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Relationship of drinking water disinfectants to plasma cholesterol and thyroid hormone levels in experimental studies

(chlorinated water/hypercholesterolemia/hypothyroidism)

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ABSTRACT **The effects of drinking water containing 2 or 15 ppm chlorine (pH 6.5 and 8.5), chlorine dioxide, and monochloramine on thyroid function and plasma cholesterol were studied because previous investigators have reported cardiovascular abnormalities in experimental animals exposed to chlorinated water. Plasma thyroxine (T4) levels, as compared to controls, were significantly decreased in pigeons fed a normal or high-cholesterol diet and drinking water containing these drinking water disinfectants at a concentration of 15 ppm (the exception was chlorine at pH 6.5) for 3 months. In most of the treatment groups, T4 levels were significantly lower following the exposure to drinking water containing the 2 ppm dose. Increases in plasma cholesterol were frequently observed in the groups with lower T4 levels. This association was most evident in pigeons fed the high-cholesterol diet and exposed to these disinfectants at a dose of 15 ppm. For example, after 3 months of exposure to deionized water or water containing 15 ppm monochloramine, plasma cholesterol was 1266 ± 172 and 2049 ± 212 mg/dl, respectively, a difference of 783 mg/dl. The factor(s) associated with the effect of these disinfectants on plasma T4 and cholesterol is not known. We suggest however that these effects are probably mediated by products formed when these disinfectants react with organic matter in the upper gastrointestinal tract.**

The use of chlorine as a disinfectant of public drinking water supplies was first practiced in the United States in 1908 (1). The benefit of chlorine on controlling levels of water-borne infectious bacteria soon became known, and the practice of chlorination spread to most large population centers within a decade. The EPA office of water supply estimates that in 1975, 192 million Americans were served by community water systems, and the majority of these systems used chlorine as a disinfectant (2). Other disinfectants used in drinking water systems include chlorine dioxide, monochloramine, and ozone.

The health benefits associated with chlorination are well established, but the possibility of harmful effects also exists. For instance, recent studies suggest that hepatotoxins and carcinogens may be generated during chlorination (3, 4). The compounds generated include chlorinated organics such as chloroform and other trihalomethanes, both of which have been observed in drinking water treated with chlorine (5). Chlorination of foods and drinking water have been associated with cardiovascular abnormalities. For example, the chlorination of flour is associated with the formation of chlorinated fatty acids, and, in animals fed these fatty acids, significant increases in heart weight have been observed (6). Revis *et al.* (7) have observed hypercholesterolemia and cardiac hypertrophy in pigeons and rabbits exposed to chlorinated drinking water. Taken together, these results suggest that chlorine and/or formed chlorinated products may adversely affect the cardiovascular system through the well-known association of plasma cholesterol levels with atherosclerosis and hypertension with cardiac hypertrophy.

To further explore the possible relationship of drinking water disinfectants to cardiovascular disease, pigeons were exposed to drinking water containing different concentrations of chlorine, chlorine dioxide, and monochloramine. The effect of these disinfectants on the cardiovascular system was determined by measuring plasma cholesterol and the presence and severity of coronary and aortic atherosclerosis. Thyroid function was determined because in previous studies we have observed a significant decrease in plasma thyroxine (T4) in pigeons treated with chlorinated drinking water. These studies were performed also because of the known association between cardiac hypertrophy and hypercholesterolemia with plasma thyroid hormone levels (8, 9).

METHODS

Male white carneau pigeons (age, 3–4 months; obtained from Palmetto Pigeon Plant, Sumter, SC) were observed for 20 days prior to treatment. The effect of chlorine, chlorine dioxide, and monochloramine on the cardiovascular system was determined by exposing groups of 12 pigeons to drinking water containing these disinfectants and various pigeon diets for 3 months. The pigeon diets were prepared by Ralston Purina (St. Louis, MO) and altered from the normal pigeon diet as follows: diet A, reduction in calcium to 0.35% (80% of the minimum daily requirement for pigeons); diet B, reduction in calcium to 0.35% and the addition of 10% lard and 0.5% cholesterol. The concentrations of cholesterol in diet A over the experimental period ranged from 0.06% to 0.09%. Animals were exposed to the diets and drinking water *ad lib.* The drinking water contained chlorine, chlorine dioxide, and monochloramine at concentrations of 2 and 15 ppm. These disinfectants were prepared daily as described (10, 11). The drinking water was changed daily, and the level of each disinfectant and the pH were measured daily and adjusted (if required) to the level and pH indicated below. The controls were given deionized drinking water (or chlorite as a control to chlorine dioxide), which was changed daily.

