Studies on the Mechanism of Action of ACNU, 1-(4-Amino-2-Methylpyrimidine-5-yl) Methyl-3-(2-Chloroethyl)-3-Nitrosourea Hydrochloride: Effects on Cultured HeLa S₃ Cells

RYUNOSUKE KANAMARU, MITSUO ASAMURA, HARUHIKO SATO, SACHIKO SAITO, AKIRA WAKUI and TATUO SAITO*

*Department of Clinical Cancer Chemotherapy, the Research Institute for Tuberculosis and Cancer, Tohoku University, Sendai 980 and *Department of Internal Medicine, the Cancer Institute Hospital, Tokyo 170*

KANAMARU, R., ASAMURA, M., SATO, H., SAITO, S., WAKUI, A. and SAITO, T. *Studies on the Mechanism of Action of ACNU, 1-(4-Amino-2-Methylpyrimidine-5-yl) Methyl-3-(2-Chloroethyl)-3-Nitrosourea Hydrochloride: Effects on Cultured HeLa S₃ Cells.* Tohoku J. exp. Med., 1980, 132 (4), 431-441 — Treatment of cultured HeLa S₃ cells with ACNU, 100 µg/ml, for 30 min inhibited the cell growth intensely. The cell number was minimum on the 4th day after the treatment and recovered gradually, but it was still 24% of control on the 14th day. The colony formation, as an index of proliferation ability of cells, was remarkably inhibited and the number of colony formed on the 14th day after the treatment was 8% of control. DNA synthesis was inhibited by 59%, whereas little or no effect was observed on RNA or protein synthesis after 24 hr. Ethylene-¹⁴C-ACNU was bound to DNA and RNA to a similar degree, and that bound to the acid-insoluble fraction within 30 min was decreased by half after 24 hr and sustained thereafter. A significant decrease in sedimentation velocity of DNA on alkaline sucrose density gradient centrifugation was observed after 24 hr, and the decrease on neutral sucrose density gradient centrifugation was noticeable already by 2 hr. From the results of this study and other reports, the mechanism of action of ACNU seems to be alkylating of DNA followed by damage to DNA, which progresses quite slowly compared with other alkylating agents, causing cell damage and cell death. — anti-cancer drug; nitrosourea; alkylation; damage to DNA; HeLa S₃ cells

Studies on anti-cancer activity of nitrosourea derivatives were started from the report in 1960 at Cancer Chemotherapy National Service Center, NIH, USA that MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) exhibited some anti-tumor activities against mouse murine leukemia L-1210 (Greene and Greenberg 1960). Thereafter, several derivatives with more potent anti-tumor activities were synthesized, and BCNU, CCNU and methyl-CCNU have been investigated clinically

Received for publication, December 19, 1979.

This paper is presented in commemoration of the retirement of Professor Tatuo Saito, the Director of the Research Institute for Tuberculosis and Cancer, Tohoku University, April, 1978.

and appraised as valuable antitumor agents (Johnston et al. 1966, 1971; Carter et al. 1972; Schepartz 1976). Since the report by Skipper et al. (1961) that MNU (N-methyl-N-nitrosourea) differs from anti-cancer agents developed so far such as 5-fluorouracil, methotrexate and mitomycin C in the effectiveness on intracerebrally implanted L 1210 cells, nitrosourea derivatives have been in limelight as anti-cancer agents which can pass the blood-brain barrier.

In relation to the mechanism of action of nitrosourea derivatives, analysis of alkylation- or carbamoylation-products has been extensively performed, and BCNU and CCNU have been reported to possess strong alkylation and carbamoylation activity (Montgomery et al. 1967; Cheng et al. 1972; Wheeler et al. 1975). In practice, however, parenteral administration of these nitrosourea derivatives has rather been hampered by their little or no solubility in water. ACNU, 1-(4-amino-2-methylpyrimidine-5-yl) methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride, which was synthesized in 1974 in the Central Research Laboratory, Sankyo Co., Ltd., Tokyo, is water-soluble and was found by Arakawa et al. (1974) to be highly active against mouse leukemia L 1210, prolonging the surviving period of tumor-bearing mice. An intraperitoneal administration of 30 to 50 mg/kg of ACNU attained about seven times prolongation, on an average, of the surviving period. In this paper, effects of ACNU on the cultured HeLa S₃ cells were studied in order to clarify the mechanism of action of ACNU.

