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EFFECT OF 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA ON NEWLY SYNTHESIZED DNA IN L-1210 CELLS

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Abstract—The synthesis of DNA in L-1210 cells was selectively inhibited by BCNU [1,3-bis-(2-chloroethyl)-1-nitrosourea, NSC 409962]. When the DNA chain growth in exponentially grown L-1210 cells was analyzed by alkaline sucrose gradient centrifugation, the initially synthesized DNA had short segments (approximately 5S) which increased in size to 30, 70 and to over 100S. When treated with BCNU, the short, newly synthesized DNA segments accumulated; the molecular weights were 5–30S. While it appears that BCNU inhibits the early elongation steps of newly synthesized DNA, the chasing experiments suggest that the drug does not affect the joining process from replicon-sized DNA (70S) to bottom peaks with a molecular weight of over 100S.

BCNU [1,3-bis(2-chloroethyl)-1-nitrosourea, NSC 409962], one of the nitrosourea derivatives, is reportedly effective against malignant diseases such as leukemia [1], malignant lymphoma [2] and brain tumor [3]. Although this drug is considered an alkylating agent [4], the detailed mechanism of action is not fully understood.

This report demonstrates the activity of BCNU toward nucleic acid metabolism in L-1210 cells and a description is given of the effect of BCNU on newly synthesized DNA.

MATERIALS AND METHODS

Chemicals. BCNU was obtained from Bristle Physiological Laboratory, Tokyo Branch, and was stored in the dark at 4°. This compound was dissolved in physiological saline immediately before use. [^{3}H]Deoxy-thymidine and [^{3}H]uridine were purchased from the Radiochemical Centre, Amersham, England.

Cells. Cultured L-1210 cells were grown in RPMI-1640 medium with 20% fetal calf serum. All experiments were performed on cells during exponential growth. The average doubling time of L-1210 cells under these conditions was 19 hr. The number of cells in the suspension used in the experiments was assessed by the standard procedure of leukocyte counting, using a diluting pipet and a hemocytometer.

Incubation. tubes containing 1 ml of the cell suspension at a concentration of 1×10^6 cells were warmed for 15 min at 37°, and a 10 μl solution of BCNU was added to the cell suspensions to give the desired concentration. The pH of the incubation mixture was not altered by adding BCNU. The control tube contained the same volume of physiological saline. After the cells were preincubated with BCNU for 15 min, [^{3}H]thymidine (5 Ci/m-mole) or [^{3}H]uridine (2.3 Ci/m-mole) was added to each tube to a final concentration of 1 μCi/ml and the incubation was continued for 30 min at 37°. The incubation was stopped by chilling the tubes on ice, and the cells were layered by gentle suction onto glass filters (Whatman GF/C, 2.4 cm in diameter) prewetted with cold 0.85% NaCl solution. The filters were washed three times with cold 0.85% NaCl solution and further washed with cold 10% Cl_3CCOOH containing 1% sodium pyrophosphate four times, dried and counted in toluene base scintillation fluids.

Under the conditions described above, the control experiment showed that survival of L-1210 cells, estimated by the ability of cells to exclude 0.025% trypan blue, was more than 90 per cent and the incorporation of the label was linear for 1 hr.

Analysis of the size of cellular DNA by alkaline sucrose gradient. Cultured L-1210 cells were incubated for 24–48 hr with [^{3}H]thymidine (5 Ci/m-mole, 1 μCi/ml). After the radioactive precursor was washed out, the resuspended cells (5×10^5 cells/ml) were cultured at 37° for 60 min in the presence of an appropriate concentration of BCNU.

For analysis of the size of the bulk DNA by alkaline sucrose gradient centrifugation, the technique developed by Sawada and Okada [5] was used with slight modifications.

Details of this method were previously described [6, 7].

