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Comparative Acute Toxicity of Chlorocitrate and Fluorocitrate in Dogs

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Comparative Acute Toxicity of Chlorocitrate and Fluorocitrate in Dogs. BOSAKOWSKI, T., AND LEVIN, A. A. (1987). *Toxicol. Appl. Pharmacol.* **89**, 97–104. The high-dose effects of chlorocitrate [(−)-*threo*-chlorocitric acid] were compared *in vivo* to another halogenated citrate analog, and a well-known inhibitor of the tricarboxylic acid (TCA) cycle, fluorocitrate. The compounds were given iv to two dogs per sex per group, and a control group received an equimolar amount of citric acid. Chlorocitrate (100 mg/kg) showed TCA cycle inhibition as did fluorocitrate (8 mg/kg) in that both caused depletion of ATP and accumulation of citrate in the liver. Chlorocitrate was a significantly weaker inhibitor of citrate metabolism than fluorocitrate as evidenced by a substantially lower accumulation of serum citrate despite a much higher dose. Both halocitrates produced a similar diabetes-like syndrome (hyperglycemia, glycosuria) mediated by a significant hyperglucagonemia and slight hypoinsulinemia. Chlorocitrate was more potent in this effect and a much greater buildup of plasma lactate ensued (18- versus 3.7-fold increase), enough to explain lethality observed in earlier studies. In contrast, fluorocitrate produced a severe life-threatening hypocalcemia (−30%), and hypercalcuria was observed. This effect on calcium distribution was only minimal with chlorocitrate. Both halocitrates had a similar depressive effect on circulation as evidenced by hypothermia, bradycardia, and elongation of the QT-interval. These changes were considered to be the result of lactic acidosis and the ongoing ion imbalance since heart ATP levels were not depleted.

Chlorocitrate [(−)-*threo*-chlorocitric acid] was recently proposed as a novel anorectic agent (Sullivan *et al.*, 1981) based on its peripheral action of delayed gastric emptying (Triscari and Sullivan, 1981). We investigated the acute toxicology of chlorocitrate by comparing it directly to the effects of a lethal dose of fluorocitrate (Bosakowski and Levin, 1986), another halogenated citrate analog. This comparative approach was deemed necessary to elucidate the mode of action of chlorocitrate toxicity since high-dose chronic treatment with the drug produced dose-related periodic collapse/recovery or lethality with no observable histologic change (Hayes *et al.*, 1983).

Fluorocitrate is a well-known and well-studied inhibitor of citrate metabolism. Peters (1952) and colleagues discovered the lethal *in vivo* synthesis of fluorocitrate from fluoroacetate whereupon fluorocitrate was found to inhibit the tricarboxylic acid (TCA) cycle at the stage where citrate is converted to *cis*-aconitate. This biochemical lesion may be the result of inhibition of the enzyme *cis*-aconitase (Peters, 1952) and/or the inhibition of mitochondrial citrate transport (Kirsten *et al.*, 1978). As a result of this blockade in the TCA cycle, large accumulations of citrate are found in the tissues and blood of intoxicated animals (Buffa and Peters, 1950; Fawaz *et al.*, 1956; Bosakowski and Levin, 1986).

Consistent with the inhibition of the TCA cycle, the production of energy in the form of ATP was arrested in the heart (Bowman, 1964; Bosakowski and Levin, 1986), liver (Buffa *et al.*, 1972), and kidneys (Simonnet *et al.*, 1980) of rats poisoned with fluoroacetate.

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However, it was recently shown that heart ATP was not depleted in dogs that were severely intoxicated with either fluoroacetate or fluorocitrate (Bosakowski and Levin, 1986). In these studies there was a marked hypocalcemia which probably reflects the cause of death in this species. In the present investigation, some of the biochemical changes that prevail during chlorocitrate intoxication are examined and compared to changes that result from an equitoxic dose of fluorocitrate. A control group was given an equimolar amount of citric acid to account for some of the possible effects which may result from the citrate portion of the molecule of both halogenated citrate analogs (halocitrates).