MATERIALS AND METHODS

HeLa S₃ cells were cultured in Eagle MEM medium containing 20% calf serum in a CO₂ incubator. ACNU was kindly supplied by Sankyo Co., Ltd. Synthesis of DNA, RNA or protein in the cells was determined by incorporation of ³H-thymidine (thymidine-6-³H, 11.4 Ci/mmol), ³H-uridine (uridine-5-³H, 17.7 Ci/mmol) or ³H-leucine (leucine-4,5-³H, 32.0 Ci/mmol) into the acid-insoluble fraction of the cell, respectively. The methods employed were described by Ono et al. (1973). The cells cultured on a Falcon dish 35 mm in diameter (Falcon Co., Ltd., USA) were incubated with 1 μ Ci of one of the above described radioactive precursors for a given period of time. Cells were collected by centrifugation. After washing twice with PBS (phosphate buffer solution; NaCl 8 g, KCl 0.2 g, Na₂HPO₄ 1.15 g and KH₂PO₄ 0.2 g in 1 liter of solution), the cells were resuspended in PBS of about 5 ml and were collected on a glass fiber filter paper disc (Whatman GF-C, 24 mm in diameter) and washed with 10 ml of PBS, 30 ml of cold trichloroacetic acid, and finally with 15 ml of 95% ethanol followed by air-drying. The filter was placed in a toluene-containing scintillation cocktail (2,5-diphenyloxazol 4 g and 1,4-bis-(5-phenyloxazolyl)-benzene 400 mg in 1 liter of toluene), and the radioactivity was measured with a liquid scintillation spectrometer (Beckman Model LS 133).

Incorporation of ¹⁴C-ACNU (ethylene-¹⁴C-ACNU, 1.14 mCi/mmol) into the acid-insoluble fraction was similarly determined. ¹⁴C-ACNU was offered from the Central Research Laboratory, Sankyo Co., Ltd. Fractionation of DNA and RNA was performed according to the method of Schmidt and Thanhauser (1945). Single strand breakage of DNA was detected by alkaline sucrose density gradient centrifugation. HeLa S₃ cells of 2 $\times$ 10⁴ labeled with ³H-thymidine for 24 hr and suspended in 0.1 ml of phosphate buffer solution (0.14 M NaCl, 0.03 M KCl, 0.01 M phosphate buffer solution, pH 7.4) were lysed by mixing with 0.2 ml of lysing solution (0.55 M NaCl, 0.4 M NaOH and 0.001 M Na₂EDTA) layered on the top of an alkaline sucrose density gradient, which was 4.7 ml of a 5–20% linear sucrose density gradient containing 0.3 N NaOH, 0.7 M NaCl, 0.01 M Na₂EDTA,

0.01 M Tris and, as a cushion, 0.2 ml of 80% sucrose underneath the gradient. The lysis of the cells was carried out at 37°C for 60 min. Centrifugation was performed in an RPS 40 rotor of Hitachi 65P ultracentrifuge at 30,000 rpm at 10°C for 120 min. Analysis of the damaged DNA by neutral sucrose density gradient centrifugation was carried out by the method of Terashima and Tsuboi (1969). Approximately, 5,000 cells were lysed by mixing with 0.2 ml of 2% sodium dodesyl sulfate solution layered on the top of 4.7 ml of the 5–20% sucrose density gradient containing 0.01 M Tris-HCl buffer at pH 7.4, 0.1 M NaCl and 0.01 M Na₂EDTA and, as a cushion, 0.2 ml of 80% sucrose solution underneath the gradient. The tubes were incubated at 37°C for 20 min and centrifuged at 25,000 rpm at 10°C for 50 min. After centrifugation, fractions were collected from the bottom of the tube on a glass fiber filter paper disc (Toyo Roshi, No. 200A), the paper disc was dried, and the radioactivity was measured in the scintillation cocktail, using a Beckman Scintillation Spectrometer. For the determination of colony forming activity, the cells were collected by centrifugation and suspended in RPMI 1640 medium (Flow Lab. Rockville, Md., USA) supplemented with 10% calf serum. ACNU dissolved in the above described medium was added to the cell suspension of 5×10^5 cells/tube at the final concentrations of 100, 10 and 1 $\mu\text{g/ml}$. After incubation in an atmosphere of 5% CO₂ in air at 37°C for 1 hr and centrifugation at 1000 rpm for 5 min, ACNU was removed and the cells were resuspended in the medium. On a 60 mm Falcon dish, 200 cells were inoculated and cultured under the same condition. Every 3 days, the medium was changed. On the 14th days of culture, the number of the colonies formed was counted macroscopically after fixation and Giemsa Staining.