Analysis of newly synthesized DNA using alkaline sucrose velocity sedimentation methods. Logarithmically growing L-1210 cells at a density of 5×10^5 cells/ml were pulse labeled for the desired times at 37° (unless otherwise noted) in 1-ml culture bottles with 5 μCi/ml of [^{3}H]methylthymidine (25 Ci/m-mole), after cells had been preincubated with or without BCNU at 37° for 15 min. In chasing experiments, unlabeled thymidine was added to the incubation medium at a final concentration of 10^{-5} M to suppress the further incorporation of [^{3}H]thymidine into DNA; then the cells were incubated at 37° for the required times. The analysis of newly synthesized DNA was carried out according to the method of Friedman *et al.* [8] with slight modifications. In this method, a linear 5–20% (w/v) alkaline sucrose gradient (4.8 ml) was formed containing 0.1 M NaOH, 0.9 M NaCl, and 0.01 M Na_2EDTA and was layered over with lysing

solution (0.45 M NaOH, 0.55 M NaCl, and 0.01 M EDTA) onto the top of the gradient. The cells were carefully placed on top of the lysing solution, and lysis was allowed to continue at 4° for 2 hr in the dark.

After cell lysis, the gradients were centrifuged at 36,000 rev/min for 240 min at 4° on a Hitachi RPS-40 swing rotor and eight droplet fractions were collected.

Acid-insoluble materials were precipitated by adding 3 ml of 10% cold Cl_3CCOOH with 1% sodium pyrophosphate solution (w/v). Bovine serum albumin (100 μg) was added as a carrier. The acid-insoluble fractions were collected on Millipore filters (type HAWP 0.45 μm, 2.5 cm in diameter), which were then washed three times with 10% Cl_3CCOOH with 1% sodium pyrophosphate.

Each filter was dried and put in a scintillation vial; then 1 ml ethylacetate was added in each vial to dissolve the filter, and toluene base scintillation fluid was added for counting.

RESULTS

Incubation of cells with BCNU. Previously, it was reported that BCNU could react with serum components in the incubation mixture, and the direct interference of BCNU with serum made the medium containing serum undesirable for drug studies [9].

We examined the difference in DNA synthesis of the cells exposed to BCNU in medium containing serum and in isotonic buffer containing no protein. As shown in Fig. 1, the preincubation of cells with BCNU in medium containing serum (RPMI-1640 medium with 20% fetal calf serum) resulted in a clear inhibition of DNA synthesis, and the inhibitory rate in Hank's balanced solution was rather steep. In addition, with Hank's solution, the incorporation of [^{3}H]thymidine into the acid-insoluble fraction (approximately 70 per cent) was less than when the medium contained serum. This suggests that experimental artifacts might occur in the analysis of the processing of newly synthesized DNA, if the cells are incubated in Hank's solution.

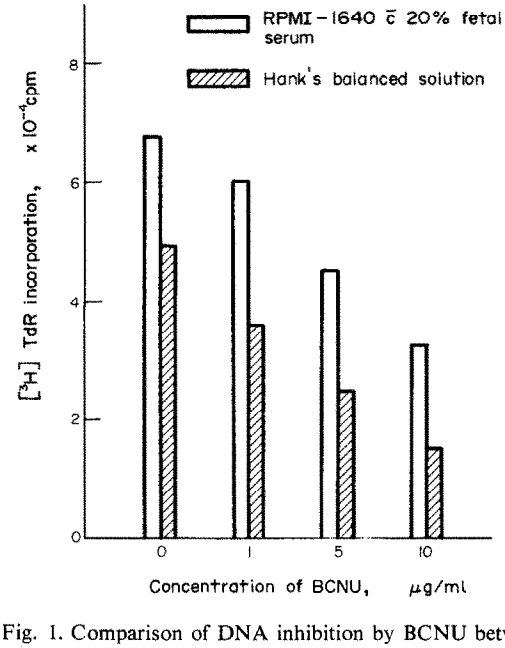

Fig. 1. Comparison of DNA inhibition by BCNU between treatments of cells in RPMI 1640 medium supplemented with 20% fetal calf serum or in Hanks' balanced solution. One ml of L-1210 cells (1×10^6 cells/ml) was incubated with BCNU for 15 min at 37° in RPMI 1640 supplemented with 20% fetal calf serum or in Hank's solution. Both cell suspensions, separately centrifuged at 400 *g* for 5 min, were resuspended in fresh RPMI 1640 medium with 20% fetal calf serum prewarmed at 37°. The cells were labeled with 1 μCi [^{3}H]thymidine for 30 min at 37°, and then acid-insoluble counts were measured. Each value is the average of duplicate samples.

