Apparent Enzyme Inhibition through Enzyme-Induction Studies as a Possible Mode of Action of Certain Cobalt Compounds

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Abstract □ The results of this investigation show that cobaltous chloride and sodium cobaltinitrite may inhibit cortisone-induced cleft palate and methylcholanthrene tumorigenesis in albino mice *via* enzyme inhibition.

Keyphrases □ Cobaltous chloride—enzyme inhibition of cortisone-induced cleft palate and methylcholanthrene tumorigenesis, albino mice, mode of action □ Sodium cobaltinitrite—enzyme inhibition of cortisone-induced cleft palate and methylcholanthrene tumorigenesis, albino mice, mode of action □ Enzyme inhibition—cobalt compounds, possible mode of action

During the past decade, much enthusiasm has been generated concerning the modification, by chemical means, of the complex processes of carcinogenesis and cleft palate inception (1–4). Out of a myriad of investigations concerned with the blocking of these two different pathological processes, one finds that cobaltous chloride and sodium cobaltinitrite used alone in the proper amount and time are both successful in reducing the incidence of methylcholanthrene-induced tumors and cortisone-induced cleft palate (1, 3). In addition, by themselves, cobaltous chloride and sodium cobaltinitrite both were shown to cause a low incidence of cleft palate.

In an attempt to explain these findings, many theories have been presented. Orzechowski *et al.* (1), in 1964, suggested that sodium cobaltinitrite reduced the amount of available oxygen to cancerous tissue through the formation of methemoglobinemia and, hence, caused inhibition of tumor growth. In 1968 (4, 5), the "ionic hormonal precursor hypothesis" was postulated to explain inhibition of cleft palate on one hand and induction on the other. In this case, it was believed that cobalt was acting in fetal tissue as a hormone and, although when used together with cortisone it interfered with its action, when used alone it mimicked the action of the parent hormone. It was suggested at that time that possibly cobalt acts like cortisone in primordial or aquatic forms of life and could be considered, therefore, as an ionic hormone performing the task in fetal tissue that cortisone would normally accomplish in later life if exogenously administered.

Many studies have shown that drug-metabolizing enzymes in liver microsomes are increased when animals are treated with various drugs and carcinogens. This increase in activity correlates directly with an increase in the concentration of enzyme protein and is referred to as "enzyme induction" (6). Conney *et al.* (7) demonstrated that pretreatment of rats with phenobarbital stimulates liver microsomal enzymes, which metabolize zoxazolamine as well as aromatic polycyclic hydrocarbons (7).

In light of the fact that specific cobalt compounds reduce the number of methylcholanthrene-induced tumors, the possibility exists that these same compounds stimulate liver enzymes which may metabolize methylcholanthrene and, hence, not allow the formation of tumors.

It is well known that certain enzymes in the liver microsomes metabolize not only drugs but also a variety of normal body substrates, such as steroid hormones, at a given rate. If the metabolizing-enzyme concentration is increased, hormonal metabolism would be increased. Therefore, because it is known that certain inorganic cobalt compounds inhibit the inception of cortisone-induced cleft palate (3), the possibility also exists that the cobalt compounds stimulate liver enzymes which may metabolize the cortisone and prevent its action in fetal tissue.

Therefore, the purpose of this investigation was to ascertain whether enzyme induction is produced by cobaltous chloride and sodium cobaltinitrite which, if observed, could account for the apparent nulling of the carcinogenic action of methylcholanthrene and the teratogenic action of cortisone.

MATERIALS AND METHODS

Ninety-six CF-1 female mice, weighing between 14 and 40 g. and averaging 25 g., were used in this experiment. These animals were divided into eight groups of 12 mice each and further divided into subgroups of six mice each. Cages were provided for each subgroup.

The diet consisted of food[1] and tap water, both supplied *ad libitum*.

All mice in each subgroup were numbered by punching holes in their ears.

All solutions were prepared of such concentrations that no animal received more than 0.45 ml. at one time. The phenobarbital solutions were prepared on the Friday prior to pretreatment injections and stored at room temperature. At that time, a 0.6% solution of phenobarbital in a 0.9% sodium chloride solution was prepared.

The sodium hexobarbital solutions were prepared 1 day prior to their use and stored at room temperature at a concentration of 1.0%.

The zoxazolamine[2] was obtained in the powdered form. The solution was prepared 1 day prior to use by adding 3.6 ml. of 1 *N* HCl to 300 mg. of zoxazolamine, which had been weighed out on an analytical balance, and diluting to 15 ml. with a 0.9% sodium

[1] Purina Laboratory Chow.
[2] Supplied through the courtesy of McNeil Laboratories, Fort Washington, Pa.

Table I—Duration of Hexobarbital Sleeping Time and Zoxazolamine Paralysis

Pretreatment Group[a]	Treatment	Duration of Action, min.
1. Phenobarbital	Hexobarbital	23[b]
Saline	Hexobarbital	125
2. Cobaltous chloride (LD)	Hexobarbital	86[b]
Saline	Hexobarbital	67
3. Cobaltous chloride (HD)	Hexobarbital	80[b]
Saline	Hexobarbital	44
4. Sodium cobaltinitrite (LD)	Hexobarbital	40
Saline	Hexobarbital	46
5. Sodium cobaltinitrite (HD)	Hexobarbital	43
Saline	Hexobarbital	47
6. Phenobarbital	Zoxazolamine	11[b]
Saline	Zoxazolamine	72
7. Cobaltous chloride (LD)	Zoxazolamine	49
Saline	Zoxazolamine	46
8. Cobaltous chloride (HD)	Zoxazolamine	46
Saline	Zoxazolamine	51

[a] Number of animals per group = 12. LD = low-dose cobaltous chloride, 10 mg./kg., and sodium cobaltinitrite, 20 mg./kg. HD = high-dose cobaltous chloride, 20 mg./kg., and sodium cobaltinitrite, 40 mg./kg. [b] p value 0.05 significantly different from saline control using Student's t test.

chloride solution. This solution also was stored at room temperature.

