Regulation of Ribosomal RNA Synthesis and Processing During Inhibition of Protein Synthesis by 1,3-Bis(2-chloroethyl)-1-nitrosourea†

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ABSTRACT: The antineoplastic agent BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea) at a concentration of 25 μg/ml inhibits initiation of protein synthesis in HeLa cells. At this low concentration of the drug, the rate of synthesis of 45S ribosomal precursor RNA (pre-rRNA) is selectively inhibited without a marked inhibition of nucleoplasmic RNA. The inhibitory effects of the drug are readily reversible upon removal of BCNU from the growth medium. Pulse-chase analysis of the labeled nucleolar RNA in sucrose gradients and acrylamide gels indicated that the 45S pre-rRNA synthesized before the addition of BCNU matures normally in the presence of the inhibitor. However, the processing of precursor RNA molecules synthesized following the addition of the drug is inhibited when incubation is continued on in the presence of 25 μg/ml BCNU. Since the formation of mature ribosomes is blocked by BCNU, the data would suggest that the effectiveness of the drug as a potent cell growth inhibitor may result from its inhibition of ribosome formation induced by inhibition of protein synthesis.

The assembly of ribosomes in mammalian cells proceeds through a number of discrete steps in the form of nucleolar ribosomal precursor particles (pre-rRNP)[1] (Kumar and Warner, 1972; Liau and Perry, 1969; Perry, 1965; Warner and Soeiro, 1967). This processing of ribosomes appears to be dependent upon the noncovalent assembly of a proper complement of proteins and ribosomal precursor RNA (pre-rRNA) (Kumar and Warner, 1972; Warner and Soeiro, 1967), since the availability of certain proteins during ribosome processing can be a rate-limiting step (Pederson and Kumar, 1971).

The close association of protein synthesis and ribosomal RNA processing has been demonstrated by inhibition of peptide chain elongation with cycloheximide which causes inhibition of 45S pre-rRNA transcription, although its maturation continues at a reduced rate (Willems et al., 1969). On premature termination of nascent polypeptides with puromycin, however, the 45S pre-rRNA continues to be transcribed and converted to the 32S intermediate species, but there is essentially no mature cytoplasmic rRNA formed (Soeiro et al., 1968).

Present studies were aimed primarily at a general understanding of the mechanism of action of BCNU at a subcellular level and its effectiveness as an inhibitor of cell growth. The results indicate that the initial effect of a low concentration of BCNU (25 μg/ml) of HeLa cells is to inhibit initiation of protein synthesis. Nucleolar RNA synthesis is selectively inhibited at this concentration of the drug. However, there is little change in the synthesis of heterogeneously sedimenting neoplasmic RNA. Since the processing of ribosomal RNA is completely inhibited in the presence of BCNU, presumably as a consequence of the inhibition of protein synthesis, no new mature ribosomes are formed. Thus the long-term effect of BCNU on the inhibition of cell growth may include a block in ribosome synthesis induced by the inhibition of protein synthesis.

Materials and Methods

(*a*) *Cell Culture and Labeling Conditions.* HeLa (S3) cells were maintained at 3–6 × 10^5 cells/ml in a spinner culture in Joklik modified Eagle's (Eagle, 1959) minimal essential medium (MEM) supplemented with nondialyzed horse serum. For radioactive labeling, cells were concentrated ten times the original cell density in fresh growth medium and incubated with either [^{3}H]- or [^{14}C]uridine at a concentration and duration as indicated in figure legends. For protein labeling cells were collected as above but in a growth medium containing one-quarter the normal concentration of amino acids plus ^{3}H-amino acids at a desired concentration. Ribosomal RNA was labeled with [*methyl*-^{3}H]methionine in methionine-free MEM supplemented with 7.5% nondialyzed horse serum and if desired, the RNA label was chased by incubation in MEM supplemented with 40 mM unlabeled L-methionine.

(*b*) *Cell Fractionation.* Isotope incorporation in cells was terminated by rapid chilling to about 4 °C with the addition of crushed frozen Earle's solution (Earle, 1943), and cells were kept at 4 °C for all subsequent procedures. Cells were washed free of the growth medium by resuspending in cold Earle's salt solution and disrupted by Dounce homogenization in RSB buffer (0.01 M NaCl, 0.0015 M $MgCl_2$, 0.01 M Tris-HCl (pH 7.4)). Nuclei were collected from the cell homogenate by centrifugation at 1000*g* for 3 min and washed twice in RSB buffer supplemented with detergent mixture (2:1 mixture of 10% (w/w) Tween 40 and 10% (w/w) sodium deoxycholate) (Penman, 1966). The combined supernatants were saved as the total cytoplasmic fraction.

For the subfraction of nucleoplasmic and the nucleolar samples, the cleaned nuclei were lysed in HSB–DNase solution (0.5 M NaCl, 0.05 M $MgCl_2$, 0.01 M Tris-HCl, pH 7.4, plus

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[1] Abbreviations used are: pre-rRNP, ribosomal precursor RNP; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MEM, minimal essential medium; pre-rRNA, ribosomal precursor RNA; EDTA, ethylenediaminetetraacetic acid.

