RESPONSE OF BONE ALKALINE PHOSPHATASE ISOZYME TO INORGANIC FLUORIDE

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We hereby recommend that the dissertation prepared under our supervision by Constance Jean Richards entitled RESPONSE OF BONE ALKALINE PHOSPHATASE ISOZYME TO INORGANIC FLUORIDE

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CHAPTER I

I N T R O D U C T I O N

As the average life span increases due to improved knowledge in such fields as nutrition and medicine, there is increased interest in the particular problems and diseases of aging. One disease which is of widespread concern in geriatrics is osteoporosis resulting in bone loss due to reduction of total bone mass.

Efforts to slow this aging process in bone which begins in approximately the fourth decade of life, have centered on therapy aimed at improving bone mineralization. Among the most promising of various therapy regimens is treatment of aging bone with inorganic fluoride.

The calcified tissues have a high capacity for the incorporation of fluoride ions which are actually deposited in the crystalline lattice of bone apatite. The resulting structure is more stable, less soluble and forms a larger crystal than the original form.

The administration of inorganic fluoride to patients with osteoporosis has been shown to depress bone resorption, decrease calcium excretion and help maintain a positive calcium balance (2). At minimal levels the fluoride

accumulates in hard tissues with no pathological effect and seems to stimulate osteoblastic activity.

Intrinsic in the problem of treating osteoporosis by diet therapy is the need of a reliable, relatively simple and accurate measurement of bone response. The present study undertakes the demonstration of bone isozyme of alkaline phosphatase as an accurate indicator of bone metabolism.

Alkaline phosphatase is a phosphohydrolase and is made up of several isozyme fractions among which is one that originates in the bone. The enzyme catalyzes the release of inorganic phosphate from organic ester-like linkage and its primary function may be to furnish the cell with inorganic phosphate and/or the appropriate substrate. The various isozymes have different sites of origin and may be related to a variety of normal and pathological conditions.

Biochemical and light microscopic evidence suggests that there is a high activity of enzyme in bone forming cells which coincides with the start of precipitation of calcium salts in the extracellular medium. The highest activity is found at times of active mineralization and decreases as the process nears completion.

Alkaline phosphatase concentrations are relatively constant and the isozyme patterns vary little in normal meta-

bolic states. Actual osteoblastic activity seems to determine the concentrations of the bone isozyme in the sera of man and the measurement of this isozyme would seem to be a more accurate indicator of actual bone metabolism than total alkaline phosphatase which may be within normal limits while masking an elevation or decrease in bone isozyme fraction.

Previous studies have used total alkaline phosphatase in serum as an indicator of bone mineralization or have commonly used heat denaturation methods for the elucidation of origin of the isozyme fractions. Such methods vary with different studies and are subject to different interpretations.

T E R M I N O L O G Y

The following are explanations of chemical symbols used in this study:

Adenosine monophosphate	AMP
Alkaline phosphatase	Alk. P'tase
Centigrade	C
Fluoride, inorganic	F^-
Grams	g
Micromolar	μM
Milligrams	mg
Milligrams per 100 milliliter	mg per cent

Milliliter	ml
Millimicron	mμ
Millimolar	mM
Molar	M
Nicotinamide adenine dinucleotide	NAD
Nicotinamide adenine dinucleotide phosphate	NADP
Normal	N
Not significant	NS
Optical density	OD
Parts per million	ppm
Phosphate, inorganic	P_i
Revolutions per minute	rpm
Serum glutamic-oxaloacetic transaminase	SGOT
Standard deviation	SD
Standard error	SE
Tricarboxylic acid	TCA

STATEMENT OF PROBLEM

In studies in progress at Texas Woman's University the effects of sodium fluoride administration on bone density of male geriatric subjects is being observed. Previous data have shown significant decreases in demineralization rate of the bone of subjects receiving daily fluoride therapy. The present study is being conducted on two sets of

subjects of approximately 40 each: a group treated daily with sodium fluoride in concentrations determined by weight and a control group.

The overall purpose of this study was to determine the practicality of using alkaline phosphatase isozyme measurements as an indicator of bone function. Specifically the response of bone to inorganic fluoride therapy was studied by measurement of bone isozyme of alkaline phosphatase by means of gel electrophoresis and bone tissue culture. These methods were compared to conventional heat denaturation methods and comparisons of the methods are discussed. In addition bone isozyme measurements were compared to other appropriate parameters to determine the value of this measurement as an indicator of the physiological state of bone. The bone cultures were examined in the presence of varying levels of inorganic fluoride, and their response was measured in terms of both total alkaline phosphatase activity and that of bone isozyme fraction.

The specific purposes of this study were:

1) To determine the influence of inorganic fluoride on the amount of bone isozyme of alkaline phosphatase in the sera of geriatric males receiving daily administration of sodium fluoride and comparisons with a control group.

