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SOME ASPECTS OF THE MECHANISM OF ACTION OF 1-PROPYL-1-NITROSOUREA

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Abstract—Activation of RNA synthesis with simultaneous suppression of DNA synthesis in experiments with Ehrlich ascites carcinoma and 22 solid hepatoma in mice was observed 20–40 min after administration of 1-propyl-1-nitrosourea (PNU) in a dose of 10 mg/kg. Under these experimental conditions DNA biosynthesis in tumor cells was inhibited earlier than in spleen and in liver cells of tumor-bearing mice and control mice.

Inhibition of synthesis of DNA and ribosomal and transfer RNA in the ascites cells, as well as of DNA and total RNA of the liver and spleen of tumor-bearing mice and controls, was observed on increasing the dose PNU to 200 mg/kg.

PNU concentration of 10^{-4} M was shown to induce RNA synthesis activation *in vitro* with simultaneous suppression of DNA synthesis in Ehrlich ascites carcinoma and leukemia L1210 cells

PNU inhibits synthesis of arginine-rich histone and activates synthesis of lysine-rich histone; synthesis of saline-soluble proteins remains unchanged (at a PNU concentration of 10^{-4} M).

IN RECENT years alkyl derivatives of nitrosourea[1, 2] have been proposed as antitumor agents. The antitumor activity of these compounds was found to be efficient against different tumors in investigating kinetics of inhibition of tumor growth.[3, 4] Some of them, such as 1·3-bis(2-chlorethyl)-1-nitrosourea (BCNU) have been successfully applied.[5, 6] Investigation of the mechanism of action of BCNU showed that it inhibits DNA synthesis at the stage of reduction of ribonucleotides to desoxyribonucleotides in tumor cells. Moreover, it inhibits the synthesis of purineribonucleotides *de novo* and consequently RNA synthesis. The inhibition of protein synthesis was mainly observed *in vitro*. Wheeler has stated[7] that diasoalkanes formed from alkyl derivatives of urea were the active components. However, it was shown later that the activity of these agents may rather be accounted for by formation of 2-chlorethylamines or 2-chlorethylisocyanates. Both compounds inhibit DNA synthesis. Moreover, it was shown that the antitumor activity of BCNU is double that of nitrogen-mustard, though its alkylating capacity is less as shown by a direct chemical reaction with 4-(*p*-nitrobenzene)pyridine.[8, 9]

This paper is devoted to a study of the mechanism of action of such an alkyl derivative of nitrosourea, 1-propyl-1-nitrosourea (PNU). Particular attention was paid to investigation of the effect of this compound on biosynthesis of DNA and RNA and of various fractions of cell proteins *in vitro* and *in vivo*. The systems used in this study were the Ehrlich ascites carcinoma, 22 solid hepatomas and ascites cells of leukemia L1210.

METHODS

Experiments in vitro. A 10 per cent suspension of tumor cells was used. It was prepared by a procedure described earlier[10] the only difference being that 0·04 M glucose dissolved in 0·05 M Tris-HCl-buffer, pH 7·6 was used as a medium for washing and for incubation. The suspension was incubated at 37° with continuous stirring. Labelled precursors were ^{14}C-formate-Na of a specific activity 80–100 mc/g and mixed L-^{14}C-amino acids (Chlorella protein hydrolysate) of a specific activity 200 mc/g. They were administered in doses of 2 and 1·66 mc/g cells, respectively. Isolation and separation of histones were carried out by the method of Johns.[11]

Experiment in vivo. Outbred albino mice of 20–22 g were taken for experiment on day 7 after inoculation of 8–10 million tumor cells. ^{14}C-formate-Na and mixed L-^{14}C-amino acids were administered in a dose of 10 μc per mouse.

