Effect of Sodium Nitrite and *p*-Aminopropiophenone on the Minimal Carcinogenic $Dose_{50}$ of Methylcholanthrene on Mouse Epidermis

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The biweekly intraperitoneal administration of sodium nitrite, in doses of 50 and 100 mg./Kg. and *p*-aminopropiophenone in doses of 5, 10, and 20 mg./Kg., failed to produce a consistent significant reduction of tumor incidence in mice exposed to the minimal carcinogenic $dose_{50}$ of methylcholanthrene. In contrast, mice which received similar injections of sodium cobaltinitrite at 80 mg./Kg. exhibited a significantly reduced incidence of epidermal tumors.

In the study of the factors which modify the complex process of carcinogenesis, attention has been given to the relationship between the availability of oxygen to the host cells and tumor development. Several investigations have shown that it is possible to modify experimental tumor formation in animals by altering the concentration of oxygen in the inspired air (1–3). These results indicated that exposure to low oxygen tensions retarded the growth of neoplasms by damaging malignant tissue more severely than normal tissue (4).

It is possible to produce an oxygen deficiency *in vivo* through the formation of methemoglobinemia, a condition which results in a decreased oxygen carrying capability of the blood (5). Thus, the use of methemoglobinemia as a method of reducing the amount of oxygen available to the tissues of the subject affords another approach to the assessment of the ability of oxygen to modify carcinogenesis.

A previous paper reported that periodic injections of sodium cobaltinitrite, a methemoglobin-forming agent, were capable of reducing significantly the incidence of epidermal tumors in mice subjected to the minimal carcinogenic $dose_{50}$ (MCD_{50}) of methylcholanthrene (6). Therefore, the possibility existed that hypoxia, a result of the formation of methemoglobinemia, was a causative factor in the reduction of the tumor incidence.

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The present investigation was undertaken to determine whether a correlation existed between the degree of methemoglobinemia and tumor inhibition. If methemoglobinemia was the cause of the tumor reduction, then similar levels of methemoglobinemia, produced by other agents, might be expected to produce a similar inhibition of tumor formation. Consequently, *p*-aminopropiophenone and sodium nitrite were utilized to provide the basis for a comparison to the results previously reported for sodium cobaltinitrite.

EXPERIMENTAL

The study was composed of two sequentially performed parts which are designated here as trials *A* and *B*. Briefly, each trial consisted of a 72-day period during which normal (control) and hypoxic mice were subjected to the MCD_{50} of methylcholanthrene. Temporary periods of hypoxia were elicited by periodic injections of one of the following methemoglobin-forming compounds: sodium nitrite, *p*-aminopropiophenone (PAPP), and sodium cobaltinitrite. Table I lists the groups of animals and the treatment each received. Detailed descriptions of the materials and procedures are in the previous papers (6–8).

All mice received biweekly topical applications of methylcholanthrene as 0.02 ml. of a 0.12% solution

Table I.—Grouping and Treatment of Mice in Trials *A* and *B*

Group	Trial *A*	Trial *B*
Controls (Methylcholanthrene)	[a]	
Exptl.		
Sodium nitrite,		
50 mg./Kg.	[a]	
100 mg./Kg.	[a]	
p-Aminopropiophenone,		
5 mg./Kg.	[a]	
10 mg./Kg.	[a]	
20 mg./Kg.	[a]	[b]
Sodium cobaltinitrite,		
80 mg./Kg.		[b]

[a] Sixty mice per group. [b] One-hundred and twenty mice per group.

TABLE II.—DEGREE AND DURATION OF METHEMOGLOBINEMIA IN MICE AFTER INTRAPERITONEAL INJECTIONS OF SODIUM NITRITE, SODIUM COBALTINITRITE, OR *p*-AMINOPROPIOPHENONE

Time, Min. after Injection	% Methemoglobin[a] Sodium Nitrite, 80 mg./Kg.	Sodium Cobaltinitrite, 80 mg./Kg.	PAPP, 10 mg./Kg.	PAPP, 20 mg./Kg.
5	...	...	51	69
10	30	15	...	...
15	...	...	55	69
20	39	32	...	...
30	...	...	38	59
40	34	39	...	...
60	30	27	17	49
90	16	16	...	...
120	4	11	12	15
180	...	3	6	15
240	...	...	...	6

[a] Expressed as per cent of total hemoglobin converted to methemoglobin. Each value listed is the mean of four to six individual determinations.