2) To determine the activity of bone isozyme as compared to bone regression slopes as determined by X-ray.

3) To determine the relation between amounts of total serum alkaline phosphatase and that of bone isozyme.

4) To determine comparison between heat inactivation methods and bone isozyme measurement on polyacrylamide gel electrophoresis.

5) To describe a method which is suitable for standard clinical analysis of alkaline phosphatase isozyme of bone.

6) To culture bone tissue from rib sections with varying levels of fluoride ion in the growth media and to measure alkaline phosphatase response.

7) To compare alkaline phosphatase levels to other biochemical parameters such as serum F^- levels.

8) To apply appropriate statistical calculations for correlation between parameters and tests of significance.

REVIEW OF LITERATURE

INTRODUCTION

Bone mass in humans increases up to the age of about 20 years. Beginning with the fourth decade of life the skeletal mass gradually decreases with age except for the skull mass. Osteoporosis frequently accompanies this decrease (93). Throughout life there is a rearrangement of the trabeculae with osteoblasts forming new bone and osteoclasts dissolving it.

Osteoporosis is thought to be a natural process of aging and is associated with lack of adequate bone formation. In general, the disease is an atrophic disorder

in which the bones become brittle and porous due to the widening of the Haversian canals, coupled with decreased thickness of the bony trabeculae. This process may be associated with deposition of calcium in tissues other than bone (15). Osteoporosis appears to be related to the rapid dissolution of bone when this process exceeds bone formation. This imbalance, particularly at the endosteal surfaces results in cortical thinning in the shafts of long bones (67).

Causative mechanisms of osteoporosis are thought to be strains of movement, malnutrition and possibly the deficiency of estrogen (154). Besides occurring in aging, osteoporosis occurs in disuse atrophy and in a variety of hormonal imbalances (131).

There is a time lag in the initiation of mineralization in response to need that increases with age. The amount of bone per unit volume becomes reduced but is normal in composition. There are increased numbers of osteons which are less completely mineralized (67).

During crystallization, bone minerals are subject to controls which produce a dynamic equilibrium involving their coalescence and dissociation into simple ions. Mineralization occurs in a ground substance produced by specific cells. These cells consist of protein molecules

which act as structural determinants for the initiation of ground substance for bone and at the site of deposition there is a depot of this loosely packed mucous ground substance (112). The ground substance consists of mucoproteins to which are added ions of calcium, magnesium, potassium, sodium, phosphate, carbonate, fluoride, chloride, mucopolysaccharides and lipids.

Various treatments have been used to reverse damage when the crystallization process is defective, among them is inorganic fluoride therapy. In an area where very low fluoride levels are found in the drinking water, it was noted that humans, particularly males, had reduced bone density ranging from 22 to 85 per cent reduction from normal levels depending on age. In those individuals with osteoporosis, bone resorption was increased and the subjects were in negative calcium balance. There was no remineralization with large intakes of calcium and it was concluded that fluoride is the most important biologic factor in osteoporosis (69).

INORGANIC FLUORIDE

Fluoride is a normal constituent of bone and tooth enamel. Calcified tissues of vertebrates have a high capacity to incorporate fluoride (191). Fluoride is quickly distributed throughout the body tissues, and accumulates

preferentially in bone (182). At minimal levels the fluoride accumulated by bone has no pathological effect. The stimulation of osteoblastic activity which occurs with fluoride ingestion is an effect that increases with age (131).

Experiments, in vivo and in vitro, show that calcified tissues are made up of either hydroxyl or fluoroapatite, depending on the availability of fluoride. As the plasma fluoride level increases, fluoride is rapidly deposited in the bones by exchange as well as by the development of new bone. In the exchange mechanism the hydroxyl ion of the hydroxyapatite exchanges with the fluoride ion to form fluoroapatite crystals at the surface of the bone (52). This exchange results in a more stable structure which is less soluble in physiological fluids, giving a stabilizing effect to the bone. This stabilizing effect results in the reduction of bone resorption (46, 2).

Formation of the apatite is controlled by three factors: lesser solubility of the crystalline phase, fluoride content, and the structural pattern of the protein molecules. The reaction mechanism of the fluoride ion is not clear but isomorphic hydroxyl groups and fluoride are able to replace each other completely (112).

X-ray diffraction studies also confirm that a rise in

the fluoride content of bone increases bone crystal size and/or decreases crystal strain. The substitution of fluoride in the apatite crystal suggest that the improvement results from increased crystal growth following a greater nucleation rate, both due to higher supersaturation resulting from the presence of fluoride in the serum and the final cyrstalline phase. More stable bone apatite is produced by improved crystallinity and isomorphic substitution of fluoride in the apatite structure (123).