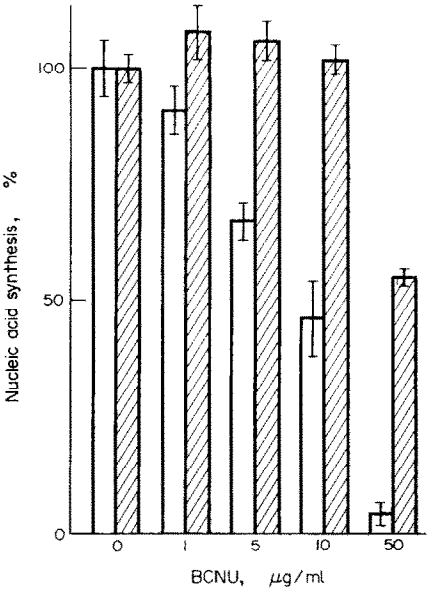

Fig. 2. Inhibition of nucleic acid biosynthesis of L-1210 cells by BCNU. L-1210 cells (1×10^6 cells/ml) were preincubated with BCNU for 15 min at 37°, then 1 μCi [^{3}H]thymidine or [^{3}H]uridine was added in an incubation mixture, and incubation was continued for 30 min at 37°. The counts incorporated into the Cl_3CCOOH-insoluble fraction were measured as described in Materials and Methods. [^{3}H]thymidine, 7.5×10^4 cpm, and [^{3}H]uridine, 1.8×10^3 cpm, were incorporated without BCNU. Open bars and hatched bars show DNA synthesis and RNA synthesis respectively.

Thus, 20% fetal calf serum added to the medium was used in all the following experiments.

Effect of BCNU on nucleic acid synthesis. L-1210 cells were incubated with [^{3}H]thymidine or [^{3}H]uridine after the cells had been preincubated for 15 min in the presence of BCNU.

As shown in Fig. 2, the incorporation of [^{3}H]thymidine into the acid-insoluble fraction was markedly inhibited by BCNU. On the other hand, the incorporation of [^{3}H]uridine into the acid-insoluble fraction was not inhibited by BCNU at concentrations of 1–10 μg/ml. These results suggest that BCNU inhibits DNA synthesis rather selectively.

The kinetics of the incorporation of [^{3}H]thymidine into the acid-precipitable fraction in the presence of BCNU are shown in Fig. 3. Exposure of cells to two different concentrations of BCNU resulted in a significant inhibition of the incorporation of [^{3}H]thymidine into the macromolecular fraction. To determine

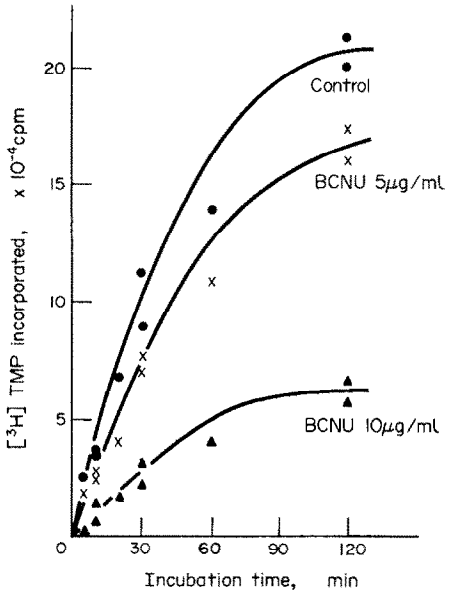

Fig. 3. Kinetics of DNA synthesis in L-1210 cells treated with BCNU. L-1210 cells (1×10^6 cells/ml) were incubated for 15 min at 37° with BCNU, and then 1 μCi [^{3}H]thymidine was added to the incubation mixture and incubation was continued for the desired time in the presence of BCNU. The counts incorporated into the Cl_3CCOOH-insoluble fraction were measured as described in Materials and Methods.