Pigeons were exposed to the diets and drinking water for 3 months; at 1-month intervals, blood samples were collected from the brachial vein into sodium citrate tubes. After the plasma was isolated, the lipoproteins were sequentially separated by flotation in a Beckman L-80 preparative ultracentrifuge with a titanium rotor. The density was adjusted to 1.063 (with NaCl) and 2.10 (with NaCl and NaBr) for the respective isolation of low density lipoproteins (LDL) and high density lipoproteins (HDL) (12). Cholesterol in the

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Abbreviations: LDL, low density lipoprotein(s); HDL, high density lipoprotein(s).

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plasma, LDL, and HDL and protein in LDL and HDL were determined (12).

After 3 months of exposure, pigeons were killed by an air embolism. After the body weight was recorded, the heart and aorta were removed and flushed with saline. Connective tissue and fat were removed from the heart before it was weighed and fixed in 10% buffered formalin. Then the aorta was stained with Oil Red "O" and washed, the number of lipid-stained plaques were counted, and the size of each plaque was determined by the square area it occupied as described (13). To distinguish between fatty streaks and atheromatous plaques of the aorta, each plaque was frozen and sectioned, and the sections were stained with Masson's trichrome for collagen deposition and with alizarin red for deposits of calcium. Atherosclerosis of the coronary arteries was determined by making blocks (≈4 mm thick) at the base, middle, and upper half of fixed heart tissue. Eight sections were made from each block, and the sections were stained with Mason's trichrome stain. Each section was viewed under a light microscope, and the number of coronary arteries with atheromatous plaques was recorded. Atheromatous plaque in aorta and coronary arteries was determined by the presence of a fibrous cap with collagen deposits, smooth-muscle-cell proliferation, lipid-droplet accumulation, and/or calcium deposits.

Thyroid hormones were measured by RIA. Plasma levels of total T4 and T3 (3,5,3′-triiodothyronine) and free T4 and T3 were made by using coat-A-coat kits provided by Diagnostic Products (Los Angeles). Within assays, the coefficient of variation ranged from 4.7% to 9.8%. The coat-A-coat assay measures the plasma level of free and total (after displacement from the appropriate binding proteins) T4 and T3 by using specific antibodies to these hormones.

Results were analyzed for statistical significance by Student's *t* test of the difference between means and by Student's *t* test of paired observations (14). The expressions $P = 0.05$, $P = 0.01$, and $P = 0.001$ are used to indicate significance at the 5%, 1%, and 0.1% levels, respectively.

RESULTS

In both control and experiment groups receiving the normal diet, plasma cholesterol increased from zero time to 3 months (Fig. 1*A*). In the controls, plasma cholesterol was increased by 35 mg/dl at 3 months. In contrast, the mean increase in plasma cholesterol averaged 64 mg/dl in pigeons given the various drinking water disinfectants for 3 months. The range of increase over the controls in these various groups was from 5 to 173 mg/dl. However, significant increases in plasma cholesterol after 3 months of exposure were only observed in pigeons given chlorine at pH 8.5 (2 and 15 ppm), chlorine dioxide (2 ppm), and monochloramine (2 ppm). Although plasma cholesterol was increased in pigeons exposed to chlorite, the increase was not statistically significant because of the relatively large standard deviation. In none of the treatment groups was a clear dose–response effect for plasma cholesterol observed.