Isolation of total RNA from washed tumor cells, liver and spleen was carried out by the Scherrer and Darnell method employing 5-fold deproteinization by phenol.[12] DNA and proteins were separated by the Georgiev method with additional purification by RN-ase.[13] Linear 5–20 per cent (w/v) sucrose gradients in 0·05 M acetate buffer (pH 5·0), 0·01 M NaCl were used. 0·2 to 0·3 mg of total RNA was layered on 5-ml gradients and centrifuged in SW-39 head in the Spinco, model *E* centrifuge at 37,000 r.p.m. for 4–5 hr.[14] After piercing the bottoms of tubes 30 fractions were collected. Three ml of water were added to each fraction and used for absorption measurements at 260 mμ in an SF-4 spectrophotometer. 0·5 mg bovine serum albumin and 1 ml 20% trichloroacetic acid were added to each fraction for radioactivity measurements. The mixture was then filtered through an ultrafilter (HUFS, 0·3–0·6 μ), washed with cold trichloroacetic acid and the filter glued to a planchette for counting. Preparative separation of *t*RNA and *r*RNA was also carried out.[15, 16] The samples DNA were isolated from washed ascites cells by deproteinization with sodium dodecylsulfate using the Doty method.[17] Prior to homogenization the ascitic cells were put into cold double-distilled water (1:10% w/w). The E(p),[19] the hyperchromic effect at 100°, the contents of RNA[20] and protein,[21] and the ratio of optical densities at 260 and 230 mμ and 260 and 280 mμ were determined for DNA samples.

PNU was dissolved in 0·9% NaCl just before the experiment. PNU was administered in a single dose considerably lower than the maximum tolerance one just before administration of labelled precursors.[22] The radioactivity measurements were performed by an end-window counter BFL-25 in all cases, except in experiments with sucrose gradients. The counting efficiency was 10 per cent.

RESULTS AND DISCUSSION

The results of experiments on the effect of PNU on biosynthesis of DNA, total RNA and total cell proteins in Ehrlich ascites carcinoma (*in vivo*) as a function of time of action of the agent are given in Fig. 1.

One can see that the effect on biosynthesis of DNA, RNA and proteins is different following administration of PNU. After a single dose of 10 mg/kg, activation of incorporation of ^{14}C-formate into total RNA was observed for 20 to 40 min (curve 3). Under the same conditions PNU causes inhibition of synthesis of DNA (curve 2) Increase in concentration of the agent up to 200 mg/kg results in inhibition of RNA synthesis immediately after administration of the agent (curve 4). No inhibition of synthesis of cellular proteins was observed within 1 hr after treatment (curve 1).

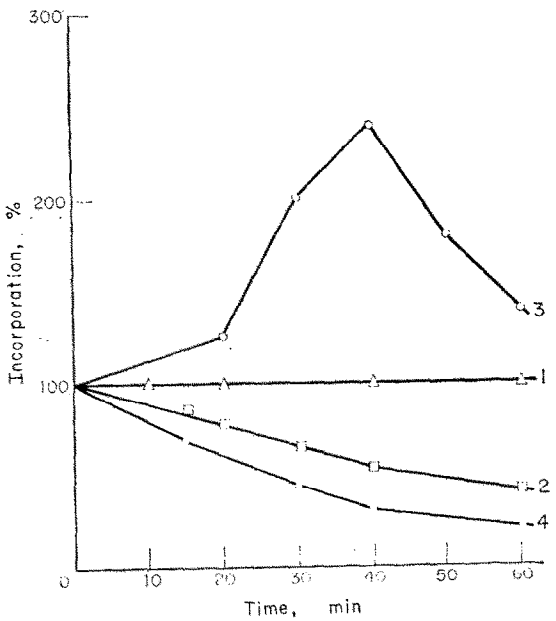

FIG. 1. Effect of PNU on incorporation of ^{14}C-formate Na and L-^{14}C amino acids into DNA, RNA and protein of Ehrlich ascites carcinoma cells.
(1) proteins, PNU 10 mg/kg
(2) DNA, PNU 10 mg/kg
(3) RNA, PNU 10 mg/kg
(4) RNA, PNU 200 mg/kg

This shows that at a certain moment administration of the agent induces suppression of DNA synthesis in a cell simultaneously with essential activation of RNA synthesis. This phenomenon is difficult to explain. However, according to Berg[23] it may suggest that direct administration of the agent in small doses causes damage to the normal relationship between DNA and RNA-polymerases, which results in competition for the DNA-template. Increase in concentration of PNU or in the time after injection *in vivo* might result in more profound changes in the DNA-template.

A similar dependence was found *in vivo* for 22 solid hepatoma in C3HA mice. The results obtained are given in Table 1.

