Effect of Chronic Oral Administration of Sodium Cobaltinitrite and Sodium Nitrite on the Minimal Carcinogenic $Dose_{50}$ of Methylcholanthrene in Albino Mice

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Chronic hypoxia induced by the oral ingestion of nitrites yielded significant reductions in the tumor incidence of animals exposed to the minimal carcinogenic $dose_{50}$ of methylcholanthrene. Sodium cobaltinitrite was a more effective antitumorigenic agent than sodium nitrite, although both agents produced comparable methemoglobin levels.

IN THE EPOCHAL quest for knowledge concerning the nature of the factors responsible for the incipiency and progression of the cancerization process, the role of oxygen lack, or hypoxia, has received considerable attention. Pertinent investigations have been performed by Urback (1), Heston and Pratt (2), and Mori-Chavez (3), who were able to demonstrate significant reductions in the incidence of lung tumors of mice subjected to carcinogenic agents in the presence of reduced oxygen tensions.

Recently, Orzechowski *et al.* (4, 5) demonstrated that hypoxia arising from the *in vivo* production of methemoglobinemia, which was induced by the intraperitoneal injection of either sodium nitrite, sodium cobaltinitrite, or *p*-aminopropiophenone, resulted in reductions of the tumor incidence in mice which were exposed to the minimal carcinogenic $dose_{50}$ (MCD_{50}) of methylcholanthrene. Of these compounds, only sodium cobaltinitrite produced consistently significant antitumorigenic effects. *p*-Aminopropiophenone, when administered at a dosage level of 20 mg./Kg., produced a slight inhibitory action, while sodium nitrite produced inconsistent effects. Therefore, insufficient data were available to draw real conclusions concerning the effectiveness of the latter compounds. All of these agents evoked variable degrees of acute hypoxia by converting hemoglobin to methemoglobin, the methemoglobin levels rising rapidly after injection, with the attainment of maximum effects within 2 hr.

This investigation was based upon the observations of Orzechowski *et al.* (4, 5) and modified to determine what effect a chronic hypoxic state, produced *in vivo*, might have on the experimental production of tumors. A preliminary study revealed that the administration of sodium cobaltinitrite and sodium nitrite *ad libitum* in drinking water produced and maintained a high level of methemoglobinemia. Therefore, this method was utilized for the production of chronic hypoxia.

EXPERIMENTAL

Three trials were performed in this experiment, two employing sodium cobaltinitrite and the other using sodium nitrite.

In each trial, 240 CF-1 mice were obtained from Carworth Farms and divided into groups of 60 each (30 males and 30 females). One of these groups served as a control and received only the methylcholanthrene applications, while the other three groups received the respective drug concentrations of 0.062, 0.125, and 0.25% *ad libitum* in their drinking water in addition to the methylcholanthrene applications. Trial *1* employed sodium cobaltinitrite and trial *2*, sodium nitrite; during trial *3*, sodium cobaltinitrite was used to reconfirm the results obtained in trial *1*. The respective nitrite concentrations remained constant throughout the three trials.

Cages employed, diet, handling, shaving, and preparation and application of the carcinogenic solution are described in earlier papers (6, 7).

At the beginning of the first two trials, three separate groups of CF-1 mice were set aside for blood studies. Each of these groups was allowed one of the nitrite concentrations *ad libitum* in their drinking water. Every Friday, at least three animals were removed from each group, and a colorimetric assay (8) was conducted on a sample of blood to determine the level or degree of methemoglobinemia that the nitrite had produced.

In trial *1*, erythrocyte counts and hematocrit determinations were performed also. The blood that was required for these determinations was obtained by direct cardiac puncture to prevent dilution of the blood with body or tissue fluids.

