Biochemical Pharmacology, 1964, Vol. 13, pp. 135–142. Pergamon Press Ltd., Printed in Great Britain.

BIOCHEMICAL EFFECTS OF THE VINCA ALKALOIDS—I. EFFECTS OF VINBLASTINE ON NUCLEIC ACID SYNTHESIS IN MOUSE TUMOR CELLS*

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(*Received* 17 *July* 1963; *accepted* 16 *September* 1963)

Abstract—The uptake of ^{3}H-uridine into the soluble RNA of Ehrlich ascites cells derived from mice treated with vinblastine is depressed about 60 per cent as compared with that of controls. Since the specific activity of both uridine and cytidine nucleotides is reduced it appears that this alkaloid inhibits the synthesis of the whole chain of soluble RNA. Synthesis of ribosomal RNA is not affected. The incorporation of ^{3}H-thymidine into DNA is reduced by high concentrations of vinblastine *in vitro* but not by therapeutic doses of this agent *in vivo*.

VINBLASTINE (VLB), an alkaloid isolated from the periwinkle plant (*Vinca rosea* Linn.),[1–3] induces metaphase arrest in dividing cells.[4, 5] This compound markedly inhibits the growth of several transplantable mouse tumors,[4, 6, 7] and its usefulness in the treatment of human trophoblastic and lymphomatous neoplasms has been established by clinical trials.[8–10] Little is known of the biochemical basis for the antineoplastic activity of VLB, but its inhibitory action is partially reversed by large doses of glutamic or aspartic acids, arginine, citrulline, and ornithine,[6, 11] and attempts have been made to utilize this property of glutamic acid clinically to reduce host toxicity.[10, 12] Both suppression of the Pasteur effect[13] and some degree of reduction in the incorporation of glycine into DNA[14] have been reported for mammalian cells incubated in the presence of VLB. Of greater interest is the observation that urinary excretion of 4-amino-5-imidazole carboxamide is reduced in both rats[15] and a human subject treated with this drug.[16] The present report indicates that VLB selectively suppresses the turnover of soluble RNA in Ehrlich ascites carcinoma cells *in vivo*.

MATERIALS AND METHODS

Chemicals. ^{3}H-thymidine, ^{3}H-uridine, and 3-^{14}C-aspartic acid were obtained from the New England Nuclear Corporation and the California Corporation for Biochemical Research. Methylated serum albumin was prepared by the action of methanol and hydrochloric acid on bovine serum albumin fraction V.[17]

Tissues. Ehrlich ascites carcinoma cells and sarcoma 180 (S-180) cells in the ascites form were maintained in Swiss white mice and harvested 4 to 6 days after inoculation, normally at the same time of day to avoid diurnal variations in the uptake of precursors.

* Supported by Grants CY 2817 from the U.S. Public Health Service and IN-31D-713 from the American Cancer Society.

Erythrocytes were lysed by exposure to a solution of sodium chloride (0·2%), and the leukocytes were washed with normal saline. Cell preparations for studies *in vitro* were routinely suspended in Krebs III buffer.[18]

Uptake of precursors in vivo. Tumor-bearing animals received single intraperitoneal injections of VLB at a level (2 mg/kg) that inhibits the growth of Ehrlich ascites carcinoma.[6, 7] At various times thereafter the mice were given 4 μc of ^{3}H-uridine (20 μc/μmole) or ^{3}H-thymidine (20 μc/μmole) and were sacrificed 30 min later. Ascites cells were removed and treated as described above. The injection schedules were arranged so that all animals were sacrificed at 10 a.m., except for those killed 7 hr after treatment with VLB.

Determinations. DNA was estimated by the method of Burton,[19] with deoxyadenosine as a standard. RNA was determined by a modification of the orcinol method.[20] Yeast RNA was used as the standard, and color development was carried out at 100° for 45 min, followed by rapid cooling in ice. Protein was measured by the biuret reaction.[21]

Incorporation of thymidine into DNA in vitro. The system consisted of cell suspension (0·5 to 1·0 ml packed cell volume), 5×10^5 cpm of ^{3}H-thymidine and VLB in the indicated amounts, made to a final volume of 10 ml with Krebs III solution.[18] The incubations were carried out in 25-ml Ehrlenmeyer flasks in a Dubnoff metabolic shaker at 37° for varying periods of time up to 60 min. Samples were withdrawn, acidified with equal volumes of 1 M perchloric acid, centrifuged, and the precipitates washed with cold 0·5 M perchloric acid until no further radioactivity was removed. The residues were washed with 80% aqueous ethanol and then suspended in a solution of sodium hydroxide (0·2 N). Radioactivity was determined in samples of this suspension with a liquid scintillation counter (Packard Instrument Corp.); where needed, internal standards were used and corrections applied for quenching. Similar incubation procedures were employed to determine the effect of VLB on the incorporation of ^{3}H-uridine (0·3 μmole/ml; 5×10^5 total cpm) into total RNA and of 3-^{14}C-aspartate (0·2 μmole/ml; 4×10^6 total cpm) into protein. In these experiments RNA was separated by the Schmidt–Thannhauser method,[22] and residual radioactivity in the proteins was determined after removal of the hot perchloric acid-soluble fraction.