MATERIALS AND METHODS

Animals. Twelve adult beagle dogs (8.6–12.1 kg) from Marshall Research Animals (North Rose, NY) were maintained daily on 400 g of commercial diet (Certified Wayne Lab Dog Diet, 8727-00) moistened with approximately 400 ml of tap water. The dogs were fasted 18 hr prior to the study. Dogs were housed individually in stainless steel cages with free access to distilled water and were maintained on a normal 12-hr light–dark cycle. The dogs were assigned into three treatment groups (two per sex per group) on the basis that no littermates were in the same group.

Dosages. All doses were administered with a 2-min iv infusion and were of equal volume. Chlorocitrate was prepared synthetically in the Chemical Research Division of Hoffmann–LaRoche, Inc. The chlorocitrate was a purified preparation containing only one of four stereoisomers [(−)-*threo*-chlorocitric acid] which was shown to have the most anorectic activity (Sullivan *et al.*, 1981). This compound was dissolved in saline and neutralized to a pH of 5.5 just prior to dosing. The final solution (50 mg/ml) was given at dose level of 100 mg/kg. Preliminary studies showed that this was the maximum dose that would produce toxicity without mortality within 4 hr posttreatment. Barium DL-fluorocitrate (Sigma Chemicals, St. Louis, MO) was prepared for injection by dilution in distilled water followed by precipitation of the barium by addition of excess sulfuric acid and centrifugation. The final solution (4 mg/ml) was neutralized to pH 5.5 and was administered at a dose level of 8 mg/kg. In previous studies (Bosakowski and Levin, 1986), this dose was shown to produce a degree of toxicity similar to that of the dose of chlorocitrate selected. Controls received an amount (92.8 mg/kg, 46.4 mg/ml) of citric acid (Mallinckrodt Chemical Works, New York, NY) equimolar to the chlorocitrate group.

Experimental. Dogs were bled from the jugular vein using sterile Vacutainers (Becton–Dickinson, Rutherford, NJ) prior to dosing and at hourly intervals up to 4 hr. Immediately after the final blood sample, an EKG recording (Burdick Corporation, Milton, WI) and body temperature (Electrotherm TM-99, Electromedics, Englewood, CO) were taken. The dogs were then anesthetized with approximately 15 mg/kg of 2% thiamylal sodium (Bio-Tal, Bio-Ceutic Laboratories, St. Joseph, MO) and respirated mechanically with oxygen (12 breaths per minute, tidal volume 700 cm^3) through an endotracheal tube. After laporotomy, heart and liver tissue samples were taken with a pneumatic biopsy drill and a rapid freezing technique as previously described (Bosakowski and Levin, 1986). Urine was collected via direct aspiration of the urinary bladder and was kept frozen (−20°C) until assayed. All surgical procedures were performed by a licensed veterinarian.

Assays. Blood was collected in serum separator Vacutainers (kept at room temperature) and in EDTA-containing Vacutainers for plasma (kept on ice). An aliquot (0.5 ml) of serum, collected anaerobically by aspirating through the stopper of the Vacutainer, was injected into an Orion SS-20 ionized calcium analyzer (Orion Biomedics, Cambridge, MA) within 1 hr after blood sampling for determination of ionized calcium. The remaining serum was refrigerated until assayed for other components.

Serum citrate was determined using the method of Moellering and Gruber (1966) with a modification for use on the COBAS-BIO automated spectrophotometer (Roche Diagnostic Systems, Nutley, NJ). Total serum calcium was analyzed by atomic absorption spectrophotometry (Perkin–Elmer 460) using lanthanum chloride as a stabilizer. Inorganic phosphorus (phosphates) was determined on the COBAS-BIO using Roche reagents (Roche Diagnostics). Sodium, potassium, and chloride were determined with the Beckman E4A ion-specific electrode (Beckman Instruments, Irvine, CA).