The solutions were all prepared according to the procedures outlined by Conney *et al.* (7).

The saline used in this experiment was obtained from 30-ml. multiple-dose vials previously prepared in this laboratory. The following drugs were always freshly prepared just prior to their injection: cobaltous chloride, 0.1 and 0.2%; and sodium cobaltinitrite, 0.2 and 0.4%.

Pretreatment consisted of sodium phenobarbital, cobaltous chloride, or sodium cobaltinitrite administered to a subgroup. For each subgroup receiving one of the test drugs, an additional subgroup was injected with a volume of saline corresponding to the dosage volume of the test drug.

The pretreatment injections were administered intraperitoneally twice a day, in the morning between 8:15 and 9:30 a.m. and in the early afternoon between 12:10 and 1:50 p.m., for 4 consecutive days, Monday through Thursday. All of the mice were weighed[3] to the nearest gram prior to the injections.

The doses of phenobarbital, hexobarbital, and zoxazolamine were those used by Conney *et al.* (7). The doses of cobaltous chloride and sodium cobaltinitrite were those that had been found to give the most significant results (1, 3) in previous experiments in this laboratory. They were as follows:

Drug	Dose
phenobarbital	1.5 mg. in 0.25 ml. of 0.9% sodium chloride solution (fixed)
hexobarbital	125 mg./kg.
zoxazolamine	100 mg./kg.
cobaltous chloride	10 mg./kg. (low dose)
	20 mg./kg. (high dose)
sodium cobaltinitrite	20 mg./kg. (low dose)
	40 mg./kg. (high dose)

On the 5th day, Friday, all animals were injected intraperitoneally, between 8:53 a.m. and 1:51 p.m., with either sodium hexobarbital or zoxazolamine. At this time, each animal was caged individually to determine the duration of action of the drug administered.

None of the drugs administered throughout the experiment was observed to be lethal to the animals.

RESULTS AND DISCUSSION

The criterion used in this investigation to observe enzyme induction was derived from a study by Conney *et al.* (7). These workers demonstrated the enzyme-inducing effects of phenobarbital upon the rate of metabolism of hexobarbital and zoxazolamine. In both cases, the duration of action was measured by determining the length of time necessary for animals to regain their righting reflexes after an intraperitoneal injection. The observed responses were hypnosis with hexobarbital and paralysis with zoxazolamine. In their experiments, animals pretreated with phenobarbital showed a significant decrease in the duration of action of both hexobarbital and zoxazolamine. In the present investigation, these results were confirmed (Table I, Groups 1 and 6) before further experimentation was attempted.

Since it is known that cobalt, in some manner, antagonizes both the tumorigenic effects of methylcholanthrene and cleft palate production by cortisone, it was hypothesized that these antagonistic effects may be brought about by increasing the concentration of enzymes that metabolize methylcholanthrene as well as cortisone.

Although no significant effect was noted when zoxazolamine (Table I, Groups 7 and 8) was used in conjunction with cobalt pretreatment, the duration of the hexobarbital response (hypnosis) appeared to be contrary to the results anticipated. Administration of hexobarbital to animals previously treated with cobaltous chloride showed an increase in the duration of hypnosis (Table I, Groups 2 and 3). Therefore, if enzyme systems are responsible for the observed results, it seems that enzyme inhibition, rather than enzyme induction, occurred.

According to Conney (6), an increase in liver microsomal enzymes parallels an increase in the rate of drug metabolism, thereby demonstrating the effect of enzyme induction. With this fact in mind, it follows that the rate of drug metabolism depends upon the concentration of metabolizing enzymes. Should this rate be decreased, it may be postulated that the concentration of metabolizing enzymes also is decreased, *via* enzyme inhibition. The action of cobaltous chloride on hexobarbital clearly demonstrates this theory (Table I, Groups 2 and 3). However, the observed effect of the cobalt compounds upon the response to zoxazolamine was not as evident. Nevertheless, enzyme inhibition may be occurring in both cases, but the means of determining this were not observed *via* duration of paralysis with zoxazolamine.

If the genesis of cancer with methylcholanthrene involves the introduction of new enzyme systems, which promote tumor growth, into the parent cell, then tumor growth would also be inhibited if these enzymes are inhibited *via* "protein deletion" (8). Accordingly, if excess cortisone produces its cleft palate effect by acting in conjunction with enzymes present in palate tissue, then cleft palate inception may be inhibited by removing these necessary enzymes. Consequently, because it was shown that both cobaltous chloride and sodium cobaltinitrite reduce the incidence of methylcholanthrene-induced tumors as well as cortisone-induced cleft palate inception (1, 3), and because apparent enzyme inhibition was observed in this present investigation, it may be hypothesized that these cobalt compounds exert their actions against cancer inception and cleft palate induction through enzyme inhibition.

[3] On a Hanson Dietetic Scale, model 1440.

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