Some Effects of Carbaryl on Ehrlich Ascites Tumor Cells *in vitro* and *in vivo*[1]

by ERNEST M. WALKER, JR., GLEN R. GALE, LORETTA M. ATKINS, and RICHARD H. GADSDEN

Departments of Pathology, Pharmacology, and Clinical Pathology
Medical University of South Carolina
and
Veterans Administration Hospital
Charleston, S.C. 29403

Carbaryl (1-naphthyl-N-methylcarbamate; Sevin), a reversible inhibitor of cholinesterase, is an agricultural pesticide used in the control of over 150 major pests (BACK 1965). It does not tend to accumulate in the environment and is rapidly metabolized and excreted by mammals, primarily via the kidney (KNAAK et al. 1965). Tissue samples from cattle sprayed with 0.3% carbaryl retained detectable levels three days after sprayings, but not after seven days; none was found in milk collected 77 hours after spraying (HURWOOD 1967). Similar results were obtained in another study using cattle, sheep, goats, and hogs (CLABORN et al. 1963). Dietary inclusion of carbaryl did not yield detectable levels of the compound or its metabolites in tissues of cows (WHITEHURST et al. 1963). When laying hens were fed a diet containing 200 ppm[2] carbaryl for a period of one week, none could be detected in the tissues, and there was no noticeable change in egg production (McCAY and ARTHUR 1962). JOHNSON and CRITCHFIELD (1963) measured carbaryl and its metabolites in chickens dusted with the compound. An average of 19.3 ppm was found in birds sacrificed one day after treatment, but only 2.15 ppm remained in birds sacrificed seven days after dusting. Eggs from treated chickens were devoid of carbaryl or its metabolites throughout the period of observation.

In contrast, chronic administration or oral ingestion of large quantities of carbaryl were found to decrease fertility in female rats and to have a deleterious effect on spermatogenesis in male rats (VASHAKIDZE et al. 1966). Orally administered carbaryl was found to produce necrospermia and sterility in male rats, and a variety of reproductive abnormalities in female rats (VASHAKIDZE 1965). It was also found to be teratogenic in guinea pigs

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[2]The following abbreviations are used: ppm, parts per million; DMSO, dimethyl sulfoxide; MEM, Eagle's minimum essential medium with Hanks' balanced salt solution; ip, intraperitoneally; TPCV, total packed cell volume of Ehrlich ascites tumor cells; DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

Correspondence to: Glen R. Gale, VA Hospital, 109 Bee St. Charleston, S. C., 29403

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(ROBENS 1968), in beagle dogs (SMALLEY et al. 1968) and in rats (REPORT OF SECRETARY'S COMMISSION 1969).

A single administration of the maximum tolerated dose of carbaryl to rats increased DNase and RNase activities in the liver and spleen; liver DNA and RNA levels decreased (ANINA 1968). It was concluded that carbaryl and related compounds disrupt nucleic acid metabolism by increasing its rate of catabolism, possibly by an induction of nuclease synthesis.

Examination of the relatively simple structure of carbaryl reveals that it possesses structural features common to urethan (ethyl carbamate); both are esters of carbamic (or N-substituted carbamic) acid (Figure 1). The antineoplastic and myelosuppressive activities of urethan have been known for more than thirty years

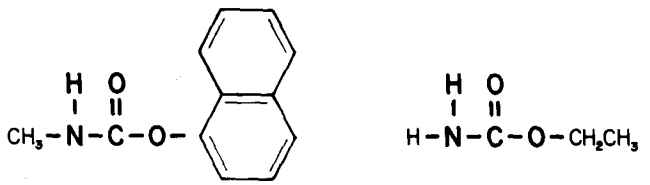

Fig. 1. Structural formulas of carbaryl and urethan.

(see HADDOW and SEXTON 1946; PATERSON et al. 1946), and, even though carcinogenic, it has been used not uncommonly in the treatment of various neoplastic states. Consideration of all of the above prompted us to examine certain effects of carbaryl on Ehrlich ascites tumor cells, with particular reference to effects on tumor development and nucleic acid synthesis.