RESULTS

Effect of ACNU on the growth of HeLa S₃ cells

Changes in cell number after the inoculation of 2×10^4 cells/dish of HeLa S₃ cells with various concentrations of ACNU are shown in Fig. 1. At a concentration not higher than 10 $\mu\text{g/ml}$, no significant difference from control was observed. At 100 $\mu\text{g/ml}$, a marked inhibition of growth was recognized. The cell

Fig. 1. Effect of ACNU on the growth of HeLa S₃ cells.

Experimental procedures were as described in the text. Each value shows mean of 5 experiments.

○—○, cells treated with 10 $\mu\text{g/ml}$ of ACNU for 30 min; △—△, treated with 100 $\mu\text{g/ml}$ of ACNU for 30 min; ●—●, control.

number decreased by half after 4 days, then recovered gradually. When proliferation ability was determined from colony formation ability, no inhibition was observed at a concentration lower than 10 $\mu\text{g/ml}$ as was the case of cell growth, but the inhibition to an extent of 7.9% of control was observed at the concentration of 100 $\mu\text{g/ml}$ (Table 1). Colonies were measured after direct fixing of cells on the Falcon dish and Giemsa staining. A colony consisted of more than 50 cells.

TABLE 1. *Effect of ACNU on colony formation of HeLa S₃ cells*

ACNU ($\mu\text{g/ml}$)	Number of colonies formed	% Control
0	270.6	100
1	301.0	111.2
10	267.2	98.7
100	21.4	7.9

Experimental procedures were as described in the text.
The values show mean of 5 experiments.

Effect of ACNU on DNA, RNA and protein syntheses

After treatment with various concentrations of ACNU for 30 min, the cells were reincubated in a drug-free medium, and incorporation of 1 $\mu\text{Ci/dish}$ of ^3H -thymidine, ^3H -uridine or ^3H -leucine for 1 hr was determined at 2, 24 and 48 hr. As shown in Table 2, DNA synthesis was inhibited already at 2 hr by 13% at the concentration of 10 $\mu\text{g/ml}$ and 59% at 100 $\mu\text{g/ml}$, whereas RNA synthesis was not influenced at all and protein synthesis was slightly enhanced. At 24 hr after ACNU treatment, DNA synthesis was recovered to the level of control in the cells treated with 10 $\mu\text{g/ml}$, while no recovery was observed in the cells treated with 100 $\mu\text{g/ml}$. In the latter cells, slight inhibition of RNA and protein syntheses was accompanied.

TABLE 2. *Effect of ACNU on DNA, RNA and protein syntheses of HeLa S₃ cells*

	ACNU ($\mu\text{g/ml}$)	2 hr (%)	24 hr (%)	48 hr (%)
DNA synthesis	0	100	100	100
	1	93	104	100
	10	97	105	65
	100	41	48	6.7
RNA synthesis	0	100	100	100
	1	112	116	114
	10	98	102	72
	100	117	89	44
Protein synthesis	0	100	100	100
	1	124	92	110
	10	126	102	66
	100	122	72	37

Experimental procedures were as described in the text.
The values show means of 3 experiments.

