Table IV shows that the cortisone-treated mice (Groups B and C) in *Parts 1 and 2* of this study had a definite retardation or even a significant decrease in mean body weight at the termination of the cortisone injections, while the control mice (Groups A and D) exhibited a definite increase in mean body weight during the same period.

SUMMARY

The administration of 0.4 mg. of cortisone acetate per 30 Gm. of body weight, injected subcutaneously five times a week over a 16-day period (12 injections) during the early phase of the experiment, caused a slight decrease in the incidence of methylcholanthrene-induced tumors.

When administered subcutaneously, cortisone acetate suspension decreased the skin irritation and subcutaneous vasodilation caused by the application of a 0.12% solution of methylcholanthrene in acetone to the interscapular region. However, this effect was noticeable only during the interval of cortisone therapy and for a short period thereafter.

The CF-1 albino mice used in this experiment also showed definite retardation or even a significant decrease in body weight during the 16-day period of cortisone acetate administration. However, when cortisone acetate was withdrawn, the mice gained weight rapidly. At the termination of the experiment, the groups of mice which had received cortisone (Groups B and C) exhibited a mean weight nearly equal to the mean weight of the control groups (Groups A and D).

The administration of cortisone acetate to the mice of Groups B and C delayed the resurgence of hair growth approximately 2 weeks beyond the resurgence of hair growth in the control groups.

REFERENCES

(1) Kendall, E. C., Mason, H. L., and Meyers, C. S., *J. Biol. Chem.*, **114**, 613(1936).
(2) Reichstein, T., *Helv. Chim. Acta*, **19**, 1107(1936).
(3) Wintersteiner, O., and Pfiffner, J. J., *J. Biol. Chem.*, **116**, 281(1936).
(4) Heilman, F. R., and Kendall, E. C., *Endocrinology*, **34**, 416(1944).
(5) Bizustowicz, R. J., Svien, H. J., Bennett, W. A., *Proc. Staff Meetings Mayo Clinic*, **26**, 121(1951).
(6) Emerson, G. A., Wurtz, E., and Zanetta, M. E., *Federation Proc.*, **9**, 357(1950).
(7) Agosin, M., Christen, R., Badinez, O., Gasic, G., Neghme, A., Pizarro, O., and Jarpa, A., *Proc. Soc. Exptl. Biol. Med.*, **80**, 128(1952).
(8) Higgins, G. M., Woods, E. A., and Bennett, W. A., *Cancer Res.*, **10**, 203(1950).
(9) Baserga, R., and Shubik, P., *ibid.*, **14**, 12(1953).
(10) Sugiura, K., Stock, C. C., Dobringer, K., and Rhoads, C. P., *ibid.*, **10**, 244(1950).
(11) Herrmann, F., Morrill, S. D., Sherwin, R. W., Rothstein, M. J., and Sulzberger, M. B., *J. Invest. Dermatol.*, **25**, 423(1955).
(12) Sherwin-Weidenreich, R., Herrmann, F., and Rothstein, M. J., *Cancer Res.*, **19**, 1150(1959).
(13) Herrmann, F., Sherwin, R. W., Morrill, S. D., Rothstein, M. J., and Sulzberger, M. B., *J. Invest. Dermatol.*, **25**, 403(1955).
(14) Morrill, S. D., and Herrmann, F., *ibid.*, **37**, 243(1961).
(15) Gautieri, R. F., and Mann, D. E., Jr., THIS JOURNAL, **47**, 350(1958).

Effect of Sodium Cobaltinitrite on the Minimal Carcinogenic Dose$_{50}$ (MCD$_{50}$) of Methylcholanthrene in Albino Mice

By RAYMOND F. ORZECHOWSKI, RONALD F. GAUTIERI, and DAVID E. MANN, JR.

The bi-weekly, intraperitoneal administration of sodium cobaltinitrite in doses of 50, 60, and 70 mg./Kg. in mice subjected to the MCD$_{50}$ of methylcholanthrene resulted in a reduction of tumor incidence to 31, 31, and 25 per cent, respectively, compared to 47 per cent for the controls.

