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INHIBITION OF THE BIOSYNTHESIS OF RAT TESTICULAR HEME BY 1,2-DIBROMO-3-CHLOROPROPANE*

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Abstract—The nematocide 1,2-dibromo-3-chloropropane (DBCP) is known to produce oligospermia and azoospermia in workers engaged in its manufacture. DBCP was examined for its potential to interfere with regulation of the rat testicular heme biosynthetic pathway. Forty-eight hours after a single dose of DBCP (200 mg/kg, orally), testicular microsomal heme and cytochrome P-450 contents were depressed by approximately 27 and 35 per cent respectively. Heme and cytochrome P-450 levels returned to control values at day 6. The incorporation of δ-[4-^{14}C]aminolevulinic acid (ALA) into heme was depressed by approximately 43 per cent at 24 hr. DBCP administration did not modify testicular heme oxygenase activity. These results indicate that DBCP inhibits testicular heme synthesis at an enzymatic reaction other than ALA synthetase. Mitochondrial ALA synthetase (EC 2.3.1.37) activity, unchanged 24 hr after DBCP administration, had increased by 66 per cent at 48 hr and had returned to control values by day 6. The increased ALA synthetase activity probably reflects a compensatory attempt to restore the depressed heme content to control levels.

1,2-Dibromo-3-chloropropane (DBCP) is a highly effective nematocide which was used extensively prior to a ban on its use in September 1977. In 1961, Torkelson *et al.* [1] demonstrated that respiratory exposure of rats to DBCP resulted in adverse effects associated with the liver, bronchioles, renal collecting tubules and the testicular germinal epithelium. Recent epidemiological studies have revealed that a depressed sperm count exists in workers who have undergone industrial exposure to DBCP [2, 3]. Testicular biopsies have demonstrated normal histology except for the reduction or total absence of spermatogenic cells [4–6]. Plasma testosterone levels were found to be within the normal range, whereas plasma levels of follicle stimulating hormone (FSH) were consistently above normal [2–6]. These studies indicate that the site of DBCP action in the testis is the germinal epithelium. The mechanism by which DBCP impairs spermatogenesis is unknown. The synthesis of heme and of hemeproteins, such as cytochrome P-450, in testicular tissue is likely to be an important, rigidly regulated cellular process susceptible to interference by environmental agents. Therefore, the effect of DBCP on rat testicular heme synthesis was investigated. The results of this investigation are presented in this report.

MATERIALS AND METHODS

Materials. Pyridoxal-5′-phosphate, disodium EDTA, sodium succinate, sorbitol, NAD, NADP, isocitric acid, hemin (Type III), albumin (human, Fraction V), porcine heart succinyl-CoA synthetase (EC 6.2.1.4), cytochrome *c* (Type III) and isocitric dehydrogenase (Type IV) (EC 1.1.1.42) were obtained from the Sigma Chemical Co., St. Louis, MO. GTP, β-aminolevulinic acid (ALA) and coenzyme A were obtained from CalBiochem, La Jolla, CA. δ-[4-^{14}C]Aminolevulinic acid (42 mCi/mmole) was purchased from the New England Nuclear Corp., Boston, MA, and [2,3-^{14}C]succinic acid from ICN Pharmaceuticals, Irvine, CA. DBCP (technical grade) was a gift from the Occidental Co., Lathrop, CA; this represents material responsible for worker exposure. The presence of a potent contaminant in DBCP formulations that was responsible for production of azoospermia and oligospermia remains a possibility. Proof, however, awaits the isolation and characterization of such a substance before evaluation. Other chemicals were reagent grade.

Treatment of animals. Male Sprague–Dawley rats (200–250 g, 60–80 days of age) were obtained from Sasco Inc., Omaha, NE. The rats were permitted food and water *ad lib.*, except for a 12-hr period before DBCP administration when food was withheld. DBCP was dissolved in mineral oil (2 ml DBCP/100 ml) and administered orally (200 mg/kg; 5 ml/kg) with a 16-gauge, curved animal feeding needle (Popper & Sons, Inc., New York, NY). The acute, oral LD_{50} in the rat is reported to range from 170 to 300 mg/kg [1, 7]. No deaths, however, were observed nor were testicular weights altered during the 6 day interval following administration of DBCP. Control animals received equal volumes of mineral oil, which did not alter the activities of the enzymes measured in this study. Animals were given DBCP and were killed at about the same time of day (8:00 to 9:00) to avoid any potential differences in effects caused by circadian rhythms.