Separation of RNA fractions. The cell pellet was suspended in a buffered saline solution composed of 7 volumes of a solution of sodium chloride (0·01 M) containing 0·01 M sodium phosphate buffer, pH 6·7; after freezing and thawing, the suspension was stirred for 1 hr at room temperature with an equal volume of 90% phenol.[23] After centrifuging for 30 min at 10,000*g*, the aqueous layer was removed. The phenol layer and the interphase were then extracted with an equal volume of buffered saline and centrifuged. The combined aqueous layers were made to 2% (w/v) with respect to potassium acetate, and 2 volumes of ethanol added to precipitate RNA. After standing for at least 1 hr at 4°, the precipitate was centrifuged, redissolved in buffered saline, and reprecipitated twice under the same conditions, in order to give a preparation consisting of soluble and ribosomal RNA (*s*-RNA and *r*-RNA). Further separation of these two species of RNA was effected by chromatography from columns of methylated serum albumin,[17, 24] using a linear gradient produced by siphoning a solution of sodium chloride (1·5 M) in 0·05 M phosphate buffer, pH 6·7, into 500 ml of 0·05 M phosphate buffer. The *s*-RNA was eluted first with solutions of sodium chloride, 0·1 to 0·2 M, and the *r*-RNA was removed at 0·8 to 1·0 M. The interphase

layer from the phenol extraction was washed twice with 5 volumes of ethanol–ether (4:1) to remove phenol. Further phenol treatment of aqueous extracts of this residue[25] yielded a fraction characterized by rapid incorporation of precursors, which presumably contained messenger RNA. This interphase fraction will be referred to as *m*-RNA.

Hydrolysis and chromatography. RNA preparations, labeled with ^{3}H-uridine, were hydrolysed overnight with potassium hydroxide (0·3 N) at 37°, neutralized with perchloric acid, and aliquots chromatographed on Whatman 1 paper with methanol–ethanol–water–concentrated hydrochloric acid (50:25:19:6, v/v)[26] as the solvent system. The areas containing UMP and CMP were eluted with water and ribose content and ultraviolet absorbance was determined. Radioactivity was measured by evaporating the eluates on planchets and counting them in a Nuclear Chicago windowless gas-flow counter.

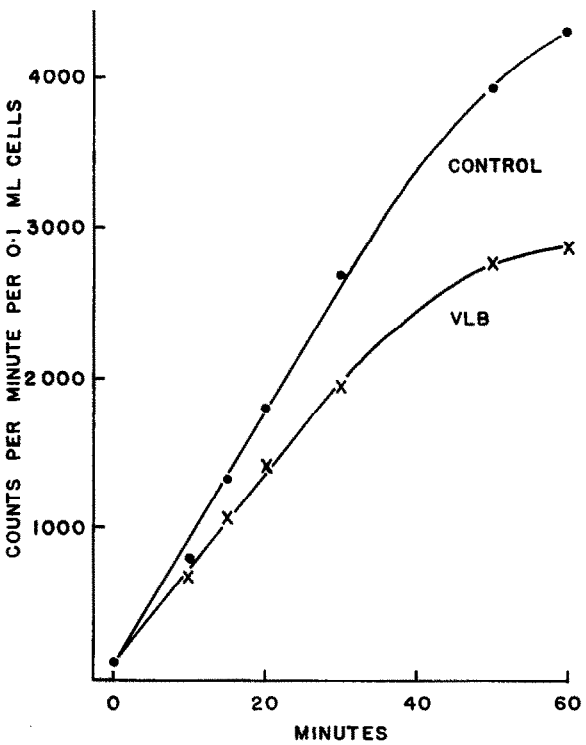

FIG. 1. Uptake of ^{3}H-thymidine into the DNA of Ehrlich ascites cells *in vitro*. Thymidine (0·25 μmole; 5 × 10^5 cpm) and 1 ml of packed cells were incubated in 10 ml of Krebs III buffer. VLB was added at a level of 0·2 μmole/ml.