FROM THE MANY diverse factors that have been shown to modify experimentally induced carcinogenesis, an intriguing concept has arisen which stresses the need for a deeper understanding of the role played by oxygen in the inception and regulation of the cancerization process. According to Warburg (1), a normal cell becomes cancerous because of irreversible damage to its respiratory mechanism. Cells which are unable to compensate immediately to the abrupt change in intracellular respiration die, while others survive only by adjusting to a fermentation type of respiration, thus becoming undifferentiated and cancerous. In short, the cancer cell derives energy from fermentation which fulfills its metabolic demands as adequately as intracellular respiration meets the requirements of the normal cell. On the other hand, Weinhouse (2) presented experimental evidence that the respiration of tumor slices is approximately equal to the oxidative metabolic activity of normal cells. In further support of these findings, isotope studies performed in 1949 (3) have shown that tumor cells can oxidize glucose to carbon dioxide at rates comparable to those of normal cells. Therefore, it

Received June 3, 1963, from the School of Pharmacy, Temple University, Philadelphia, Pa.
Accepted for publication July 19, 1963.
This investigation was supported by Grant CA-06239 from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
The authors are indebted to Drs. Marion W. McCrea and William R. Sabes, Henry Isaiah Dorr Department of Histopathology, Temple University, for their valuable assistance.
Presented to the Scientific Section, A.PH.A., Miami Beach meeting, May 1963.

appears unlikely that an impairment of oxygen consumption or utilization is an aberration common to all neoplasms.

The present investigation was undertaken to observe the effect that an *in vivo* oxygen deficiency might produce on the growth of chemically induced tumors in mice—specifically on squamous cell carcinomas resulting from the topical application of methylcholanthrene. The basis for this study was an established method which has elicited a consistent tumor response. Accordingly, the bi-weekly application of 0.02 ml. of a 0.12% methylcholanthrene solution to the shaved interscapular area for 10.4 weeks has repeatedly resulted in tumor formation in approximately 50% of the treated animals. This dose/response relationship has been termed the minimal carcinogenic dose$_{50}$ (MCD_{50}) of methylcholanthrene (4) and has provided a satisfactory procedure for the evaluation of various factors, such as the effects of gonadectomy and/or estrogen (5), and cortisone (6), on controlled tumor production.

The present investigation was undertaken to observe the tumor incidence in normal and hypoxic mice subjected to the MCD_{50} of methylcholanthrene. A temporary hypoxic state was produced through the formation of methemoglobinemia, a condition which results in a decreased oxygen-carrying capability of the blood. Several groups of agents are known to induce methemoglobinemia, including nitrites and *p*-amino phenol derivatives. Sodium cobaltinitrite, $Na_3Co(NO_2)_6$, was chosen because it is superior to sodium nitrite as an antidote for cyanide poisoning in mice, presumably from its ability to convert hemoglobin to methemoglobin (7).

EXPERIMENTAL

The experiment was divided into three separately performed parts, designated as trials *1*, *2*, and *3*. In each trial, 300 or 360 mice (CF-1) were divided into groups of 60 mice, each with 30 males and 30 females. Table I lists these groups and the treatment which each received. Cages employed, diet, handling, shaving, and method of application of the carcinogen were described in a previous paper (4). Ampuls containing 10 ml. of 0.12% methylcholanthrene (Eastman-Kodak) in acetone were prepared and refrigerated until used. The carcinogen was applied twice weekly (Tuesday and Thursday); freshly prepared aqueous solutions of sodium cobaltinitrite were injected intraperitoneally on Mondays and Fridays. This schedule of injections was begun on the Friday of week 1 of each trial; therefore, the mice had been exposed to two previous applications of methylcholanthrene. Concentrations of the cobaltinitrite ranged from 0.5 to 1.6%, depending on the dose to be administered, so that the volume of solution injected would not exceed 0.3 ml.

The intraperitoneal LD_{50} of sodium cobaltinitrite, determined by the method of Litchfield and Wilcoxon (8), was 159 (149 to 170)mg./Kg. Since the animals were to receive a total of 20 injections over a period of 72 days, it was necessary to choose dosage levels which, while producing a definite methemoglobinemia, would not elicit toxic symptoms. After a single intraperitoneal injection of 50 to 100 mg./Kg., mice exhibited ataxia, reduced respiratory activity, and a moderate degree of cyanosis within 5 minutes. However, noticeable toxic manifestations were not apparent 1 or 2 hours later; the animals appeared normal in all respects. When doses greater than 100 mg./Kg. were administered, marked cyanosis accompanied by gasping and occasional convulsions