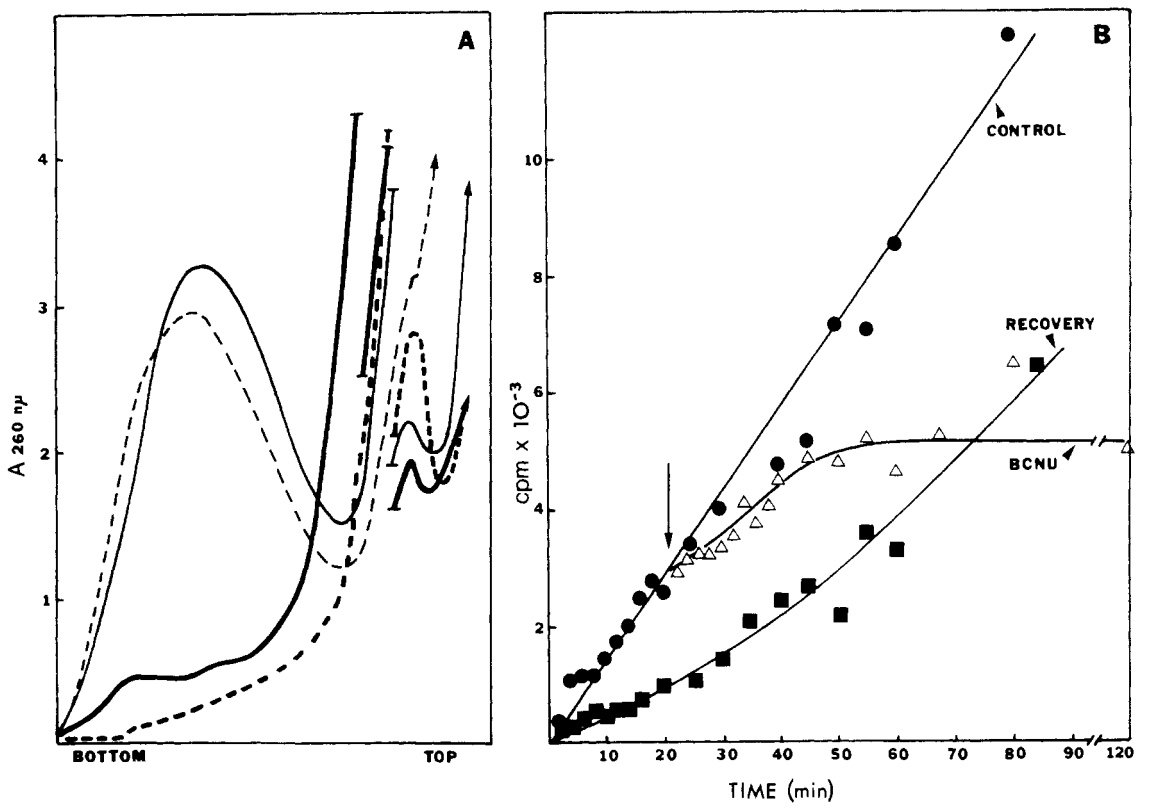
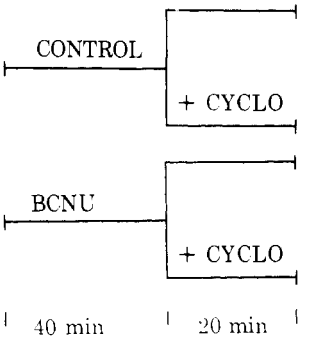


FIGURE 1: (A) Breakdown of polyribosomes during BCNU treatment. A culture of HeLa cells at a cell density of 5×10^5/ml was divided into two equal aliquots of 10^8 cells and incubated at 37 °C for 1 h with or without the addition of 25 μg/ml of BCNU. The drug treated and untreated control cultures were further divided into equal aliquots and to half of each of the cultures, 100 μg/ml of cycloheximide was added and incubation continued for 20 min, as outlined:



Cells were harvested and washed in cold Earle's solution and lysed in the presence of nonionic detergent in isotonic buffer (0.6% nonidet-P-40 in 0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.0015 M $MgCl_2$). The cell lysate was centrifuged at 12 000*g* for 10 min and the ribosomal fractions from the supernatant were resolved through 7–47% (w/w) sucrose gradients in HSB buffer. The gradients were centrifuged at 26 000 rpm for 3.5 h at 4 °C in a Spinco SW27 rotor and eluted through a continuous-flow-recording spectrophotometer. The thin continuous line represents the absorbance profile of control samples and the thin broken line that of control cells treated with cycloheximide. The thick continuous line represents BCNU-pretreated samples and the thick broken line, drug-treated samples to which cycloheximide was added. (B) Inhibition of protein synthesis by BCNU. An aliquot of cell culture was divided into two-thirds and one-third portions and incubated at 37 °C without any additions to the larger portion and with the addition of 25 μg/ml of BCNU to the smaller portion. After 30 min of incubation, the cells from the drug-treated culture were collected by centrifugation at 37 °C, washed twice, and resuspended at the original cell density in fresh growth medium. ^{3}H-L-amino acid mixture at a concentration of 0.25 μCi/ml was added to both cultures and equal aliquots removed at indicated times. After 20 min of incorporation, to a fraction of the untreated culture 25 μg/ml of BCNU was added and aliquots were taken for acid-insoluble counts, as in the case of the other two samples. The arrow indicates the time of addition of the drug.

50 μg/ml of DNase I) as described by Penman (1966). Nucleoli were collected from the lysate by centrifugation at 12 000*g* for 10 min. RNA was purified from various cell fractions by a phenol–chloroform procedure and resolved on sucrose density gradients or acrylamide gel electrophoresis, as previously described (Weinberg et al., 1967; Weinberg and Penman, 1970). The details of the conditions for each experiment are described in the figure legends.