RESULTS

Epilation was noted in all of the animals after the third application of methylcholanthrene. This

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TABLE I.—EFFECT OF SODIUM COBALTINITRITE AND SODIUM NITRITE ON THE INCIDENCE OF EXPERIMENTAL TUMORS PRODUCED BY THE MCD_{50} OF METHYLCHOLANTHRENE IN ALBINO MICE

Group	Drug. Concn., %	Tumors/Nontumors	% Tumors	% Tumor Inhibition
		Trial *1*[a]		
A	Control	29/56	52	...
B	0.06	22/60	37	29[d]
C	0.12	6/60	10	81[e]
D	0.25	10/59	17	67[e]
		Trial *2*[b]		
E	Control	29/52	56	...
F	0.06	13/58	22	61[e]
G	0.12	12/57	21	63[e]
H	0.25	17/57	30	46[e]
		Trial *3*[c]		
I	Control	28/57	49	...
J	0.06	19/58	33	33[e]
K	0.12	6/57	11	78[e]
L	0.25	12/58	21	57[e]

[a] Trial *1* employed the use of sodium cobaltinitrite. [b] Trial *2* employed the use of sodium nitrite. [c] Trial *3* employed the use of sodium cobaltinitrite. [d] $p > 0.10$. [e] $p < 0.005$.

condition persisted for 2–3 weeks and was characterized by a complete loss of hair on the application site in the majority of the animals. The appearance of the skin, after several applications of the methylcholanthrene solution, was erythemic due to local cutaneous vasodilation. As the experiment progressed, the epidermis developed a bluish discoloration which could be attributed to the gradual increase in the methemoglobin levels in response to the ingestion of the nitrite compounds.

Resurgence of hair growth occurred approximately 21–23 days after the initial applications of the methylcholanthrene solution in all of the groups. The hair was matted and coarse in texture, which made the clipping procedure more difficult. In addition, it lacked the natural luster typical of the hair elsewhere and failed to conform with the natural contours of the body. When the experiment was terminated, examination of the clipped interscapular area of the mice revealed that the skin of the control animals in all trials was affected profoundly by the methylcholanthrene applications and characterized by a scaly abraded appearance, while the skin of the animals which had received the nitrites appeared almost normal in texture and general appearance.

In Table I, which shows the effect of sodium cobaltinitrite and sodium nitrite on the incidence of tumors produced by the application of the MCD_{50} of methylcholanthrene, it can be noted that in trial *1*, the control group (*A*) exhibited a tumor incidence of approximately 52%, while test groups *B*, *C*, and *D*, which received the low, intermediate, and high concentrations of sodium cobaltinitrite, respectively, in their drinking water, exhibited respective tumor incidences of 37, 10, and 17%, representative of respective tumor inhibitions of 29, 81, and 67%. Table I also shows that in trial *2*, the control group (*E*) had a tumor incidence of approximately 56%, in good agreement with the results of trial *1*. The test groups *F*, *G*, and *H*, which received the low, intermediate, and high concentrations of sodium nitrite, exhibited respective tumor incidences of 22, 21, and 30%, representative of respective tumor inhibitions of 61, 63, and 46%. Trial *3* (Table I) shows that the control group (*I*), had a tumor incidence of 49%, while test groups *J*, *K*, and *L*, which received the low, intermediate, and high concentrations of sodium cobaltinitrite, respectively, exhibited respective tumor incidences of 33, 11, and 21%, representative of respective tumor inhibitions of 33, 78, and 57%.

Table II, which lists the hematocrit readings of the sodium cobaltinitrite treated animals, indicates

TABLE II.—EFFECT OF SODIUM COBALTINITRITE ON THE HEMATOCRIT OF THE CF-1 ALBINO MICE

Wk.	(*A*) Control	(*B*) 0.06%	(*C*) 0.12%	(*D*) 0.25%
1	44[a]	45	41	45
2	45	47	48	48
3	45	48	47	44
4	..	..	..	..
5	44	44	49	50
6	..	..	..	..
7	45	48	49	56
8	46	49	48	54
9	44	50	44	50
10	45	48	46	49
11	44	44	44	47

[a] All values shown represent the average value obtained from at least three experimental animals and are expressed as percentages.