in acetone. In addition, the treated groups of mice received biweekly injections (intraperitoneal) of an appropriate dose of a methemoglobin-forming compound. Freshly prepared aqueous solutions were used in concentrations so that the volume of injected solution did not exceed 0.3 ml. The dosage levels were selected on the basis of preliminary toxicity studies and blood-methemoglobin determinations on separate groups of mice. The intraperitoneal LD_{50}'s of PAPP and sodium cobaltinitrite, determined by the method of Litchfield and Wilcoxon (9), were 108 (88 to 133) mg./Kg., and 159 (149 to 170) mg./Kg., respectively. Blood methemoglobin levels were determined by the colorimetric method of Evelyn and Malloy (10). Table II shows the results of this study. Maximal methemoglobin concentrations were attained within 1 hour with all three agents and the duration of methemoglobinemia varied with the dose and the agent. Sodium nitrite produced the most transient response, followed by sodium cobaltinitrite and PAPP. The latter substance, at a dose of 20 mg./Kg., elicited detectable methemoglobinemia in mice as long as 4 hours post-injection. PAPP was more potent than the nitrite compounds in terms of degree and duration of methemoglobinemia achieved.

The epidermal reaction to methylcholanthrene was similar to that previously described, *i.e.*, epilation, followed by hyperplasia and a resurgence of hair growth (8). Tumors were initially noted on a few mice during the sixth week of each trial. At the termination of each 72-day period, all mice were examined closely for the presence of tumors. Growths, which measured at least one dimension (width versus height) of 1 mm. or greater, were drawn and recorded on individual data sheets. Mice were chloroformed, and the tumors were excised and immersed in 10% formalin in preparation for eventual sectioning, mounting, and histopathological examination.

A comparison of the combined groups of mice was made to determine if the tumor incidence in the treated groups differed significantly from that of the controls. The chi^2 test was employed, and p values less than 0.05 were accepted as indicative of a statistically significant difference between controls and treated groups.

RESULTS

The tumor incidence obtained for the various groups of mice at the conclusion of both trials is recorded in Table III. The table also lists the composite results of both trials. The effective total of mice in each group is less than the original number, due to deaths which occurred during the trial period. Mice that died before tumors were noted in the group were not counted in the effective total. When a death occurred after a measurable growth had appeared, it was included in the total. The results of the trials were as follows.

Trial A.—Control mice, which received the MCD_{50} of methylcholanthrene, responded with a 54% tumor incidence. Five test groups received biweekly injections of either PAPP in doses of 5, 10, and 20 mg./Kg. or sodium nitrite in doses of 50 and 100 mg./Kg. The tumor responses elicited in each of the PAPP-treated groups were, in order of increasing doses, 32, 43, and 46%. The two groups which received sodium nitrite showed tumor percentages of 28 and 35.

Trial B.—This trial was composed of four groups, each containing 120 mice. In addition to the controls, three experimental groups were injected with either 20 mg./Kg. PAPP, 100 mg./Kg. sodium nitrite, or 80 mg./Kg. sodium cobaltinitrite. Tumor incidence values were: controls, 45%; PAPP, 34%; sodium nitrite, 48%; sodium cobaltinitrite, 26%.

The combined data of the two trials revealed that,

TABLE III.—EFFECT OF SODIUM NITRITE, *p*-AMINOPROPIOPHENONE, AND SODIUM COBALTINITRITE ON TUMOR INCIDENCE IN MICE RECEIVING THE MCD_{50} OF METHYLCHOLANTHRENE

Group	Trial A Tumor Incidence[a]	Trial A % Tumors	Trial B Tumor Incidence	Trial B % Tumors	Total Trials Tumor Incidence	Total Trials % Tumors
Controls	31/57	54	54/119	45	85/176	48
Exptl.						
Sodium nitrite						
50 mg./Kg.	17/60	28	...	...	...	...
100 mg./Kg.	19/55	35	57/118	48	76/173	44
p-Aminopropiophenone						
5 mg./Kg.	19/59	32	...	...	...	...
10 mg./Kg.	24/59	43	...	...	...	...
20 mg./Kg.	25/55	46	41/119	34	66/174	38
Sodium cobaltinitrite						
80 mg./Kg.	...	...	31/118	26	31/118	26[b]

[a] Ratio expressed as $\frac{\text{No. of mice bearing one or more tumors}}{\text{effective total of mice in group}}$. [b] Significant reduction of tumor incidence compared to control value (chi^2 test, $p<0.01$).

of 176 control mice, 85 developed tumors of 1 mm. or greater in one dimension. This corresponds to a 48% tumor incidence, a result which is very close to the anticipated value of 50%.