(*c*) *Isotopes and Chemicals.* L-[*methyl*-^{3}H]Methionine (sp act. 290 mCi/mmol), [2-^{14}C]uridine (sp act. 57 mCi/mmol), [5-^{3}H]uridine (sp act. 25 mCi/mmol), and ^{3}H-L-amino acid mixture (NET-250) were purchased from New England Nuclear. Actidione (cycloheximide) was from Nutritional Biochemical, Cleveland, Ohio, and actinomycin D was a gift from Merck, Sharp, and Dohme, West Point, Pa. The nonionic detergent Tween 40 (polyoxyethylene sorbital monopalmitate) was purchased from Shell Chemicals. BCNU and camptothecin were kindly provided by Dr. H. B. Wood, Jr. of the

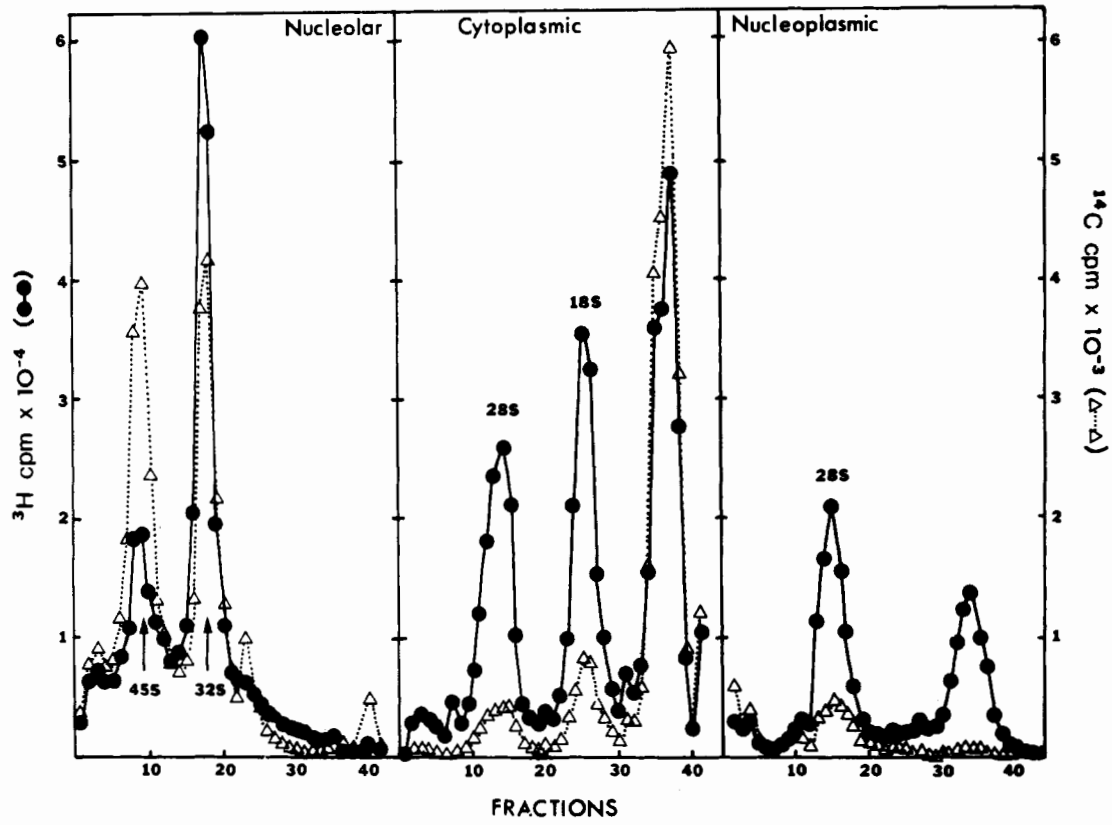


FIGURE 2: Inhibition of ribosome synthesis by BCNU. Two cultures of 8×10^7 cells were incubated at 37 °C, one with the addition of 25 μg/ml of BCNU and the other without any drug. After 30 min, [^{14}C]uridine (0.1 μCi/ml) was added to the BCNU treated culture and [^{3}H]uridine (2 μCi/ml) to the untreated control. After 90 min, isotope incorporation in both samples was stopped by rapid chilling and the controls and drug-treated cells were fractionated together into nucleolar, nucleoplasmic, and cytoplasmic RNAs as described (Kumar and Wu, 1973; Wu et al., 1971). Nucleolar RNA was centrifuged through a 15–30 (w/w) sucrose gradient buffer (0.01 M Tris-HCl, pH 7.4, 0.01 M EDTA, 0.1 M NaCl, 0.2% sodium dodecyl sulfate) at 20 500 rpm for 15 h at 25 °C in a Spinco SW27 rotor. The cytoplasmic and nucleoplasmic RNAs represent only half of the samples centrifuged in similar sucrose gradients at 23 000 rpm for 16 h. Gradients were fractionated and prepared for the determination of acid-insoluble radioactivity as described (Kumar and Warner, 1972; Kumar and Wu, 1973; Wu et al., 1971).

National Cancer Institute. The drug was stored at −20 °C until the appropriate amounts were added directly to the cell growth medium at 37 °C.

Results

(*a*) *Inhibition of Protein Synthesis.* The cellular distribution of active ribosomes engaged in protein synthesis (polyribosomes) is determined by displaying their sedimentation pattern in sucrose gradients. The results illustrated in Figure 1A compare the distribution of active ribosomes, i.e., those in polyribosomes, in untreated and BCNU-treated HeLa cells. In the drug treated cells there is a marked loss of ribosomes from the polysome population within 1 h of incubation with 25 μg/ml of BCNU. This amount of the drug was the minimum concentration needed to achieve the effects described in this report.

The rate at which ribosomes associate with mRNA, i.e., initiate protein synthesis, is reflected in the size distribution of polyribosomes. For example, a reduced rate of initiation results in slower sedimenting polyribosomes which are reformed if the rate of protein chain elongation is slowed down after addition of cycloheximide (Leibowitz and Penman, 1971; McCormick and Penman, 1969). As is shown in Figure 1A, there is a noticeable shift toward the heavier polysomes after a 20-min incubation of growing HeLa cells with 100 μg/ml of cycloheximide. However, if the cells are treated with 25 μg/ml of BCNU before the addition of cycloheximide, there is no indication of re-formation of the dissociated polyribosomes, suggesting that protein chain initiation is blocked by BCNU. The dissociation of polyribosomes by BCNU could also result from the degradation of messenger RNA. However, there is little indication of enhanced ribonuclease activity in BCNU treated cells (see below).

