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EFFECT OF 1,3-*BIS*-(2-CHLOROETHYL)-1-NITROSOUREA ON EHRLICH ASCITES TUMOR CELLS*

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Abstract—The effects of 1,3-*bis*-(2-chloroethyl)-1-nitrosourea on the synthesis of deoxyribonucleic acid, ribonucleic acid, and protein in Ehrlich ascites tumor cells was investigated *in vitro* by measuring rates of incorporation of radioactive precursors into the acid-insoluble fraction of the cells. Effect of the drug on the concentration of diphosphopyridine nucleotide (NAD) was determined by fluorometric measurement of the nucleotide. At concentrations compatible with those obtainable *in vivo*, there was a marked reduction in the rate of DNA synthesis, accompanied by an increase in the rate of RNA synthesis. Protein synthesis was affected only slightly at the lower drug concentrations. At higher concentrations all three parameters were inhibited. The concentration of NAD in the cells was increased at low drug concentrations and depressed at higher levels.

SCHABEL *et al.*[1] recently reported quantitative therapeutic studies of 1,3-*bis*-(2-chloroethyl)-1-nitrosourea (NSC-409962; BCNU) which revealed that this compound possesses marked activity against intraperitoneal L1210 leukemia when administered intraperitoneally, subcutaneously, or orally. In addition, this class of compounds is the first to be observed to possess an encouraging degree of activity against intracerebrally implanted L1210 leukemia. In a clinical trial of the drug, Rall *et al.*[2] noted rather severe toxicity in monkeys and humans, but nevertheless found that meningeal leukemia was controlled in 100% of five patients. Goldin *et al.*[3] found a marked effectiveness of the drug against a number of experimental leukemias over a wide range of treatment schedules, and urged detailed studies of its mechanism of action.

The action of BCNU on certain aspects of the metabolism of the Ehrlich ascites tumor has now been investigated; a report follows.

EXPERIMENTAL

The design of the experiments was to investigate with Ehrlich ascites tumor cells the syntheses of deoxyribonucleic acid, ribonucleic acid, and protein by measuring the rates of incorporation of thymidine-methyl-^{3}H, uracil-5,6-^{3}H, and L-leucine-uniformly labeled-^{14}C, respectively, in the presence of a range of concentrations of BCNU. In addition to the uptake of isotopic precursors, the effect of the drug on NAD content of the cells was also determined, in view of the findings of Dold *et al.*[4] regarding the alteration of pyridine nucleotide content of cells by alkylating agents.

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The isotopic thymidine, uracil, and L-leucine, with specific activities of 6·7, 1·41 and 0·223 c/mmole, respectively, were obtained from New England Nuclear Corp., Boston, Mass. BCNU was obtained from the Drug Evaluation Branch of the Cancer Chemotherapy National Service Center, Bethesda, Md., and was stored at −20°. The source of tumor cells and preparation of these for studies *in vitro* were substantially as described earlier,[5] except that a final cell suspension of 1% (v/v) in NCTC-109 medium was used.

For the series of experiments described in Fig. 1, 4·5 ml of the 1% tumor cell suspension plus 0·5 ml of BCNU in 0·9% NaCl, or 0·9% NaCl alone, were added to flat-bottomed test tubes (20 mm o.d.) and incubated in a water bath at 37° with gentle agitation. After 60 min, tubes with and without BCNU received 25 μc thymidine-^{3}H, 25μc uracil-^{3}H, or 1 μc L-leucine-^{14}C, each in 0·5 ml of 0·9% NaCl, and were returned to the water bath. At 30 min and 60 min after addition of the isotopes, 2·0 ml was withdrawn from each of the six tubes and immediately mixed with an equal volume of 3% trichloroacetic acid. The cells were sedimented by centrifugation in conical tubes and the pellets washed three times with 15 ml water by resuspension and sedimentation. After the final washing, the pellets were suspended in 0·5 ml methyl alcohol, and 2·0 ml hydroxide of Hyamine was added to each. The suspensions were allowed to incubate overnight for solubilization, after which each was transferred quantitatively to 15 ml of a toluene solution of PPO–POPOP phosphor (Packard Instrument Co., Downers Grove, Ill.). Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer, and the activity expressed as counts per minute (cpm) in the acid-insoluble residue contained in the 2·0-ml aliquot. In studies of the time course of inhibition by BCNU of the uptake of thymidine, the procedure was the same except that each mixture was prepared in triplicate, and the isotope was added immediately after addition of BCNU. Replicate tubes permitted removal of two 2·0-ml aliquots from each of three tubes which contained a total volume of 5·5 ml each. Samples were withdrawn for processing and radioactivity determinations at intervals over a period of 120 min, and expression of cpm was as above.

