THE EFFECTS OF DIETARY DDT ON PLASMA LEVELS OF CALCIUM AND MAGNESIUM IN RATS

by

M. ANNE LIEDTKE

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Approved by:

Clifton E. Meloan

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

STATEMENT OF PROBLEM

The effects of dietary DDT (2,2-bis-(p-chlorophenyl)-1,1,1-trichloroethane) on the plasma calcium and magnesium levels in rats were evaluated in an attempt to clarify the mechanism of poisoning. Both the total ion and the ultrafiltrable (free and complexed) ion were measured.

INTRODUCTION

Rationale for the study

Since the discovery in 1939 of the effectiveness of DDT as an insecticide researchers have been attempting to establish how DDT works. Although much more information is available today the question is still unanswered. Some of this information is presented and related to a research project to study the effect of DDT on blood levels of calcium and magnesium.

Mullins (1955) and Gunther, Blinn, Carman, and Metcalf (1954) proposed that DDT possessed insecticidal action because of its structural properties. Using this idea Holan (1969) reasoned that if DDT had the proper steric properties to fit in the nerve membrane-pore channel, then other molecules of similar steric properties should also be effective insecticides. Holan was successful in using the structural theory to synthesize new pesticides.

Evidence for formation of two charge-transfer complexes of DDT with components of cockroach nerve was presented by O'Brien and Matsumura (1964). Matsumura, Bratkowski, and Patil (1969) definitely identified a complex but were unable to establish that it was of the charge-transfer type.

The work reviewed above was all done on insects. Barnola, Camejo, and Villegas (1971) used squid and human material to establish that DDT accumulates in the plasma membranes of nerve fibers. The membrane lipids provide an appropriate medium for the interaction of DDT with membrane proteins. Spectral evidence for a complex between DDT and nerve membrane proteins in the presence of membrane lipids was presented.

DDT alters the observed electrical activity of nerve. Gordon and Welsh (1948) reported repetitive firing and spontaneous activity from DDT-poisoned nerve in the crayfish. This effect could be inhibited by calcium or magnesium ions.

Other workers have found that DDT delays the process turning off the peak transient (sodium) current and inhibits the steady-state (potassium) current in voltage clamped axons. Narahashi (1967) used lobster nerve and Hille (1968) used frog nerve. Hille further reported that a fraction of the sodium channels remained open for a longer time than normal following depolarization. Barnola, et al. (1971) suggested that changes in the sodium current may be due to the effect on potassium. The observation of Matsumura (1966) that cockroach nerve poisoned by DDT has an increased ability to take up sodium or to lose potassium is surely related to the observed electrical characteristics.

Based on a rather large body of evidence, some of which will be presented, Matsumura, et al. (1969) suggested that the nerve enzyme adenosine triphosphatase (ATPase) might actually be the site of DDT attack. This suggestion is based on the close correlation between ATPase inhibition by DDT and nerve symptoms of DDT poisoning.

Two kinds of ATPase are of concern and have been identified (S. Puskin, Berl, E. Puskin and Clarke, 1968). Both kinds are dependent upon the presence of certain ionic species. One requires Na^{+}, K^{+}, and Mg^{++} and is referred to as the Na-K-Mg type. The other requires Ca^{++} or Mg^{++}.

The two types of ATPase have been associated with different functions. The Na-K-Mg type is assigned a governing role in cation transport across the cell membrane (Germain and Proulx, 1965 and Skou, 1965). Kadota, Mori, and Imaizumi (1967) have found the Ca-Mg type in the synaptic vesicle fraction and Germain and Proulx (1965) suggested that it may be involved in the release of acetylcholine.

DDT definitely inhibits ATPase. Matsumura, et al. (1969) found inhibition of the Na-K-Mg type in rat brain. Koch (1969) and Koch, Cutkomp, and Do (1969) reported inhibition of both types of ATPase in cockroach, honey bee and rabbit

nerve. Inhibition of the ATP-P exchange was demonstrated using mosquito by Gonda, Kaluszymer, and Avi-Dor (1959).

Very little work has been done on relating DDT poisoning to ionic changes. Gordon and Welsh (1948) found that the toxic effect of DDT was altered by changes in concentration of calcium, magnesium, and potassium ions in the physiological saline surrounding the nerve of crayfish. In a study of rat muscle tissue, a 35% increase in potassium ions and a 21% decrease in sodium ions was reported in the sarcoplasm by Fudel-Osipova, Rodidnov, and Sokur (1972). Apparently no work has been done on magnesium and all of the calcium (Pujman et al. 1970; Bitman et al. 1969; Peakall 1969 and 1970; and Simpson et al. 1972) has been for total not ionic calcium in birds and no changes were detected.

In a study of human blood plasma, Walser (1961) reported that calcium and magnesium ions occur in several forms. The predominant species are the free ion and the protein-bound ion. Several complexed forms occur, the monohydrogen phosphate and citrate complexes being the only two Walser identified.

It was decided that an investigation of both the ionic and total levels of calcium and magnesium should be undertaken. Using rats as the experimental animal this research sought to establish whether or not a difference occurs and in what portion of the plasma ion. Plasma was chosen over serum and whole blood since calcium would be lost in forming a clot and the concentration of magnesium in red blood cells is three times that found in plasma.

Rationale for the choice of method

Complexation of calcium and magnesium with heparin. The addition of heparin to blood to prevent coagulation has been observed to lower the apparent concentration of ionic calcium. Measuring Ca^{++} with an ion-selective electrode, Moore (1970) found that heparinized whole blood was significantly less than corresponding serum in calcium ion concentration. This effect was apparently

due to the formation of a calcium-heparin complex. Similar findings were reported by Pederson (1970) who was also able to demonstrate that the complex was not ultrafiltrable.

The relative affinities of several cations are reported to be $Na^{+} < K^{+} < Mg^{++} < Sr^{++} < Ba^{++} < Ca^{++}$ (Dunstone, 1962). For calcium solutions less than 400 ppm a maximum of 21 to 22 calcium ions was found to be bound per mole of heparin (Lages and Stivala, 1973).

Because of the implications of this work in using heparinized blood samples, it was decided to first study the complexation of heparin with both calcium and magnesium. Since the complex was not ultrafiltrable it could be removed from the solution and the amount of ion complexed determined by the difference between the ultrafiltrate of a heparin-containing sample and a heparin-free sample.

Sulphate Determination. Heparin is a highly sulphated mucopolysaccharide having alternating units of hexosamine and hexuronic acid (Foster and Huggard, 1955). Of the five sulphate groups per tetrasaccharide, three are sulphate ester groups on oxygen (Wolfrom and Wang, 1967) and two are sulphamino groups (Gibbons and Wolfrom, 1962). Essentially all nitrogen is present as the sulphamino group. The probably repeating tetrassaccharide unit of heparin (Lindahl, 1970) is given in Figure 1.

In a study on the relative affinity of various cations, Dunstone (1962) concluded that the association of cations with acid mucopolysaccharides was due to mainly electrostatic interactions. Although both the sulphate and carboxylic acid groups are available for cation interaction, Dunstone believed the sulphate group gave a stronger interaction. However, Lages and Stivala (1973) believed Ca^{++} bound to carboxylic acid groups first but also to sulphate at high concentrations.