The effect of BCNU on the rate of accumulation of newly synthesized proteins is shown in Figure 1B. There is a lag of about 15–20 min before the incorporation into acid-insoluble proteins is completely inhibited following the addition of 25 μg/ml of BCNU. This result suggests that already initiated peptide chains are allowed to complete in the presence of BCNU. Upon removal of BCNU by washing the cells in fresh growth medium, the inhibition of protein synthesis is readily reversed as seen by re-formation of polyribosomes (not illustrated), as well as the resumption of protein synthesis (Figure 1B), although the recovery is by no means complete.

The initial effect of BCNU on the inhibition of protein synthesis, however, may not be the primary reason for its long-term effects as an inhibitor of cell growth. Because of the strict dependence of ribosome processing on protein synthesis in mammalian cells (Pederson and Kumar, 1971; Soeiro et al., 1968; Willems et al., 1969) and because of the central role of ribosomes in protein synthesis and cell growth, the next series of experiments were designed to show the effects of BCNU specifically on the regulation of ribosomal RNA transcription and processing.

(*b*) *Inhibition of Ribosomal RNA Synthesis.* The extent of ribosomal RNA accumulation, as determined by continuous incorporation of uridine into nucleolar, nucleoplastic, and cytoplasmic ribosomal RNA, is shown in Figure 2. After 90

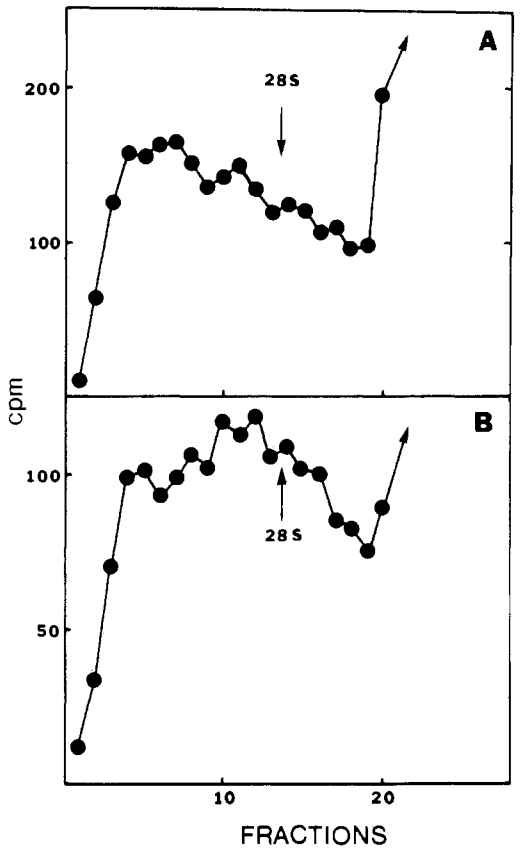


FIGURE 3: Effect of BCNU on nucleoplasmic RNA synthesis. Of the two cultures, each containing about 10^8 cells, one was incubated with 25 μg/ml of BCNU and the other without any additions for 30 min at 37 °C. Cells in both cultures were then pulse labeled with [^{14}C]uridine (0.1 μCi/ml for 10 min), and the nucleoplasmic RNA prepared and centrifuged through 15–30% sucrose gradients in NETS buffer, as before, at 19 000 rpm for 16 h in a Spinco SW 25.3 rotor at 25 °C. Gradients were fractionated and prepared for determination of acid-insoluble radioactivity. Panel A shows the untreated control and B the nucleoplasmic RNA from 30 min BCNU pretreated cells. The radioactivity in drug-treated nucleoplasmic RNA is 80% that of the untreated sample. The arrows in each case indicate the sedimentation of added unlabeled 28S RNA.

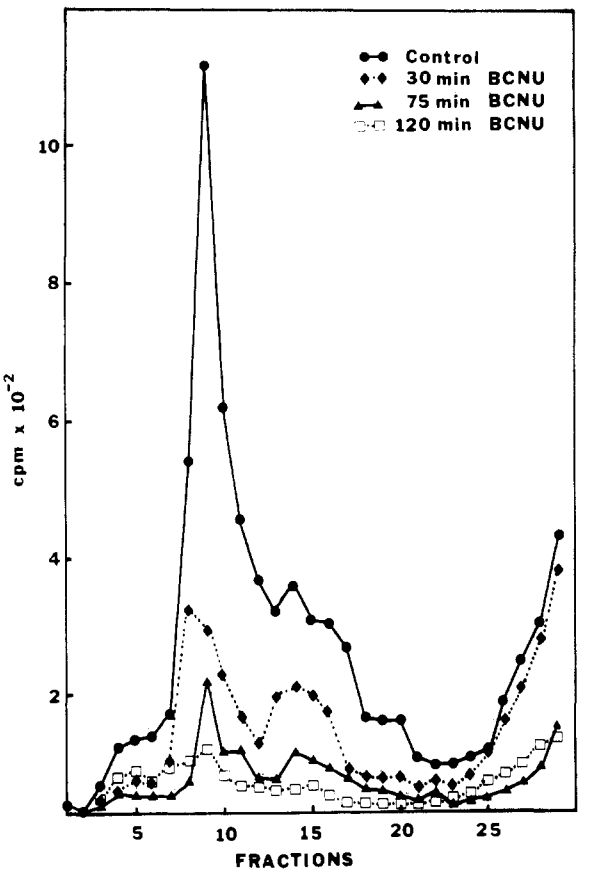


FIGURE 4: Rate of inhibition of 45S pre-rRNA by BCNU. A culture of HeLa cells was divided into two portions containing 2×10^7 cells and 6×10^7 cells and incubated at 37 °C. To the larger portion 25 μg/ml of BCNU was added, and one-third aliquots were removed after 30, 75, and 120 min and pulse labeled with [^{3}H]uridine (2 μCi/ml) for 15 min. The untreated cells were similarly pulse labeled for 15 min with 2 μCi/ml of [^{3}H]uridine. The nucleolar RNA was fractionated from each of the four samples and analyzed on NETS–sucrose gradients as before. Gradients were centrifuged in a Spinco SW41 rotor at 18 500 rpm for 19 h at 25 °C. Sucrose gradient fractions were prepared for acid-insoluble radioactivity as before.