The observed activation of incorporation of the labelled precursor may be explained by direct action of PNU on the RNA synthesis or by changes in DNA-template.

The following experiments were carried out to examine changes in properties of the DNA of Ehrlich ascites carcinoma cells after administration of PNU to the animals. The DNA samples were isolated from three groups of animals: controls (without PNU, DNA_I); mice to which PNU was administered at a dose of 10 mg/kg 30 min previously, i.e. under conditions of optimal activation of RNA synthesis with simultaneous suppression of DNA synthesis DNA_{II}), and a group of animals treated with PNU at a dose of 200 mg/kg during 30 min previously, thus ensuring conditions for 50 per cent inhibition of RNA synthesis (DNA_{III}).

It follows from Table 2 that there is no essential difference in the three samples of DNA. Moreover, we have shown that the samples do not differ in molecular weight

TABLE 1. EFFECT OF PNU ON INCORPORATION OF ^{14}C-FORMATE Na INTO DNA AND RNA OF HEPATOMA 22

Fraction	PNU concentration (mg/kg)	Time after injection (min) 30 counts/min	30 % control	60 counts/min	60 % control
DNA control*	10	122‡	100	192	100
exp.†		60	50	71	37
RNA control		510	100	980	100
exp.		632	124	680	69
DNA control	100	555	100	640	100
exp.		254	46	326	51
RNA control		612	100	850	100
exp.		430	70	400	47
DNA control	200	200	100	280	100
exp.		40	20	56	20
RNA control		737	100		
exp.		365	50		

* The DNA and RNA samples were isolated from control groups of animals.
† The DNA and RNA samples were isolated from mice to which PNU was administered intraperitoneally.
‡ Mean of five determinations.

TABLE 2. PROPERTIES OF DNA SAMPLES EXTRACTED FROM EHRLICH ASCITES CARCINOMA CELLS OF ANIMALS INJECTED WITH PNU

Characteristic of sample	Data from reference 27 (%)	DNA_I (%)	DNA_{II} (%)	DNA_{III} (%)
Protein	1·0	traces	traces	traces
RNA	0·5	1·0	1·0	1·0
$E_{(P)}$·$_{260}$ mμ	6140	6599	6334	6320
D_{260} mμ/D_{230} mμ	2·17	2·19	2·11	2·19
D_{260} mμ/D_{280} mμ	1·88	1·90	1·88	1·92

(near 10^7) or in hyperchromicity values. No difference was observed in electron micrographs of these samples.

It was of interest to establish the RNA types responsible for the effects observed. The preparations of total RNA were layered on sucrose-gradients and centrifuged. RNA samples were taken from two groups of animals: controls (without PNU) and experimental mice injected with the agent at a dose of 200 mg/kg, 4 hr, i.e. conditions for inhibition of RNA synthesis were ensured.

Figure 2 shows the sucrose gradient pattern of the RNA fractions after 240 min labelling *in vivo*. Injection of PNU at a dose of 200 mg/kg may be seen to inhibit synthesis both of *s*RNA and *t*RNA. Similar results were obtained in preparative fractionation of ribosomal and transport RNA.

We also studied *in vivo* the PNU effect on DNA and RNA biosynthesis in liver and spleen of mice bearing tumors and of normal mice. Data from these experiments are given in Table 3.