Plasma samples were stored at −80°C until assayed. Plasma glucose and lactate were determined on the COBAS-BIO using Roche reagents and a Sigma kit (Sigma Diagnostics, NJ), respectively. Aliquots of plasma for insulin were stabilized with sodium fluoride (5 mg/ml) and were analyzed by radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA). Aliquots of plasma for glucagon were stabilized with 500 KIU units/ml of Trasylol (Sigma) and were assayed using the kit from Radioassay Systems Laboratories (Carson, CA). Urine was assayed for citrate, calcium, potassium, sodium, chloride, phosphorus, and glucose using the same methods described above. Nitroprusside strips (Ketostix, Miles Laboratories, Elkhart, IN) were used for detection of excessive ketones (acetoacetate) in the urine. Urine creatinine was determined on the COBAS-BIO using Roche reagents. Tissue ATP and citrate were assayed as previously described (Bosakowski and Levin, 1986).

Statistics. All data were subjected to parametric statistical analysis on the RS/1 software system (BBN Soft-

TABLE 1

CHANGES IN HEART RATE, QT-INTERVAL, AND BODY TEMPERATURE 4 hr AFTER INFUSION OF CHLOROCITRATE, FLUOROCITRATE, OR CITRIC ACID

	Heart rate (BPM)	QT-interval (s)	Body temperature (°C)
Chlorocitrate	74*	0.24**	36.6**,†
(100 mg/kg)	(12.4)	(0.018)	(0.85)
Fluorocitrate	86	0.23**	37.8***
(8 mg/kg)	(8.1)	(0.006)	(0.33)
Citric acid	106	0.19	38.9
(92.8 mg/kg)	(4.7)	(0.004)	(0.50)

** $p < 0.01$, *** $p < 0.05$, * $p < 0.10$, compared to citric acid group.

† $p < 0.05$ that chlorocitrate and fluorocitrate are from same population.

Values are means ($N = 4$) with SD in parentheses.

ware Products Corp., Cambridge, MA) using a Student–Newman–Keuls multiple range test (Zar, 1974). Urine data was log-transformed to approximate a normal distribution prior to multiple range testing (Zar, 1974). Heart rate and QT-interval were quantified from EKG recordings by taking the average of the first five peaks that were readily distinguishable.

RESULTS

Chlorocitrate and fluorocitrate caused a significant hypothermia 4 hr after infusion compared to dogs receiving citric acid (Table 1). This hypothermia was associated with a significantly elongated QT-interval and mild bradycardia (not significant at $p < 0.05$) with both halocitrates. One female dog that received chlorocitrate exhibited a frank convulsion just prior to the EKG and 4-hr blood sample. All dogs survived the 4-hr period prior to surgical biopsy.

Chlorocitrate and fluorocitrate produced high levels (six- and eightfold, respectively) of citrate in the liver 4 hr postinfusion relative to citric acid treatment (Fig. 1). Hepatic citrate accumulation was associated with a significant depletion of liver ATP reserves for both halocitrates. In contrast to the liver, the heart was not affected by either halocitrate since citrate and ATP concentrations were not significantly altered.

Hourly blood chemistry levels showed that treatment with both halocitrates resulted in a steady increase in serum citrate up to 4 hr (Fig. 2) although fluorocitrate produced a significantly greater ($p < 0.05$) citrate accumulation than did chlorocitrate at 2, 3, and 4 hr after dosing. The control dogs, infused with citric acid, showed the reverse condition with an early peak (3.5-fold) in serum citrate at 1 hr and a steady decline to pretreatment levels. Plasma glucose levels were significantly increased by treatment with both halocitrates