Preparation of tissues. Animals were decapitated

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and the testes were removed, decapsulated and weighed. The testes from each animal were pooled, and testicular homogenates (10%, w/v) were prepared in 0.25 M sucrose. The method of Cammer and Estabrook [8] was used to prepare the mitochondrial and microsomal fractions. Spectrophotometric assay indicated that the preparations were devoid of hemoglobin. Protein was determined by the method of Bradford [9], using bovine serum albumin as the standard.

Microsomal heme determination. Microsomal heme was determined from the difference spectrum of the oxidized/reduced pyridine hemochromogen between 541 and 557 nm, using a millimolar extinction coefficient of 20.7 $mM^{-1} cm^{-1}$ [10].

Microsomal cytochrome P-450 determination. Levels of cytochrome P-450 were determined from the carbon monoxide difference spectrum (450–490 nm) of dithionite-reduced microsomes using an extinction coefficient of 91 $mM^{-1} cm^{-1}$ [11].

ALA synthetase activity. Testicular mitochondrial ALA synthetase activity was determined by a radioisotopic method employing [2,3-^{14}C]succinate and a succinyl-CoA generating system previously developed in this laboratory [12]. Recovery of ALA was 91 per cent, and the ^{14}C-labeled material was confirmed to be the [^{14}C]ALA-pyrrole, by thin-layer chromatography in two different solvent systems with an authentic ALA-pyrrole standard [12]. Rat testicular ALA synthetase activity was found to be localized predominantly in the mitochondrial fraction [12].

Heme oxygenase activity. Testicular microsomal heme oxygenase activity was determined by the method of Schacter [13], using a partially purified biliverdin reductase preparation from rat liver as described by Tenhunen *et al.* [14]. Enzyme activitiy was determined from the increase in absorbance at 468 nm using a millimolar extinction coefficient of 60 $mM^{-1} cm^{-1}$. Product formation was found to be linear for 15 min with up to 2 mg protein/ml.

Sorbitol dehydrogenase activity. Testicular sorbitol dehydrogenase activity was determined in the cytosolic fraction by the method of King and Mann [15], using a millimolar extinction coefficient of 6.22 $mM^{-1} cm^{-1}$.

NADPH-cytochrome c reductase activity. NADPH-cytochrome *c* reductase activity was determined in testicular microsomes by the method described by Baron and Tephly [16], using a millimolar extinction coefficient of 19.1 $mM^{-1} cm^{-1}$.

Incorporation of [4-^{14}C]ALA by microsomal heme. The relative rates of testicular heme synthesis were measured in rats 24 hr after a single, oral dose of DBCP in mineral oil (200 mg/kg; 5 ml/kg) or of mineral oil. Rats received 4 μCi of [4-^{14}C]ALA [i.p.; 1 ml/kg in 50 mM Tris–HCl (pH 7.4)–0.9% NaCl] and were killed 90 min later. Controls received an equal volume of vehicle. Incorporation of [^{14}C]ALA by testicular microsomal heme was linear for 120 min. Testes were pooled from two animals and microsomes were prepared by the method of Cammer and Estabrook [8]. Heme was extracted into ethyl acetate/acetic acid (4:1, v/v), and washed in water and 1.5 N HCl by the method of Bonkowsky *et al.* [17]. Aliquots of the heme extract were added to 6 ml of Amersham ACS scintillation fluid and counted for radioactivity in a Beckman LS8000 liquid scintillation counter (93 per cent efficiency).