min of incubation with [^{3}H]uridine, very few completed ribosomes appear in the cytoplasm when the cells are incubated in the presence of 25 μg/ml of BCNU, as compared to the untreated culture. The nucleolar pre-rRNA sample, on the other hand, shows some accumulation of counts in 45S pre-rRNA; its conversion to the 32S pre-rRNA stage is not as efficient as in the untreated cells, since a larger proportion of newly synthesized 45S pre-rRNA does not transfer to the 32S RNA in the presence of 25 μg/ml of BCNU. This is shown by comparison of the [^{3}H]uridine counts incorporated into untreated samples with the [^{14}C]uridine incorporation in the nucleolar RNA from BCNU treated samples (Figure 2). The inhibition of the rate of processing of 45S pre-rRNA is further illustrated by experiments described below.

Following its cleavage from the nucleolar 32S pre-rRNA, the nascent 28S rRNA appears in the nucleoplasm as a 50S ribonucleoprotein particle, before its transfer to the cytoplasm as a mature large ribosomal subunit (Vaughan et al., 1967). Nearly one-third of the nuclear 28S rRNA is purified along with the nucleolar fraction (Vesco and Penman, 1968), although in sucrose density gradients nucleolar 28S RNA is not resolved from the 32S species (Figure 2). By base ratio determinations, the nucleolar and nucleoplasmic 28S rRNAs appear to be quite similar (Willems et al., 1968; Vaughan et al., 1967), and they possibly represent the stages in transfer of the newly synthesized 28S rRNA from nucleus to the cytoplasm. The specific activity of untreated 45S pre-rRNA (^{3}H-labeled in Figure 2) was 139 900 cpm/A_{260nm} and that of the 32S species was 74 384 cpm/A_{260nm}, whereas in the BCNU pre-treated cells (labeled with [^{14}C]uridine), the values were 23 290 cpm/A_{260nm} for 45S and 5995 cpm/A_{260nm} for the 32S RNA region. Therefore, the ribosomal precursor RNA transferred to the 32S stage is considerably reduced in the presence of BCNU. Examination of the nucleoplasmic 28S rRNA from the BCNU-treated cells showed that little new

TABLE I: Inhibition of the Rate of 45S Pre-rRNA Synthesis.

Treatment	cpm (% Control)[a]
None (control)	3267 (100)
30 min BCNU	1104 (33.8)
75 min BCNU	505 (15.45)
120 min BCNU	412 (12.6)

[a] Counts in the 45S region of the gradient (fractions 7–12) were estimated assuming the untreated control 45S RNA counts as 100%.

TABLE II: Inhibition of the Rate of Pre-rRNA Processing by BCNU.

	45S[a] (cpm)	32S[a] (cpm)	45S Counts Remaining after Chase (%)	45S Counts Transferred to 32S (%)
		Experiment 1[b]		
Control Pulse	29 200	785		
Chase	4 450	14 750	15	56
15 min BCNU Pulse	15 750	320		
Chase	11 400	4 400	72	28
30 min BCNU Pulse	6 250	150		
Chase	9 150	345		3
60 min BCNU Pulse	2 625	120		
Chase	5 275	1 500		
		Experiment 2[c]		
Control Pulse	2 690			
Chase	214	965	8	39
15 min BCNU Pulse	1 190			
Chase	680	52	57	10
30 min BCNU Pulse	374			
Chase	376	45	100	2
60 min BCNU Pulse	210			
Chase	197	35	94	3

[a] Counts in the 45S and 32S pre-rRNA regions (as shown in Figure 7) were estimated above the background counts. [b] In this experiment (as shown in Figure 7) the cells were pulse labeled with 10 μCi of [^{3}H]uridine/ml and chased with the addition of 5 μM camptothecin for 15 min following the preincubation in BCNU for indicated period. [c] Pulse labeled with 2 μCi of [^{3}H]uridine/ml for 10 min and chased with 5 μg of actinomycin D/ml for 15 min. In each case the concentration of BCNU was 25 μg/ml and the cells were preincubated for the indicated period.

28S rRNA is formed as compared to the untreated cells (Figure 2). Therefore, the inhibition of ribosomal RNA processing due to BCNU treatment must be completed before the nucleoplasmic stage. As will be shown in a later section, the rate of synthesis of 45S pre-rRNA is also inhibited during BCNU treatment.

The same concentration of BCNU (25 μg/ml) causes only about 20% inhibition of the labeling of nucleoplasmic RNA during 10 min of incorporation with [^{14}C]uridine following a 30-min pretreatment with the drug (Figure 3). Therefore, at this low concentration of the drug, the inhibition of ribosomal RNA synthesis is rather specific.

(*c*) *Effect of BCNU on the Rate of Transcription of 45S pre-rRNA.* Continuous incorporation of uridine as described in the previous section does not permit a clear distinction between the effects of BCNU on the rate of transcription of 45S pre-rRNA and its processing. The effect of BCNU treatment on the efficiency of 45S pre-rRNA synthesized within 15 min of [^{3}H]uridine pulse labeling after incubation with BCNU for different periods is illustrated in Figure 4. A summary of these results is presented in Table I. There is a marked inhibition of the rate of 45S rRNA synthesis following the addition of 25 μg/ml of BCNU. After a 60-min pretreatment with the drug, 45S pre-rRNA synthesis is inhibited to about 90% (Table II). However, since the residual amounts of 45S pre-rRNA synthesized in the presence of BCNU are inhibited in its maturation to the 32S RNA stage, a higher proportion of 45S pre-rRNA accumulates on continued incubation in BCNU (Figure 2). The inhibition of 45S pre-rRNA synthesis due to BCNU appears to be rapidly reversible upon resuspension of the cells in fresh growth medium (Figure 5).