When dilute fluoride solutions react with hydroxyapatite, hydroxyl ions are liberated. At higher fluoride concentrations double decomposition occurs due to the low solubility of calcium fluoride (77). In studies measuring the growth of calcium phosphate from stable supersaturated solutions in the presence of fluoride ions it was found that the fluoride is absorbed at actively growing sites on the bone matrix. The reasons for increased growth in the presence of fluoride is uncertain; when small amounts of fluoroapatite are formed they may aid in the deposition of further apatite material (2).

Fluoride, in vivo, follow the physiochemical principles of ion exchange in deposition and mobilization. Homeostatic deposition of fluoride in bone may be a marker for the physiological state of the intact animal (188). The

effects of fluoride on the intact animal appear to be related to the basic properties of the fluoride ion. Besides being exchanged irreversibly with the hydroxyl group of hydroxyapatite to form the less soluble crystal, fluoride also stimulates certain enzymes such as alkaline phosphatase, concerned with mineralization if given below inhibition levels (81). Fluoride is present in small amounts in most tissues but is concentrated in the skeleton and kidneys(2).

Administration of fluoride results in a reduction of specific surface of bone due to the production of larger crystals. Specific surface is a measure of the relation between surface area and bone mass and this decrease in relative surface is directly correlated with increases in fluoride content of bone (191).

It is widely accepted that the primary effect of fluoride administration is the stimulation of osteocytes which induce new bone formation with increased numbers of osteoblasts, decreased resorption and increased crystal size (74). When immature rats were used to study the effect of fluoride administration on bone, an increase in osteoblastic formation was observed which exceeded resorption rate. There were three primary effects: an increase in periosteal matrix and bone formation, the inhibition of

mineralization of young bone and increased endosteal bone resorption. After 113 days of fluoride administration there was a generalized increase in cross-sectional dimensions with a decrease in susceptibility to bone stress (12).

In human subjects with a mean age of 74 years, demonstrating senile osteoporosis, the structural changes and turnover rates were measured. The application of fluoride induced augmentation of bone substance over a period of 20 weeks and increased volumetric density was brought about by gradual deposition of new slowly mineralizing osteoid tissue along the surface of the trabeculae. The main effects were increased volumetric density of cancellous bone which showed large increases, about twice the original volume after 80 weeks of treatment, mainly due to the apposition of new bone along the surface of pre-existing trabeculae. A positive skeletal balance was caused by increased stimulation of bone formation averaging 17 times the original values. Other effects were the retardation of osteoid mineralization and the acceleration of osteoblastic activity (148).

Comparing areas with high fluoride containing water with areas of low content, there were significantly greater incidence of osteoporosis, reduced bone density, collapsed vertebrae and bone pain among individuals residing in the area of low availability. The results of this study were

taken as overwhelming evidence that fluoride is therapeutically desirable for the prevention of these conditions (15). Similar results were demonstrated by other studies (131, 93).

In studies on rabbit bone the principal effect of fluoride was to increase trabecular and cortical bone and thicken the periosteum. There was an increase in the length of cartilage columns due to increases in the number of fully grown cells indicating the arrest of resorption (47). These results were confirmed in rat studies in which daily administration of fluoride increased the mineralization rate of bone counteracting the decrease in bone thickness, ash content and specific gravity lowering induced by immobilization without changing the calcium to phosphorus ratio in ash (56, 113).

Rats which were given a daily dose of fluoride amounting to 30 parts per million in their drinking water had 2000 to 4000 parts per million in bones. The bone withstood a higher breaking stress and there was a significant correlation between fluoride intake and bone strength with increased resistance to fracture. Two possibilities were noted, the formation of mixed hydroxy-fluoroapatite during the administration of inorganic fluoride and the increase of bone solubility inhibition of osteoclastic bone

resorption (135).

The primary effect in weanling rats given 50 parts per million of fluoride in their drinking water was a decrease in loss of alveolar bone and calculus (193). X-ray emission, diffraction and electron microscopic studies in mice show that with a level of 0.03 milligrams of inorganic fluoride per day for two to four months the distribution of fluoride is non-homogenous with heavy deposition in the bone (11). The pelvic bones of both heavy and light boned mice showed increased bone crystallinity with the administration of 50 parts per million of fluoride. This effect was observed over a period of 16 months (195).

In swine it was found that adding fluoride to the diet increased the radiographic density of bone. Osteoporosis was not prevented but was compensated for by increased periosteal bone formation. An increased cross-linking of bone mineral in fluoride treatment was observed. When calcium and phosphorus were added as supplements in the diet, an additional increase in bone formation occurred and it was concluded that the addition of these minerals enhance the effects of fluoride and that the fluoride either affects the solubility of bone crystals or the movement of calcium through the cell membrane (126).

Studies with dogs demonstrated that with increased

fluoride there was an increase at sites of active bone formation per unit area. A positive correlation between fluoride intake and bone remodeling was noted, and it was concluded that fluoride increases the rate of bone turnover by action on the osteoprogenitor cells (44).