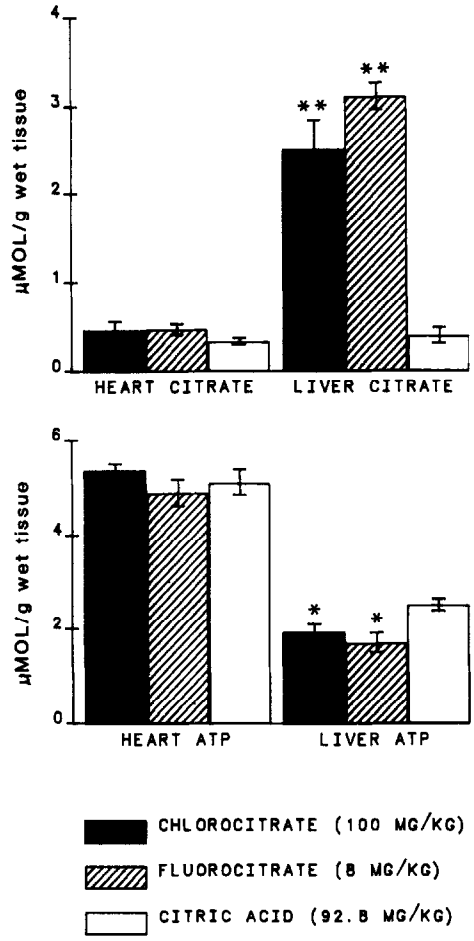

FIG. 1. Citrate and ATP concentrations from heart and liver of dogs 4 hr after infusion with chlorocitrate, fluorocitrate, or citric acid. Values are means ± SEM. ** $p < 0.01$, * $p < 0.05$ (statistically different from citric acid group).