It appears that the efficiency of ribosomal precursor RNA synthesis is markedly reduced in the presence of BCNU. However, it is not clear if the precursor molecules synthesized prior to the addition of BCNU would form mature ribosomes in the presence of the inhibitor. For example, addition of BCNU may cause reduced amounts of the ribosome processing enzymes resulting in the inhibition of completed ribosomes. Alternately, incubation of HeLa cells with BCNU could result in the alteration of the components of the ribosomal precursors. Thus, the "incomplete precursor" would not mature efficiently.

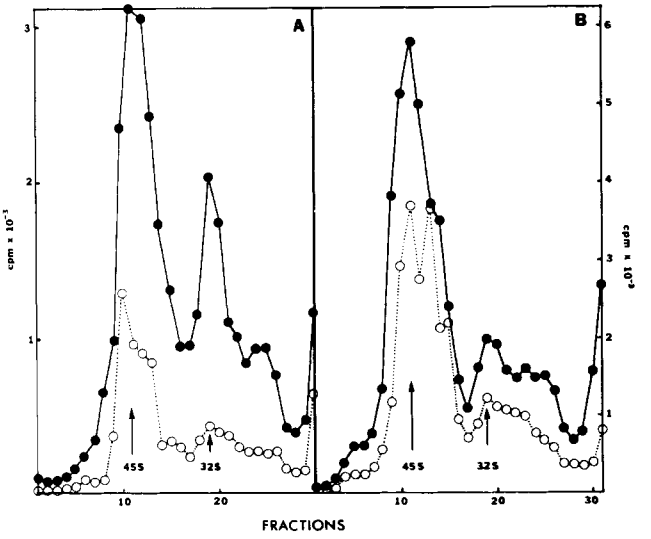


FIGURE 5: Reversibility of the inhibition of 45S pre-rRNA synthesis due to BCNU treatment. Two 150-ml spinner cultures with a cell density at 5×10^5/ml were incubated at 37 °C for 60 min, one with 25 μg/ml of BCNU and the other without. Half of the drug-treated and control cultures were removed and pulse labeled with [^{3}H]uridine (2 μCi/ml) for 20 min at 37 °C. Cells from the other halves were collected by centrifugation at 1000*g* for 3 min at 37 °C, washed twice, resuspended, and pulse labeled in fresh growth medium with 2 μCi of [^{3}H]uridine/ml for 15 min as above. Nucleolar RNA was purified and analyzed in 15–30% NETS–sucrose gradients as described. Gradients were centrifuged in a Spinco SW41 rotor at 19 500 rpm for 16.5 h at 25 °C. Fractions were prepared for the determination of acid-insoluble radioactivity as before. For a direct comparison, the radioactivity profiles of BCNU-treated (--●---●--) and control (—●—●—) samples before (A) and after removal (B) of the drug effect are superimposed.