TABLE I.—GROUPING AND TREATMENT OF MICE IN TRIALS *1*, *2*, AND *3*

Group[a]	Treatment
Trial *1:* Mar. 20 to May 31, 1962	
A-1	Methylcholanthrene (controls)
B-1	Methylcholanthrene + sodium cobaltinitrite,[b] 30
C-1	Methylcholanthrene + sodium cobaltinitrite, 40
D-1	Methylcholanthrene + sodium cobaltinitrite, 50
E-1	Methylcholanthrene + sodium cobaltinitrite, 60
Trial *2:* June 7 to Aug. 18, 1962	
A-2	Methylcholanthrene (controls)
D-2	Methylcholanthrene + sodium cobaltinitrite, 50
E-2	Methylcholanthrene + sodium cobaltinitrite, 60
F-2	Methylcholanthrene + sodium cobaltinitrite, 70
G-2	Methylcholanthrene + sodium cobaltinitrite, 80
Trial *3:* Sept. 25 to Dec. 6, 1962	
A-3	Methylcholanthrene (controls)
B-3	Methylcholanthrene + sodium cobaltinitrite, 30
C-3	Methylcholanthrene + sodium cobaltinitrite, 40
D-3	Methylcholanthrene + sodium cobaltinitrite, 50
E-3	Methylcholanthrene + sodium cobaltinitrite, 60
F-3	Methylcholanthrene + sodium cobaltinitrite, 70

[a] Each group consists of 60 mice, equally divided regarding sex. [b] All doses (mg./Kg.) of sodium cobaltinitrite are by intraperitoneal injection.

TABLE II.—FORMATION AND DURATION OF METHEMOGLOBINEMIA IN MICE AFTER INTRAPERITONEAL INJECTIONS OF SODIUM COBALTINITRITE (80 mg./Kg.) OR SODIUM NITRITE (80 mg./Kg.)

After Injection, min.	% Methemoglobin[a] Sodium Cobaltinitrite	Sodium Nitrite
10	15 ± 3	30 ± 2
20	32 ± 4	39 ± 2
40	39 ± 6	34 ± 3
60	27 ± 5	30 ± 2
90	16 ± 7	16 ± 4
120	11 ± 3	4 ± 3
180	3 ± 2	...

[a] Expressed as per cent of total hemoglobin converted to methemoglobin. Each value listed is the mean of six different determinations ± standard error.

TABLE III.—EFFECT OF INTRAPERITONEAL INJECTIONS OF SODIUM COBALTINITRITE ON TUMOR INCIDENCE IN MICE RECEIVING THE MCD_{50} OF METHYLCHOLANTHRENE

Treatment (mg./Kg.) and Group No.	Trial 1 Tumor Incidence[a]	Trial 1 Tumors, %	Trial 2 Tumor Incidence	Trial 2 Tumors, %	Trial 3 Tumor Incidence	Trial 3 Tumors, %	Total Trials Tumor Incidence	Total Trials Tumors, %
Methylcholanthrene controls (A-1, A-2, A-3)	30/59	51	24/58	41	28/59	48	82/176	47
Na Cobaltinitrite, 30[c] (B-1, B-3)	27/59	46	...	..	20/58	35	47/117	40
Na Cobaltinitrite, 40 (C-1, C-3)	26/60	43	...	..	20/59	34	46/119	39
Na Cobaltinitrite, 50 (D-1, D-2, D-3)	20/58	35	13/60	22	21/59	36	54/177	31[b]
Na Cobaltinitrite, 60 (E-1, E-2, E-3)	16/58	28	24/57	42	14/59	24	54/174	31[b]
Na Cobaltinitrite, 70 (F-2, F-3)	...	..	16/59	27	14/59	24	30/118	25[b]
Na Cobaltinitrite, 80 (G-2)	...	..	18/59	31	...	..	...	..

[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 with control value. (Chi² test, $P < 0.01$). [c] Dose is mg./Kg.

occurred. Death usually ensued within 2 hours. On the basis of these preliminary observations, it was decided not to exceed the 80-mg./Kg. dose level.

A blood study was conducted on a separate group of mice to determine the extent and duration of the methemoglobinemia produced by sodium cobaltinitrite (80 mg./Kg.) and by sodium nitrite at a comparable dose in terms of nitrite present. The colorimetric method of Evelyn and Malloy (9) was employed; the results of this experiment are listed in Table II. The data indicated that a peak methemoglobin concentration was achieved within 60 minutes after the time of injection and decreased until negligible concentrations remained after 3 hours. This peak value represented the conversion of approximately 40% of the total hemoglobin to methemoglobin. It should be kept in mind that 80 mg./Kg. was the highest dose of sodium cobaltinitrite utilized, and that the methemoglobinemic response was, naturally, less at the lower dosage schedules. Several determinations at a dose of 60 mg./Kg. gave results indicating peak values of approximately 25% methemoglobinemia, which diminished appreciably by the end of 2 hours.