For NAD determinations, identical tubes as above were prepared except that 0·5 ml of 0·9% NaCl replaced the 0·5 ml of isotope solution. After 120-min incubation the contents of each tube were transferred to a chilled conical centrifuge tube, washed three times with cold 0·9% NaCl, and the cell pellet was extracted with 2·0 ml of 3% TCA. The supernatant solutions were removed after centrifugation and analyzed for NAD by the fluorometic method[6] in a Farrand fluorometer.

RESULTS

Figure 1 shows the effects of BCNU on the synthesis of DNA, RNA, and protein over a 50-fold concentration range. In the following descriptions of the curves, when per cent inhibition is stated the two values in each case refer to per cent of control at 30 min and 60 min respectively.

At 50 $\mu g/ml$, a concentration perhaps considerably greater than that obtainable *in vivo*, inhibition of synthesis of DNA and of protein was virtually complete, while that of RNA was depressed, but to a lesser degree.

At 25 $\mu g/ml$, synthesis of DNA and of protein was still inhibited markedly, and RNA synthesis was strongly enhanced.

At 10 $\mu g/ml$, which may be considered the upper limits of theoretical concentrations

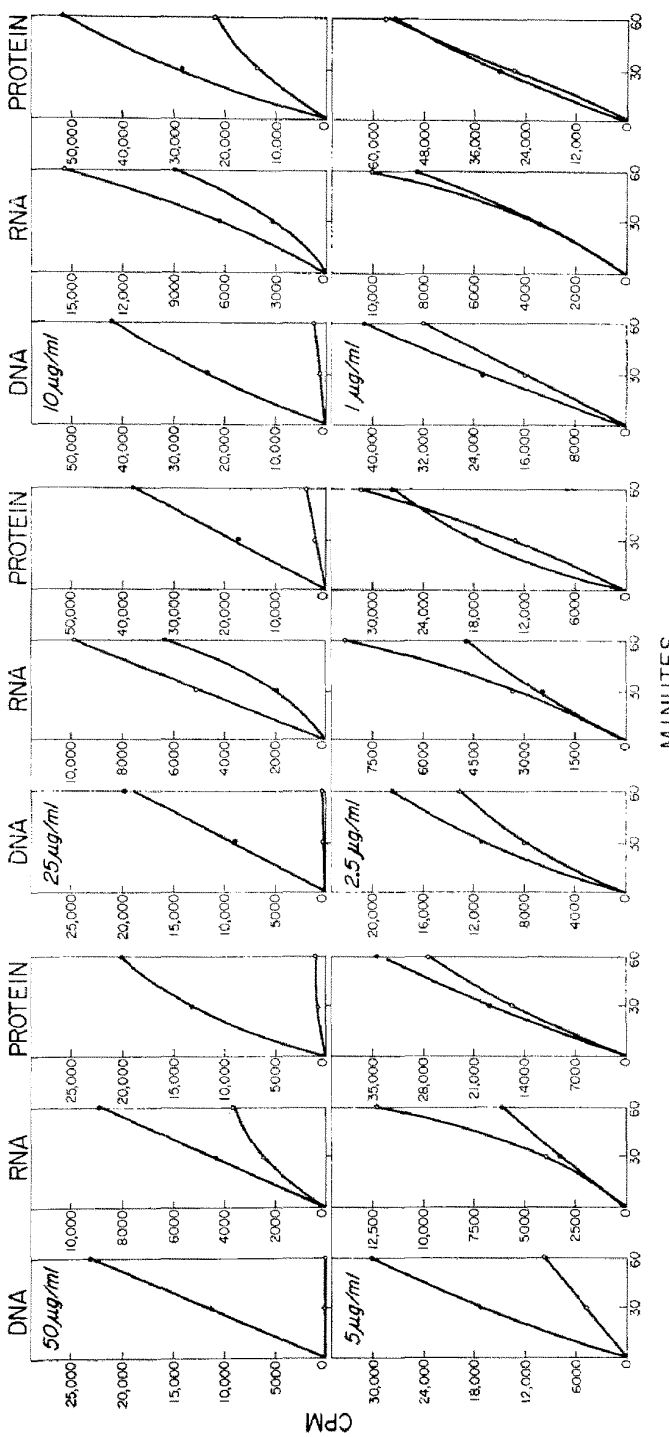

FIG. 1. Effects of several concentrations of BCNU on synthesis of DNA, RNA, and protein in Ehrlich ascites tumor cells; ●, control; ○, with BCNU at the concentration designated in each triad of curves. Cells were preincubated with the drug 60 min prior to addition of radioactive precursor.

obtainable *in vivo* upon chronic administration, striking differences in responses of the three parameters were evident. DNA synthesis was depressed 94% at both sampling intervals, while protein synthesis was depressed only 51% and 57%. Inhibition of these processes was in contrast to the 94% and 71% increases in rate of RNA synthesis.