MATERIALS AND METHODS

Carbaryl was obtained in a highly purified form (99.9%) from the Pesticides Research Laboratory, Perrine, Florida. Thymidine-methyl-^{3}H, L-leucine-^{14}C, and uridine-5-^{3}H, were obtained from New England Nuclear Corporation. Ehrlich ascites tumor cells were maintained in BALB/c mice. The investigation included the following methods which have been described in detail in earlier reports as indicated: development of the Ehrlich ascites tumor *in vivo* (GALE et al. 1970), and rates of synthesis of DNA, RNA, and protein in tumor cells *in vitro* (GALE and HYNES 1968; GALE et al. 1967). Carbaryl was dissolved in DMSO. The volume of DMSO injected was 0.1 ml per 40 gm body weight of each mouse. In the *in vitro* experiments the final concentration of DMSO was 1% (v/v). Control mice as well as control reaction vessels in the

in vitro experiments received the same amounts of DMSO as the experimentals.

Daily ip injections of various doses of carbaryl in DMSO were administered to groups containing 8 to 10 mice; the first injection was given 24 hr after mice received 2 x 10^6 Ehrlich ascites tumor cells ip, and were given each day for 5 or 6 days. Mice were sacrificed 24 hr after the last injection and the average TPCV was determined.

RESULTS AND DISCUSSION

Effects of carbaryl on development of the Ehrlich ascites tumor *in vivo* are shown in Table 1. With three of the five dose regimens used, a reduced tumor mass was found which was significant at the 95% confidence level or better. At two of these dose levels, however, toxicity was evident by death of some of the treated mice. At the other two dose regimens, the level of significance was barely outside the limits of the 95% confidence level.

No inference should be made regarding any potential clinical utility of carbaryl in chemotherapy. The Ehrlich ascites tumor is virtually totally confined to the peritoneum following ip transplantation; ip injections of any agent consequently yield a high level of exposure of the cells to the injected material.

To detect any effect of carbaryl on nucleic acid and protein synthesis, tumor cells were incubated in MEM *in vitro* for various periods after addition of a single concentration of the compound. Following these intervals, replicate tubes received the appropriate radioactively-labeled precursor to monitor the rate of synthesis of DNA, RNA, or protein. At 10^{-4} M carbaryl, a time-dependent onset of inhibition of incorporation of each of the three precursors was noted; protein synthesis was the least sensitive of the three parameters monitored (Table 2).

Dose-dependency of this inhibitory action is shown in Table 3. At a concentration corresponding to 2.0 ppm, virtually no effect on DNA synthesis was found. However, at levels of about 20 ppm and above, the degree of inhibition of both nucleic acid and protein synthesis became pronounced, and substantially total inhibition occurred at just under 200 ppm.

To determine if inhibition of macromolecular synthesis was reversible, replicate cell suspensions were incubated with carbaryl at 2 x 10^{-4} M for 3 hr. Parallel replicate control suspensions without carbaryl were incubated simultaneously. After 3 hr, the control cells were washed with fresh MEM by sedimentation and resuspension. The vessels containing carbaryl were divided into two equal groups; cells in one group were washed with MEM containing carbaryl at 2 x 10^{-4} M, and those in the other group were

TABLE 1

EFFECT OF CARBARYL ON DEVELOPMENT OF THE EHRLICH ASCITES TUMOR IN VIVO

Dose mg/kg/day	Number of injections[a]	Average weight change (gm.) T/C[b]	Toxic deaths C	Toxic deaths T	Average TPCV[c] T/C, ml.	% inhibition	S.D.[d] T/C	p[e]
11	6	0.2/1.6	0/10	0/8	1.451/1.744	17	0.230/0.320	<0.05
12	5	3.6/3.2	0/9	0/9	1.387/1.572	12	0.213/0.265	<0.10
13	5	2.9/3.2	0/9	0/9	1.185/1.572	25	0.495/0.265	<0.10
18	5	2.4/5.4	0/8	1/8	0.900/1.954	54	0.831/0.162	<0.01
18	6	2.9/5.8	0/8	2/8	1.281/2.000	36	0.292/0.330	<0.001

[a]Injected ip, once per day
[b]Treated/Control
[c]Total packed cell volume
[d]Standard deviation of the TPCV of the treated/control groups
[e]Level of significance of difference, T vs. C

TABLE 2

Effects of carbaryl on the incorporation of radioactively-labeled precursors into DNA, RNA, and protein in Ehrlich ascites tumor cells *in vitro*.[a]