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Figure 1:

Heparin Structure: Repeating Tetrasaccharide Unit

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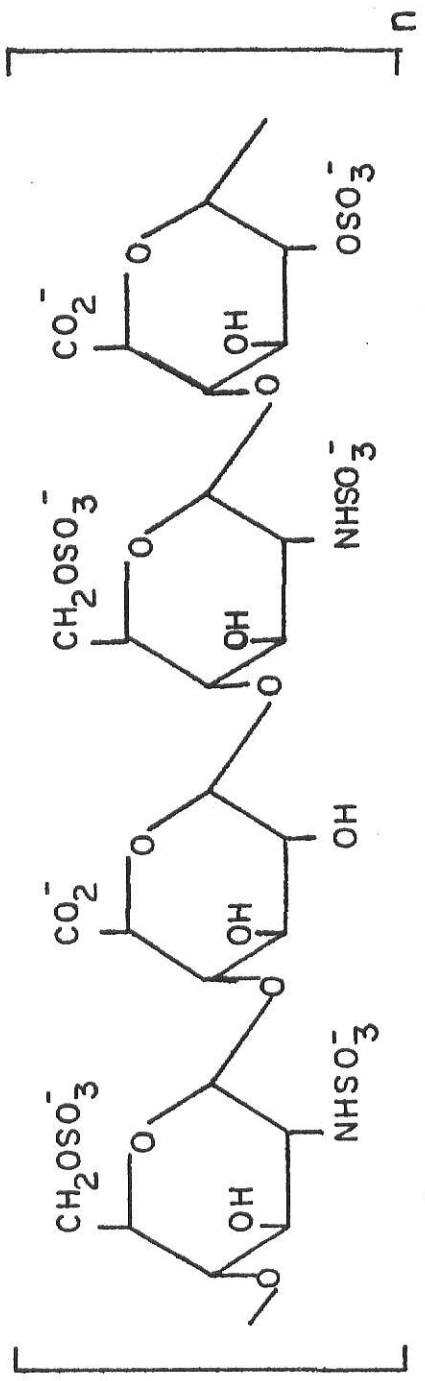

n
CO_2^-
OH
O
OSO_3^-
$CH_2OSO_3^-$
$NHSO_3^-$
OH

The determination of sulphate content was thus believed to be a handle for comparing heparin samples. The method of Dunstone (1962) was used to cleave the sulphate groups by refluxing in acid. The sulphate could then be determined by titration with barium perchlorate once the sample had been freed of potentially interfering ions by passing through an alumina column and then a cation exchange column (Fritz, Yamaura, and Richard, 1957).

Determination of Free Ion Concentration. Calcium and magnesium exist in blood in three different forms: protein-bound, complexed, and the free ion. It has been long recognized that the ionic form was the physiologically active form which has posed certain analytical problems. How does one analyze for only the ionic species?

The two basic approaches which have been used to solve the problem are techniques measuring only the free ion and techniques allowing separation of the free ion prior to analysis. Included in the first are the use of ion selective electrodes (for calcium only) and some spectrophotometric techniques. Separation techniques include ultrafiltration and ion exchange paper.

Ultrafiltration (Farese, Mager, and Blatt, 1970) involves passing the sample through a membrane which passes only low molecular weight materials. The free and complexed ions are thereby separated from the protein bound.

Pederson (1969, 1970) has investigated several variables which affect the amount of ultrafiltrable ion. The amount of ultrafiltrable calcium was decreased by heparin, increased pH, increased temperature, and decreased ionic (sodium and/or potassium) strength. Ionic strength and pH affected magnesium similarly but the temperature effect was variable.

Similar results were obtained by Munday and Mahy (1964) for temperature. These workers also found it necessary to control the carbon dioxide tension at physiological levels since ultrafiltration in air depressed the amount of ultra-

filtrable calcium and magnesium.

Ion exchange strips with aryl sulphonic acid groups were successfully used to remove calcium and magnesium from blood (Frizel, Malleson, and Marks, 1967). Simply by equilibration with whole blood the authors were able to separate out the free ion forms.

Steinberg (1944) investigated the use of an ion exchange resin to prevent blood coagulation by removing calcium. The method was successful and Quick (1947) used to obtain calcium free blood for coagulation studies. It was decided to evaluate the possibility of separating out the free ion form by ion exchange resin.

Atomic Absorption Method. Considerable disagreement was found in the literature on the best method for determining calcium and magnesium in blood. The method of Sunderman and Carroll (1965) was adopted since it eliminates all known or suspected sources of interference. It consisted of removing protein by trichloroacetic acid precipitation and adding strontium chloride to prevent phosphate interference. Standards also contained sodium chloride to correct for its enhancing effect.

ABSTRACT OF RESULTS

The following data was found to support the idea that DDT alters the amount of total calcium and magnesium without affecting the ultrafiltrable amount of either ion. Total calcium dropped after initial feeding but rose to a higher than normal level after feeding two or more days. Time was not a factor for total magnesium but rats fed at the 0.075% level did not differ from controls while rats fed at the 0.125% level showed raised magnesium levels.

EXPERIMENTAL

Chemicals

DDT. The DDT used was obtained from Montrose Chemical Corporation of California. The analysis of the 100% technical grade powder is given in Table 1, and the gas chromatographic analysis is found in Figure 2, with the calculations and operating parameters in Table 2. Technical grade DDT was selected since it is the form used in commercial applications.

Heparin. Isotonic saline solutions of heparin, 1000 U.S.P. units/ml, were used as anticoagulants and for the determination of complex formation. Although not affecting anticoagulant characteristics, different lots of heparin solution were found to vary greatly in the extent of complex formation with calcium. The solution of choice was Sodium Heparin Injection, U.S.P. Aqueous (Lipo-Hepin) by Riker Laboratories, Inc. of Northridge, CA, lot number 33726, since it complexed insignificant amounts of calcium and magnesium.

Human blood plasma. Twenty milliliters of human venous blood was drawn and 0.5 ml of heparin solution added to prevent coagulation. The blood was centrifuged and the plasma isolated for use in the separation studies.

Ion exchange resins. For the sulphate determination a sulphonic acid resin (Rexyn 101 (H) by Fisher Scientific) was used in the hydrogen form.

Three resins were tested for separating protein-bound from complexed and free ions. One was a sulphonic acid resin, Amberlite IR-120 by Fisher Scientific. Two resins were of the carboxylic acid type, Rexyn 102 (H) (Fisher Scientific) and Bio-Rex 70 (Bio-Rad Laboratories of Richmond, CA). The resins were tried in the hydrogen, sodium and ammonium forms.

Chemicals. Reagent grade chemicals were used unless otherwise specified. Those used for sample preparation had to be tested for calcium and magnesium levels. Wherever possible new reagents were employed since they showed lower

Table 1:

DDT Analysis

Setting point	93.0°
Organic chlorine	49.8%
Hydrolysable chlorine	9.8%
p,p'-DDT isomer	76.7%
M.P. of separated isomer	pass
Chloral hydrate	standard
Acidity	nil
Acetone insolubles	0.03%
Water content	0.02%

Note: Analyzed by W. A. Carey of Montrose Chemical Corporation according to procedures detailed by WHO in "Specifications for Pesticides".