Studies with cattle indicate that the fluoride administered enters the bone at a rate that decreases with time and gradually reaches an equilibrium state. From previous studies dosage levels were calculated which were based on blood fluoride concentrations (167).

In human subjects the concentration of fluoride in the bones increases linearly with increases in fluoride intake up to the level of four parts per million (194). When patients with osteoporosis, Paget's disease and idiopathic osteoporosis were given varying doses of fluoride in amounts varying from 50 to 200 milligrams per day, a grossly positive calcium retention was initiated through increased absorption. Bones showed increased mineralization, increased bone accretion rate and there was relief of bone pain (16). In 33 patients with osteoporosis, dietary rickets or parathyroid disorders, serum alkaline phosphatase, calcium accretion rate and calcium resorption correlated well among individuals with these major bone diseases (76, 125, 58, 140).

Patients ranging in age from 49 to 86 years who were suffering from bone atrophy due to osteoporosis were studied. Seven patients received 30 milligrams of sodium fluoride for 200 days and six received from 30 to 70 milligrams for 140 to 300 days. Blood calcium levels and osteoblastic activity as measured by alkaline phosphatase increased significantly. Regression studies showed a significant and positive relationship between length of treatment and blood alkaline phosphatase levels. There was a decrease in calcinuria, increase in hydroxyproline and increase in serum alkaline phosphatase suggesting a modification of balance between resorption and deposition in favor of the latter. More than 50 per cent of the subjects were relieved of bone pain and histological examination showed that fluoride induced the formation of compact and cancellous bone with increased osteocytes. About 50 per cent of the administered fluoride was retained in the bone (171).

The idea of treatment of aging bone with fluoride is based on the observation that 20 to 80 milligrams of fluoride per day is associated with an increase of bone mass. The length of time of observable chance is inversely proportional to intake. The use of fluoride to treat bone disease is still in the experimental stage, but early studies on human subjects have indicated that at the

recommended levels of fluoride there is improvement in bone formation, the quality of which is subject to different interpretations (30, 136, 74, 14).

Not all studies on the effects of fluoride on bone produce positive results. Among those studies which yielded negative results was one in which seven osteoporotic patients were given 35 milligrams per day of sodium fluoride. A minimal response was noted and negative calcium balances were reduced for only a short time but it was concluded that long term effects were questionable (144). In induced osteoporosis in dogs in which increasing levels of fluoride were added to the diet, no beneficial effects were observed (72). In osteoporotic patients there was no increase in the size of the exchangable calcium pool or accretion rate with the administration of fluoride. There was no significant increase in total body calcium and it was concluded that this is not an effective treatment for osteoporosis or that longer studies are needed for evaluation (31).

In spite of the negative studies the preponderance of evidence suggests that fluoride therapy for osteoporosis is effective and relatively safe. Therapy usually results in increased bone formation and potentially reverses the effects of osteoporosis by increasing bone mass. If adequate calcium is given to insure normal bone mineralization

there is no evidence that fluorotic bone is not as strong as normal bone. Five year studies indicate that at doses of 25 milligrams per day of fluoride, osteoporotic bone mass may increase by as much as 15 per cent (139).

It is possible that fluoride administration between the ages of eight and 25 is a plausible preventative measure against physiological aging resulting in osteoporosis (40). In studies with children between the ages of six and 16 with abnormal bone mineralization, those treated with fluoride showed an increase in bone formation amounting to 8.1 per cent over untreated controls. Radiographic bone density was highly correlated with calcium content and total bone mineral (89).

ABSORPTION AND IN VIVO REGULATION

Using human volunteers, the results of fluoride uptake was studied. One hour after injection it was found that 40 per cent of the ^{18}F was contained in the extracellular fluid and 40 per cent was taken up by the tissues. Most of the fluoride was found in the bones, amounting to 200 to 1300 parts per million depending on age of the subject. About one part per million was located in the soft tissues (75). Urinary excretion of fluoride apparently has no relationship to age except that mobilization is greater in children (96). Mobilization from bones and teeth occur

indicating that the ion in mineralized tissues exists in labile as well as stabile form (192).

Studies on the uptake of fluoride in water show that where water contains one part per million of the fluoride ion there is three to 30 per cent of this amount organically bound in vivo (55). Passage of fluoride into intracellular fluid varies with the nature of the tissue. The ion penetrates muscle, liver and tendon but not the brain in which there is a barrier to passage. Fluoride affects the permeability of cell membranes and 20 to 40 parts per million in the blood causes human red blood cells to shrink and become resistant to osmotic hemolysis due to the rapid loss of potassium from the cell. It also causes the leakage of nucleotides from the membrane. Fluoride affects the ion transport system due to changes in permeability which could affect a modified ion transport mechanism (29).