At 48 hr after ACNU treatment, syntheses of DNA, RNA and protein were inhibited to 65, 72 and 66% of control, respectively, in the cells treated with 10 $\mu\text{g}/\text{ml}$. On the other hand, the cells treated with 100 $\mu\text{g}/\text{ml}$, DNA synthesis was inhibited to 6.7% of control, and RNA and protein syntheses were also strongly inhibited to 44% and 37% of control, respectively.

Binding of ^{14}C -ACNU to high molecular substances in the cells

In order to examine the duration of binding of ACNU to high molecular substances in the cells, HeLa S₃ cells were incubated with 100 $\mu\text{g}/\text{ml}$ of ethylene- ^{14}C -ACNU for 30 min, and the radioactivity in the acid-insoluble fraction was determined after re-incubation in ^{14}C -ACNU-free medium. As shown in Table 3, the radioactivity decreased as time elapsed and to about half of control after 24 hr and this level was sustained thereafter. In this table, the radioactivity was measured per dish. As the cell number did not decrease until 48 hr after the treatment with 100 $\mu\text{g}/\text{ml}$ of ACNU as shown in Fig. 1, the radioactivity per cell was considered to decrease similarly. Binding of ^{14}C -ACNU to either DNA or RNA was not significantly different at 4 and 24 hr after the treatment as shown in Table 4, suggesting the saturation of the binding of ACNU to nucleic acids in the cells rather in a short period. The quantities of ^{14}C -ACNU bound to DNA and RNA were not significantly different.

TABLE 3. *Binding of ethylene- ^{14}C -ACNU to acid-insoluble fraction of HeLa S₃ cells*

Incubation period (hr)	Radioactivity (cpm/dish)	% Control
1	5685	100
5	4089	71.9
24	2955	52.0
48	2876	50.6
72	2666	46.9

Experimental procedures were as described in the text.
The values show means of 3 experiments.

TABLE 4. *Binding of ethylene- ^{14}C -ACNU to DNA and RNA of HeLa S₃ cells*

Incubation period (hr)	DNA (dpm/mg ENA)	RNA (dpm/mg RNA)
4	3794	5252
24	4987	4441

Experimental procedures were as described in the text.
The values show means of 3 experiments.

Analysis of DNA of HeLa S₃ cells treated with ACNU by alkaline sucrose density gradient centrifugation

Fig. 2a shows the sedimentation profile of DNA of the cells immediately after the treatment with ACNU for 30 min. The main peak shifted slightly towards the

Fig. 2. Sedimentation profile on alkaline sucrose density gradient centrifugation of DNA of HeLa S_2 cells (a) immediately after, (b) 24 hr after and (c) 48 hr after the treatment with ACNU for 30 min.

---- cells treated with 10 $\mu\text{g/ml}$ of ACNU; $\circ$ — $\circ$, cells treated with 100 $\mu\text{g/ml}$ of ACNU; — control.

top and became broad in the cells treated with 100 $\mu\text{g/ml}$ of ACNU, indicating the beginning of fragmentation of single strand DNA. After 24 hr, this tendency became quite definite as shown in Fig. 2b. The peak of control was located at the 10th fraction, while those treated with 10 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ moved to the 15th and 20th fractions, respectively, indicating marked breakage of single strand DNA

by ACNU. After 48 hr, the peak of DNA in the cells treated with 10 $\mu\text{g/ml}$ coincided roughly with that of control, but that of the cells treated with 100 $\mu\text{g/ml}$ was still broadened towards the top. These results suggested that the damage of DNA in the cells treated with 10 $\mu\text{g/ml}$ of ACNU was repaired almost completely, and that the damage in the cells treated with 100 $\mu\text{g/ml}$ of ACNU was only partially repaired (Fig. 2c). Table 5 shows the unscheduled DNA synthesis of the

TABLE 5. *Unscheduled DNA synthesis in HeLa S₃ cells 24 hr after the treatment with various concentrations of ACNU for 30 min*

ACNU ($\mu\text{g/ml}$)	³ H-TdR incorporated/10 ⁴ cells (cpm)		+HU/-HU* (%)
	(-Hydroxyurea)	(+Hydroxyurea)	
0	3560	434	12.2
10	3344	551	16.5
100	2216	943	42.6

* Ratio of incorporated radioactivity of ³H-thymidine in the presence of 10⁻² M of hydroxyurea where scheduled DNA synthesis in the S-phase was inhibited (+Hydroxyurea) and in the absence of hydroxyurea (-Hydroxyurea) was determined.