The mean average increases in plasma cholesterol in all groups exposed to the high-cholesterol diet (Fig. 1*B*) were from 168 ± 8 (0 time) to 1505 ± 215 (at 3 months). The 3-month value represents an ≈9-fold increase in plasma cholesterol in 90 days. Although increases compared to controls were observed in pigeons given 2 ppm chlorine (pH 6.5 and 8.5), significant increases at 3 months were only observed in pigeons given 15 ppm chlorine (pH 8.5), chlorine dioxide, and monochloramine. After 3 months of exposure to deionized water or 15 ppm monochloramine, the mean plasma cholesterol was 1266 ± 172 and 2049 ± 212, respectively—a difference of 783 cholesterol mg/dl. The range of increase over the controls in pigeons given the various

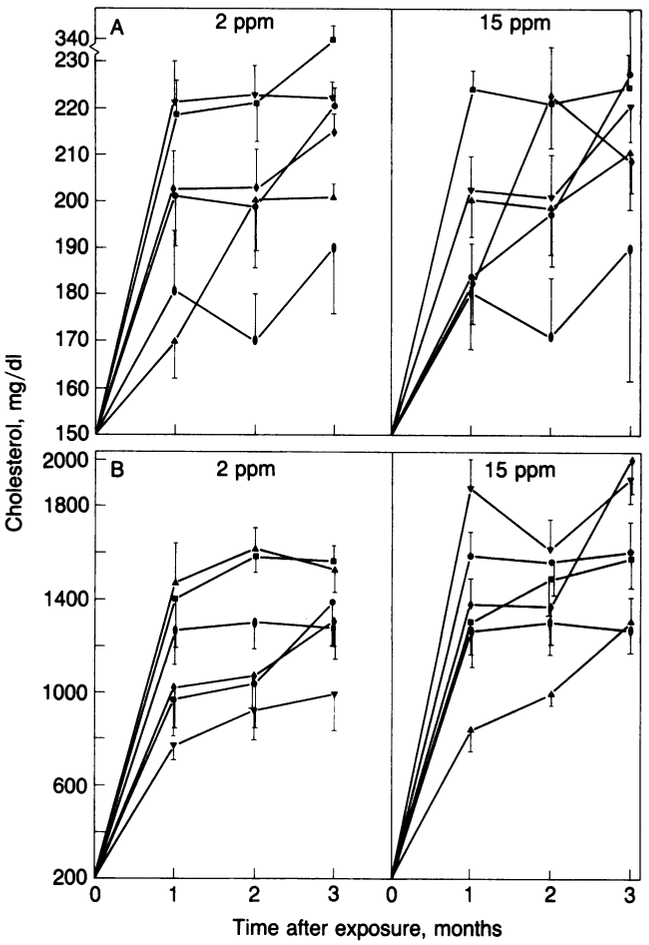

FIG. 1. The effect of drinking water disinfectants on plasma cholesterol. Values are means ± SEM for plasma cholesterol in pigeons exposed through the drinking water to deionized water (0) or 2 (*Left*) and 15 (*Right*) ppm chlorine, chlorine dioxide, or monochloramine and fed a diet deficient in calcium (*A*) or a calcium-deficient diet with 0.5% cholesterol added (*B*). Plasma cholesterol was determined at 0 time and at 1-month intervals for 3 months during the exposure to these drinking waters and diets. In each exposure group, 10 pigeons were used. 0, Control; ▲, chlorine at pH 6.5; ■, chlorine at pH 8.5; ▼, chlorine dioxide; ●, chlorite; ◆, monochloramine.

drinking water disinfectants at 15 ppm was from 42 to 738 mg/dl. Although a dose–response effect was not observed, results suggest that the 15 ppm dose was more effective in increasing plasma cholesterol than was the 2 ppm dose.

Fig. 2 shows the changes in the LDL and HDL cholesterol. Total plasma cholesterol is included in these figures to show the relationship of these two lipoproteins to the total plasma cholesterol. Both LDL and HDL cholesterol increased in response to these drinking water disinfectants (Fig. 2*a*). However, significant increases were only observed in the LDL cholesterol. Neither the disinfectant dose nor the exposure period appeared to be important determinants of the observed effects. For example, 2 ppm chlorine (pH 8.5) and chlorine dioxide induced a significant increase in the LDL cholesterol at 1 and 3 months of exposure, whereas significant changes were not observed with a dose of 15 ppm for these exposure periods.