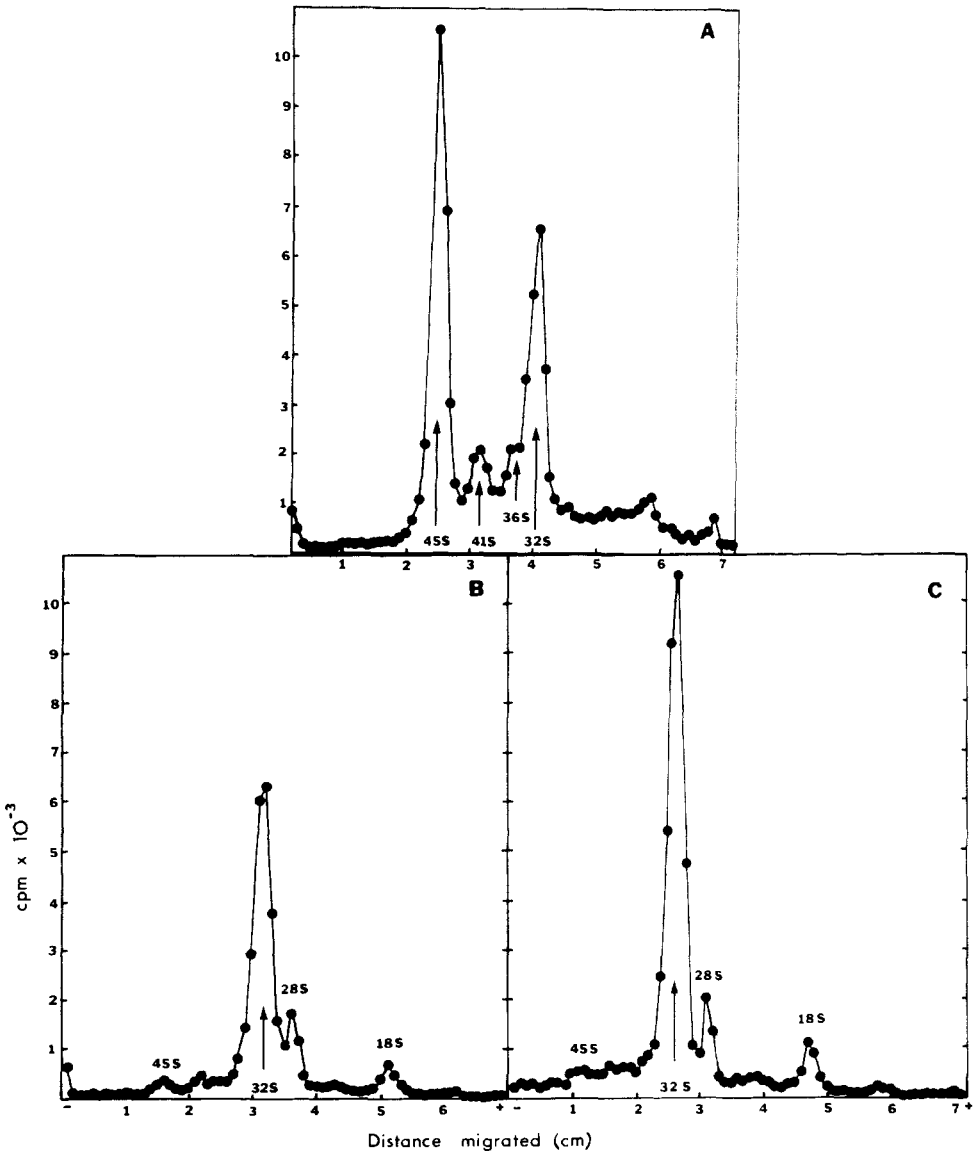


FIGURE 6: Maturation of preformed 45S pre-rRNA is not inhibited in the presence of 25 μg of BCNU/ml. 3×10^8 cells were concentrated tenfold in methionine-free growth medium and incubated for 10 min at 37 °C. Following that [*methyl*-^{3}H]methionine was added at a concentration of 20 μCi/ml and incubation continued for 20 min; one-third of the aliquot (illustrated in Panel A) was rapidly chilled by pouring over frozen Earle's solution. The remaining pulse-labeled cells were divided into two aliquots; unlabeled methionine was added to both to make 40 mM, and 25 μg of BCNU/ml to one of them (shown in panel C), and incubation continued for 45 min at 37 °C. The chased samples (control chase, panel B; BCNU-chase, panel C) were fractionated and nucleolar RNA purified as above. The RNA samples were analyzed by acrylamide gel electrophoresis (2.7% acrylamide, 5 mA/gel for 5.5 h at room temperature), fractionated into 1-mm slices, and prepared for liquid scintillation counting, as described (Weinberg and Penman, 1970). (A) Control pulse; (B) cold methionine chase; (C) cold methionine chase in the presence of inhibiting dose of BCNU.

In the latter case, there will be a marked inhibition of the efficiency of processing of 45S pre-rRNA transcribed after BCNU treatment.

The results illustrated in Figure 6 show that nucleolar pre-rRNA labeled with [*methyl*-^{3}H]methionine for 20 min (Figure 6A) is quantitatively chased to the 32S and 28S species after incubation of the cells in a growth medium supplemented with excess unlabeled methionine (Figure 6B). The efficiency of processing of the [^{3}H]methionine-labeled precursor RNA appears to be unchanged even in the presence of an inhibiting concentration of BCNU (Figure 6C). Therefore, the processing of preformed 45S pre-rRNA seems unimpaired in the presence of inhibiting amounts of BCNU.

(*d*) *Effect of BCNU on the Efficiency of Maturation of 45S pre-rRNA.* As shown before with longer incubation in BCNU, the rate of synthesis of 45S pre-rRNA is markedly inhibited. A similar result is shown in Figure 7. We then attempted to examine the efficiency of maturation of 45S pre-rRNA made in the presence of BCNU. Cells were pulse labeled with [^{3}H]uridine after different periods of preincubation with BCNU. The newly transcribed 45S pre-rRNA in each case was allowed to mature to 32S RNA in the absence of new 45S RNA synthesis, due to 5 μM camptothecin, an inhibitor of RNA synthesis which does not interfere with the maturation of pre-rRNA (Kumar and Wu, 1973; Wu et al., 1971). In untreated control cells, almost all of the counts from the 45S region disappear and over 40% is transferred to the 32S RNA, whereas in the cells preincubated with BCNU, an increasingly higher proportion of the newly synthesized 45S pre-rRNA remain, even after the camptothecin chase, and very little of the 45S RNA counts appear in the 32S region (Figure 7 and Table II).

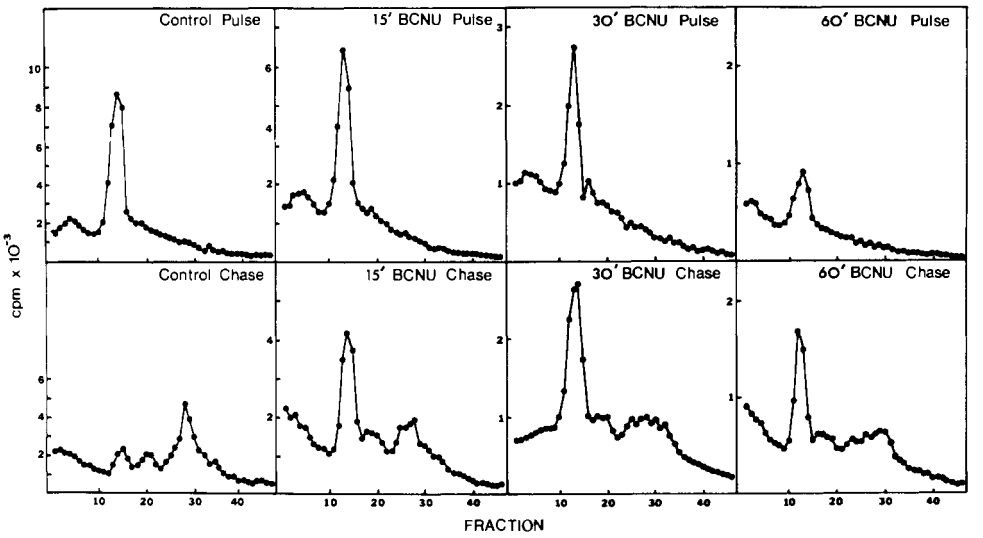


FIGURE 7: Inhibition of the rate of pre-rRNA processing by BCNU. 2.5×10^8 cells at a density of 5×10^5 cells/ml were concentrated tenfold in fresh medium and incubated at 37 °C for 10 min. Following that, the cell suspension was divided into one-fourth and three-fourth portions. To the larger portion BCNU was added to make 25 μg/ml and equal aliquots were removed after 15, 30, and 60 min of incubation at 37 °C. To each of the four drug-treated and untreated cultures, [^{3}H]uridine (10 μCi/ml) was added to pulse label 45S pre-rRNA. After 10-min pulse labeling, half of each of our samples were collected on frozen crushed Earle's solution and to the other half 5 μM camptothecin was added, and the incubation continued for 15 min. Nucleolar RNA fractions were analyzed by acrylamide gel electrophoresis as described for Figure 6.