The values show mean of 3 experiments.

cells treated with ACNU. The effect of ACNU on the incorporation of ³H-thymidine in the presence of 10⁻² M hydroxyurea where DNA synthesis in the S-phase was inhibited almost completely was compared with that in the absence of hydroxyurea. The incorporation of ³H-thymidine into the cells treated with ACNU was definitely high in the presence of hydroxyurea. This means that the unscheduled DNA synthesis occurred in the cells treated with ACNU. In other words, repair of DNA occurred 24 hr after the treatment with ACNU.

Analysis of DNA of HeLa S₃ cells treated with ACNU by neutral sucrose density gradient centrifugation

Andoh and Ide (1972) reported that damage of DNA-protein complex in the cells treated with 4-nitroquinoline-1-oxide was demonstrated by neutral sucrose density gradient centrifugation. It was also reported from our laboratory that the change of sedimentation velocity of DNA-protein complex of the cells treated with carboquone on the neutral sucrose density gradient centrifugation coincided well with the cell growth (Kanamaru et al. 1978). Similar analysis of HeLa S₃ cells treated with ACNU showed that the peak moved significantly towards the top right after the treatment for 30 min at 100 $\mu\text{g/ml}$ (Fig. 3a) and more markedly after 24 hr (Fig. 3b). Even after 48 hr, the peak was located at the 18th fraction (Fig. 3c). Considering from changes of cell number, the extent of damage to DNA-protein complex (perhaps double strand breakage being included) coincided seemingly more sensitively with cell growth.

Fig. 3. Sedimentation profile on neutral sucrose density gradient centrifugation of DNA of HeLa S₃ cells (a) immediately after, (b) 24 hr after and (c) 48 hr after the treatment with ACNU for 30 min.

--- cells treated with 10 µg/ml of ACNU; - · - · - ○ cells treated with 100 µg/ml of ACNU; — control.

DISCUSSION

Studies on the metabolism, mechanism of action, anti-tumor effects in experimental animals, and clinical utility of several nitrosourea derivatives, especially BCNU, CCNU and methyl-CCNU have been broadly carried out mainly in USA. Studies on nitrosourea derivatives started from the investigations on anti-tumor effects against mouse leukemia L 1210 of MNNG, which possessed carcinogenicity rather than anti-tumor activity (Greene and Greenberg 1960; Carter et al. 1972; Schepartz 1976). Since then, studies on the mechanism of action have been extensively carried out.

From the standpoint of chemical structure and the pattern of metabolism,

alkylation and carbamoylation were mainly studied as the mechanism of action (Montgomery et al 1967; Cheng et al. 1972; Wheeler et al. 1975). At first, it was considered on the basis of alkylation caused by BCNU that among metabolites, diazoalkane alkylated DNA and isocyanate reacted with free NH₂ group to produce cytotoxicity. However, diazoalkane is produced only at unphysiologically high pH. BCNU possesses two chloroethyl groups, but unlike nitrogen mustard, urea nitrogen of BCNU is considered not to be able to cause alkylation because of low electron density of carbon. Montgomery et al. (1967) demonstrated that BCNU reacted in water to produce ethylenediazohydroxide and chloroethylisocyanate and the former acted as an alkylator and the latter as a carbamoylator. CCNU is considered to react in water similarly to BCNU and to have a similar mechanism of action.

The metabolism and action mechanism of ACNU have not yet been fully clarified, but three metabolites were demonstrated in the urine by Tanaka, Sankyo Co., Ltd. (unpublished). From the fact that either pyrimidine-¹⁴C-ACNU or ethylene-¹⁴C-ACNU showed almost no binding to polylysine, albumin or cytochrome C in vitro and that ethylene-¹⁴C-ACNU was bound rather strongly to nucleic acids, especially to DNA, unlike BCNU, CCNU and methyl-CCNU, ACNU was proved to possess a quite low carbamoylating ability.