In comparing the cobaltinitrite response with that produced by sodium nitrite, a similar overall picture was observed. Peak levels were practically identical and occurred at approximately the same time. However, 2 hours after injection, the 80-mg./Kg. cobaltinitrite-treated mice exhibited higher methemoglobin values than the corresponding nitrite group. Thus, the one difference attributed to sodium cobaltinitrite—compared with sodium nitrite—was a slight prolongation in duration of methemoglobinemia.

It is unnecessary to describe in detail the macroscopic changes which occurred in the mice as they were exposed to repeated methylcholanthrene applications, since these observations have been published (4). It is sufficient to mention that the epidermal reactions were similar and consisted of an initial period of epilation which lasted for about 3 weeks. This was followed by another period of renewed hair growth, at which time the animals were left with longer hair in the interscapular region than in other areas of their bodies.

The appearance of tumors was first noted on a few mice during the sixth week of each trial. Both

TABLE IV.—INITIAL AND FINAL WEIGHTS OF MICE

Group	Initial Wt. (Gm. ± S.D.)	Final Wt. (Gm. ± S.D.)
Trial *1*		
A-1	25 ± 3	33 ± 4
B-1	25 ± 5	31 ± 3
C-1	26 ± 3	33 ± 3
D-1	25 ± 2	32 ± 3
E-1	25 ± 2	31 ± 6
Trial *2*		
A-2	20 ± 2	30 ± 4
D-2	21 ± 2	29 ± 3
E-2	21 ± 2	29 ± 3
F-2	20 ± 2	28 ± 3
G-2	22 ± 3	30 ± 3
Trial *3*		
A-3	25 ± 4	30 ± 3
B-3	24 ± 3	30 ± 2
C-3	25 ± 3	29 ± 3
D-3	26 ± 3	30 ± 3
E-3	26 ± 3	29 ± 3
F-3	25 ± 2	29 ± 3

control and cobaltinitrite groups responded at the same time; *i.e.*, no outstanding delay in tumor onset could be attributed to the methemoglobinemia, although at a given time there were usually less tumors in the test groups than in the controls. It was not our purpose to study the chronological progression of tumor growth, for it was apparent that a close daily examination of 360 mice would be extremely impractical. Rather, the experiment was designed to use the established method of producing tumors in a 72-day period, and to compare the tumorous mice in the test groups with the number of tumorous mice in the controls at the end of this time.

At the termination of each trial, all growths which measured at least one dimension (width *versus* height) of 1 mm. or greater were drawn and recorded on individual data sheets. All mice were chloroformed and the tumors excised and immersed in Bouin's fixative. Histological examination revealed that these growths were true cancers of the type that are produced on the epidermis by methylcholanthrene, *viz.*, squamous cell carcinomas.

DISCUSSION

Table III contains the complete data with respect

to tumor incidence in the separate groups at the termination of each trial and lists the composite results of the three trials. These totals were obtained by combining the corresponding individual groups of each trial, thus giving a single "per cent tumor" value for the control mice and for each of the five cobaltinitrite groups. The effective total of mice in each group was, in most cases, less than the original number of 60. Obviously, this was due to deaths which occurred during the trial period. Of the 960 mice employed in all three phases of the experiment, only 28 died. Mice which died before tumors were noted in the group were not counted in the effective total. When a death occurred after growths had appeared, the animal was examined for the presence of a tumor, and the data obtained were incorporated into the final results. No evidence of cumulative toxicity was ever apparent throughout the experiment; the animals seemed normal in all respects. Table IV, which shows the initial and final weights of the various groups, fails to reveal any differences that might indicate some toxic manifestation.

Table III shows that of 176 control mice (those which received only the methylcholanthrene), 82 developed tumors of 1 mm. or greater in one dimension. This corresponds to a 47% tumor incidence, a result which is very close to the expected value of 50% and which reconfirms the MCD_{50} of methylcholanthrene. The mice which had received the two lower doses (30 and 40 mg./Kg.), responded to the MCD_{50} with a slight but insignificant decrease in tumors. However, at the 50-mg./Kg. dose level and higher, a significant reduction of tumor incidence was seen. Thus, bi-weekly, intraperitoneal injections of 50, 60, and 70 mg./Kg. of sodium cobaltinitrite were capable of reducing the per cent of tumor bearing mice to 31, 31, and 25, respectively, compared to the 47% control value.