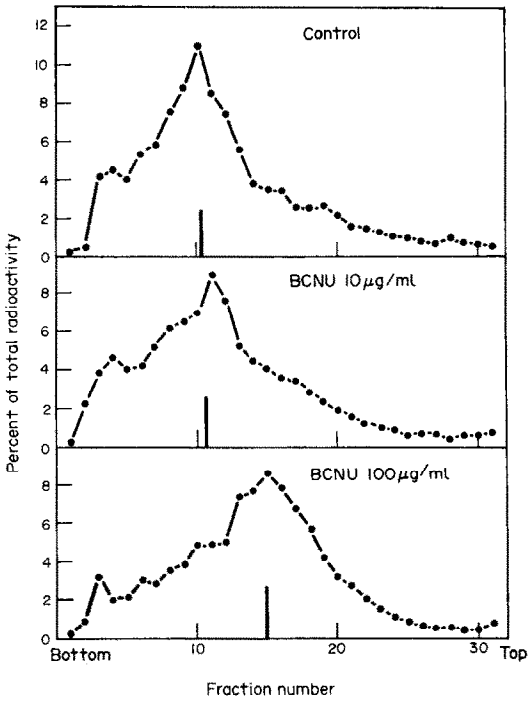

Fig. 4. Effect of BCNU on sedimentation profile of L-1210 cells DNA on alkaline sucrose density gradient centrifugation. Experimental details are described in Materials and Methods. Total counts in each profile recovered were: (cpm) control, 3.2×10^4; BCNU, 10 μg/ml, 3.1×10^4; and BCNU, 100 μg/ml, 2.8×10^4.

whether or not BCNU would affect the uptake of the DNA precursor into the cells, the uptake and conversion of [^{3}H]thymidine into nucleotides in L-1210 cells were measured. As shown in Table 1, the incorporation of [^{3}H]thymidine into the acid-soluble fraction was not inhibited by BCNU at a concentration of 10 μg/ml, which gave about 70 per cent inhibition on DNA synthesis.

Moreover, the radioactivity of nucleotides in the acid-soluble fraction in the drug-treated cells was almost the same as in the control, suggesting that BCNU did not affect the uptake of thymidine into the cells and its conversion into nucleotides in L-1210 cells.

Effect of BCNU on the size of cellular DNA. Attempts were then made to investigate the effect of BCNU on the size of a cellular DNA strand by alkaline sucrose gradient centrifugations. The size of the DNA

Table 1. [^{3}H]thymidine uptake into the acid-soluble pool of L-1210 cells and its conversion to deoxythmidine nucleotides *

BCNU (μ/ml)	Per cent inhibition of DNA synthesis	Acid-soluble $\times 10^{-3}$ cpm	Per cent recovery†	Per cent of nucleotides‡
Control		26.4	100	69
1	18	21.4	80	82
5	39	21.0	79.8	79
10	68	33.7	128	85

* L-1210 cells (1×10^6 cells/ml) were preincubated with 0, 1, 5 and 10 μg/ml of BCNU for 15 min at 37°; then the cells were labeled with [^{3}H]thymidine for 30 min at 37°. The cells were chilled on ice and washed twice with cold 0.85% NaCl solution. The acid-soluble fraction was extracted by cold 0.5 N perchloric acid, neutralized by 1 N KOH, and counted in toluene base scintillation fluid. Approximately 2×10^4 cpm of this fraction was spotted on Toyoroshi No. 51 paper with unlabeled deoxythymidine, dTMP and dTTP as carriers. The nucleoside and nucleotide were separated by paper chromatography as described by Sawada *et al.* [6]. The acid-insoluble fraction was solubilized from the residue of the acid-soluble fraction by treatment with 0.5 N perchloric acid at 100° for 20 min. Each value is the average of triplicate analyses. A value of 1×10^5 cpm was obtained in an acid-insoluble fraction without BCNU treatment.

† Per cent recovery of acid-soluble fraction.

‡ Per cent of nucleotides in acid-soluble fraction.