If all of the 32S pre-rRNA is derived from the 45S precursor, the radioactivity transferred from the 45S to the 32S RNA species following the inhibition of transcription of new 45S RNA precursor should be proportional to their molecular weights. In an untreated culture about 45% of the counts from 45S pre-rRNA are transferred to the 32S RNA under a chase condition when no new rRNA is being synthesized in the presence of actinomycin D (Willems et al., 1969). A summary of the experiments utilizing either actinomycin D or camptothecin to chase-preformed 45S pre-rRNA into 32S RNA is shown in Table II. As is shown (Figure 7), the fraction of 45S RNA counts transferred to 32S species after the chase is drastically reduced in cells preincubated with BCNU. After the chase, a larger fraction of the 45S pre-rRNA counts remain in the 45S region, indicating a slower rate of transfer from 45S to 32S RNA species (Table II). Thus, it appears that the rate of maturation of the 45S pre-rRNA synthesized in the presence of BCNU is markedly inhibited and most of them, which are unable to transfer to the next step in the maturation pathway, are degraded in the nucleolus.

Discussion

The antineoplastic activity of BCNU has been most useful in the treatment of Hodgkins disease, gastrointestinal carcinomas, and malignant brain tumors (Chabner et al., 1975). However, the mode of action of this inhibitor is still unclear. Among the nitrosoureas, BCNU ($ClCH_2CH_2N(NO)C(=O)NHCH_2CH_2Cl$) has two chloroethyl groups with alkylating capability, and an isocyanate portion which has been shown to bind to proteins (Kann et al., 1974). Because of the marked instability of BCNU and the reactivity of its cleavage products, it is assumed that the unique antitumor activity of this inhibitor, in large part, is due to its alkylating and isocyanate products (Chabner et al., 1975; Kann et al., 1974).

The results described in this report demonstrate that the initial effect of BCNU in cultured human cells is a complete breakdown of polyribosomes engaged in protein synthesis. Already initiated polypeptides are presumably allowed to complete, since the incorporation of amino acids into the acid-insoluble fraction in the drug-treated culture continues in parallel with the untreated cells for about 10 min before a total inhibition of protein synthesis. The inhibition of protein synthesis is presumably mediated through a short-lived cleavage product of BCNU, since removal of the drug readily reverses its inhibitory effect on protein synthesis. However, the long-term effect of BCNU as a potent inhibitor of cell growth appears to be due to its selective inhibition of ribosomal RNA transcription and processing caused by the inhibition of protein synthesis.

In a previous study it was shown that, although the synthesis of ribosomal proteins is independent of the transcription of 45S pre-rRNA, its utilization in ribosome formation is strictly dependent upon the concurrent transcription of ribosomal RNA (Craig and Perry, 1971; Wu et al., 1971). Similarly, in a number of studies it has been shown that inhibition of protein synthesis leads to severe derangement of the maturation of ribosomal precursors (Pederson and Kumar, 1971; Soeiro et al., 1968; Willems et al., 1969). Thus, the processing of ribosomes in mammalian cells is closely regulated by the noncovalent assembly of a proper complement of ribosomal RNA and proteins. The results presented in this report suggest that the inhibitory effects of BCNU on cell growth might be mediated by its initial inhibition of translational initiation, that in turn blocks the formation of mature ribosomes. First of all it is clear that a low dose of BCNU (25 μg/ml) rapidly and reversibly inhibits the synthesis of 45S pre-rRNA. At this concentration of BCNU, the effect on the transcription of heterogeneously sedimenting nuclear RNA is minimal. The fact that removal of the inhibitor from growth medium rapidly restores both the transcription of ribosomal RNA and the rate of protein synthesis suggests that the inhibitory effect of the drug might be mediated through a complex formation between short-lived cell components and the breakdown product of BCNU. The inhibition of protein synthesis by BCNU is not likely to be due to the degradation of existing messenger RNA molecules, because of the rapid reversal of the inhibition of protein synthesis (Figure 1). Furthermore, since most mammalian mRNA is long-lived (Greenberg, 1972; Perry and Kelley, 1973; Singer and Penman, 1972; Singer and Penman, 1973), it appears that, upon reversal of the inhibition of

translational initiation, the existing mRNA in the cells are translated at normal rates.

Unlike the earlier reports of the effects of protein synthesis inhibition on ribosome formation, treatment with a low dose of BCNU leads to a rapid inhibition of the efficiency of transcription of 45S pre-rRNA. In addition, it is clear that the rate of maturation of the residual amounts of pre-rRNA synthesized in the presence of 25 μg/ml of BCNU is also markedly reduced as compared to the untreated cells (Figure 7 and Table II). However, there is no inhibition of the maturation of pre-rRNA synthesized before the addition of BCNU to the culture medium (Figure 6), suggesting that there is no significant change in the maturation enzymes responsible for ribosome processing due to BCNU. Therefore, it appears that the inhibition of protein synthesis by BCNU must result in suboptimal RNA–protein interaction in the ribosome precursors that are incapable of completing maturation. General inhibition of cellular protein synthesis by the drug is likely to induce metabolic derrangements other than in ribosome synthesis, which accounts for its effective cell growth inhibitory property.

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