TABLE III.—EFFECT OF SODIUM COBALTINITRITE ON THE BLOOD METHEMOGLOBIN LEVELS IN CF-1 ALBINO MICE

Wk.	(*B*)[a] 0.06%	(*C*) 0.12%	(*D*) 0.25%
1	3.8 ± 1.4[b]	4.7 ± 1.8	4.8 ± 1.5
2	28.1 ± 2.1	3.6 ± 2.0	14.3 ± 1.7
3	30.0 ± 8.2	8.2 ± 1.8	11.6 ± 2.1
4			
5	4.0 ± 2.1	28.9 ± 1.2	22.7 ± 2.3
6			
7	66.1 ± 1.8	58.8 ± 1.3	21.0 ± 2.9
8	60.4 ± 2.9	59.6 ± 1.4	24.3 ± 1.9
9	58.7 ± 2.1	64.0 ± 2.9	29.7 ± 2.4
10	51.3 ± 2.3	60.5 ± 1.9	31.3 ± 2.9
11	45.6 ± 2.1	65.9 ± 2.9	34.3 ± 2.4

[a] Control (*A*) was 0.0 in all cases. [b] All values are expressed as per cent methemoglobin and represent the average value obtained from at least three experimental animals.

TABLE IV.—EFFECT OF SODIUM NITRITE ON THE BLOOD METHEMOGLOBIN LEVELS IN CF-1 ALBINO MICE

Wk.	(F)[a] 0.06%	(G) 0.12%	(H) 0.25%
1	4.9 ± 1.3[b]	3.7 ± 1.7	4.8 ± 1.9
2	9.6 ± 2.1	12.6 ± 2.2	8.5 ± 2.1
3	12.6 ± 1.6	19.2 ± 1.5	16.6 ± 1.4
4	20.4 ± 3.1	30.3 ± 1.0	24.8 ± 2.3
5	25.6 ± 2.0	39.4 ± 1.7	31.6 ± 1.4
6	29.8 ± 1.5	42.6 ± 2.5	32.5 ± 2.7
7	34.2 ± 1.7	49.0 ± 2.8	38.2 ± 2.4
8	38.6 ± 2.1	53.6 ± 1.4	40.3 ± 1.2
9	39.2 ± 1.8	56.2 ± 1.1	43.8 ± 1.6
10	42.6 ± 2.8	57.3 ± 1.0	44.6 ± 1.7
11	45.6 ± 2.5	60.3 ± 2.6	42.8 ± 1.9

[a] Control (E) was 0.0 in all cases. [b] All values are expressed as per cent methemoglobin and represent the average value obtained from at least three experimental animals.

TABLE V.—EFFECT OF EXPERIMENT ON THE WEIGHT OF THE CF-1 MICE

Group	Av. Wt. of Animals at Beginning of Expt., Gm.	Av. Wt. of Animals at Termination of Expt., Gm.	Av. Wt. Gain, Gm.
	Trial 1		
A	23.4 ± 1.4	29.5 ± 2.0	6.1
B	23.9 ± 2.1	26.1 ± 1.6	2.2
C	23.8 ± 1.3	27.8 ± 1.8	4.0
D	22.9 ± 1.4	26.3 ± 2.2	3.4
	Trial 2		
E	22.6 ± 2.1	30.3 ± 2.6	7.7
F	22.2 ± 1.6	29.3 ± 1.2	7.1
G	21.8 ± 2.4	32.3 ± 2.4	10.5
H	21.3 ± 1.1	29.4 ± 1.6	8.1

that at the termination of the experiment, the control group (A) had a hematocrit reading of 44%, while the test groups B, C, and D had readings of 44, 44, and 47%, respectively.

Table III depicts the effects of sodium cobaltinitrite in producing methemoglobinemia and shows that the methemoglobin levels rose as the experiment progressed. At the termination of the experiment, the control group (A) showed 0.0% methemoglobin, while the test groups B, C, and D had respective methemoglobin levels of 45.6, 65.9, and 34.3%.