In the control pigeons fed a normal diet, LDL cholesterol represented 40% and HDL represented 50% of total cholesterol. In contrast, in pigeons fed the high-cholesterol diet, LDL cholesterol was 72% and HDL was 15% of the total plasma cholesterol; these results suggest that in the pigeon hypercholesterolemia is associated with hyper-β-lipoprotein-

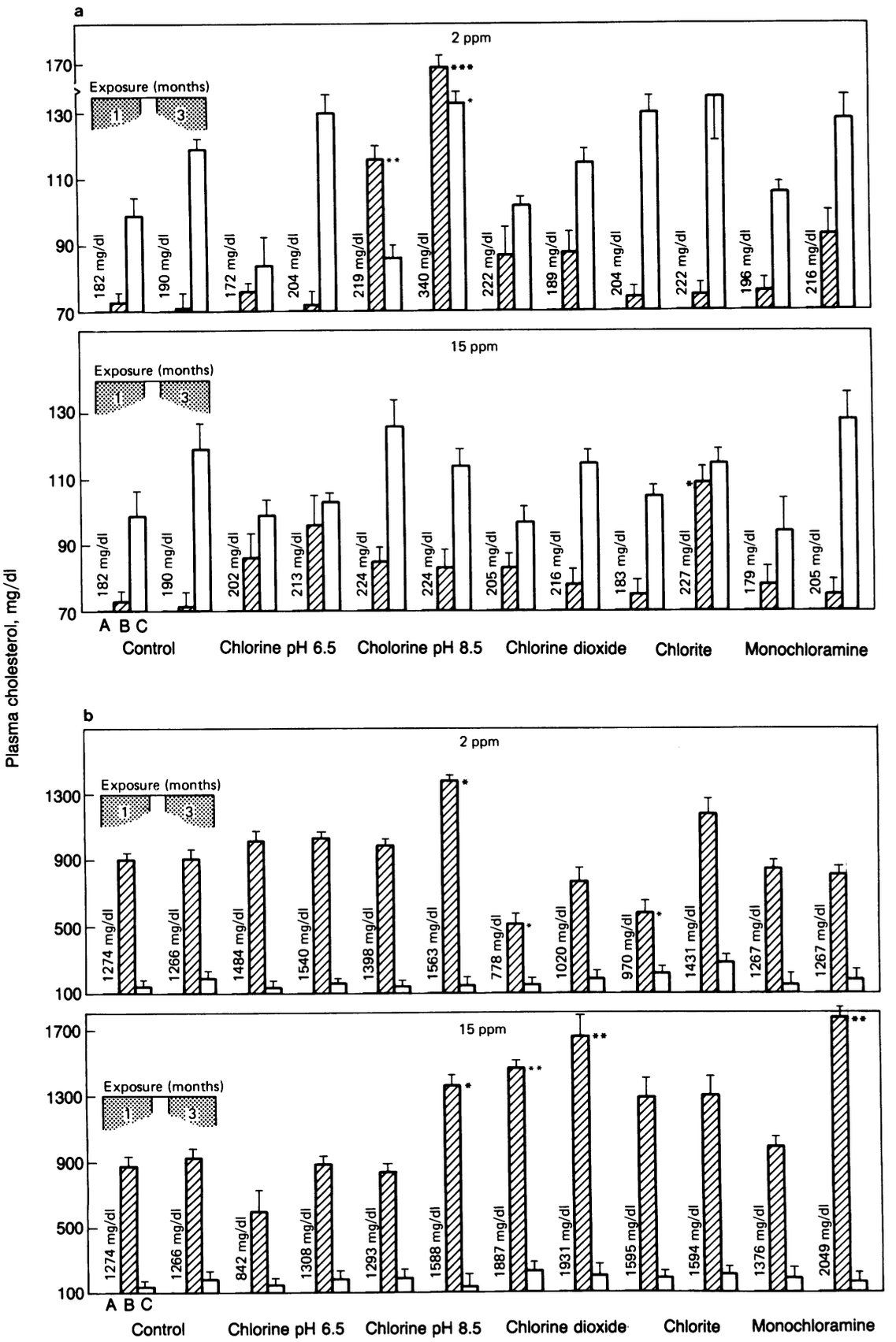

FIG. 2. The effect of drinking water disinfectants on LDL and HDL cholesterol. Data are means ± SEM (10 pigeons per group) for the cholesterol of LDL (hatched bars; columns B) and HDL (blank bars; columns C). The means for total plasma cholesterol for the various groups are also shown in columns A. Pigeons were exposed to a calcium-deficient diet (*a*) or a calcium-deficient diet with 0.5% cholesterol added (*b*) and the drinking water disinfectants at concentrations of 2 and 15 ppm. Cholesterol was determined in the plasma and on the isolated lipoproteins at 1 and 3 months of exposure. *, $P < 0.05$; **, $P < 0.01$; ***, $P = 0.001$.

emia. Only modest increases were observed in the HDL cholesterol (Fig. 2*b*). The only significant change observed in the LDL cholesterol after the exposure to the 2 ppm dose was an increase in animals given chlorine (pH 8.5) for 3 months and a decrease in pigeons given chlorine dioxide for 1 month. When the dose was increased to 15 ppm, significant increases in LDL cholesterol were observed in animals given chlorine pH 8.5 (3 months), chlorine dioxide (1 and 3 months), and monochloramine (3 months).

Aortic atherosclerosis appeared to be associated with the change in LDL cholesterol (Table 1). Significant increases in the mean plaque size of aortic plaques in pigeons fed the normal diet were only observed in the group exposed to chlorine pH 8.5 (2 and 15 ppm) and chlorine dioxide (2 ppm). In pigeons fed the high-cholesterol diet, significant increases in aortic plaques were observed in pigeons given 15 ppm chlorine (pH 8.5), chlorine dioxide, and monochloramine. These latter results suggest a relationship between mean aortic plaque size and plasma cholesterol levels. Significant increases in coronary atherosclerosis were not observed in this relatively short-term study. The ratio of heart weight to body weight was increased in pigeons exposed to either diet and 2 and 15 ppm chlorine (pH 8.5), chlorine, and monochloramine.