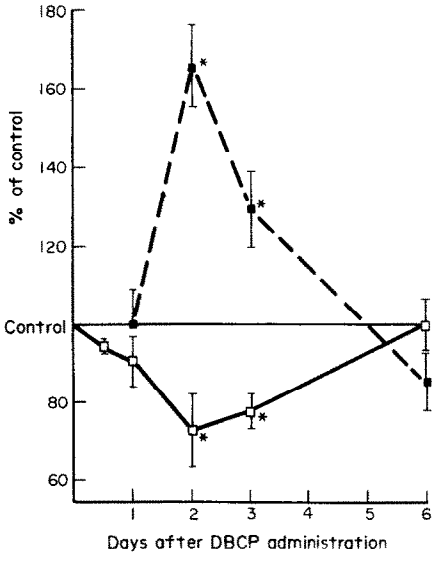

Fig. 1. Effect of DBCP on rat testicular mitochondrial ALA synthetase activity (■ – – – ■) and microsomal heme content (□ ——□). Animals were killed at the specified time intervals after a single, oral dose of DBCP (200 mg/kg). The mean control value for ALA synthetase activity was 183 nmoles ALA/g protein/hr. Each point for ALA synthetase activity is the mean ± S.E.M. of six determinations. The mean control value for microsomal heme was 0.386 nmole heme/mg protein. Each point for microsomal heme is the mean ± S.E.M. for at least three determinations. An asterisk indicates that the difference between the DBCP-treated and control values was statistically significant ($P < 0.05$).

Statistics. Data were analyzed by Student's *t*-test to determine the significance of differences between means.

RESULTS

The time courses of the effect of a single, oral dose of DBCP (200 mg/kg) on rat mitochondrial testicular ALA synthetase activity and on microsomal heme content are shown in Fig. 1. Microsomal heme levels decreased for 2 days following DBCP administration and returned to control levels by day 6. ALA synthetase activity did not differ from controls until day 2, when activity was 166 per cent of control levels. After the maximum increase at day 2, ALA synthetase activity, like microsomal heme, returned to control values. The effect of DBCP on testicular microsomal cytochrome P-450 exhibited the same temporal pattern as that of microsomal heme (Table 1). Microsomal cytochrome P-450 levels exhibited a maximal depression 2 days after DBCP administration and returned to control values at day 6.

Several compounds have been shown to stimulate the degradation of hepatic heme through the induction of hepatic oxygenase [18, 19]. Therefore, one mechanism by which DBCP might decrease testicular

Table 1. Effect of DBCP on rat testicular microsomal cytochrome P-450 content*

Days after DBCP treatment	Microsomal cytochrome P-450 (% of control)
1	81 ± 9
2	65 ± 12†
3	70 ± 12†
6	82 ± 12

* Rats received a single dose of DBCP (200 mg/kg, orally) at time 0 and were killed at the intervals indicated; testicular microsomal cytochrome P-450 was determined as described in Materials and Methods. The levels of cytochrome P-450 were measured in controls at each interval, and did not differ significantly. The mean control value for microsomal cytochrome P-450 was 0.167 nmole P-450/mg protein. Each value is the mean ± S.E.M. of eight determinations.

† Indicates that the difference between the DBCP-treated and control values was statistically significant ($P < 0.05$).

microsomal heme would be by the induction of testicular heme oxygenase. However, testicular heme oxygenase activity was not altered by DBCP treatment (Table 2).

The observation that DBCP did not enhance the rate of heme breakdown by stimulation of heme oxygenase activity suggested that the reduced microsomal heme content might have been produced by an impairment of the synthesis of heme. Therefore, the rate of incorporation of [4-^{14}C]ALA into testicular microsomal heme was measured for DBCP-treated and control rats. The effect of DBCP on the incorporation of [4-^{14}C]ALA into microsomal heme is shown in Fig. 2. It is evident that, 24 hr after DBCP treatment, there was a decrease of 43 percent in the [4-^{14}C]ALA incorporation into microsomal heme.

It is possible that a chemical such as DBCP depresses heme and cytochrome P-450 levels by nonspecific inhibition of testicular protein synthesis. In order to test this possibility, activities of the non-hemedependent enzymes, sorbitol dehydrogenase and NADPH-cytochrome *c* reductase, were measured. Sorbitol dehydrogenase is localized in the testicular cytosolic fraction associated with the seminiferous tubules and has been used as a marker for testicular protein synthesis [20], whereas NADPH-cytochrome *c* reductase is a microsomal flavoprotein. As shown in Table 3, the activities of rat testicular sorbitol dehydrogenase and NADPH-cytochrome *c* reductase were not altered by DBCP administration. Testicular weight was not changed by DBCP treatment during the 6-day period, which also suggests that DBCP does not act merely by inhibition of protein synthesis.