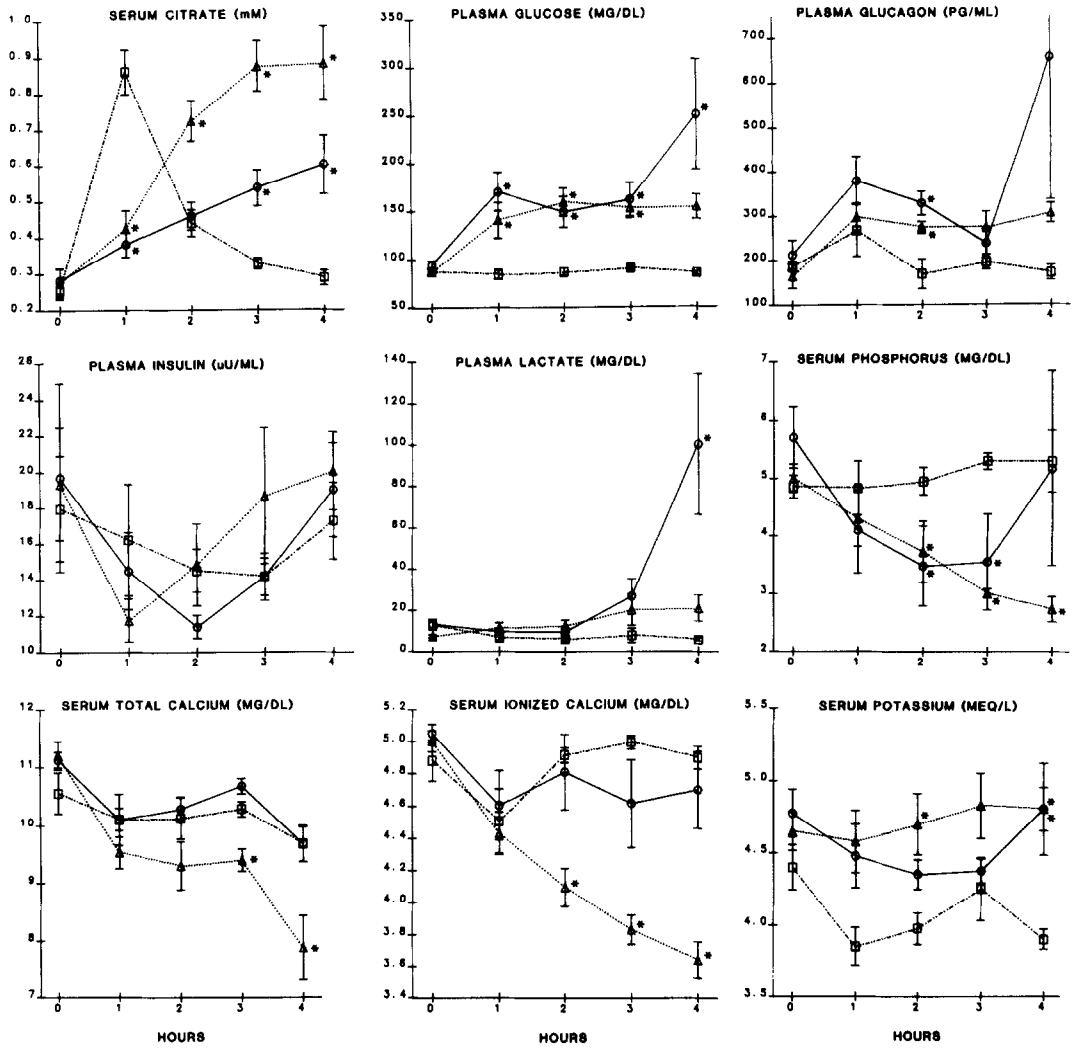

FIG. 2. Clinical chemistry values for dogs up to 4 hr after infusion with chlorocitrate (○, 100 mg/kg), fluorocitrate (△, 8 mg/kg), or citric acid (□, 92.8 mg/kg). Values are means ± SEM. *Indicates statistically different than citric acid group by at least $p < 0.05$.

and correlated with significant (2 hr) rises in plasma glucagon and a slight depression (not significant) in plasma insulin levels. Plasma lactate was significantly increased with chlorocitrate (18-fold) by 4 hr, but the increase observed with fluorocitrate (3.7-fold) was not significant at $p < 0.05$ because of the effects of the large variance from the chlorocitrate group on the Student–Newman–Keuls test. (The fluorocitrate group was significantly different from the control group at 4 hr when tested with a Student t test.) Both halocitrates produced similar losses of serum phosphorus, except at 4 hr where chlorocitrate treatment showed a wide variability. Decreases in serum calcium levels were significant only with fluorocitrate; i.e., serum total calcium was reduced at 3 and 4 hr after dosing and serum-ionized calcium was reduced at 2, 3, and 4 hr. Serum potassium levels remained within normal limits for both halocitrates but was significantly decreased in the citric acid controls. Sodium and chloride serum levels were similar among groups (data not shown).

TABLE 2

MEAN URINE CHEMISTRY VALUES 4 hr AFTER INFUSION WITH CHLOROCITRATE, FLUOROCITRATE, OR CITRIC ACID (RANGE IN PARENTHESES)

	N	Calcium (mg/dl)	Potassium (MEQ/l)	Phosphorus (MEQ/l)	Glucose (mg/dl)	Citrate (mM)	Creatinine (mg/dl)
Chlorocitrate (100 mg/kg)	3	24.9*** (16–41)	133.5 (40–233)	311.6 (126–311)	1077.7* (5–2966)	1.74 (1.5–2.1)	65.6 (42–80)
Fluorocitrate (8 mg/kg)	4	59.6*** (19–96)	110.0 (54–173)	116.9 (31–232)	1176.5*** (10–2108)	1.46 (1.0–2.2)	50.4 (12–83)
Citric acid (92.8 mg/kg)	4	7.6 (1–25)	77.8 (50–95)	229.1 (84–315)	5.0 (4–6)	1.08 (0.3–2.4)	100.1 (84–134)

*** $p < 0.05$, * $p < 0.10$, compared to citric acid group (statistical analysis was performed after base 10 log transformation of the above data).

Analysis of urine 4 hr postinfusion showed significantly higher concentrations of glucose and calcium for dogs treated with both halocitrates (Table 2). Urine citrate and potassium also appeared to be increased but were not statistically significant, possibly because of the small sample size involved. In addition, urine creatinine was slightly decreased with both halocitrates indicating a slight diuresis. If the citrate and potassium values are corrected for creatinine then the magnitude of the increase was even greater than that indicated by the final urine sample concentrations. No obvious pattern for urine levels of phosphorus, chloride, or sodium was observed (data not shown). The nitroprusside reaction for ketones (acetoacetate) was negative for all urine samples.