Hypercholesterolemia is frequently associated with hypothyroidism. In an attempt to determine if the observed increase in plasma cholesterol is associated with thyroid function, the plasma levels of T4 and T3 were determined (Table 2). Significant changes in the level of these hormones were observed. Plasma T4 levels were significantly decreased in animals fed the normal or high-cholesterol diets and given chlorine (pH 8.5), chlorine dioxide, and monochloramine at 2 and 15 ppm. The exceptions were monochloramine at 2 ppm in the normal diet and chlorine dioxide at 2 ppm in the high-cholesterol diet. The plasma level of T3 appeared to be increased at 2 ppm and decreased at 15 ppm in both dietary groups. Consistent changes in free T4 and T4 were not observed.

DISCUSSION

In the so-called atherosclerotic-resistant laboratory animal such as the dog and rat, plasma cholesterol is transported primarily by the HDL (15). In contrast, in the atherosclerotic-susceptible species such as swine and monkey (*Erythrocebus patas*), plasma cholesterol is primarily transported by the LDL (16). However, even though plasma cholesterol in the pigeon is transported primarily in the HDL, this species is known to develop spontaneous atherosclerotic lesions in the aorta and coronary arteries (17, 18). The development of such lesions in the pigeon may be associated with the ability of the LDL to respond to variations in the level of plasma cholesterol. This is not to say that the HDL does not respond, but rather that the LDL is more responsive to changes in the plasma cholesterol level. The suggestion is supported by the results shown in Fig. 2. Increases in the level of plasma cholesterol were significantly greater in the LDL fraction compared with that in the HDL fraction. Such increases in the LDL cholesterol resulted in a decrease in the ratio of HDL/LDL. A decrease in this ratio has been associated in human and experimental animals with atherosclerosis (19–21). Thus, the pigeon may be susceptible to this disease because of the response of the LDL to changes in plasma cholesterol.