Figure 2:

Gas Chromatogram of DDT

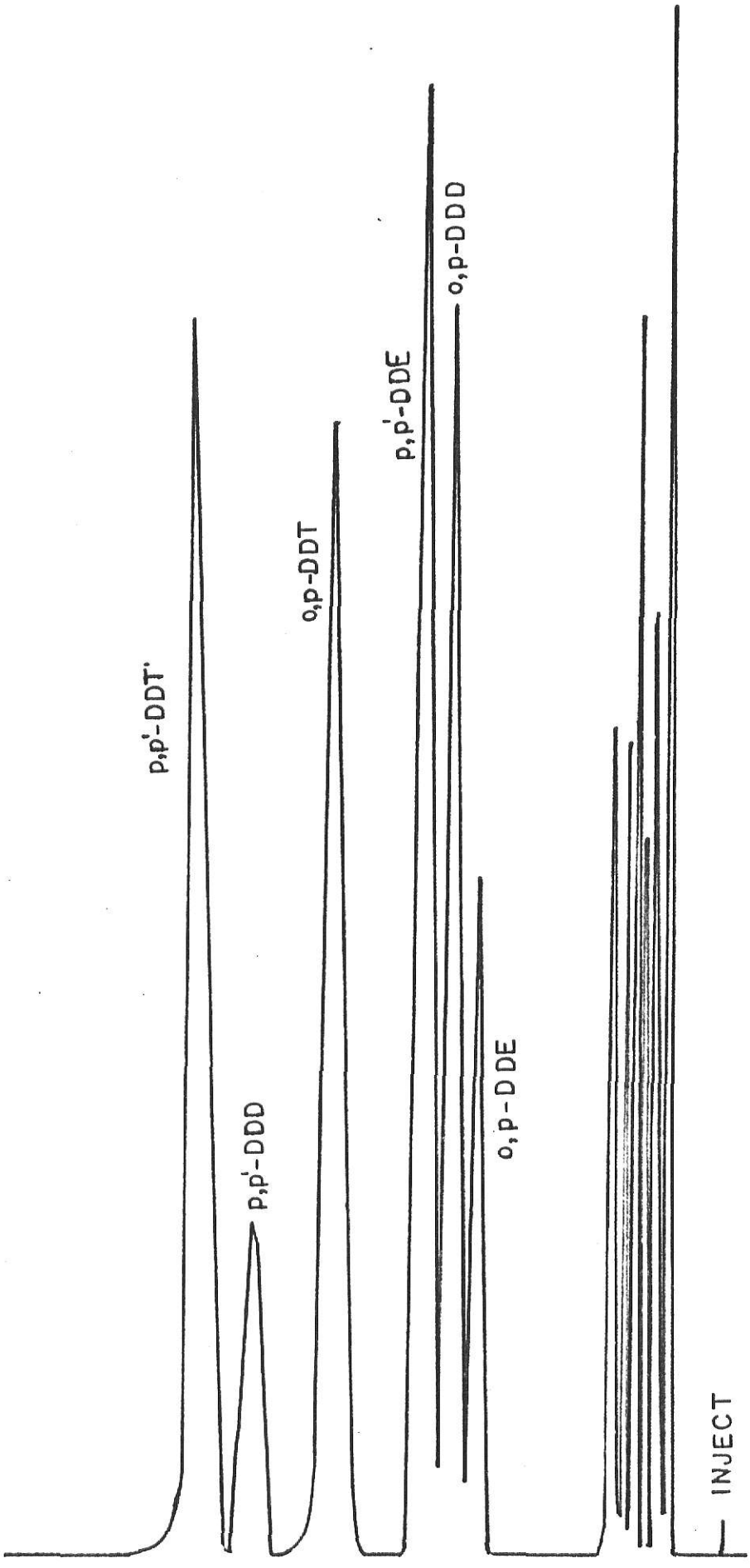

p,p'-DDT'
p,p'-DDD
o,p-DDT
p,p'-DDE
o,p-DDD
o,p-DDE
INJECT

Table 2:

Gas Chromatographic Parameters and Calculations for DDT Analysis

Operating Parameters

Instrument: Varian 600 D with flame ionization detector

Column: 3% QF-1 on Varaport 30 (100/120 mesh), 10 ft. x 2 m.m.

Carrier Gas: Helium, 40 ml/min

Flow Rates: hydrogen 40, air 300 (60 psig)

Temperatures: column 210°C, injector 215°C

Isomer Calculations

isomer	ht		wt½		attenuation		relative area	%
p,p'-DDT	38.2	x	0.65	x	(32x1)	=	794.6	76.2
p,p'-DDD	10.3	x	0.75	x	(1x1)	=	7.7	0.7
o,p'-DDT	35.1	x	0.65	x	(8x1)	=	182.5	17.5
p,p'-DDE	45.2	x	0.50	x	(16x0.1)	=	36.3	3.5
o,p'-DDD	38.6	x	0.50	x	(2x0.1)	=	3.9	0.3
o,p'-DDE	21.0	x	0.40	x	(2x0.1)	=	1.7	0.2
UNKNOWN	COMPOSITE AREA					=	17.5	1.6

backgrounds.

Considerable amounts of calcium and magnesium were found in concentrated hydrochloric acid. Efforts at cleaning up the acid by distillation actually increased the magnesium level. A new bottle of acid was found to be free of both calcium and magnesium and, after transfer to a polyethylene bottle, was used for all subsequent work.

Solutions. All solutions were prepared with *distilled, deionized* water and stored in polyethylene containers. To minimize introduction of impurities all glassware used in solution preparation was rinsed with deionized water.

a. Stock solutions

NaCl, 70 meq. Diluted 4.09g NaCl (U.S.P. grade) to one liter.

Ca, 200 ppm. Diluted 1.46736g $CaCl_2 \cdot 2H_2O$ to two liters.

Mg, 100 ppm. Diluted 1.67224g $MgCl_2 \cdot 6H_2O$ to two liters.

b. Sulphate determination

Barium perchlorate, 0.01 M. Barium perchlorate trihydrate, 3.9 grams, was dissolved in 200 ml of water and 800 ml methanol. The solution was standardized against 0.005 M sulphuric acid.

Sulphuric acid, 0.005 M. Prepared by dilution of concentrated sulphuric acid and standardized against 0.02 N sodium hydroxide.

Sodium hydroxide, 0.02 N. The supernatant from a 1:1 sodium hydroxide solution was diluted and standardized with primary standard potassium hydrogen phthalate.

Thorin I. A 0.2% solution of Thorin I [2(2-hydroxy-3,6-disulfo-1-naphthylazo) benzenearsonic acid] in water was the indicator for the barium perchlorate titration.

Ammonium hydroxide, 1 M and 0.1 M. Concentrated ammonium hydroxide was diluted to prepare both concentrations.

Hydrochloric acid, 2 M. Prepared by dilution of concentrated acid.

c. Heparin complexation

Calcium, 100 ppm. In a 100 ml volumetric was placed 10 ml 70 meq NaCl and 50 ml 200 ppm Ca. After diluting to volume the pH was adjusted to 7.40 ± 0.05 with sodium hydroxide.

Magnesium, 25 ppm. Ten milliliters of 70 meq NaCl and 25 ml of 100 ppm Mg were diluted to 100 ml. The pH was adjusted to 7.40 ± 0.05 with sodium hydroxide.

d. Ion exchange separation

Mock Serum. Into a 2 l volumetric were placed 16.38g NaCl, 0.5044g $MgCl_2 \cdot 6H_2O$, and 0.7400g $CaCl_2 \cdot 2H_2O$. When diluted to volume this approximated the physiological concentrations of sodium, magnesium, and calcium.

EDTA, 2%, pH7. Two grams of the all-hydrogen form of EDTA were diluted to 100 ml and the pH adjusted to 7 with ammonium hydroxide.

e. Atomic Absorption Standards

Strontium chloride. Concentrated hydrochloric acid, 17.5 ml, and strontium chloride hexahydrate, 15.2g, were diluted to one liter with deionized water.

Trichloroacetic acid, 10% and 22.5% (w/v).

Working standard solutions for heparin complexation and ultrafiltration.

Calcium: In a 1 l volumetrics, 100 ml of 70 meq NaCl and 0, 2.5, 5, 7.5, 10, 15, 20, 25, 30, 35, 40 or 45 ml of 200 ppm Ca were added to obtain solutions corresponding to 0, 20, 30, 40, 60, 80, 100, 120, 140, 160, and 180 ppm in original solution.