ACNU gave little influence on RNA and protein syntheses and inhibited DNA synthesis; however, the concentration of ACNU to get significant inhibition of DNA synthesis is 100 µg/ml, quite higher than those of other alkylating agents such as carboquone and mitomycin C. Namely, the same degree of inhibition of DNA synthesis was obtained at 1 µg/ml of carboquone and 10 µg/ml of mitomycin C (Kanamaru et al. 1975a). Provided that the toxicity of ACNU is due to its alkylating ability, the maximum tolerable dose of 40 mg/kg of ACNU, 2.5 mg/kg of mitomycin C or 1 mg/kg of carboquone for rat is related to their alkylating ability (Sato and Ichimura 1971).

In the study of anti-tumor effects determined by the prolongation of surviving period of rats bearing various strains of Yoshida ascites hepatoma at maximum tolerable dose, carboquone and mitomycin C showed marked effects against AH 13, AH 272, AH 44 and AH 7974, but ACNU showed an effect only against AH 130 (Ichimura 1975). Thus, ACNU showed different anti-tumor spectra from other alkylating agents.

In the previous experiment to examine the prolongation of surviving period of AH 109A-bearing rats obtained by various anti-cancer drugs at the maximum tolerable dose, carboquone was most effective, then mitomycin C followed by Trenimon. However, these results on three anti-tumor agents possessing closely related structures are not related to the inhibitory potencies on DNA synthesis (Kanamaru et al. 1975b). As mentioned above, alkylating potency is closely related to cyto- or bio-toxicity and not to anti-tumor activity determined by the prolongation of surviving period of tumor-bearing animals. As the direct action of ACNU on DNA, breakage of single strand of DNA and damage to DNA-protein complex

were observed similarly to other alkylating agents (Kanamaru et al. 1978), though only at a higher concentration of 100 $\mu\text{g/ml}$.

Clinically, effectiveness and side effects of nitrosourea appear slowly. In our experience, decreases of circulating thrombocytes and leucocytes appeared 4 to 6 weeks after the administration of ACNU. As shown in the present experiments, the inhibition of DNA synthesis and breakage of single strand of DNA by ACNU seemed to appear characteristically later than those by other alkylating agents. This would be partially related to delayed-type bone marrow toxicity. Nakamura (unpublished) reported that the enzymatic activity of DNA polymerase incubated with ACNU was not affected. Regarding it as a lack of carbamoylation of enzyme protein, it will be the evidence of low carbamoylating activity of ACNU on protein moieties.

Considering from several experimental results obtained until now, the main mechanism of ACNU will be a characteristic alkylation, that is to say, quite slow alkylation of intracellular DNA molecules, which induces fragmentation of DNA strands and damage to DNA-protein complex followed by inhibition of DNA synthesis, then by inhibition of RNA and protein syntheses. However, anti-tumor effects which cannot be explained by the above described mechanism of action were also observed in experimental tumors and clinical investigations. Further studies including passage of ACNU through the blood-brain barrier are required.