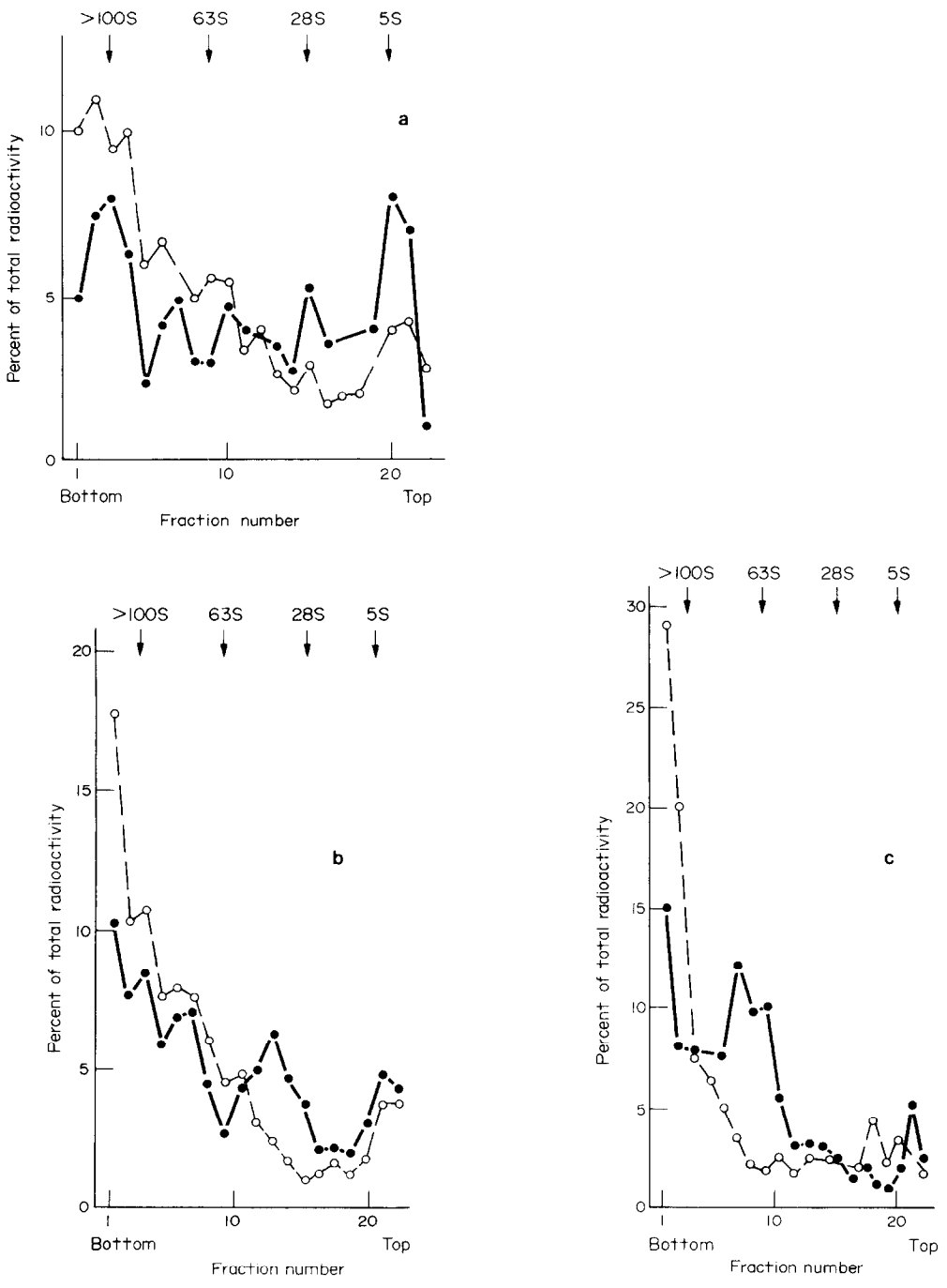

Fig. 5. Alkaline sedimentation of newly synthesized DNA. Experimental details are described in Materials and Methods. ^{32}P-labeled T_4 phage DNA was used as a marker. (a) the cells were labeled at 26° with 30 μCi/ml of [^{3}H]thymidine for 30 sec (●——●), and then chased with unlabeled thymidine for 5 min (○——○). (b) The cells were labeled at 37° with 5 μCi/ml of [^{3}H]thymidine for 20 min (●——●), and then chased with unlabeled thymidine for 20 min (○——○). (c) The cells were labeled at 37° with 1 μCi/ml of [^{3}H]thymidine for 20 min (●——●), and then chased with unlabeled thymidine for 2 hr (○——○). Total radioactivity (cpm) (a) ● (528), ○ (1405); (b) ● (3561), ○ (6076); and (c) ● (16,237), ○ (32,474).

strand in control cells observed by these methods has been estimated to be 170S with the reference of T_4 phage DNA.