Continous exchange between bone and body fluids takes place and if the amount in bone exceeds 5000 parts per million there is an alteration in cellular behavior (2).

In rats given 2.94 milligrams of fluoride in one dose, a rapid absorption was observed and this absorption was 29 per cent complete at five hours. At 50 hours 88 per cent was absorbed with 70 per cent of this being deposited in the skeleton (28). Fluoride administered orally is absorbed

in the gastrointestinal tract. With normal fluoride intake intestinal absorption averages about 93 per cent. The absorption rate depends on the solubility of the substance ingested. Fluoride with high solubility is absorbed between 90 and 100 per cent with the unabsorbed portion being excreted in the feces. Rigid homeostasis of the fluoride ion is maintained by the body and even high doses of fluoride cause only temporary increases in blood levels. The constant blood level is maintained by excretion and storage in the bone. Differences in excretion and fluoride absorption is determined by bone storage (3).

Increases in bone forming activity results in elevations of serum alkaline phosphatase which correlate with serum fluoride concentrations. These serum fluoride levels appear to be a good indicator of levels of fluoride adjustment between calcified and non-calcified tissues (157). While plasma concentrations of fluoride are normally low, very large amounts of ingested fluoride may overwhelm this homeostatic mechanism.

Human serum contains exchangable and non-exchangable or fixed portions of fluoride. The fixed portion makes up 80 to 90 per cent of the total with only 0.02 to 0.04 parts per million available for exchange (118). Comparison of fluoride levels in plasma and erythrocytes indicate that

75 per cent is contained in the plasma. Less than five per cent of plasma fluoride was found to be bound to plasma solutes under physiological conditions (24).

There is a positive correlation between dosage of fluoride and changes in bone formation which appear to be related to the amount of calcium present in the diet, and there is a negative correlation between bone resorption and calcium supplementation (83). When 30 young healthy male monkeys were given 10 milligrams per day of sodium fluoride per kilogram of body weight, fluorosis developed. Fluoride administered alone produced rachitomimetic effects and when low protein diets were also added there was a synergistic effect noted. When subjects were placed on low calcium, low vitamin C diets with sodium fluoride administration, the serum alkaline phosphatase levels were elevated. Levels of alkaline phosphatase do not change in scurvy and elevated levels in the low vitamin C fed group were thought to be the result of fluoride intoxication. Low intakes of dietary calcium along with low ascorbic acid levels resulted in osteoporosis (129). These fluorotic monkeys were then measured for ^{45}Ca turnover and calcium and phosphorus balance studies were done with different levels of dietary protein, calcium, vitamin C and fluoride. Fluoride increased the accretion rate and exchangable pool size of

calcium in animals fed adequate diets. On diets which were low in calcium and vitamin C the accretion rates were further increased. Low protein diets with or without fluoride resulted in decreases in the exchangable pool size and decreased accretion rate. Fluoride administered with adequate diets increased cumulative retention of ^{45}Ca (128).

Fluoride added to the diet results in a small increase in ash and magnesium. In osteoporotic human subjects, increases in fluoride incorporation into bone accompanied slight increases in magnesium content and decreases in carbon dioxide (58). There is also a small but persistent decrease in sodium and carbonate and approximately a 30 per cent decrease in citrate in the bone. Ions showing change are believed to be positioned outside the crystalline lattice of apatite (191).

Fluoride at elevated levels can produce changes in calcification when added to magnesium deficient diets and in some cases to magnesium supplemented diets. Fluoride appears to prevent calcification of soft tissues produced by magnesium deficiency, and trace elements such as molybdenum, manganese, vanadium or selenium may indirectly effect the deposition of fluoride (191). Fluoride alleviates magnesium deficiency by molybdenum sparing action which also prevents the decrease of circulating alkaline phos-

phatase (66).

FLUORIDE-DISCONTINUED THERAPY

When fluoride was administered as the sodium salt at an intake level of 9.4 milligrams per day to humans, the average amount retained was 5.4 milligrams. After cessation of therapy the urinary and fecal excretion was markedly decreased. Data indicate that in man the high retention of fluoride is maintained over fairly long periods (159). It is possible that fluoride is held in stable configuration for long periods after the cessation of therapy. Indirect data in humans reveal that individuals drinking water previously containing eight parts per million of fluoride but defluorinated to one part per million show concentrations of two parts per million in the urine (191).

In human fluoride therapy studies when the ion is administered and then discontinued under strictly controlled dietary conditions, the urinary fluoride varies but is constant for each individual. Urinary fluoride excretion increases slightly with time in the experimental phase of fluoride therapy but all fluoride balances during the study were positive. After cessation of therapy urinary excretion was slightly higher than in control subjects and fluoride balances were slightly lower. In comparisons of the same individuals during a control phase, a therapy phase and

after discontinuation of therapy, net absorption figures were 94, 94, and 92 per cent of the amount absorbed during therapy. Results show that the retention of fluoride increased when supplements were given and varied little during high or low intake levels. The body store of fluoride accepts and releases fluoride for prolonged periods (161).