Table III also shows that of 174 mice which received 20 mg./Kg. of PAPP, 66 (38%) developed tumors. Similarly, doses of 100 mg./Kg. of sodium nitrite yielded a tumor ratio of 76/173 or 44%. Both of these values do not differ significantly from the control value. In contrast, a significant reduction of tumor incidence occurred in the group of mice which had received 80 mg./Kg. of sodium cobaltinitrite. Thus, intraperitoneal injections of this agent were capable of reducing the per cent of tumor bearing mice to 26%, compared to the 48% control value.

DISCUSSION

The purpose of this investigation was to use methemoglobinemia as a means of inducing transient hypoxia in mice to determine the effects of this condition on the formation of tumors. A previous paper reported that the biweekly injection of sodium cobaltinitrite in doses of 50 to 80 mg./Kg. in mice subjected to the MCD_{50} of methylcholanthrene resulted in a significant reduction of tumor incidence compared to controls (6). Methemoglobin determinations confirmed that sodium cobaltinitrite produced transient periods of methemoglobinemia. Therefore, the possibility existed that hypoxia, a result of the formation of methemoglobinemia, was a causative factor for the reduction of tumor incidence. On the other hand, the relatively short periods of methemoglobinemia, which lasted no longer than 3 hours and were elicited twice weekly, could not be unquestionably concluded as being the cause of tumor inhibition. It is quite possible that some metabolic effect other than hypoxia was responsible for, or contributed to, the decreased tumor incidence.

The use of other methemoglobin-forming agents was necessary to determine whether any degree of parallelism existed between methemoglobinemia and suppression of tumor formation.

Interestingly, methemoglobinemia, induced by PAPP and sodium nitrite, has been shown to exert a protective action against lethal doses of X-radiation in mice (11). Gray *et al.* (12) reported that a correlation existed between the degree of methemoglobinemia and protection against radiation and that the reduced mortality was due possibly to the decreased supply of oxygen to the tissues.

PAPP and sodium nitrite were utilized in the present study for comparing their results to those previously reported with sodium cobaltinitrite. It was reasoned that if methemoglobinemia was the sole cause of the tumor inhibition observed, then similar levels of methemoglobinemia produced by either sodium nitrite or PAPP might be expected to produce a comparable degree of inhibition of tumor formation. However, the results of this investigation suggested that this was not the case. Mice which received 20 mg./Kg. of PAPP responded with a 38% tumor incidence, an insignificant deviation from the control value. The level of methemoglobinemia produced by this dose of PAPP greatly exceeded the responses obtained with sodium cobaltinitrite (Table II). Similarly, a tumor incidence of 44% was recorded in the animals which had received 100 mg./Kg. of sodium nitrite. In contrast, the group of 120 mice which received 80 mg./Kg. of sodium cobaltinitrite responded with a significant reduction (26%) of tumor incidence. Thus, it appears that methemoglobinemia was not primarily responsible for the tumor inhibition observed in mice injected with sodium cobaltinitrite.

Interesting results were obtained in the few groups of mice subjected to lower doses of PAPP and sodium nitrite. Table III shows that a low incidence of tumors was exhibited in two groups of animals which received 5 mg./Kg. of PAPP and 50 mg./Kg. of sodium nitrite. However, because the data were comprised of small numbers of mice, it would be necessary to repeat the experiment with these doses of PAPP and sodium nitrite in order to arrive at conclusions.

The nature of the factor(s) involved in the inhibitory response produced by sodium cobaltinitrite remains to be elucidated; consequently, an attempt to explain this phenomenon must be hypothetical. It is possible, for example, that the cobalt moiety plays a part in the inhibitory action of sodium cobaltinitrite. In this respect, the uptake of radioactive cobalt has been shown in proliferating cells to be higher than in cells at rest (13), due to the formation of chelated compounds with the newly formed proteins and nucleoproteins of dividing cells. If these cobalt–protein complexes resulted in enzyme inhibition, then cell growth and metabolism would be suppressed sufficiently to reduce the formation of tumors. It must be emphasized that the foregoing explanation is merely speculative and that arrival at valid conclusions must await further experimentation with other cobalt compounds.

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