Magnesium: Ten milliliters of 70 meq NaCl and 0, 0.5, 1, 1.5, 2, 2.5, 3.0, or 3.5 ml of 100 ppm Mg were mixed in one liter volumetrics. After

dilution these gave standards of 0, 10, 20, 30, 40, 50, 60 and 70 ppm in original solution.

Working standards for total ion determination. Prepared as for ultrafiltration with the addition of strontium chloride solution (Ca: 500 ml, Mg: 50 ml) and 22.5% trichloroacetic acid solution (Ca: 200 ml, Mg: 20 ml).

Equipment.

a. Atomic Absorption Spectrophotometer. A modified Perkin-Elmer model number 290 atomic absorption spectrophotometer was used for all calcium and magnesium analyses. Modifications made were tipping the instrument, inserting flowmeters, and changing the burner head.

The back of the instrument was raised on wooden blocks so that it was 3.0 cm higher than the front, giving an eight per cent rise. The manufacturer indicated that this would increase sensitivity by promoting drainage of large water droplets from the nebulizer.

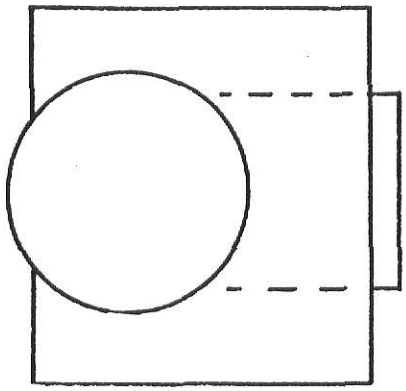
Flowmeters reading from 1 to 36 1/min were inserted into the air and acetylene lines to obtain more reproducible flows. The manufacturer was Roger Gilmont Instruments, Inc. of Great Neck, NY, catalog number F 1400, size number 4. The air flowmeter was fitted with a shield, catalog number F-1465-A, size number 4, as a safety measure due to the large pressure drop across the flowmeter.

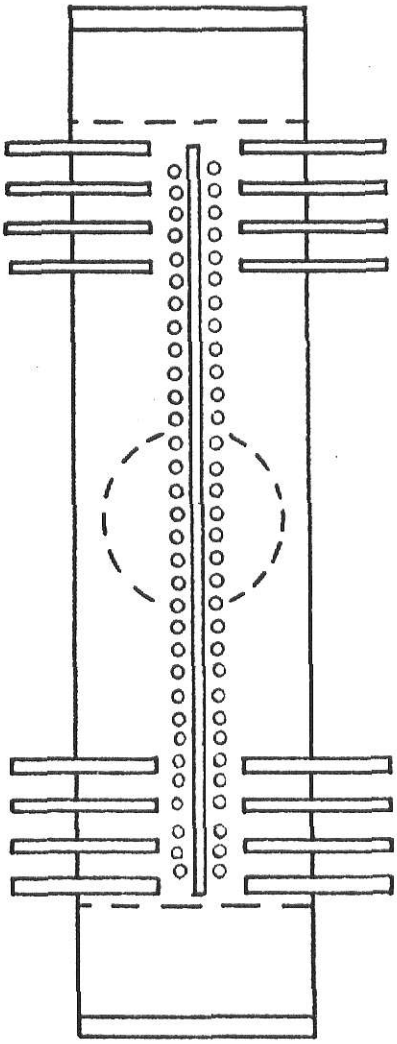
The burner head was a Wang burner (Mid-Continent Scientific of Prairie Village, KS) having a center slot 10 cm by 0.08 cm. Along either side of the slot were 32 holes 0.08 cm in diameter and 0.08 cm from the slot. Four aluminum cooling fins were located at each end of the burner. (See Figure 3.)

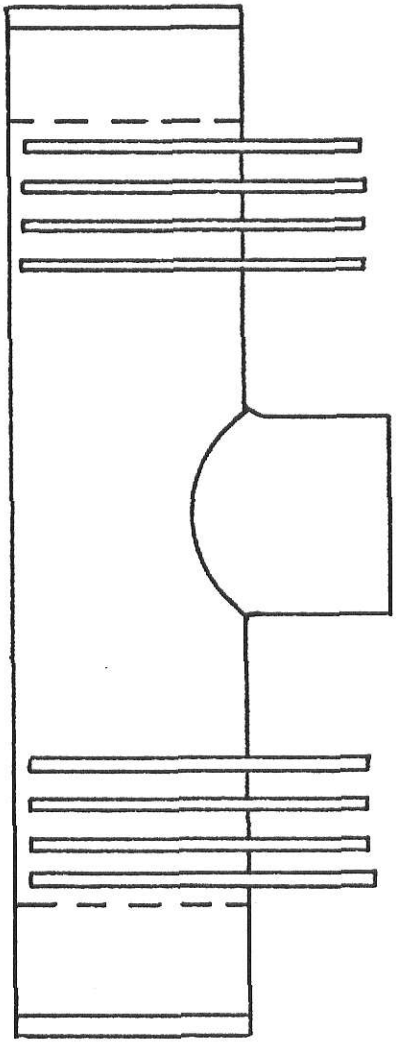
b. Membrane Ultrafilters. Centriflo membrane ultrafilters, Amicon Corp. of Lexington, MA, were used to separate protein-bound ion from the free and complexed forms. The ultrafilter has a molecular weight cut off at 50,000 MW

Figure 3:

Wang Burner Head







with essentially no retention below that. Shaped into a cone, the ultrafilter was used in a conical support fitted into a centrifuge tube (see Figure 4). Both support and tube were obtained from Amicon Corporation.

c. Fraction Collector. For the ion exchange separation studies a Golden Retriever Pup Fraction Collector, model 1100, Instrumentation Specialties Company of Lincoln, NE, was used to collect eluent fractions. Used in the drop counting mode the required fractions were collected in 13 mm by 100 mm test tubes.

d. Centrifuges. Three table top centrifuges were used for the project. An Adams Sero-Fuge (Clay-Adams, Inc. of New York, NY) was used for isolating plasma from the blood cell mass. For spinning down the precipitated plasma proteins a Fisher Scientific Safety Centrifuge was used. The centrifuge used for ultrafiltration was a Senior Centrifuge by Clay-Adams, Inc.

e. Vortex mixer. A Vortex Jr. Mixer by Scientific Industries of Queens Village, NY, was used to obtain uniform mixing in the total ion determinations.

f. pH Meter. To obtain solutions of physiological pH a Beckman Expanded Scale pH Meter, model 76, was employed.

Animals.

a. Rats. Twenty-four of the rats used were hooded females (Long-Evans) and two were albinos (Sprague-Dawley). One of the albinos was male. The rats were ten to twelve months old and averaged 259g (standard deviation= 28g). The females were paired and housed in wire cages; the male was housed singly in a metal cage.

b. Feed. The feed contents and preparation are summarized in Table 3. A typical proximate analysis is shown in Table 4. Feed not containing DDT was pelleted.