As shown in Fig. 4, when the cells were treated with 10 μg/ml of BCNU, the profiles for labeled DNA from BCNU-treated L-1210 cells demonstrated almost the same sedimentation rate as that from the control, suggesting that a low dose of BCNU does not produce a manifest degradation of bulk DNA. On the other hand, increasing the BCNU concentration up to 100 μg/ml caused a breakdown of DNA in the cells, as shown in the bottom panel of Fig. 4.

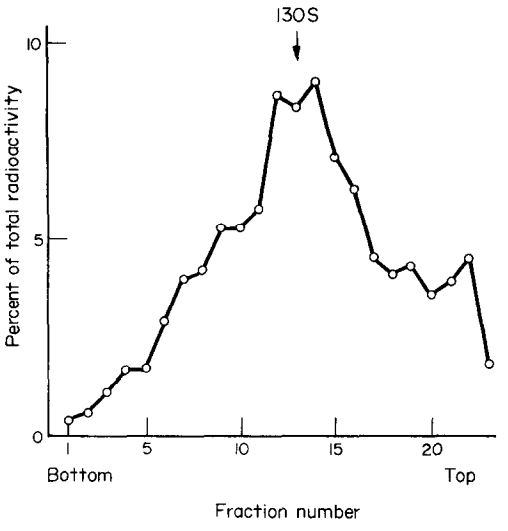

Fig. 6. Size of bottom peak of newly synthesized DNA. L-1210 cells (5×10^5 cells/ml) were labeled with [^{3}H]thymidine (1 μCi/ml) for 60 min and then the cells were lysed on alkaline sucrose gradient for 2 hr at 4° in the dark and then centrifuged at 30,000 rev/min for 2 hr at 4° on a Hitachi RPS-40 rotor. ^{32}P-labelled T_4phage DNA was used as a marker. Total radioactivity (cpm): 61,454.

Effect of BCNU on the newly synthesized DNA. To investigate the kinetics of DNA chain growth, pulse-labeled DNA was analyzed by the alkaline sucrose sedimentation method with a short lysis time (2 hr). When the cells were incubated with [^{3}H]thymidine for 30 sec at 26°, the centrifugation of pulse-labeled DNA revealed three components: slow sedimenting (5S), several small peaks at regions from 30 to 70S, and fast sedimenting (100S). After a 5-min chase at 26° (Fig. 5a), the 5S peak declined, and radioactivity in the high molecular weight region (50S) of the gradient increased. Figure 5b shows the sedimentation profiles of newly synthesized DNA labeled for 2 min at 37° and then chased for 20 min at 37°. The sedimentation of DNA immediately after a 2-min pulse showed a peak at the 30S portion. Broad peaks at high molecular weight regions around 70S and 5S peak were also visible. After a 20-min chase, the 30S peak disappeared and the radioactivity moved onto the high molecular weight regions.

The late stage of replication is shown in Fig. 5c. Immediately after the 20-min labeling period, most of the DNA sedimented in 50–70S portions and moved into the bottom peak after a 2-hr chase. The above results suggest that the initially synthesized DNA in L-1210 cells is in short segments with a molecular weight of approximately 5S and that these segments increase in size to 30, 70 to over 100S. For estimation of the size of the bottom peak, the cells labeled with [^{3}H]thymidine for 60 min were lysed on the alkaline sucrose gradient for 2 hr as described above; then the gradients were centrifuged at 30,000 rev/min for 2 hr at 4°. As shown in Fig. 6, the peak appeared to sediment at about 130S. In order to examine the effects of BCNU on replication of DNA, the L-1210 cells were incubated