Synthesis of	Sample[b]	Hours of incubation[c]	Average CPM[d]	% Inhibition
DNA	Control	0	109,167	
	Carbaryl	0	101,676	7
	Control	1.5	55,262	
	Carbaryl	1.5	48,409	12
	Control	3.0	22,333	
	Carbaryl	3.0	12,350	45
RNA	Control	0	45,868	
	Carbaryl	0	45,530	1
	Control	1.5	21,961	
	Carbaryl	1.5	20,016	9
	Control	3.0	17,451	
	Carbaryl	3.0	11,378	35
Protein	Control	0	3,917	
	Carbaryl	0	3,718	5
	Control	1.5	2,339	
	Carbaryl	1.5	2,088	11
	Control	3.0	1,299	
	Carbaryl	3.0	1,039	20

[a]Radioactively-labeled precursors and final activities were: for DNA, thymidine-methyl-^{3}H (10 µCi/ml); for RNA, uridine-5-^{3}H (10 µCi/ml); and for protein, L-leucine-^{14}C, uniformly labeled (2 µCi/ml). Period of labeling was 20 min.

[b]Concentration of carbaryl, 10^{-4} M.

[c]Time interval between addition of carbaryl and addition of precursor to cell suspensions.

[d]Averages of three separate experiments, each of which was done in duplicate.

TABLE 3

Effects of various concentrations of carbaryl on the incorporation of radioactively-labeled precursors of DNA, RNA, and protein in Ehrlich ascites tumor cells *in vitro*.[a]

Synthesis of	Incubation with carbaryl at [M][b]	ppm	Average CPM	% Inhibition
DNA	0		37,418	
	10^{-5}	2.0	36,850	2
	4×10^{-5}		35,049	6
	8×10^{-5}		29,907	20
	10^{-4}	20.1	25,986	31
	4×10^{-4}		5,569	85
	8×10^{-4}		262	99
	10^{-3}	201	150	99
RNA	0		26,480	
	10^{-5}	2.0	20,987	21
	4×10^{-5}		21,667	18
	8×10^{-5}		21,416	19
	10^{-4}	20.1	16,934	36
	4×10^{-4}		4,437	83
	8×10^{-4}		954	96
	10^{-3}	201	463	98
Protein	0		2,630	
	10^{-5}	2.0	2,400	9
	4×10^{-5}		2,572	2
	8×10^{-5}		2,581	2
	10^{-4}	20.1	2,167	18
	4×10^{-4}		768	71
	8×10^{-4}		86	97
	10^{-3}	201	78	97

[a]Same as footnote a of Table 2.
[b]Cells were incubated with carbaryl for 3 hr prior to addition of radioactively-labeled precursors.
[c]Values represent averages of 6 reaction vessels for each control and 2 vessels for each carbaryl concentration.

washed with MEM devoid of carbaryl. Assessment of the rates of synthesis of DNA, RNA, and protein revealed that washing with carbaryl-free MEM those cells which had been incubated with the inhibitor for 3 hr completely restored the rates to those observed with cells which had been incubated without carbaryl.

In the final group of experiments, tumor cell suspensions were pulse-labeled in vitro with radioactively-labeled thymidine, uridine, or leucine. After 20 min, the unincorporated precursors were removed by washing the cells followed by resuspension in fresh MEM. One group of replicate suspensions was then incubated with 2×10^{-4} M carbaryl for up to 3 hr, while the other group served as controls. At intervals, an equal volume of cold 10% trichloroacetic acid was added to control and experimental tubes, the insoluble material was washed twice with cold 5% trichloroacetic acid, and radioactivity of the acid-insoluble material was determined. No difference whatsoever was found between control and experimental cells, and it was concluded that under these experimental conditions there was no increase in DNase and RNase activities as had been found in vivo by ANINA (1968).

Obviously no claim can be made that the biochemical lesions induced by carbaryl in vitro, described herein, bear any relationship to the effects of this compound on rapidly dividing cells (VASHAKIDZE et al. 1966; VASHAKIDZE 1965; ROBENS 1968; SMALLEY et al. 1968; REPORT OF SECRETARY'S COMMISSION 1969). However, these data may serve to call attention to a pharmacologic action of this compound quite unrelated to its action on cholinesterase, and may serve as a basis for more elaborate investigations of the mechanism of its teratogenic actions and other adverse effects on reproduction.

SUMMARY

Carbaryl produced moderate but significant inhibition of the development of the Ehrlich ascites tumor in mice and reduced appreciably the rates of incorporation of isotopically-labeled precursors into ribonucleic acid, deoxyribonucleic acid, and protein in Ehrlich ascites tumor cells in vitro.

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