DISCUSSION

Chlorocitrate was found to have a variety of effects comparable to the classic inhibitor of citrate metabolism, fluorocitrate, yet there were a few marked differences between the toxic reactions to these two halocitrates. Clinically, fluorocitrate poisoning produces emesis, tremors, tetany, and convulsions in dogs (Bosakowski and Levin, 1986), whereas high doses of chlorocitrate produce emesis, ataxia, weakness, tremors, collapse, bradycardia, and infrequent convulsions (Hayes *et al.*, 1983).

Both halocitrates produced a similar elongation of the QT-interval, slight bradycardia, and a significant hypothermia. Roy *et al.* (1980) and Taitelman *et al.* (1983) found that fluoroacetate, a metabolic precursor of fluorocitrate, caused an elongation of the QT-interval in cats which was accompanied by decreases in blood pressure. Despite the cardiac effects reported here and elsewhere, neither chlorocitrate nor fluorocitrate was found to cause an inhibition of the TCA cycle in the heart of dogs, since ATP and citrate levels remained essentially unaffected. However, in the liver, both halocitrates produced a similar degree of TCA cycle inhibition as attested by the large accumulations of citrate and significant depletion of ATP. Similarly, liver ATP depletion has been reported with fluoroacetate (Buffa *et al.*, 1972), and monochloroacetate has been shown to inhibit acetate oxidation *in vitro,* although apparently by different kinetics than fluoroacetate (Hayes *et al.*, 1973).

Consistent with TCA cycle inhibition, citrate accumulation was also observed in the serum of dogs treated with either halocitrate. Serum citrate has previously been shown to be a reliable indicator of toxicity for fluoroacetate and fluorocitrate (Bosakowski and Levin, 1986). Even though the dose of chlo-

rocitrate used in this study was 12.5 times higher than fluorocitrate, serum citrate accumulation was appreciably higher with fluorocitrate. The differences in doses required to produce a given increase in citrate are indicative of the greater potency of fluorocitrate to inhibit citrate oxidation than chlorocitrate. Note also that 8 mg/kg of a racemic mixture of fluorocitrate was administered as opposed to 100 mg/kg of a single stereoisomer of chlorocitric acid.

A similar pattern of hyperglycemia and glycosuria was produced with both halocitrates. Hyperglycemia is one of the cardinal features of poisoning with fluoroacetate (Cole *et al.*, 1955; Engel *et al.*, 1957) and fluorocitrate (Bosakowski and Levin, 1986). Buffa *et al.* (1972) demonstrated that fluoroacetate caused a rapid glycogenolysis in the liver of rats which the authors believed was responsible for the hyperglycemic effect. In this study, both halocitrates significantly increased plasma glucagon levels and slightly decreased insulin which would explain (reviewed by Unger and Orci, 1976, p. 807) the liver glycogenolysis and subsequent hyperglycemia described above. This bihormonal response is characteristic of stress hyperglycemia (Unger and Orci, 1976) which might result indirectly from the failure of the TCA cycle to produce adequate levels of ATP. Bowman (1964) showed that fluoroacetate-induced citrate accumulation had an inhibitory effect on phosphofructokinase activity, and Cole *et al.* (1955) speculated that reduced ATP levels might inhibit hexokinase activity. The present experiment tends to rule out these factors as major causes of hyperglycemia since chlorocitrate produced a significantly greater hyperglycemia but produced a significantly smaller amount of citrate accumulation than fluorocitrate.