It is difficult to accept that the reduction in tumor incidence is the result of the transient hypoxia produced by sodium cobaltinitrite. Periods of methemoglobinemia which lasted no longer than 3 hours and were elicited twice weekly certainly should not be unquestionably concluded as being the sole cause of this tumor inhibition. To reiterate the initial statements, the role of oxygen in the inception of the cancerization process is obscure, as is the basic mechanism of methylcholanthrene-induced carcinogenesis. It is quite possible that some metabolic effect other than hypoxia is responsible for or contributes to the decreased tumor incidence observed.

As a consequence of the theory that sulfhydryl enzymes may be involved in the induction of chemically-induced carcinogenesis by a fixation of the carcinogen to cellular proteins through sulfur linkages (10) an interesting concept arises. Lusky, *et al.* (11), obtained significant tumor reduction by applying dimercaprol (BAL) to mice during painting with 3,4-benzpyrene and reasoned that the additional SH-groups might compete with cellular SH for the carcinogen. Coupling this theory to the report by Vollmer and Carey (12)—that methemoglobinemia produced by sodium nitrite and *p*-amino propiophenone induces an increase in blood SH-enzyme levels—leads to the similar possibility that the increased SH-groups in the blood might also compete with the SH-groups of epidermal cell proteins, thereby affecting the neoplastic changes.

REFERENCES

(1) Warburg, O., *Science*, **123**, 309(1956).
(2) Weinhouse, S., "Advances In Cancer Research," Vol. III, Academic Press, Inc., New York, N. Y., 1955, p. 303.
(3) Olson, R. E., *Cancer Res.*, **11**, 571(1951).
(4) Gautieri, R. F., and Mann, D. E., THIS JOURNAL, **47**, 350(1958).
(5) *Ibid.*, **50**, 556(1961).
(6) Mancini, R. T., Gautieri, R. F., and Mann, D. E., *ibid.*, **53**, 385(1964).
(7) Goldenberg, M. M., and Mann, D. E., *ibid.*, **49**, 210 (1960).
(8) Litchfield, J. T., and Wilcoxon, F., *J. Pharmacol. Exptl. Therap.*, **96**, 99(1949).
(9) Evelyn, K. A., and Malloy, H. T., *J. Biol. Chem.*, **126**, 655(1938).
(10) Rondoni, P., "Advances In Cancer Research," Vol. III, Academic Press, Inc., New York, N. Y., 1955, p. 188.
(11) Lusky, L. M., Braun, H. A., and Woodard, G., *Cancer Res.*, **7**, 667(1947).
(12) Vollmer, E. P., and Carey, M. M., *J. Pharmacol. Exptl. Therap.*, **111**, 114(1954).

Nonketol Reduction of Tetrazolium Salts in Pharmaceutical Analysis

By EDWARD F. SALIM†, PETER E. MANNI‡, and JOSEPH E. SINSHEIMER

Nonketol compounds of pharmaceutical interest have been tested for reduction of tetrazolium salts; the quantitative applications of these reactions have been evaluated.

IN CONTRAST TO most organic compounds, the reduction of tetrazolium salts yields highly colored compounds. Based essentially upon this property, the production of these formazans by various reducing systems has led to a diverse range of biological and chemical applications. Lakon (1) has used triphenyltetrazolium chloride to detect viability of seeds, while Straus, Cheronis, and Straus (2) have applied tetrazolium salts in the study of carcinomatous tissue.

Chemical applications of tetrazolium salts include the quantitative analysis of reducing sugars (3), ketol steroids (4), and ascorbic acid (5). Rosenkrantz (6) has compared the effect of struc-

Received May 6, 1963, from the College of Pharmacy, University of Michigan, Ann Arbor.
Accepted for publication July 16, 1963.
Abstracted in part from a thesis submitted by Edward F. Salim to the Graduate School, University of Michigan, Ann Arbor, in partial fulfillment of Doctor of Philosophy degree requirements.
† Lilly Endowment Fellow. Present address: Drug Standards Laboratory, A.PH.A. Foundation, Washington, D.C.
‡ American Foundation for Pharmaceutical Education Fellow.