In Table IV, which illustrates the effect of sodium nitrite on methemoglobin levels, the control group (E), had 0.0% methemoglobinemia at the termination of the experiment, while test groups F, G, and H had respective levels of 45.6, 60.3, and 42.8%.

Table V shows that all of the groups throughout the 10.4 weeks' duration of the experiment, exhibited an increase in weight. Groups A, B, C, D, E, F, G, and H had respective weight increases of 6.1, 2.2, 4.0, 3.4, 7.7, 7.1, 10.5, and 8.1 Gm. Of all of the groups employed, only group H appeared to exhibit a higher distribution of tumors in female mice (Table VI).

Table VII illustrates the effect of sodium cobaltinitrite on the erythrocyte counts of the experimental animals and reveals that the control group had an average count of 6,500,000 cells per cubic millimeter. Also, this table shows that the highest erythrocyte count in the groups receiving sodium cobaltinitrite occurred between the third and fourth week, then slowly fell for 2–3 weeks, eventually becoming somewhat constant. At the termination of the experiment, groups B, C, and D had respective erythrocyte counts of 9,652,000, 8,621,000, and 8,942,000 per cubic millimeter of blood.

During the course of the experiment, several deaths were encountered in the different groups. In the control groups (A) and (E), four and eight mice died before the termination of the experiment. Groups D, F, G, and H had one, two, three, and three deaths, respectively.

DISCUSSION

When the control groups of all three trials were totaled, as recorded in Table I, the cumulative total of 165 mice revealed that 52% had developed measurable tumors, measuring at least 1 mm. in any dimension (width *versus* height), thus confirming the MCD_{50} of methylcholanthrene.

Table I also reveals that during the first trial the administration of sodium cobaltinitrite *ad libitum* in drinking water caused a significant inhibition of tumorigenesis in all of the treated groups, except group B. The latter group exhibited only a 29% tumor inhibition which, according to the χ^2 test, gave a p value of greater than 0.10. In contrast, the p values of the other groups were less than 0.005.

In the second trial, which was identical to the first except that sodium nitrite replaced sodium cobaltinitrite in the treated groups, significant tumor inhibitions of 61, 63, and 46%, respectively, were attained with increasing concentrations of the drug.

TABLE VI.—COMPARISON OF THE TUMOR INCIDENCE BY SEX

Group	M	F
	Trial 1	
A	14	15
B	10	12
C	2	4
D	4	6
	Trial 2	
E	15	14
F	7	6
G	5	7
H	6	12

TABLE VII.—EFFECT OF SODIUM COBALTINITRITE ON THE ERYTHROCYTE COUNT OF THE CF-1 ALBINO MICE

Wk.	(B)[a] 0.06%	(C) 0.12%	(D) 0.25%
1	9,710	6,890	8,056
2	10,860	11,132	10,253
3	10,231	10,236	9,562
4			
5	10,211	9,325	10,826
6			
7	9,896	9,234	9,032
8	8,260	9,136	8,096
9	8,926	8,236	9,546
10	9,105	8,216	8,014
11	9,652	8,621	8,942

[a] In all cases, the control (A) was 6,500 ± 100 expressed in thousands.

In trial *3*, repeating sodium cobaltinitrite in the identical concentrations employed in the first trial, respective tumor inhibitions of 33, 78, and 57% were obtained, thus reconfirming the effectiveness of this compound in inhibiting tumorigenesis. According to the χ^2 test, all of these tumor inhibitions were significant.