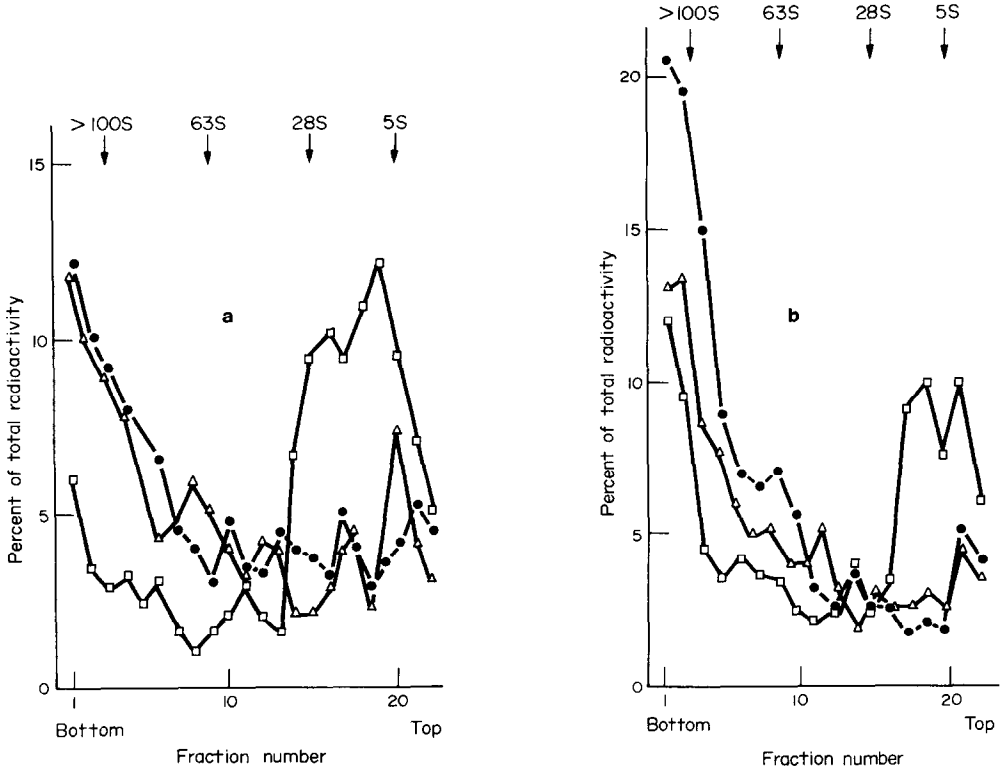

Fig. 7. Effect of BCNU on the newly synthesized DNA in L-1210 cells. Cells were preincubated with BCNU for 15 min at 37° and then labeled with [^{3}H]thymidine (5 μCi/ml) for 2 min (a), and then chased with unlabeled thymidine for 20 min (b). Total radioactivity (cpm):
(a) control (●——●) 8035; BCNU, 1 μg/ml (△——△) 6231; and BCNU 5 μg/ml (□——□) 2234;
(b) control (●——●) 17550; BCNU 1 μg/ml (△——△) 14,264; and BCNU 5 μ/ml (□——□) 5370.

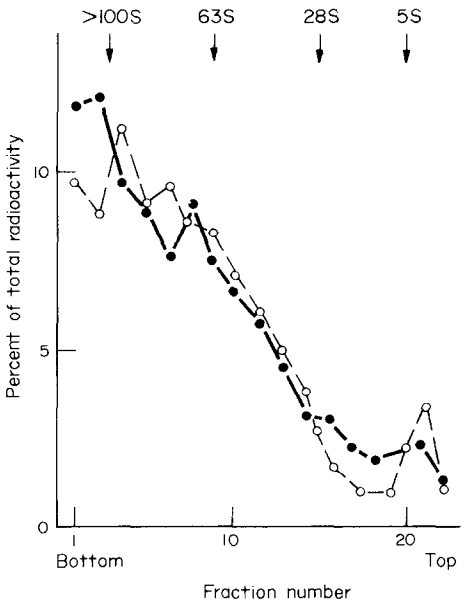

Fig. 8. Effect of BCNU on the late stage of DNA replication. The cells were labeled with [^{3}H]thymidine (5 μCi/ml) for 2 min and then were treated with 5 μg/ml of BCNU for 20 min in the presence of excess unlabeled thymidine (10^{-5} M). The cells were then lysed and centrifuged through an alkaline sucrose gradient as in Fig. 5. Total radioactivity (cpm): control 53,834 cpm (●—●), and BCNU 5 μg/ml 50,420 cpm (○···○).

with [^{3}H]thymidine for 2 min at 37° after being preincubated with BCNU for 15 min, and the sedimentation patterns of pulse-labeled DNA were compared with the control, using the same alkaline sedimentation procedure.