RELATIONSHIP BETWEEN FLUORIDE, ALKALINE PHOSPHATASE AND BONE

Biochemical and light microscopic histochemical evidence indicate that the high activity of alkaline phosphatase in bone forming cells coincides with the start of precipitation of calcium salts in the extracellular medium. The exact role in calcification is obscure. When studied by electron microscopy of the rat callus, the alkaline phosphatase activity was located in fibroblasts, chondroblasts, hypertrophic chondrocytes and osteoblasts. No activity was noted in osteoclasts. The greatest localization was in the osteoblasts with less in the chondrocytes and chondroblasts. The enzyme was also observed in small cytoplasmic vesicles immediately below the plasma membrane which may be associated with the endocytic vesicles. There was also slight activity in the Golgi apparatus and on membranes bordering intracellular vacuoles containing collagen. The highest activity was observed during calci-

fication and decreased as the process neared completion (61). There is an association between alkaline phosphatase levels and the calcification process (115).

Elevation of serum alkaline phosphatase is usually a reflection of increased bone activity, biliary obstruction or gastrointestinal disease (99). Increase in calcification by the administration of fluoride may be accompanied by increased levels of the enzyme which reflects mineralization as high activity of the enzyme is found in calcifying tissues. It is thought that the enzyme may serve to keep phosphate esters away from the site of mineralization as these esters inhibit the process. High alkaline phosphatase activity is found in the stratum intermedium and subodontoplastic layer in developing molars and low activity in ameoloblasts and odontoblasts (176).

Alkaline phosphatase activity was responsive to decalcification of bone as early as five days with peak activity at 20 days after the origin of the process. At 40 days the activity had declined to 15 per cent of maximum in cells which proliferate and later differentiate into bone and bone marrow. When cortical bone was implanted into rat muscle tissue, deposition of calcium began 10 to 12 days after implant and increased to 20 days. Alkaline phosphatase was used as an indicator of bone cell function (51).

Alkaline phosphatase is sensitive to high concentrations of fluoride. Inhibition occurs at levels of 10^{-2} molar. Experiments with dairy heifers demonstrated close correlation between fluoride ion ingested, fluoride in the bone and alkaline phosphatase activity. In the form of sodium fluoride, at levels of 49 parts per million, there were decreased enzyme levels (104). In porcine bone alkaline phosphatase levels in the serum closely correlated with the rate of new bone formation and ingestion of sodium fluoride resulted in increased alkaline phosphatase levels. Several mechanisms were postulated for this response: the interaction of bone crystal, the stimulating effect of fluoride on the parathyroid which may act directly or indirectly by effects on calcium metabolism, the increase in osteoblastic activity and osteoid formation in the presence of adequate calcium or the reduction of the rate of movement of calcium through the cell membrane and the slowing of the combination of calcium with calcium-binding protein or receptors on the cell surface (158).

Fluoride exerts similar effects on many enzymes in common with other halogens. It is not known whether the action by fluoride on enzymes is produced by the physical presence of the ions or by secondary changes in the ambient solution. When sodium fluoride was administered to humans

at levels of five milligrams per day, the alkaline phosphatase blood levels were reduced for the first six weeks, averaging a 16 per cent decline in humans. At eight weeks the levels were similar to the control subjects. This activity was explained on the basis of the inhibition of the enzyme by fluoride. Adaptation suggests some actual enzyme change and perhaps protein configuration alteration. Lowered blood levels may be due to decreased loss from cells affected by lower transport rate. Return to normal levels may result from the induction of alternate alkaline phosphatase pathways in the tissue of origin (104). The normal levels of circulating fluoride at physiological concentrations is not an enzyme inhibitor *in vitro* (49).

GENERAL PROPERTIES OF ALKALINE PHOSPHATASE

> Among esters of phosphoric acid possessing biochemical interest there are known those in which the phosphoric group is believed to be combined with a hexose molecule. Formation of the precipitate when studying hydrolysis of the esters by enzymes using calcium and barium salts suggest that a reaction operates concerned with bone formation.

One of the first definitive studies of alkaline phosphatase appeared in 1923 in which Robert Robison defines alkaline phosphatase in terms of its action on bone (143). By using rabbit bone it was shown that ossifying cartilage contained the active enzyme which rapidly hydrolyzed hexose-monophosphoric esters and glycerophosphoric esters. This enzyme was

also localized in the kidney.

Interest in alkaline phosphatase has been demonstrated recently by a number of review articles that have appeared in the literature (85, 86, 151). The alkaline phosphatases are a group of enzymes sharing the capacity to hydrolyze phosphate from various esters in an alkaline medium. It is a metalloenzyme composed of two identical subunits having serine as its active center (149).