RESULTS

Studies in vitro

In preliminary studies, suspensions of Ehrlich ascites and S-180 cells were incubated with ^{3}H-uridine, ^{3}H-thymidine, or 3-^{14}C-aspartate, and the amounts of radioactivity that were incorporated into RNA, DNA, and protein, respectively, were determined. Of these three synthetic processes, only the uptake of thymidine into DNA was affected by moderate concentrations of VLB (Fig. 1), but the minimal levels required

for inhibition were considerably greater than any that might be achieved therapeutically, even if very low concentrations of thymidine were employed (Table 1). Inhibition of the uptake of aspartate into protein was not obtained with VLB concentrations of 0·3 μmole/ml and below, but at 0·7 μmole/ml, incorporation was reduced by 51 per cent. The over-all synthesis of RNA was not affected by concentrations of VLB up to 0·6 μmole/ml, but the distribution of radioactivity in the different RNA species, which might have changed, was not studied.

TABLE 1. EFFECT OF VLB ON THE INCORPORATION OF THYMIDINE INTO THE DNA OF S-180 CELLS *IN VITRO*

Thymidine, μmole/ml	VLB, μmoles/ml	Incorporation,* mμmoles/ml cells/hr
0·003	0	1·08
0·003	0·04	1·05
0·003	0·08	0·99
0·003	0·15	0·81
0·003	0·40	0·37
0·333	0	8·31
0·333	0·30	5·92
0·333	0·60	3·51
0·333	1·30	1·15

* Results are given as the means of two determinations at each VLB concentration. Thymidine (5×10^5 counts/min) and 0·5 to 1·0 ml of packed cells were used in a final volume of 10 ml of Krebs III buffer.[18]

Incorporation of uridine and thymidine into nucleic acids in vivo

The incorporation of thymidine into the DNA of Ehrlich ascites cells was studied at 1, 3, and 13 hr after an intraperitoneal injection of VLB. Apart from a transitory fall in specific activity at 1 hr, this agent does not appear to affect significantly the synthesis of DNA (Table 2). In view of the marked variation in rates of uptake of

TABLE 2. THE EFFECT OF VLB UPON THE UPTAKE OF THYMIDINE INTO THE DNA OF EHRLICH ASCITES CELLS *IN VITRO**

After injection, hr	Sample	Specific activity, cpm/μmole deoxyribose
1	control	3,410
	treated	2,680
3	control	3,740
	treated	3,650
13	control	3,290
	treated	4,490

* The mice were treated with 2 mg VLB/kg by intraperitoneal injection, and 30 min before sacrifice received an injection of 4μc ^{3}H-thymidine (20 μc/μmole).

radioactive precursors into the different species of RNA, it was decided to separate these fractions. Accordingly, the incorporation of ^{3}H-uridine was studied at various time intervals up to 48 hr after an injection of 2 mg VLB/kg *m*-RNA, which possessed the highest specific activity of any fraction, showed greater uptake of uridine in

the evening (7-hr sample) than in the morning, presumably a reflection of variation in mitotic rate during the day. After treatment with VLB, the specific activity of the *m*-RNA showed an initial stimulation; this was followed by a slow decline and then a marked secondary rise to a maximal level at 24 hr (Fig. 2), which probably reflects

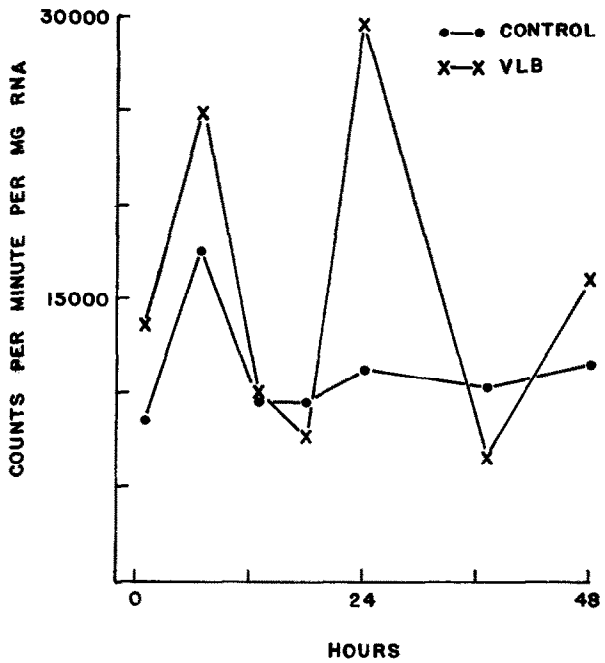

FIG. 2. Specific activity of the interphase RNA fraction from Ehrlich ascites cells. The mice were treated with VLB (2 mg/kg) and sacrificed at the indicated times, 30 min after having received ^{3}H-uridine (4 μc; 0·2 μmole). Only the 7-hr sample was harvested in the afternoon, and specific activities at this time were invariably higher than in the morning. Each point is the mean of two experiments involving 6 mice each.