Table 2. Effect of DBCP on rat testicular microsomal heme oxygenase activity*

Treatment	Heme oxygenase activity (nmoles bilirubin formed/mg protein/hr)
Control	7.8 ± 0.7
DBCP, 24 hr	7.7 ± 0.8
DBCP, 48 hr	7.3 ± 0.9

* Rats received a single dose of DBCP (200 mg/kg, orally) and were killed at the specified time intervals. Microsomal heme oxygenase activity was measured as described in Materials and Methods. Each value is the mean ± S.E.M. of three determinations.

DISCUSSION

At the present time, impairment of spermatogenesis is the only known pathological consequence to the testis associated with human industrial exposure to DBCP [2, 3]. Plasma testosterone levels do not appear to be affected [4, 5], nor has any specific biochemical effect of DBCP on the human testis been described. In this paper we report that administration of DBCP to rats decreased testicular microsomal heme and cytochrome P-450 content and decreased the rate of [4-^{14}C]ALA incorporation into microsomal heme. DBCP administration did not modify the activity of heme oxygenase. These results suggest that DBCP lowers testicular heme and cytochrome P-450 content by inhibiting the synthesis of heme.

Table 3. Effect of DBCP on the activity of rat testicular NADPH-cytochrome *c* reductase and sorbitol dehydrogenase*

Treatment	NADPH-cytochrome *c* reductase activity (nmoles cytochrome *c* reduced/mg protein/min)	Sorbitol dehydrogenase activity (nmoles NADH formed/mg protein/min)
Control	42 ± 2	2.1 ± 0.1
DBCP, 24 hr	43 ± 5	2.5 ± 0.2
DBCP, 48 hr	38 ± 3	2.4 ± 0.1

* Rats received a single dose of DBCP (200 mg/kg, orally) and were killed at the specified time intervals. Microsomal NADPH-cytochrome *c* reductase and cytosolic sorbitol dehydrogenase activities were measured as described in Materials and Methods. Each value is the mean ± S.E.M. of three determinations.

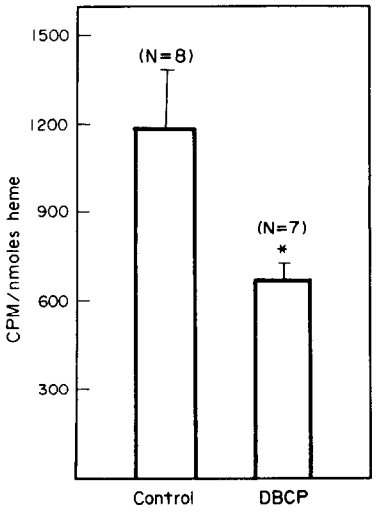

Fig. 2. Effect of DBCP on the incorporation of [^{14}C]ALA into rat testicular microsomal heme. Rats received 4 μCi of [^{14}C]ALA intraperitoneally 24 hr after administration of DBCP (200 mg/kg, orally). Rats were killed 90 min later, and the microsomal heme was extracted, assayed, and the radioactivity measured as described in Materials and Methods. Numbers above the bars denote the numbers of animals. Values represent means ± S.E.M. An asterisk indicated that the difference between the DBCP-treated and control values was statistically significant ($P < 0.05$).

The previously reported inverse relation between heme levels and the activity of ALA synthetase [21–23] offers a plausible explanation for the increased activity of ALA synthetase following depression of heme content (Fig. 1). The reduced heme content caused by DBCP inhibition of the synthesis of heme would be a feedback signal for induction of ALA synthetase to restore cellular heme levels [22]. Menard *et al.* [24] have demonstrated that 71–86 per cent of rat testicular microsomal cytochrome P-450 is located in the Leydig cells. This relatively large amount of microsomal heme in the Leydig cells, if unaffected by administration of DBCP, would have a tendency to mask any decrease in microsomal heme in the seminiferous tubular cells. Human studies have indicated that the site of DBCP action is the seminiferous tubules and not the Leydig cells [2, 4–6]. If this is also the site of DBCP action in the rat testis, small decreases in either total testicular microsomal heme or cytochrome P-450 would actually represent much larger relative decreases in heme levels of cells of the seminiferous tubules. Future research investigations will be concerned with the effects of DBCP on heme synthesis in the specific cell types of the rat testis and identification of the enzymatic step(s) of the testicular heme biosynthetic pathway that are impaired by this chemical agent.

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