To add the DDT, a ten gram sample of DDT was thoroughly mixed with 100 g

Figure 4:

Membrane Ultrafilter, Support and Centrifuge Tube

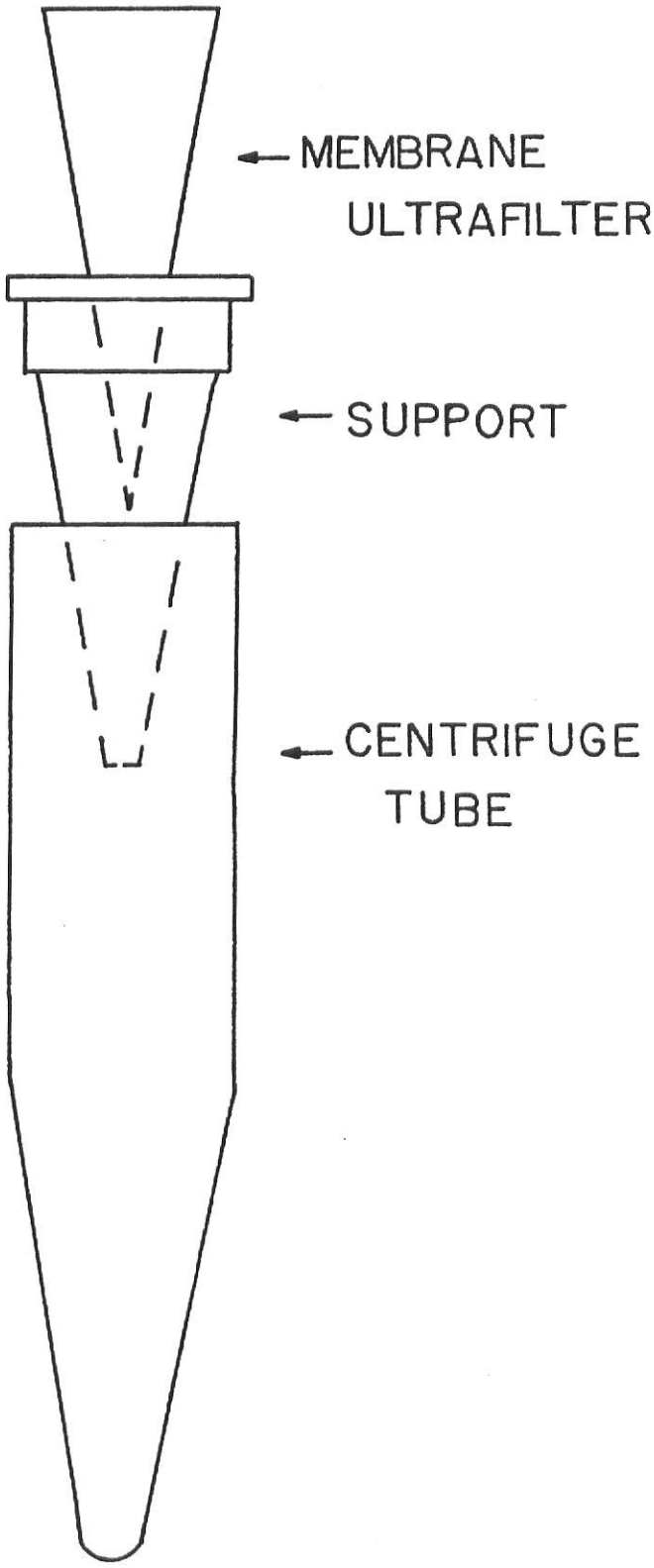

MEMBRANE
ULTRAFILTER
SUPPORT
CENTRIFUGE
TUBE

Table 3:

Food Mixture

Bulk Mixture	
Soybean meal (44%)	180 lb
Ground yellow corn	200 lb
Alfalfa meal (17%)	400 lb
Wheat midds	120 lb
Corn oil	20 lb
Wheat germ oil	2 lb
Premix A	
Dicalcium phosphate	20 lb
Sodium chloride	10 lb
Brewer's yeast	20 lb
Stapel	20 lb
Premix B	
Vitamin D_3 (15,000 IU/gm)	90 g
Vitamin B_{12} (Proferm 20)	20 g
Aurofac-10	1362 g
Trace minerals (Z-5)	227 g
Ground corn	2841 g

Mix each part separately. Combine and mix Premixes A and B. Add to bulk mixture and mix thoroughly.

Table 4:

Proximate Analysis of Food Mixture

Moisture	9.2%
Protein	17.5%
Fiber	13.5%
Fat	4.1%
Ash	8.3%

soybean oil (solvent) and 100g peanut butter (appetizer). This was mixed with 500g meal and then sufficient meal was added to bring the total weight to one kilogram. Three one kilogram batches of meal were added and thoroughly mixed after each addition. The resultant mixture was 0.25% DDT and was diluted further with meal to obtain the desired concentration.

Food containing DDT was placed in stationary pans in amounts of 35-40 g/day/rat. The rats averaged an intake of approximately 25 g/day/rat.

c. Level of DDT. Two levels of DDT were chosen, 0.075% and 0.125%. At the 0.075% level, rats showed few external symptoms and no deaths occurred even when feeding for as long as 56 days. The 0.125% level was lethal to two rats at five and six days but several lived for two weeks. Clinical symptoms of mild tremors were observed and prior to death tetany was seen.

Feeding at the 0.2% level was tested on eight rats. Mild tremors occurred on the second or third day and tetany followed a day later. One animal lived fourteen days, the rest were dead on or before the seventh day.

d. Blood sampling. All animals were etherized prior to obtaining a sample. Those expected to survive had a two milliliter sample removed from the heart with a heparinized syringe. If a sample was not readily obtainable the animal was bypassed to reduce the possibility of death from internal bleeding. Less difficulty occurred when the sample was taken through the side rather than alongside the sternum.

Animals to be sacrificed for maximum amount of sample were bled from the posterior vena cava and/or the heart, the choice being one of convenience. Heparinized syringes were again employed.

Procedure.

a. Sulphate content of heparin. The sulphate groups were cleaved from the heparin by refluxing 0.5 ml heparin solution in 25 ml 2N hydrochloric acid

for four hours. An alumina column was prepared by making an alumina-water slurry and packing a 1 cm diameter column to a height of 6 cm. The alumina was washed with 50 ml 1 M ammonia, several 5- ml portions of 0.1 M ammonia and 50 ml water. Ten milliliters of 2N hydrochloric acid were passed through the column which was then ready to receive the refluxed sample. The sample was followed by 10 ml 1:20 hydrochloric acid and 5-5 ml portions of water. The eluent was collected while passing through 5 ml 1 M ammonia, 20 ml 0.1 M ammonia, 4-5 ml portions of 0.1 M ammonia and 25 ml water.

The alumina column eluent was passed through an ion exchange column 3 inches high in a 1 inch diameter column. The sample was followed by sufficient water to fill a 100 ml volumetric flask.

A ten milliliter aliquot of the ion exchange eluent was diluted with 40 ml methanol and titrated with barium perchlorate. Thorin I was the indicator.

b. Heparin complexation. One half milliliter of heparin solution and 4.5 ml of complexation solution (either calcium or magnesium) were combined in a 50 ml plastic beaker. A teflon coated stir bar was used to obtain good mixing. Since no difference was found between stirring for 10 minutes or 1 hour, the shorter time was preferred.

Membrane ultrafilters were centrifuged for 30 minutes to remove excess water. The sample was then placed in the filter and centrifuged 30 minutes. The ultrafiltrate was diluted 1 in 20 for calcium and one in 200 for magnesium. See above for atomic absorption procedure.

c. Ion exchange separation. Twenty-five milliliters of ion exchange resin suspended in water were placed in a 2 cm diameter column. The resin was Bio-Rex 70, a carboxylic acid type, 20-50 mesh. Supplied in the sodium form, the resin was converted to the ammonium form with ammonium chloride.

A one milliliter sample was placed on the column and washed through with

three ten milliliter portions of deionized water. EDTA was most effective in stripping calcium and magnesium off the column. Twenty milliliters of 2%, pH7 EDTA, five milliliters water, and 5 milliliters of 2%, pH7 EDTA were passed through the column. Three 10 ml portions of water washed the EDTA through the column.

Fractions were collected in 13x100 mm test tubes using the fraction collector to get equal volumes. These were analyzed for sodium, calcium, and magnesium to locate the appearance of sample in the eluent.