References

- 1) Andoh, T. & Ide, T. (1972) A novel DNA-protein complex in cultured mouse fibroblasts, strain LP3. *Exp. Cell Res.*, **73**, 122-128.
- 2) Arakawa, M., Shimizu, F. & Okada, N. (1974) Effect of 1-(4-amino-methylpyrimidine-5-yl) methyl-3-(2-chloro-ethyl)-3-nitrosourea hydrochloride on leukemia L 1210. *Gann*, **65**, 191.
- 3) Carter, S.K., Shabel, F.M., Jr., Broder, L.E. & Johnston, T.P. (1972) 1,3-bis (2-chloro-ethyl)-1-nitrosourea (BCNU) and other nitrosoureas in cancer treatment: a review. *Advanc. Cancer Res.*, **16**, 273-332.
- 4) Cheng, C.J., Fujimura, S., Grunberger, D. & Weinstein, I.B. (1972) Interaction of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (NSC 79037) with nucleic acid and proteins in vivo and in vitro. *Cancer Res.*, **32**, 22-27.
- 5) Greene, M.O. & Greenberg, J. (1960) The activity of nitrosoguanidine against ascites tumors in mice. *Cancer Res.*, **20**, 1166-1171.
- 6) Ichimura, H. (1975) Experimental studies on a screening system of anti-cancer drugs employing the rat ascites hepatoma spectrum. *Gan to Kagakuryoho* **2**, 605-610. (Japanese)
- 7) Johnston, T.P., McCaleb, G.S., Opliger, P.S. & Montgomery, J.A. (1966) The synthesis of potential anti-cancer agents. XXXVI. N-nitrosoureas. II. Haloalkyl derivatives. *J. med. Chem.*, **9**, 892-911.
- 8) Johnston, T.P., McCaleb, G.S., Opliger P.S., Laster, W.R. & Montgomery, J.A. (1971) Synthesis of potential anticancer agents. 38. N-nitrosoureas, 4. Further synthesis and evaluation of haloethyl derivatives. *J. med. Chem.*, **14**, 600-614.
- 9) Kanamaru, R., Asamura, M., Sato, H. & Saito, T. (1975a) Comparative studies of effects of carbazilquinone and mitomycin C on DNA, RNA and protein synthesis of Yoshida sarcoma and Yoshida ascites hepatomas. *Sci. Rep. Res. Inst. Tohoku Univ. -C*, **22**, 18-24.

- 10) Kanamaru, R., Asamura, M., Hayashi, Y., Sato, H. & Saito, T. (1975b) Comparative studies of effects of carbazilquinone, mitomycin C and Trenimon on DNA, RNA and protein synthesis of AH 109A cells and on survival of AH 109A-bearing rats. (a preliminary note). *Sci. Rep. Res. Inst. Tohoku Univ. -C*, **22**, 66-68.
 - 11) Kanamaru, R., Asamura, M., Hayashi, Y., Sato, H. & Saito, T. (1978) Damage of DNA and its recovery in AH 109A cells treated with carboquone in vivo. *Tohoku J. exp. Med.*, **124**, 331-337.
 - 12) Montgomery, J.A., James, R., McCaleb, G.S. & Johnston, T.P. (1967) The mode of decomposition of 1,3-bis(2-chloroethyl)-1-nitrosourea and related compounds. *J. med. Chem.*, **10**, 668-674.
 - 13) Ono, T., Kurata, S., Wakabayashi, K., Saito, M. & Ogawa, H. (1973) Inhibitory effect of a streptococcal preparation (OK 432) on the nucleic acid synthesis in tumor cells in vitro. *Gann*, **64**, 59-69.
 - 14) Satoh, H. & Ichimura, H. (1971) The spectrum of various anti-cancer drugs in Yoshida ascites hepatomas of rat. *Nihon Rinsho*, **29**, 1818-1826. (Japanese)
 - 15) Schepartz, S.A. (1976) Early history and development of the nitrosoureas. *Cancer Treat. Rep.*, **60**, 647-649.
 - 16) Schmidt, G. & Thanhauser, S.J. (1945) A method for the determination of deoxy-ribonucleic acid, ribonucleic acid and phosphoprotein in animal tissue. *J. biol. Chem.*, **161**, 83-89.
 - 17) Skipper, H.E., Schabel, F.M., Jr., Trader, M.W. & Thomson, J.R. (1961) Experimental evaluation of potential anticancer agents. VI. Anatomical distribution of leukemic cells and failure of chemotherapy. *Cancer Res.*, **21**, 1154-1164.
 - 18) Terashima, T. & Tsuboi, A. (1969) Mammalian cell DNA isolated with minimal shearing. A sensitive system for detecting strand breaks by radiation. *Biochim. biophys. Acta (Amst.)*, **174**, 309-314.
 - 19) Wheeler, G.P., Bowdon, B.J. & Struck, R.F. (1975) Carbamoylation of amino acids, peptides and proteins by nitrosoureas. *Cancer Res.*, **35**, 2974-2984.
-