some degree of synchronization resulting from the period of mitotic arrest. In contrast, the specific activity of the total *r*- and *s*-RNA preparation fell markedly to about 40 per cent of control levels, from which it recovered at about 18 hr, reached a maximum at 24 hr, and finally returned to normal by 48 hr after treatment (Fig. 3). In order to identify the fraction the turnover of which was inhibited by VLB, the combined ribosomal and soluble preparation was separated on a methylated serum albumin column. It was found that the amount of radioactivity associated with *s*-RNA was strikingly reduced, whereas the activity of the ribosomal peak was unaffected (Fig. 4). Samples of the unfractionated *r*- and *s*-RNA preparations, derived from Ehrlich ascites cells harvested 7 and 13 hr after treatment with VLB *in vivo*, were subjected to alkaline hydrolysis. The specific activities of both 2′(3′)-UMP and CMP isolated from these hydrolysates were depressed, as compared with those of control preparations (Table 3).

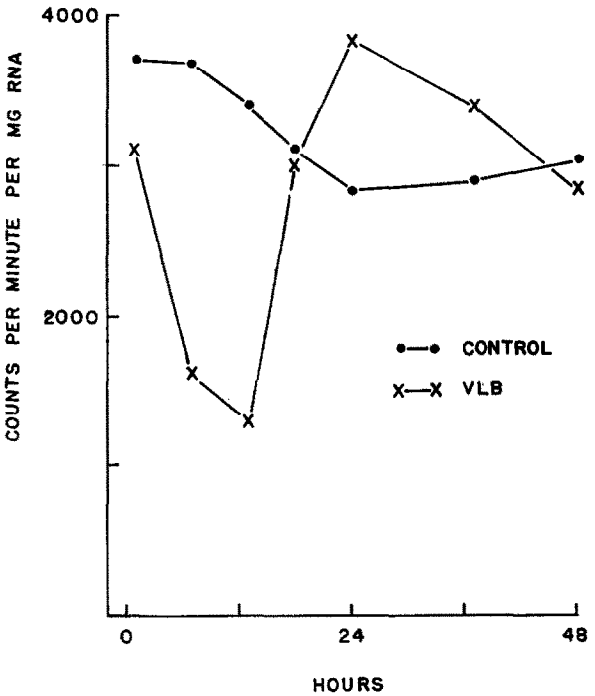

FIG. 3. Specific activity of the combined ribosomal and soluble RNA fractions. Animals were treated as described in Fig. 2. Specific activities of the control samples fell with increasing age of the tumor population.

DISCUSSION

The present studies provide evidence that VLB is able to inhibit two distinct biochemical processes—the synthesis of DNA and the turnover of *s*-RNA. Inhibition of DNA synthesis, however, requires relatively high concentrations of VLB and is probably not a factor in the antineoplastic action of this compound unless, of course, other tissues are acutely more sensitive in this respect. Conceivably, this represents a secondary effect that results from interference with respiration, which has been described previously at these drug levels.[13]

Of greater significance is the inhibition of *s*-RNA synthesis that is apparent *in vivo* after moderate doses of VLB. That it is the synthesis of the whole chain, rather than the independent turnover of the terminal–pCpCpA group,[27, 28] which is interrupted, is suggested by the depression in the specific activity of both the uridine and cytidine nucleotides isolated from the RNA. The action of VLB appears to be similar to, but more specific than, that of actinomycin D, which inhibits all DNA-dependent synthesis of RNA except the turnover of the end groups of *s*-RNA.[29] In order to characterize further the action of VLB, experiments at the enzymic level are essential, and these are in progress at present. In addition, the intimate involvement of *s*-RNA in protein synthesis[27, 28, 30] suggests the value of studies of the effect of VLB upon protein synthesis and amino acid metabolism, a line of research also indicated by the reversal experiments with glutamic and aspartic acids[6, 11] that were mentioned above.