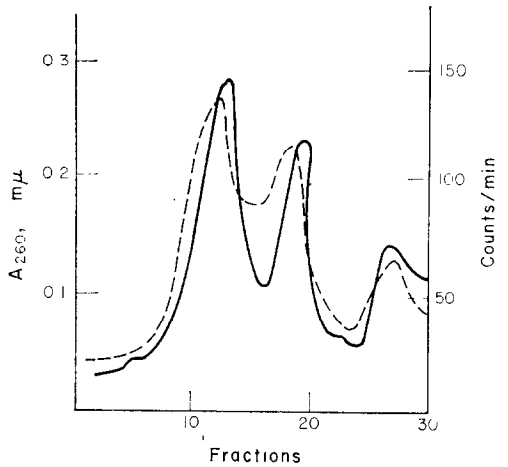

FIG. 2A

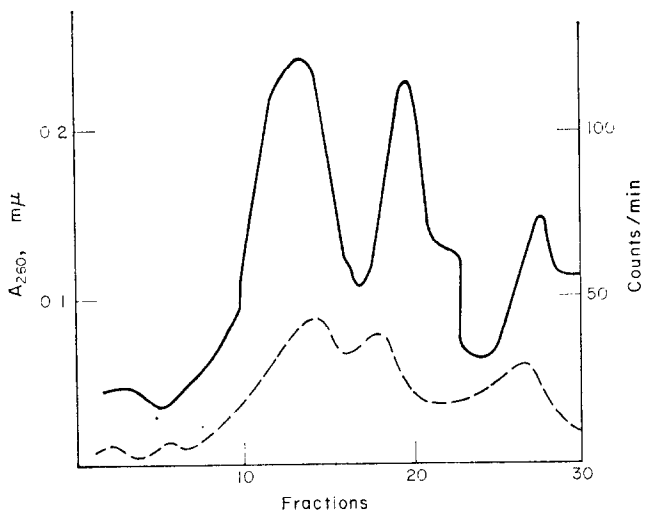

FIG. 2B

FIG. 2. Sucrose gradient pattern of the total RNA fractions isolated from Ehrlich ascites carcinoma cells labelled during 240 min *in vivo*.

(a) RNA sample isolated from control mice

(b) RNA sample isolated from Ehrlich ascites carcinoma cells on mice; the latter had been injected with PNU dose of 200 mg/kg, for 4 hr.

———— the absorption at 260

---------- radioactivity mμ.

Injection of PNU in large doses into both mice bearing tumors and normal ones induces suppression of DNA and RNA biosynthesis in liver and spleen to a similar degree. A somewhat stronger inhibition of incorporation of ^{14}C-formate-Na into DNA was observed in Ehrlich carcinoma cells. More essential differences, under the same experimental conditions, were observed with hepatoma-bearing animals. The incorporation of ^{14}C-formate-Na into DNA of the hepatoma was inhibited by 85 per

TABLE 3. EFFECT OF PNU ON INCORPORATION ^{14}C-FORMATE INTO DNA AND RNA OF ASCITIC CELLS, LIVER AND SPLEEN OF NORMAL MICE AND MICE WITH TUMORS

Organ	Fraction	PNU, concentration (mg/kg)	Normal mice		Mice with tumor	
			counts/min	% control	counts/min	% control
Liver	DNA	Control	244‡	100	108	100
		10*	334	137	145	134
		200†	142	58	47	43
	RNA	Control	172	100	180	100
		10	418	243	265	147
		200	53	31	67	37
Spleen	DNA	Control	1100	100	770	100
		10	1881	171	1430	186
		200	583	53	309	40
	RNA	Control	2904	100	2071	100
		10	2149	74	4140	200
		200	784	27	642	31
Ehrlich ascites carcinoma	DNA	Control			540	100
		10			432	80
		200			160	30
	RNA	Control			4200	100
		10			6720	160
		200			1470	35

*, † The time after treatment (30 min).
‡ Mean of five determinations.

cent, into DNA of the liver, by 60 per cent, and into DNA of the spleen, by 30 per cent only.

The most marked differences in the nature of PNU effect on DNA biosynthesis were found at small doses of the drug. Inhibition of biosynthesis of tumour DNA was accompanied by marked activation of incorporation of the labelled precursor into organs of normal and tumor-bearing mice; activation of RNA synthesis under similar conditions was observed in all experiments except normal mice spleen.

Thus, it cannot be stated for certain that PNU selectively inhibits DNA and RNA biosynthesis in tumor cells. However, it will be stated that inhibition of DNA biosynthesis in tumor cells occurs earlier than in cells of spleen and liver of tumor-bearing mice and normal mice.

Experiments with suspensions of Ehrlich ascites carcinoma cells and leukemia L1210 were carried out in order to determine the mechanism of PNU action *in vitro* (Table 4).

It may be seen that *in vitro* as well as *in vivo* small doses of PNU (10^{-4} M) inhibit DNA synthesis and activate RNA synthesis. With an increase in PNU concentration to 10^{-3} M both processes are inhibited.