Alkaline phosphatase shows little specificity for any of the phosphomonoesters that it hydrolyzes (7). Hydrolysis by bacterial alkaline phosphatase around its active center suggests structural as well as functional analogy with other esterases. The enzyme is also able to act as a phosphotransferase (149). Being a fairly nonspecific phosphomonoesterase it enables the cell to utilize organic phosphates when these are the only source of phosphate present in the medium.

The alkaline phosphatase apoenzyme does not bind phosphate or form any phosphoryl enzyme at any pH. The addition of two zinc cations per molecule to the apoenzyme induces tight binding of one phosphate dianion. The magnitude of binding is affected by ionic strength, pH and protein concentration. Cobalt, zinc and cadmium all induce tight binding of one phosphate per dimer but only zinc and cobalt induce enzymic activity. One catalytically active site per

dimer can be measured by equilibrium dialysis against inorganic phosphate, a competitive inhibitor (149).

Alkaline phosphatase is a glycoprotein but the exact carbohydrate content has not been agreed upon. The intestinal isozyme contains hexose and hexosamine but no sialic acid. The alkaline phosphatases from other tissues contain bound sialic acid. Removal of the sialic acid residues from the enzyme affects the electrophoretic mobility but does not impair enzymic action. The role of the carbohydrate units are not understood but exist with heterogenous complexity and may represent specific genetic characteristics. There is no evidence that these units participate in catalysis (115). Present evidence indicates that the carbohydrate content is responsible for variation in electric charge on which electrophoretic mobility depends. Treatment with neuraminidase reduces anodic migration of the isozymes. This finding is interpreted as meaning that there is a terminal sialic acid residue on those isozymes in which electrophoretic movement is altered which is absent when treatment with neuraminidase does not affect mobility. Its removal reduces the magnitude of the negative charge on the enzyme molecule. Intestinal isozyme is unaffected by neuraminidase, and it is proposed that sialic acid is not terminal or is occluded in the three dimensional structure

of the enzyme. Treatment with neuraminidase has little effect on the kinetic properties of the enzyme, and it appears that the difference in structure affecting electrophoretic mobility does not involve the active center of the enzyme (183).

Covalent binding of inorganic phosphate to serine at the active site is a reaction common to alkaline phosphatases in which an intermediate phosphoryl enzyme is formed. Experiments with the enzyme from E. coli show that the phosphate esters have initial rapid phosphorylation of approximately 30 per cent that of total activity. Inorganic phosphate added to the substrate results in a rise to steady state levels. Dephosphorylation does not seem to be a rate-limiting step (130).

Alkaline phosphatase is unusually stable, but can be deactivated by chelating agents, urea and mercaptoethanol, as well as low pH. These agents dissociate the active dimer into inactive subunits (149). The bacterial enzyme can be reduced by thioglycolate and 6.0 molar urea to yield inactive monomers which, under appropriate conditions, can be reactivated to active dimers (88).

In the pathway from subunit to enzyme five states have been demonstrated. The first consists of an extended coil unfolded monomer with the chain containing no significant

non-covalent interaction. The globular unfolded monomer at pH 4.0 has a net positive charge which is partially neutralized and results in a smaller hydrodynamic volume. At pH 6.0 the folded monomer of all alpha-helical configuration of active dimers is restored and four tyrosine residues per monomer are associated with hydrophobic regions. The apodimer at neutral pH forms an inactive zincless dimer which forms the active enzyme with the addition of zinc ions that give additional conformational change (149). The active tetramer has a high protein concentration and high zinc content. In this form the enzyme has a doubled molecular weight and higher turnover number. The alkaline phosphatase of E. coli has a molecular weight of 86,000 and contains two identical subunits linked through disulfide bonds (88). The enzyme can hydrolyze a variety of primary phosphate esters irrespective of their organic radicals. Optimum pH for the hydrolytic action of the enzyme increases with an increase in the initial concentration of the substrate (115).

Alkaline phosphatase possesses hydrogen donating centers and an electron-rich core. The electron attraction by substituents, the inductive effect toward oxygen and the opening of one of the bonds of phosphate to oxygen make phosphorus atoms acquire a positive charge. Possibly ionic oxygen which supplies the electrons to phosphorus are mini-

mized by participation in the formation of hydrogen bonds.

Phosphorus atoms may form a strong bond with the enzyme and attachment of phosphorus to the enzyme is stronger than that of the hydrogen bonds. The enzyme may undergo structural change when combining with any of its substrates (111).

FUNCTIONS OF ALKALINE PHOSPHATASE

It is uncertain as to whether the hydrolytic action, transphosphorylation or pyrophosphatase activity is of primary importance in alkaline phosphatase activity (70). Alkaline phosphatase may have a key role in calcification by removing the layer of pyrophosphate which covers the bone mineral surface. This pyrophosphate retards the rate of deposition of calcium and phosphorus (and perhaps fluoride) onto the bone surface.