During fluoroacetate intoxication, the increased availability of glucose is utilized anaerobically as evidenced by an increased lactate to pyruvate ratio in fluoroacetate poisoning (Taitelman *et al.*, 1983). In the present study, both fluorocitrate and chlorocitrate also caused elevations in plasma lactate, although chlorocitrate was fivefold more potent in this regard—enough to explain lethality observed in previous studies. According to Oliva (1970), lactate levels of 63–72 mg/dl are usually associated with a fatal outcome. In this study, three of four dogs intoxicated with chlorocitrate had lactate levels above 100 mg/dl by 4 hr. In contrast, the highest lactate level observed with fluorocitrate was 39 mg/dl. Excessive lactate production, with consequent acidosis, is commonly associated with circulatory failure and shock (Weisburg, 1974) and indeed, it is interesting to note that Engel *et al.* (1957) described a shock-like condition of fluoroacetate-poisoned rats. Oliva (1970) contends that it is exceedingly difficult to tell whether excess lactic acid is the cause or result of circulatory insufficiency. We speculate that, with chlorocitrate and fluorocitrate intoxication, circulatory depression (and bradycardia) occurred secondarily to hyperlactatemia since heart ATP and citrate levels remained essentially normal. This conclusion is supported by several studies which demonstrated that infusion of lactic acid in dogs caused a marked reduction in ventricular contractile force and bradycardia (Wildenthal *et al.*, 1968; Silberschmid *et al.*, 1966). Although the source of lactic acid was not investigated in our studies, the inhibition of the TCA cycle and subsequent dependence on glycolysis for ATP production would presumably result in increased lactic acid formation in the liver and other affected tissues.

Hypocalcemia has been previously shown to be a significant side effect of fluorocitrate in dogs (Perez and Frindt, 1977; Bosakowski and Levin, 1986) as demonstrated by decreases in total serum calcium. Serum-ionized calcium was reported to be decreased in cats (Roy *et al.*, 1980) and rats (Saitou, 1984) treated with fluoroacetate, although surprisingly, total serum calcium remained normal (Saitou, 1984) or increased (Bosakowski and Levin, 1986) in rats. In the present investigation, fluorocitrate markedly decreased both total (−30%) and ionized calcium (−27%) in dogs while chlorocitrate had only a marginal effect on serum calcium at best. These differ-

ences in calcium distribution correlate with the degree of citrate accumulation in the serum and the degree of hypercalcuria (both substantially higher with fluorocitrate). According to Resnick (1972), tetanic seizures in dogs are associated with a 20% decrease in serum calcium, thereby supporting our hypothesis that hypocalcemia is the mechanism of death in dogs intoxicated with fluorocitrate. Since citrate is a potent chelator of many divalent ions, elevated serum citrate could explain reductions in ionized calcium while greater concentrations of citrate and calcium in the urine should explain decreases in total calcium. Early attempts with calcium rescue therapy had a slight alleviating effect on fluorocitrate-poisoned pigeons (Hastings *et al.*, 1955) and, more recently, Roy *et al.* (1980) were able to double survival time in fluoroacetate-poisoned cats. Nonetheless, calcium therapy was not enough to prevent convulsions and eventual death in either experiment. Hastings *et al.* (1955) postulated that in addition to calcium alterations, there may be a breakdown in the intracellular–extracellular potassium gradient. Although serum potassium appeared to remain within normal limits with chlorocitrate and fluorocitrate intoxication, it was higher than observed in the citric acid controls. Tasker (1980) warns that serum potassium values can be seriously misleading during acidosis as cells tend to take up hydrogen ions and release potassium into the extracellular fluid. Of interest, Chenoweth *et al.*, (1951) found that fluoroacetate-poisoned animals were more susceptible to KCl injections, thus suggesting an ongoing ion imbalance.

Based on the results of these studies demonstrating liver ATP reductions and citrate elevations in liver and serum, it appears that chlorocitrate has similar TCA cycle-inhibiting properties as fluorocitrate but is less potent. The greater potency of fluorocitrate was evidenced by a substantially higher accumulation of citrate in the serum despite a 12.5-fold smaller dose than chlorocitrate. Both halocitrates produce a similar diabetes-like syndrome (hyperglycemia, glycosuria) mediated by a significant hyperglucagonemia and slight hypoinsulinemia. Chlorocitrate is a more potent inducer of hyperglycemia and the greater degree of hyperglycemia appears to be temporally related to the severe life-threatening lactic acidosis that develops (18-fold increase). In contrast, fluorocitrate produces only a 3.7-fold increase in lactate but causes a significantly greater accumulation of citrate than chlorocitrate, with death believed to result from a severe hypocalcemia due to chelation of calcium by citrate. The circulatory depression noted in the later stages of poisoning with both halocitrates was attributed to lactic acidosis and the ongoing ion imbalance since heart ATP levels were not depleted.

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