It is noteworthy that groups *C*, *G*, and *K*, which received the intermediate concentration of each nitrite, had the highest degree of tumor inhibition—81 and 78% for sodium cobaltinitrite and 63% for sodium nitrite. A parallel situation arose in the blood studies which revealed that these groups also had the highest degrees of methemoglobinemia—65.9 and 60.3%, respectively. This can be explained readily by the realization that the degree of methemoglobinemia is actually dependent upon two factors: (*a*) the concentration of the methemoglobin forming agent in the drinking solution and (*b*) the amount that is consumed. If one were to assume that the degree of methemoglobinemia was based upon concentration alone, then it would be anticipated that the 0.25% solution would produce the greatest degree of methemoglobinemia. However, this was not the case, for the highest concentrations were undoubtedly distasteful to the animals and forced them to imbibe only enough to maintain their minimum daily requirements. On the other hand, the weakest solution, 0.062%, probably was consumed in the greatest amount, for it would be the least objectionable in taste; yet it failed to produce the highest level of methemoglobinemia because it contained insufficient quantities of the drug to achieve this response.

As noted in Table VII, sodium cobaltinitrite consistently produced an elevation in the erythrocyte count. This effect might have occurred either in response to the methemoglobin incurred hypoxia, as a compensatory polycythemia, or as a result of a direct stimulating effect of the cobalt moiety of the cobaltinitrite molecule on the erythropoietic system. In either case, the polycythemic state undoubtedly served to intensify the tissue hypoxia due to the increased blood viscosity which resulted.

It should be emphasized that the tumor inhibition observed in the nitrite treated mice was probably due to the hypoxia elicited by methemoglobin rather than to the direct effect of methemoglobin *per se*. This premise is supported by the previously mentioned studies of Urback (1), Heston and Pratt (2), and Mori-Chavez (3), which showed that a simple reduction of the amount of atmospheric oxygen available to the mice caused a reduction in tumor incidence.

Table I reveals that sodium cobaltinitrite was more effective in inhibiting tumorigenesis than sodium nitrite, even though both compounds produced comparable degrees of methemoglobinemia. The increased effectiveness of the cobaltinitrite appears to be due to the presence of the cobalt moiety of the molecule which may act at an enzymatic level. This concept is supported by the fact that cobalt complexes (with 8-azaquanine and histidine) have produced regressions of certain tumors (9). Recently, cobalt, as vitamin B_{12}, has been shown to exert a temporary remission of leukemia in children when given in massive doses (10).

It is also interesting that cobalt is found in high concentrations in neoplastic tissue, which also displays a more accelerated uptake of blood-borne cobalt than normal tissue (11). This phenomenon may indicate either a severe hematopoietic disturbance or a fundamental metabolic change in the cancer cell. Because it is known that cobalt complexes with proteins, nucleoproteins, and enzymes, it is suggested that an interference with certain metabolic pathways may occur, indicated by the observation that cobalt compounds interfere with the cytochrome-oxidase system of the cancer cell to disrupt its respiration (12). Consequently, as a result of these observations, it can be postulated that either sodium nitrite or sodium cobaltinitrite may inhibit tumorigenesis through the production of a hypoxic state. In addition, sodium cobaltinitrite may exert an additional action manifested by a direct inhibitory effect exerted by the cobalt moiety upon certain enzymatic processes.

SUMMARY

1. The chronic administration of sodium cobaltinitrite *ad libitum* in drinking water, in the respective concentrations of 0.12 and 0.25%, resulted in a significant tumor reduction in two separate trials in mice which also received the MCD_{50} of methylcholanthrene.

2. The chronic administration of sodium nitrite *ad libitum* in drinking water in concentrations identical to those employed for cobaltinitrite, resulted in a significant reduction in the tumor incidence in mice subjected to the MCD_{50} of methylcholanthrene.

3. Both sodium nitrite and sodium cobaltinitrite produced comparable methemoglobin levels, although sodium cobaltinitrite yielded a greater degree of tumor inhibition, thus indicating that the cobalt moiety of the molecule may also influence the potency of this compound.

4. All of the CF-1 mice gained weight throughout the experiment, an indication that the test drugs used were relatively nontoxic in the concentrations employed.

5. It was demonstrated also that sodium cobaltinitrite had little effect on the hematocrit reading of the animals, despite the fact that it consistently produced polycythemia.

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