At 5 *μg/ml*, the pattern was similar, except that synthesis of both DNA and protein was less affected; the former was decreased 72% and 68%, while that of the latter was reduced only 17% and 21%. Rate of RNA synthesis was elevated 19% and 84%. The progressive increase of the RNA curve at this drug concentration suggests a delayed action of the drug per se, or a secondary effect due to a possible primary inhibition of DNA synthesis.

At 2·5 *μg/ml*, DNA synthesis was inhibited 30% at both intervals, RNA formation was increased 34% and 76% (again progressive), and the curve of protein synthesis in the presence of the drug was biphasic, being first inhibited 25%, and then stimulated 14%.

At 1·0 *μg/ml*, the pattern was much the same as at 2·5 μg/ml. DNA synthesis was depressed 29% and 22%, and RNA synthesis was elevated by 0% and 21%. The curve of protein synthesis was again biphasic, showing 11% inhibition followed by 4% stimulation. Even though the significance of this latter value may be questioned, the value is given in view of the similarity of the response to that obtained at 2·5 μg/ml.

The time course of the depression of DNA synthesis by BCNU at two concentrations. is shown in Fig. 2. At 2·5 μg/ml, there appeared to be no significant lag in onset of

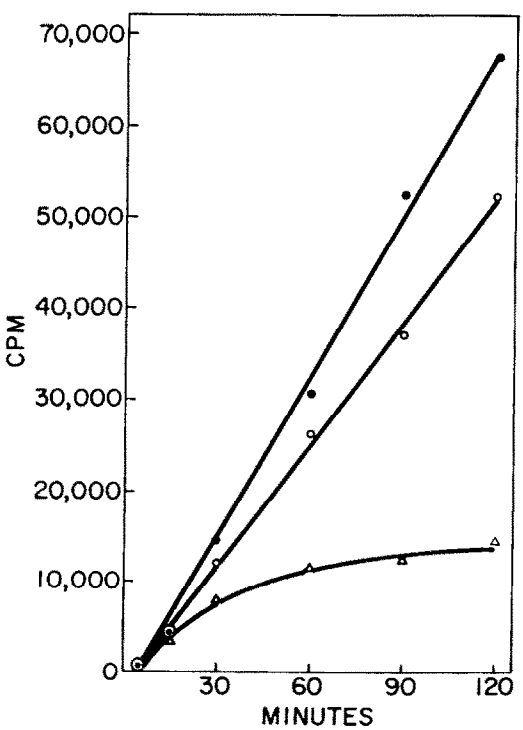

FIG. 2. Time course of the inhibition by BCNU of DNA synthesis in Ehrlich ascites tumor cells; ●, control; ○, 2·5 μg BCNU/ml; △, 10 μg BCNU/ml. BCNU added immediately prior to thymidine-^{3}H.

inhibition, and the curve was virtually linear over the 120-min period of measurement. The extent of inhibition was 23% at 120 min. At 10 μg BCNU/ml, some depression of synthesis was evident at the earliest sampling period, but increased in magnitude with time. The reductions were 36, 31, 46, 63, 76, and 78% at 5, 15, 30, 60, 90, and 120 min, respectively.