To recover the sample, the first forty milliliters of eluent were discarded and the next fifty collected in a 50 ml volumetric flask. Although a small portion of the desired ions remained behind, this procedure gave the optimum concentration for calcium determination.

d. Ultrafiltration of plasma. A 0.5 ml plasma sample was diluted to 10 ml, divided into two portions and placed in a membrane ultrafilter which had been spun dry for twenty minutes. Most samples required one hour for ultrafiltration, a few required longer. The ultrafiltrate was then ready for calcium analysis but required a 1 in 10 dilution for magnesium analysis.

e. Total ion determination. One half milliliter of plasma was pipetted into 4.5 ml 10% trichloroacetic acid (the reverse order does not work well). After mixing with a vortex mixer the sample was allowed to stand for ten minutes. The precipitated protein was centrifuged out with a 20 minute centrifugation time.

Dilution of 2 ml supernatant with 2 ml strontium chloride solution prepared the sample for calcium analysis. A further dilution of 1 in 10 was required for magnesium analysis.

f. Atomic absorption analysis. The flame was lit at an acetylene flow of 1.5 1/min and an air flow of 7 1/min. Once a stable flame was obtained the

air was turned down to 6 1/min to increase the calcium sensitivity. The hollow cathode lamp was operated at 8 mA. Calcium was analyzed at its resonance line, 422.7 nm, and magnesium at its resonance line, 285.2 nm. For solutions not containing strontium it was possible to make a quasi-quantitative sodium analysis from flame colour.

No change in standards was found as a function of time. Due to the sensitivity of the analysis it was found that traces of ion left by incomplete washing of glassware greatly altered the results. It was found necessary to thoroughly wash all containers and rinse several times with deionized water before use.

Calculations.

a. Least squares fit of standard curves. The slope and intercept of all standard curves were calculated by computer. Points more than two standard deviations away were discarded and the slope and intercept recalculated. The resulting slope and intercept were used to calculate the ppm in the sample.

b. Means and standard deviations. Groups of data were averaged and the standard deviation calculated by computer. Any data point more than two standard deviations from the mean was discarded. The recalculated mean and standard deviation are reported and the number of discarded data points is given in parentheses following the number of retained data points.

c. Statistical significance. The t-test for two independent means was used for determining statistical significance. The formula for calculating t is

$$t = \frac{\left|\bar{X}_1 - \bar{X}_2\right|}{\sqrt{\frac{n_1 s_1^2 + n_2 s_2^2}{n_1 + n_2 - 2}\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

where $\bar{X}$ is the mean, n the number of samples, and s the standard deviation.

For the data

$\bar{X}_1 = 113$, $n_1 = 15$, $s_1 = 8.6$

$\bar{X}_2 = 107$, $n_2 = 7$, $s_2 = 4.0$,

t would be calculated to be

$$t = \frac{|113-107|}{\sqrt{\frac{15(8.6)^2 + 7(4.0)^2}{15 + 7-2}\left(\frac{1}{15} + \frac{1}{7}\right)}}$$

$$= 1.67.$$

For $df = n_1 + n_2 - 2 = 20$, this is significant at the 0.05 level, reported as $p < 0.05$.

RESULTS AND DISCUSSION

Sulphate content

Four heparin samples were analyzed and found to have an average sulphate content of 1.79×10^{-8} moles of sulphate per unit of heparin (see Table 5). The standard deviation of 0.28×10^{-8} probably represents no more than the limitations of the technique. Therefore it was concluded that there was no difference in sulphate content of heparin samples 1, 2, and 3 which complexed significant amounts of calcium and sample 4 which did not. Since sulphate is considered one of the probably binding sites (Dunstone, 1962) the lack of difference in sulphate content failed to clarify the reason for differences in extent of complex formation.

Heparin complexation

The amount of calcium removed by 100 units heparin/ml is shown in Table 6 for the different heparin samples used. Six magnesium determinations were made with heparin sample 3 giving a loss of 1.5 ppm magnesium.

Heparin blood levels of 1 unit/ml are reported to make blood incoaguable (Physicians' Desk Reference, 1972). It was reasoned that even at levels of 10 units/ml less than half a part per million calcium or magnesium would be lost due to heparin sample 3. This was far less than the variation in ion level anticipated from the limitations of the method. For all blood samples subsequently drawn heparin sample 3 was used and no correction for complexation was needed.

Separation of Ion Species

Ion Selective Electrode. The use of a calcium ion selective electrode and a divalent selective electrode were considered. It was believed that the free calcium ion concentration could be obtained directly and the free magnesium ion concentration from the difference between divalent and calcium. However, the manufacturer indicated that this would not be a suitable method and

Table 5:

Sulfate Content of Heparin

Sample	$SO_4^=$ Molarity	moles SO_4/ unit heparin
1	7.73×10^{-4}	1.55×10^{-8}
2	10.1×10^{-4}	2.02×10^{-8}
3	10.2×10^{-4}	2.04×10^{-8}
AVG	9.34×10^{-4}	1.87×10^{-8}
s	1.40×10^{-4}	0.28×10^{-8}

Table 6:

Calcium Complexed by 100 units/ml Heparin

Heparin Sample	# Determinations	PPM Ca Lost
1	9	18.5
2	3	12.7
3	6	2.7

the idea was abandoned.

Ion Exchange Resin. Three ion exchange resins were evaluated for use in separating the ionic forms of calcium and magnesium in blood plasma. A sulphonic acid resin was rejected since it retained the ions so well that removal required excessive amounts of eluting agent. The hydrogen form of the resin collapsed the bead to an extent that made exchange with the relatively large calcium and magnesium ions too difficult. Therefore, either the sodium or ammonium form of a carboxylic acid resin was preferred.

Removal of calcium and magnesium by simple displacement with sodium or ammonium ions was fairly difficult. The use of EDTA as a complexing agent to strip off the desired ions was extremely effective. The pH of the EDTA was found to be an important factor with best results obtained at pH7 and satisfactory results obtainable at pH 9.5. Using two portions with a small portion of water in between decreased the amount of tailing seen with a single portion of EDTA.

Three milliliter fractions were collected and analyzed for the presence of sodium and the amount of calcium and magnesium. Typical results of a one ml mock serum sample are shown in Figure 5.

It was believed that protein-bound ion would appear as soon as the column volume had been displaced. However, a one milliliter sample of human blood plasma gave essentially the same result as mock serum (Figure 6). Fractions four through seven had the faint brown colour of the plasma sample but contained no detectable sodium, calcium, or magnesium. It was concluded that the resin could effectively compete with the protein for calcium and magnesium.

Recovery studies on different sizes of mock serum samples are summarized in Table 7. It can be seen that the recoveries tended to be on the low side which was due to failure to collect all of the sample tail.