Hydroxyurea is reported to inhibit synthesis only of the histone fraction of proteins (especially lysine-rich) and have no effect on synthesis of saline-soluble proteins.[25] It was of interest to observe the effect of PNU on synthesis of histones. According to Johns[11] the histone fractions are: f_1, lysine-rich histone, f_{2a}, slightly lysine-rich histone; f_3, arginine-rich histone. Results of experiments on histone fractionation are given in Table 5.

TABLE 4. EFFECT OF PNU ON BIOSYNTHESIS OF DNA, RNA AND PROTEIN IN THE CELLS OF LEUKEMIA L1210 AND EHRLICH ASCITES CARCINOMA

Tumor cell type	Additions	Fraction					
		DNA		RNA		Protein	
		counts/min	% control	counts/min	% control	counts/min	% control
Leukemia L1210	None	1456‡	100	4860	100	4100	100
	PNU (10^{-4} M)*	1080	74	6800	140	2880	70
	PNU (10^{-3} M)†	150	10	2118	43	1640	40
Ehrlich ascites carcinoma	None	5200	100	5275	100	1000	100
	PNU (10^{-4} M)	2440	47	7915	150	1000	100
	PNU (10^{-3} M)	1000	20	2110	40	570	57

*, † The time after treatment (30 min).
‡ Mean of ten determinations.

TABLE 5. EFFECT OF PNU ON INCORPORATION OF ^{14}C-AMINO ACIDS INTO SALINE-SOLUBLE PROTEIN AND INTO DIFFERENT FRACTIONS OF HISTONE OF EHRLICH ASCITES CARCINOMA CELLS (*IN VITRO*)

Fraction	Additions	Time (min)			
		30		60	
		counts/min	% control	counts/min	% control
f_1	None	1370 ± 96*	100	5450	100
	PNU (10^{-4} M)	2260 ± 153*	165	4900	90
	PNU (10^{-3} M)	1640	120	4360	80
f_{2a}	None	1530	100	4950	100
	PNU (10^{-4} M)	1715	112	5300	108
	PNU (10^{-3} M)	1520	100	3160	64
f_3	None	2740 ± 192*	100	13,000	100
	PNU (10^{-4} M)	1700 ± 119*	62	10,600	80
	PNU (10^{-3} M)	2190	80	7800	60
Saline soluble protein	None	4750 ± 333*	100	12,900	100
	PNU (10^{-4} M)	4740 ± 330*	100	11,870	92
	PNU (10^{-3} M)	4750	100	9000	70

* Values significant at the 5% level.
All other values are the mean of five determinations.

It may be seen that when PNU (10^{-4} M) is added to suspension of ascites cells and the incubation is of short durations the saline-soluble the reaction of protein and histones to addition of the agent is different: a marked inhibition of synthesis of arginine-rich histones (f_3) was observed simultaneously with activation of lysine-rich histone (f_1). Synthesis of saline-soluble proteins remained unchanged. The selectivity of action disappeared with increase in PNU concentration: inhibition of synthesis was observed for all fractions.

Since various fractions of histones are often believed to take a direct part in regulation of DNA and RNA synthesis, inhibition of DNA synthesis or activation with subsequent inhibition of RNA synthesis in tumor cells under the action of PNU

may be suggested to be accounted for by the effect of this compound on synthesis of histone-repressors. Some authors have found that the lysine-rich histone fraction (f_1) served as inhibitor for DNA synthesis, while the arginine-rich fraction (f_3) inhibited RNA synthesis.[26-28] This might provide an explanation for the effects observed by us. Indeed, we always observed activation of RNA synthesis and suppression of DNA synthesis at a PNU concentration of 10^{-4} M. This was accompanied by 40 per cent inhibition of synthesis of the arginine-rich histone fraction (f_3) and activation of lysine-rich histone synthesis (f_1) (Table 5). This decrease in the concentration of repressors of RNA synthesis resulting from inhibition of synthesis of the f_3 fraction. could result in activation of RNA biosynthesis. On the other hand, activation of synthesis of the f_1 fraction results in an increase in concentration of DNA synthesis repressors, which is reflected in suppression of DNA synthesis. With increase in concentration of drug and increasing time after treatment when both DNA synthesis and RNA synthesis are inhibited, perhaps, some other mechanisms start acting. Studies on the mechanism of PNU action continue.

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