 Inhibition of RNA and protein synthesis in cultures treated with 0.4 μg/ml puromycin relative to logarithmically growing controls. Calculated on basis of total culture content.
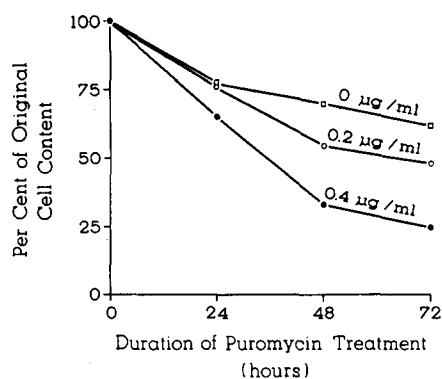
FIGURE 15 Cell content of acid-soluble, UV light-absorbing compounds, expressed as per cent of the initial value.

TABLE I

Content of RNA and Protein Expressed as per cent of the Initial Value (RNA-ca. 40 x 10⁻¹² g per cell, Protein-ca. 400 x 10⁻¹² g per cell), at Intervals During Exposure to Puromycin (0.25 μg/ml).

Cell volume at 24 hours was 3000 μ^3 and at 48 hours ca. 1000 μ^3. The values are means and standard errors of three experiments.

	RNA		Protein	
	24 hrs.	48 hrs.	24 hrs.	48 hrs.
Unfractionated cells	82 ± 9	32 ± 14	103 ± 5	37 ± 10
Nuclei	93 ± 11	71 ± 7	117 ± 35	81 ± 5
Cytoplasmic fraction	59 ± 14	24 ± 16	80 ± 9	30 ± 13

therefore, that nucleolar changes take place only when the inhibition of DNA and RNA synthesis is direct.

The effects of puromycin on the dividing cell illustrate clearly that the late stages of mitosis are most susceptible to inhibition and that the point of inhibition shifts back to an earlier stage of the replication process as the severity of treatment increases. Thus, the first effect after administration of low doses of puromycin is a slower rate of mitosis; this is followed by metaphase arrest. More severe inhibition by puromycin appears to prevent the cells from entering the visible stages of mitosis, and from synthesizing DNA as suggested by Powell (24) and Mueller et al. (18). Some insight into the metabolism of DNA in cells treated with puromycin can be gained by consideration of the theoretical effects of continued synthesis of DNA and inhibition of cell division in an asyn-

chronous, logarithmically growing culture. A proportion of the cells in such a culture contains the postmitotic amount of DNA(d). The DNA content of cells in the G2 period, prophase, and metaphase will be 2d, and cells in the synthetic period (S) will contain intermediate amounts. Assuming that the synthesis of DNA is approximately linear, the average cell content in S period may be taken as 1.5d; thus, since the measured value of DNA per cell was 16 × 10⁻¹² g in the present experiments, the amount of DNA contained in a postmitotic cell (d) will be given by the equation,

$$\frac{T + G^1}{\Sigma t} d + \frac{S}{\Sigma t} \times 1.5d + \frac{G^2 + P + M + A}{\Sigma t} \times 2d$$

$$= 16 \times 10^{-12} g,$$

where the symbols for each period represent its duration; e.g., P is the duration of prophase, M is the duration of metaphase, etc., and Σt is the total

generation time. This equation is derived from the consideration that at any point of time the fraction of cells in any phase of the growth cycle is directly proportional to the duration of that phase, relative to the total time of the growth cycle. A more complex but formally correct equation presented by Stanners and Till (28) gives essentially the same results.

Substituting the values obtained experimentally by Painter and Drew (21) for S3 clone of HeLa cells growing with a generation time of 25 hours, we have

$$\frac{14}{25}d + \frac{6 \times 1.5}{25}d + \frac{5 \times 2}{25}d = 16 \times 10^{-12}\,g$$

which gives $d \simeq 12 \times 10^{-12}\,g$, i.e., the DNA content of the postmitotic cell. Thus, if cells no longer divide and remain in the G1 period, the DNA content per cell should be in the region of 75 per cent of the average amount in exponentially growing cultures. A decrease of this magnitude was described by Salzman in the stationary phase of HeLa cell cultures (26). Cultures with cells entirely in metaphase arrest or in G2 period can have up to $24 \times 10^{-12}\,g$ per cell (150 per cent). Such changes in mean DNA content per cell were found in puromycin-treated cells (Fig. 4 a and b). In the lower concentrations, puromycin does not, therefore, appear to have any direct effect on syn-

thesis of DNA. It does, however, lead to inhibition of mitotic division, and, as a result, indirectly affects the DNA content of the cell.

In this work, we have studied the changes caused by puromycin in mammalian cells and have correlated cytochemical and biochemical findings. The results show that both sets of observations are mutually complementary, but they also illustrate the obvious truth that such correlations cannot be applied too rigorously since the biochemical result gives a mean value for the cell population, and "an average cell" does not exist as such. Among the complicating factors which must be considered in the interpretation of biochemical data are changes in cell size, change in distribution of the cells among the various phases of the life cycle, and altered fragility of cell components caused by the action of the agent studied. Furthermore, in experiments in which a proportion of cells detach from culture during the course of treatment, cells remaining for analysis may well constitute a selected population. This, however, should be paralleled by detachment of cells during fixation for cytological studies.

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