Figure 5:

Fraction Study of 1 ml Mock Serum

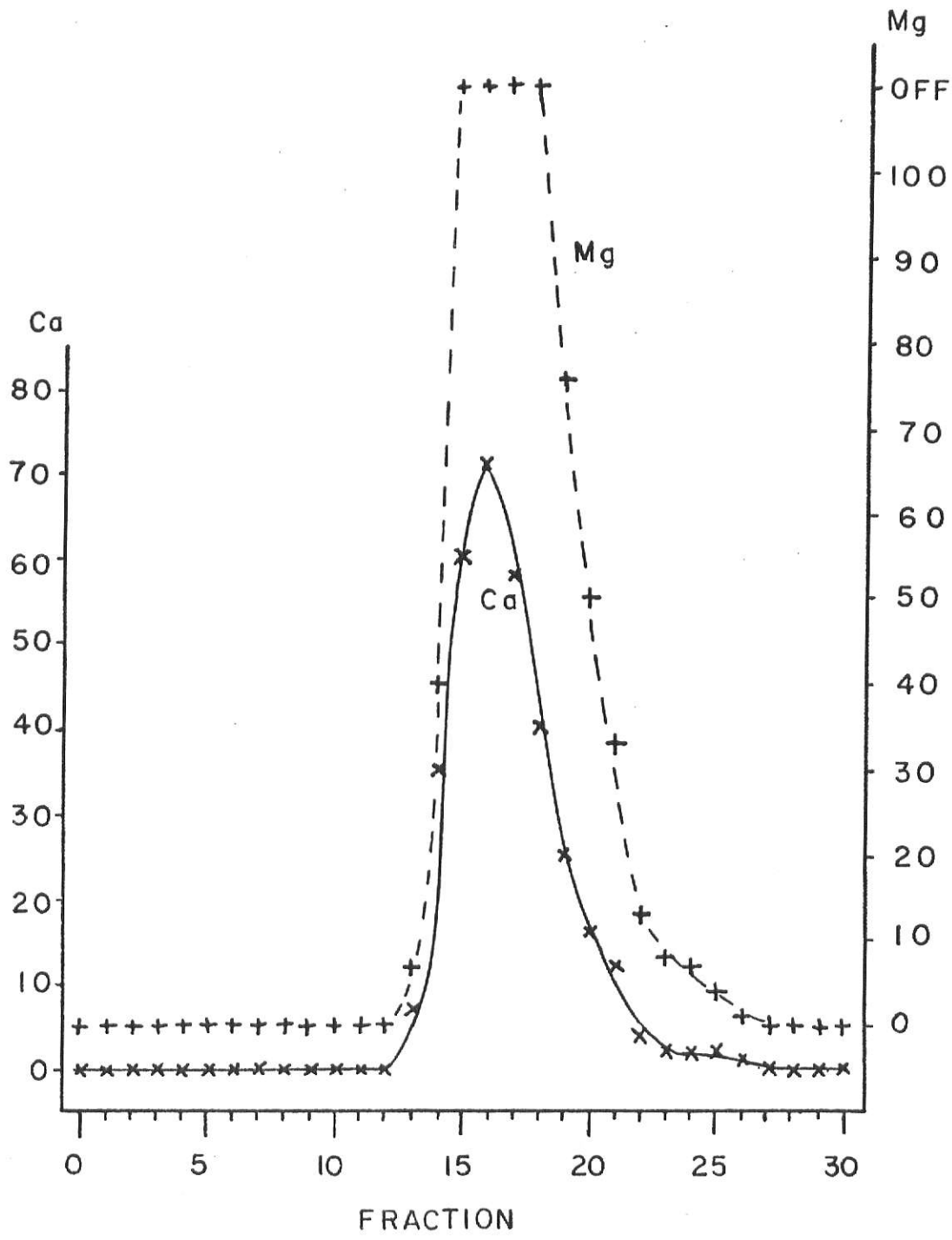

Mg
OFF
100
90
80
70
60
50
40
30
20
10
0
Mg
Ca
80
70
60
50
40
30
20
10
0
Ca
0
5
10
15
20
25
30
FRACTION

Figure 6:

Fraction Study of 1 ml Human Plasma

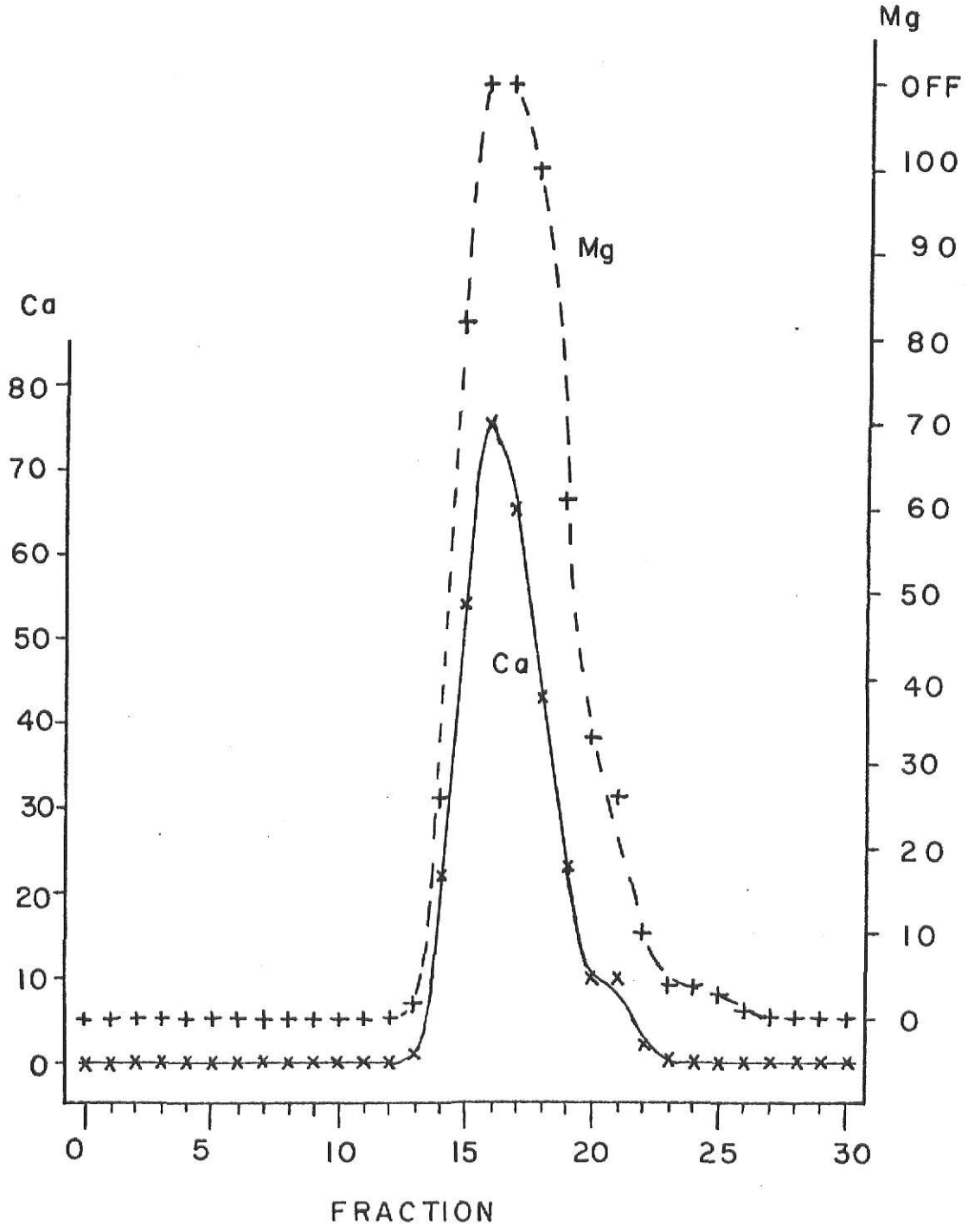

Mg
OFF
100
90
80
70
60
50
40
30
20
10
0
Mg
Ca
80
70
60
50
40
30
20
10
0
Ca
0
5
10
15
20
25
30
FRACTION

Table 7:

Column Recovery of Calcium and Magnesium

		1.0 ml MS Control	0.5 ml MS	1.0 ml MS	2.0 ml MS
Ca	mg	0.106	0.054	0.100	0.209
	%		102%	94%	99%
	n	4	3	6	3
Mg	mg	0.023	0.010	0.023	0.043
	%		87%	100%	93%
	n	4	3	6	3

Ultrafiltration. Before ultrafiltration was used for the rat studies it was tested on mock serum and human blood plasma. Two mock serum samples gave 102% recovery of calcium and 120% recovery for magnesium.