Table 1. Effects of drinking water disinfectants on atherosclerosis in the white carneau pigeon

Treatment	Dose, ppm	Plaque size, mm²	
		Normal diet	High-lipid diet
Control		1.29 ± 0.48	4.75 ± 0.77
Chlorine pH 6.5	2	1.28 ± 0.28	5.43 ± 1.40
	15	1.31 ± 0.27	4.61 ± 0.91
Chlorine pH 8.5	2	1.93 ± 0.11*	7.75 ± 0.40
	15	2.41 ± 0.48†	12.0 ± 1.58†
Chlorine dioxide	2	1.99 ± 0.14*	5.37 ± 0.43
	15	1.81 ± 0.39	11.95 ± 1.55†
Chlorite	2	1.46 ± 0.17	4.78 ± 0.48
	15	1.42 ± 0.18	5.11 ± 1.08
Monochloramine	2	1.28 ± 0.34	7.33 ± 0.66
	15	1.62 ± 0.80	10.99 ± 0.72†

Values are means ± SEM for at least 10 pigeons per group. The normal and high-lipid diet and scoring for atherosclerosis were as described.
*$P < 0.05$.
†$P < 0.01$.

Significant increases in plasma cholesterol were observed in pigeons given the various drinking water disinfectants. However, a clear dose–response effect was not observed, although pigeons were more responsive when given the 15-ppm dose and the high-cholesterol diet or 2 ppm and the normal diet. In pigeons fed the normal diet, chlorine (pH 8.5) and chlorine dioxide (2 ppm) were both effective in the induction of an increase in plasma cholesterol, whereas in the high cholesterol diet studies, three disinfectants [i.e., 15 ppm chlorine (pH 8.5), chlorine dioxide, and monochloramine] were associated with significant increases in plasma cholesterol. Pigeons fed the two diets and exposed to chlorine (pH 6.5) showed insignificant increases in plasma cholesterol. Since the pH of the solutions containing chlorine, chlorine dioxide, and monochloramine ranged from 7.6 to 8.5, this suggests a relationship between pH, disinfectant dose, and plasma cholesterol. In previous studies we have not observed significant changes in plasma cholesterol in pigeons exposed to deionized water buffered to pHs of 7.0, 7.5, 8.5, and 9.5. This suggests that it is the disinfectant itself rather than the pH of the drinking water that is responsible for the observed plasma cholesterol effect.

The factors associated with the effect of these disinfectants on plasma cholesterol are not known. However, because of the relative reactivity of these disinfectants with organic matter, it would seem unlikely that they would directly affect cholesterol absorption and/or metabolism. Thus, the formation of chloroorganics in the upper gastrointestinal tract as these animals drink the disinfectant water seems a likely factor in explaining the observed change in plasma cholesterol. The formation of a variety of chlorinated products is possible because these disinfectants react with proteins, lipids, nucleic acids, and carbohydrates. In several publications Cunningham has shown that chlorinated lipids and proteins stimulate an increase in heart weight (22). Cunningham has also observed increased heart weight in animals fed chlorinated fatty acids (6). In pigeons treated with chlorinated water, we have observed in past and present studies increased heart weight and plasma cholesterol and a decrease in plasma T4 (23). The association of hypothyroidism with increases in heart weight and plasma cholesterol (24, 25) suggests that both the results of Cunningham and those observed in the present studies may be related to an effect by chloroorganics (i.e., products formed when these disinfectants react with organic matter in the upper GI tract) on thyroid function.