The effect of BCNU on the content of NAD in the tumor cells is shown in Fig. 3. Data are expressed as per cent of control NAD in cells to which no BCNU was added. After a slight increase in the nucleotide at lower drug concentrations, there was a decrease of 16% at 25 μg/ml, and 66% at 50 μg/ml.

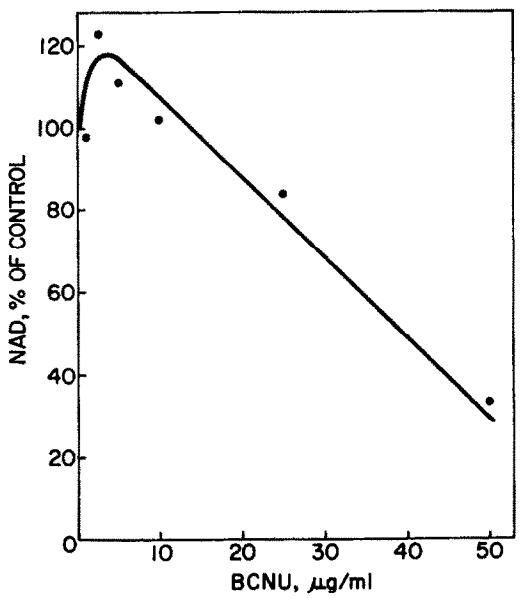

FIG. 3. Effect of BCNU on NAD content of Ehrlich ascites tumor cells.

DISCUSSION

In determining the effect of BCNU on intraperitoneally implanted L1210 leukemia, Schabel *et al.*[1] found that a plot of all available data suggested an optimal dose of 8·5 mg/kg/day, and Wheeler *et al.*[7] found that BCNU labeled with ^{14}C in the carbonyl or the two 2-chloroethyl groups was found in various tissues of mice after intraperitoneal injection. Assuming equal distribution of the drug in total body water, and assigning a value of 70% to the latter, it can be calculated that the highest theoretical concentration that could be obtained upon injection of 8·5 mg/kg would be approximately 12 μg/ml in the body water. The concentration of drug in tissues of mice drops rapidly during the first few hours after injection but changes only slightly between 24 and 48 hr.[7] In general, Wheeler *et al.*[7] found from 6% to 32% of the injected dose present in the tissues after 6 hr, while values at 24 hr ranged from 4% to 12%, with considerable variations occurring among the different tissues. Thus the 50 μg/ml and 25 μg/ml curves in Fig. 1 are of academic interest only and likely have no relationship to the situation *in vivo*, excluding the possibility of an as yet undemonstrated accumulation of the drug at some site.

Consequently the other four sets of curves may be examined for suggestions as to mode of action of the drug. At 10 μg/ml, synthesis of DNA was markedly depressed while that of protein was decreased to just over half that of the control rate. RNA synthesis was stimulated. At still lower concentrations protein synthesis was less affected, and the parameters displaying the greatest magnitude of change were DNA and RNA syntheses. With BCNU at 1 μg/ml, per cent depression of DNA formation and per cent elevation of RNA synthesis were the same. Thus of these three processes, it would appear that the one most likely to be involved in the carcinostatic activity of BCNU is the inhibition of DNA synthesis; more extensive investigations seem warranted to determine whether the elevated rate of RNA formation is a primary action of the drug or is in some way secondary to the block in DNA synthesis.

The fact that NAD was slightly elevated by drug concentrations corresponding to probable levels *in vivo* tends to rule out this effect as a significant aspect of the action of the drug.

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