Data from the four human plasma samples are presented in Table 8. When compared to the literature values, the values obtained correspond most closely with the free ion rather than the non-protein bound.

The ultrafiltrate should contain all of the non-protein bound material since the membrane has a molecular weight cut off around 50,000. The explanation for failure to measure the complexed forms was believed to be interferences, primarily from phosphate. Strontium chloride was added prior to ultrafiltration but then values approximating the total ion concentration were obtained. The discrepancy was therefore allowed to remain.

Rat blood plasma studies

Ultrafiltrable Ion. No statistically significant differences were found for ultrafiltrable calcium or magnesium. The results obtained are summarized in Tables 9 and 10. Acute refers to animals fed 0.125% for one day only and 0.075% and 0.125% refer to animals fed at that level of DDT for two or more days. Animals in the acute group invariably showed severe tremors while animals in the other groups showed a range of symptoms, including none.

Total Calcium. Both length of time fed DDT and the level of DDT fed were important variables in the total calcium level. Table 11 gives the amount of calcium measured as a function of group. The groups are compared in Table 12, and all differences are seen to be statistically significant.

Animals fed 0.125% DDT showed a drop in total calcium after one day but after two or more days on DDT the total calcium was higher than controls. The highest level of total calcium was seen in the group fed 0.075% for two or more days. No animals on the 0.075% level were run after only one day's exposure to

Table 8:

Ultrafiltration of Human Blood Plasma

Sample	PPM Ca	PPM Mg
1	49	12
2	43	11
3	50	12
4	43	10
AVG	46	11
Literature: non-protein bound	54	16
% of non-protein bound	85%	69%
Literature: free ion	47	13
% of free ion	98%	85%

Reference: M. Walser (1961)

Table 9:

Ultrafiltrable Calcium in Rat Blood Plasma

Group	Mean PPM Ca	Standard Deviation	No. of samples
Control	60	5.7	11
Acute	65	-	2
0.075%	59	-	2
0.125%	58	7.3	14
All DDT	59	6.8	18
All Rats	60	5.3	28 (1)

Note: numbers in parentheses refer to number of samples discarded as being more than two standard deviations from the mean.

Table 10:

Ultrafiltrable Magnesium in Rat Blood Plasma

Group	Mean PPM Mg	Standard Deviation	No. of Samples
Control	11	1.3	10
Acute	14	-	2
0.075%	10	-	2
0.125%	12	1.5	14
All DDT	12	1.6	18
All Rats	12	1.4	27 (1)

Note: numbers in parentheses refer to number of samples discarded as being more than two standard deviations from the mean.

Table 11:

Total Calcium in Rat Blood Plasma

Group	Mean PPM Ca	Standard Deviation	No. of Samples
Controls	113	8.6	15
Acute	107	4.0	7 (1)
0.075%	123	3.4	13
0.125%	119	10.1	37 (4)

Note: numbers in parentheses refer to number of samples discarded as being more than two standard deviations from the mean.

Table 12:

Differences Between Calcium Groups

Groups	Difference Between Means PPM Ca	p
acute - controls	-6	<0.05
0.075% - controls	+10	<0.0005
0.125% - controls	+6	<0.01
0.075% - acute	+16	<0.0005
0.125% - acute	+12	<0.001
0.075% - 0.125%	+4	<0.05

Note: Minus sign indicates first named group is lower than second.

DDT.

Five pairs of rats were run in more than one group. The data from these pairs is compared to the group means in Table 13. All pairs showed the same trends as the overall groups did.

Total Magnesium. Length of time fed DDT was not a variable in total magnesium level but level of DDT fed was. Tables 14 and 15 summarize the group data and compare the groups.

Animals fed at the 0.125% level (includes acute group) showed raised levels of magnesium. Feeding at the 0.75% level did not significantly alter the total magnesium level.

The pairs of animals appearing in more than one group showed two exceptions to the mean trends (see Table 16). However, not enough pairs were studied to draw any conclusions.

Recommended further study

To further clarify and establish the interaction between dietary DDT and blood levels of calcium and magnesium, several areas of research suggest themselves.

First, it would appear advisable to run a series of studies in which individual rats were followed through control, acute, and chronic stages. The rat might also be removed from DDT-containing feed and plasma levels monitored to determine if and when the control level is again established.

The present study supported the hypothesis that it is the protein-bound ion that changes. However, this is not proven due to the ambiguity of the ultrafiltrable data which should represent all non-protein bound but may actually represent only free ion. Therefore it appears further work should be done to see which ion form(s) changes. Electrophoresis could be used to study the plasma proteins and a suitable method for separating the ultrafiltrable components

Table 13:

Animals in More than One Group; Total Calcium

Animal	Control	Acute	0.075%	0.125%
Reference: means of all rats	113	107	123	119
Hooded Pair C	-	-	122	118
Hooded Pair K	114	-	-	116
Hooded Pair D	108	-	-	115
Hooded Pair F	118	104	-	-
Hooded Pair A	111	102	-	115

Table 14:

Total Magnesium in Rat Blood Plasma

Group	Mean PPM Mg	Standard Deviation	No. of Samples
Controls	19	1.9	15 (1)
Acute	22	3.3	8
0.075%	20	2.6	13
0.125%	23	5.1	40 (1)
Acute + 0.125%	22	4.5	47 (2)

Note: numbers in parentheses refer to number of samples discarded as being more than two standard deviations from the mean.

Table 15:

Differences Between Magnesium Groups

Groups	PPM Mg Difference Between Group Means	p
acute - controls	+3	<0.01
0.075% - controls	+1	n.s.
0.125% - controls	+4	<0.001
0.075% - acute	-2	<0.05
0.125% - acute	-1	n.s.
0.075% - 0.125%	-3	<0.025
acute + 0.125% - controls	+3	<0.005
acute + 0.125% - 0.095%	+2	<0.05

Notes: n.s. means not significant

minus sign indicates first named group is lower than second

Table 16:

Animals in More than One Group; Total Magnesium

Group	Control	Acute	0.075%	0.125%
Reference: group means	19	22	20	23
Hooded Pair C	-	-	19	19
Hooded Pair K	18	-	-	26
Hooded Pair D	18	-	-	22
Hooded Pair F	20	22	-	-
Hooded Pair A	22	18	-	24

should be found.

The technical grade DDT actually contained six isomers, of which the p,p'-DDT was the major component. It is not known which of the six components is (or are) capable of effecting the observed changes. Feeding of the purified individual isomers would establish which components are involved.

CONCLUSION

This study has established that dietary DDT alters the total plasma levels of calcium and magnesium. The observed changes were not reflected in the ultrafiltrable ion which represented the free ion form and, possibly, the complexed form of the ion. It appeared that protein-bound calcium was affected by duration and amount of DDT fed while only feed level influenced the protein-bound magnesium.

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THE EFFECTS OF DIETARY DDT ON PLASMA LEVELS OF CALCIUM AND MAGNESIUM IN RATS

by

M. ANNE LIEDTKE

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ABSTRACT

The effects of dietary DDT on the plasma calcium and magnesium levels in rats were evaluated in an attempt to clarify the mechanism of poisoning. Both the total ion and the ultrafiltrable (free and complexed) ion were measured. Total calcium dropped after initial feeding but rose to a higher than normal level after feeding two or more days. Time was not a factor for total magnesium but rats fed at the 0.075% level did not differ from controls, while rats fed at the 0.125% level showed raised magnesium levels. Neither time nor level of feeding affected the amount of calcium or magnesium ultrafiltered.