In previous studies we have observed an increase in cholesterol absorption in pigeons exposed to chlorinated drinking water (23). Since plasma cholesterol would be elevated with an increase in absorption, this suggests that the observed effect of these disinfectants on plasma cholesterol may be associated with increased absorption and/or

Table 2. Effect of drinking water disinfectant on pigeon plasma thyroid hormone levels

Treatment	Dose, ppm	Levels in pigeons fed the normal diet, mean ± SD: N	Total T4, μg/dl	Free T4, ng/dl	Total T3, ng/dl	Free T3, pg/dl	Levels in pigeons fed the high-lipid diet, mean ± SD: N	Total T4, μg/dl	Free T4, ng/dl	Total T3, ng/dl	Free T3, pg/dl
Control	—	12	2.54 ±0.14	1.04 ±0.12	123.0 ±9.9	53.0 ±11	19	2.78 ±0.17	0.92 ±0.090	130.4 ±8.5	42.5 ±7.00
Chlorine pH 6.5	2	12	2.58 ±0.29	1.00 ±0.24	118.0 ±15	21.0* ±0.086*	11	2.49 ±0.13	1.34* ±0.18	164* ±12.1	40.5 ±11.1
	15	9	2.26 ±0.16	1.00 ±0.143	152.0 ±14	8.60‡ ±4.6	11	2.35 ±0.13	0.98 ±0.19	182* ±18.0	6.50‡ ±3.1
Chlorine pH 8.5	2	10	1.82† ±0.18	0.83 ±0.079	167.0† ±8.1	11.4† ±4.4	10	2.31* ±0.079	0.75 ±0.08	152 ±15.0	74.0* ±17.0
	15	11	1.80‡ ±0.12	0.60* ±0.096	96* ±7.60	3.43‡ ±1.7	10	1.86‡ ±0.15	0.62* ±0.11	66† ±9.7	5.8† ±7.9
Chlorine dioxide	2	11	1.86† ±0.19	0.86 ±0.15	150.5 ±19.6	77.5 ±21.0	12	2.40 ±0.15	1.05 ±0.13	182† ±11.0	74.0† ±12.0
	15	10	2.05* ±0.12	0.75* ±0.073	102.4 ±8.1	12.5† ±5.1	11	2.02† ±0.20	0.70 ±0.12	96.0* ±9.9	18.6* ±7.9
Chlorite	2	10	2.69 ±0.18	1.06 ±0.089	90.0* ±10.5	20.2* ±7.20	12	2.45 ±0.13	1.21 ±0.16	143.33 ±16.0	50.9 ±11.9
	15	11	2.19 ±0.18	0.90 ±0.068	110 ±10.3	42.9 ±11.1	12	2.78 ±0.14	1.35 ±0.14	140 ±20.8	67.0 ±23.4
Monochloramine	2	10	2.42 ±0.14	1.20 ±0.180	119.1 ±10.2	33.3 ±8.50	12	2.22* ±0.16	0.88 ±0.089	131.33 ±20.0	22.7 ±7.7
	15	12	2.15* ±0.13	0.78 ±0.11	120.2 ±6.4	13.4† ±4.20	12	2.07† ±0.17	0.83 ±0.095	81.9† ±10.8	7.10† ±5.9

N, number of pigeons per experimental group.
*$P < 0.05$.
†$P < 0.01$.
‡$P < 0.001$.

hypothyroidism. Further studies are required to determine if hypothyroidism is associated with increases in the intestinal absorption of endogenous and exogenous cholesterol. Such information would help to explain whether the effect of these disinfectants on plasma cholesterol is associated with increases in cholesterol absorption or thyroid function.

We have observed significant increases in plasma cholesterol and aortic atherosclerosis in pigeons exposed to three commonly used drinking water disinfectants. The atherosclerotic effects observed in these studies are probably related to increases in plasma cholesterol. The observed decrease in plasma T4 appeared to be associated with an increase in plasma cholesterol, which would support human and experimental studies in which hypercholesterolemia is frequently associated with hypothyroidism. However, for reasons not understood, hypercholesterolemia is not always observed in hypothyroid patients or in experimental animals. Results presented above are consistent with this observation in that we observed a consistent decrease in T4 in animals given either diet and 2 and 15 ppm chlorine (pH 8.5), chlorine dioxide, or monochloramine. However, plasma cholesterol in these groups was not consistently higher than the controls. The factor(s) associated with the effects of these disinfectants on plasma T4 and cholesterol is presently not known. We suggest that these effects are probably mediated by products formed when these disinfectants react with organic matter. Confirmation of this suggestion awaits further studies.

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