As shown in Fig. 7a, when the cells were treated with 1 μg/ml of BCNU, the sedimentation profile for newly synthesized DNA was almost the same as that of the control. However, it was found that 5–30S fragments of DNA were accumulated significantly in L-1210 cells treated with 5 μg/ml of BCNU. Figure 7b shows that the number of short segments of DNA in the BCNU-treated cells declined and was replaced by increasing radioactivity in the bottom peak after the cells had been chased for 20 min by adding excess unlabeled thymidine. This suggests that some of the joining process may occur even in BCNU-treated cells. To determine the effect of BCNU on the late stage of DNA replication, the cells were labeled with [^{3}H]thymidine for 2 min, and were then treated with 5 μg/ml of BCNU for 20 min in the presence of excess unlabeled thymidine. As shown in Fig. 8, the sedimentation pattern from BCNU-treated cells was almost the same as the control, thereby suggesting that the drug did not affect the late stage of DNA replication.

DISCUSSION

Although BCNU, the well-known chemotherapeutic agent [1–3], is considered to be an alkylating agent [4], much evidence suggests that the molecular mechanism responsible for the antitumor activity of BCNU is not a simple alkylation and that multiple points of nucleic acid metabolism are involved. For example, it has been reported that this nitrosourea compound inhibits DNA polymerase II activity [10], nucleotidyltransferase activity [11], ribosomal RNA maturation [12], DNA repair activity [13] and initiation of protein synthesis [14]. Our results suggest that BCNU inhibits DNA synthesis selectively in L-1210 cells, which is in accord with previous reports [15, 16]. However, our results reveal that this drug neither affects the uptake into the cells nor the metabolism of [^{3}H]thymidine. Our results also suggest that BCNU does not result in a manifest degradation or produce an alkaline labile bond in cellular DNA, unless the cells are treated with high doses of BCNU (100 μg/ml). Such may induce an artifact breakdown of DNA as the result of cell damage.

We used the alkaline sedimentation procedure developed by Friedman *et al.* [8] with slight modifications and we found that the growth of replicating DNA chains in log phase L-1210 cells may be divided into three phases: Most of the initially synthesized DNA is in short segments that sediment approximately at 5S, although the possibility that some of the synthesis occurs directly at the end of long strands cannot be excluded. The intermediate stage of chain growth is the joining of short segments to form 30S intermediate strands and 50–70S pieces which may represent complete replicons. The third phase is the joining of complete replicons to form larger strands which sediment approximately at 130S. Such is in agreement with the findings of Friedman *et al.* [8]. When the cells were treated with BCNU (5 μg/ml), the sedimentation distribution revealed that short segments of DNA (5–30S) were significantly accumulated in the cells. Thus, BCNU may not primarily inhibit the initiation of replicons but rather the polymerization process to the size of replicons. From the chasing experiments, it seemed likely that the joining of replicons to form bulk DNA may not be inhibited by this drug. This step of DNA replication was shown to be inhibited specifically by hydroxyurea [17].

Although the molecular mechanism of DNA synthesis is not entirely understood, our results may show that BCNU could react with maternal DNA and induce structural modifications which would induce inhibition of DNA chain growth. It has already been reported that BCNU can produce altered bases and also DNA cross links as the results of alkylation [18–20]. We also have investigated the antibacterial activity of BCNU using isogenic strains of *Escherichia coli* with a normal or defective DNA repairing system [21]. The results showed that growth of the strains lacking the recA gene was inhibited by BCNU in a much lower concentration in the case of those possessing it, and some mutation was also detected in the recA$^+$ strain by BCNU (Tashima *et al.* unpublished data). The above results further support the view that BCNU probably interacts with DNA and inhibits the processing of DNA synthesis.

Although Erickson *et al.* [22], applying the technique of alkali elution, showed that BCNU caused single strand breaks in normal and SV-40 transformed human embryo fibroblasts, we did not detect any decrease in bulk DNA size in L-1210 cells treated with the concentration of BCNU which induced the accumulation of short, newly synthesized DNA. It had already been reported that alkylation of DNA did not necessarily cause detectable single strand breaks [23].

Our results may show that the primary effect of BCNU on DNA synthesis in L-1210 cells is the accumulation of short, newly synthesized DNA. However, it remains to be clarified whether or not the same mechanism is responsible for its activity in normal cells.

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