It is of particular interest that a compound such as VLB, the chief biological effect of which is to inhibit the progress of cells, from metaphase, should intefere with RNA

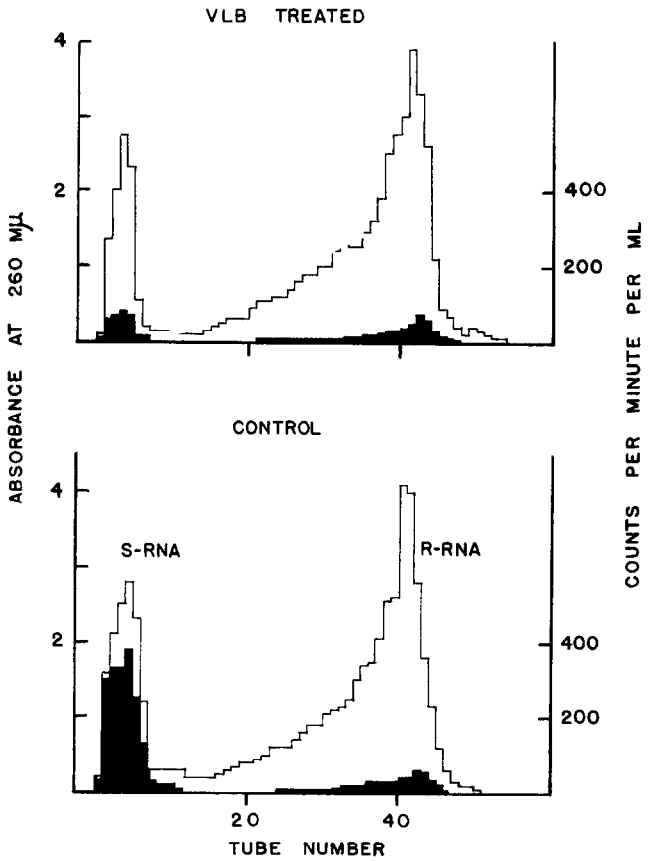

FIG. 4. Separation of soluble and ribosomal RNA on methylated serum albumin columns. A linear gradient from 0·1 to 1·5 M NaCl in 0·05 M phosphate buffer, pH 6·7, was used with a 50-g column of Super-cel and methylated serum albumin; 260 optical density units of *s*- and *r*-RNA preparations, derived from control cells and cells harvested 13 hr after administration of 2 mg VLB/kg, were applied to the columns. The animals had received ^{3}H-uridine 30 min before sacrifice, and the radioactivity derived from this is shown in black. Ultraviolet absorbance is represented by the white area.

TABLE 3. SPECIFIC ACTIVITIES OF 2′(3′)-URIDYLIC AND CYTIDYLIC ACIDS ISOLATED FROM THE RNA OF CONTROL AND VLB-TREATED ASCITES CELLS*

Experiment	Specific activity, cpm/μmole UMP		CMP	
	Control	Treated	Control	Treated
1— 7 hr	9,140	3,090	3,290	1,566
13 hr	11,500	6,490	2,675	1,295
2— 7 hr	10,700	4,660	2,014	1,213
13 hr	13,100	5,740	5,130	1,830

* Samples of the combined *r*- and *s*-RNA preparations were hydrolysed at 37° in potassium hydroxide solution (0·3 N) for 18 hr. After neutralizing with perchloric acid and centrifuging, the supernatant fractions were subjected to paper chromatography as described under Methods; 2′(3′)-UMP and CMP were eluted with water, and after determination of the ultraviolet absorbance of the eluates, evaporated to dryness in planchetes and counted in a Nuclear Chicago windowless gas-flow counter.

metabolism. Preliminary experiments have shown that the related alkaloid, vincristine, itself an arrester of mitosis, also exerts some inhibitory action on the synthesis of *s*-RNA. It is not unlikely, therefore, that *s*-RNA plays an important role in the formation and maintenance of the mitotic apparatus,... and that inhibition of the synthesis of *s*-RNA is a feature common to other agents that interfere with mitosis.

In terms of practical chemotherapy, an understanding of the mode of action of VLB is essential to the logical use of this drug in combination or sequential therapy designed to obtain enhanced antitumor activity. The increased number of cells temporarily synchronized as a result of mitotic arrest is, of course, one factor that might lead to increased incorporation of an antimetabolite into nucleic acid. The data presented here suggest that combinations of VLB with other agents that affect RNA synthesis, or exposure of the cells to a compound that enters RNA at a time when synthesis of the latter is maximal, should be the most promising approaches.

Acknowledgements—We are indebted to Professor A. D. Welch for his interest in and support of this project. Vinblastine used in these studies was generously provided by Doctor I. S. Johnson of the Eli Lilly Company. We would like to thank Doctor A. C. Sartorelli for providing mouse tumors.

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