There is evidence for the identity of pyrophosphatase with alkaline phosphatase in *E. coli*, human liver, intestine and bone. It is possible that the two species are manifestations of the same enzyme (176). Studies of inhibitors of liver and intestinal alkaline phosphatase show that each type of substrate, orthophosphate and pyrophosphate, is a competitive inhibitor of the other. Magnesium activates orthophosphate but inhibits pyrophosphate except at low concentrations, indicating the presence of a single enzyme

with one active center (41).

Alkaline phosphatase from several tissues not only demonstrates pyrophosphatase activity but also acts as a phosphotransferase. In kidney, intestine and bone the catalytic activities seem to be the result of the same enzyme (177). The transphosphorylation activity implies that during catalysis the enzyme is phosphorylated and acts as a transphosphorylase. Dephosphorylation of the enzyme is the rate controlling step (5).

In studies, in vitro, of enzymic hydrolysis of pyrophosphate by bone extract and comparison with alkaline phosphatase activity it was observed that the latter was inhibited by L-histidine but not fluoride while the reverse was true of the pyrophosphatase. Magnesium concentrations exceeding 3.0 millimolar inhibited both enzymes. The conclusions drawn from these studies were that alkaline phosphatase may be an index of osteoblastic activity and pyrophosphatase may have a similar role in calcification or be synergistic to alkaline phosphatase. The activity of the two enzymes may be dependent on pH at the site of calcification (173).

Using embryonic chick femurs the participation of alkaline phosphatase and pyrophosphatase in ossification was studied. Results tend to confirm the observation that

there may be a close parallel between the two activities involving a reciprocal relationship (116). Other studies confirm this observation (184, 50, 106, 34, 42). Evidence for the similar activities of the two enzymes is demonstrated by the inability to separate them by ion-exchange chromatography and gel filtration. It is suggested that there is a single enzyme acting simultaneously on two substrates (27).

It is likely that alkaline phosphatase is part of the transport system for phosphate and/or calcium ions which are influenced by the parathyroid hormone (70). Function in the transport system is suggested by the localization of alkaline phosphatase to absorbative surfaces of the proximal convoluted tubules of the kidney, small intestinal mucosa and cell wall. A possible function is the movement of phosphate across the cell mass as demonstrated by the binding of alkaline phosphatase to inorganic phosphate. This function may be connected with protein synthesis in the cell and with nucleoprotein and nucleotide metabolism. It is known that the enzyme has the ability to hydrolyze RNA and DNA and this function may influence DNA synthesis and growth (177).

In studies of living cells it was found that bone-tissue interactions occur within the enzyme systems con-

cerned with calcium and phosphate in the presence of large amounts of bone material. The active site of the enzyme may be approached by the substrate from outside the osteocytic cell. Alkaline phosphatase has been found in the perfusion medium of in vitro studies and its activity apparently originates in the enzyme bound to the outer cell membrane (70).

Intestinal isozyme may function in the absorption of fatty acids as well as in calcium absorption. A further possible role is in the hydrolysis of dietary sphingomyelin and its conversion to ceramide and water soluble compounds (177)

In the presence of human serum, NADP is hydrolyzed to NAD by the action of alkaline phosphatase which acts as a catalyst. The conversion of NADP to NAD may interfere in the assay of various NADP-linked enzymes in the serum. As alkaline phosphatase levels increase, the conversion also increases but amounts differ with the different isozymes. It is possible that the enzyme may function in the regulation of tissue levels of NADP produced in the cells by NAD kinase or in the regulation of ratios of NADP to NAD (119).

LOCALIZATION

Alkaline phosphatase has been detected histochemically in human liver, bone, kidney, intestine, placenta and

leucocytes and erythrocytes. It is not found free in the cytoplasm but is attached to subcellular structures (106). In human red blood cells the enzyme is bound to the cell membrane or stroma, as demonstrated by the hydrolysis of alpha-napthylphosphate by ghost cells (98). The enzyme is found in the red blood cells of humans, bovine and porcine species (71).

In studies of the liver in normal and disease conditions, the enzyme increased significantly in the non-soluble cell fraction in liver disease. In subcellular studies the main enzyme activity was found in the non-soluble fraction with only limited amounts in the soluble fraction. Affinity of alkaline phosphatase from the two fractions for the substrate para-nitrophenylphosphate was identical (101).

There is not much available information on the cellular ultrastructure localization of alkaline phosphatase. The activity in homogenates and subcellular fractions show reproducible and specific patterns for each tissue. In general, the microsomal fraction has the highest specific activity while the supernatant or soluble fraction has the lowest content relative to protein level. The activity of other subcellular fractions varies. The most marked difference in thermostability of the fractions is between the

microsomal and soluble fractions in normal tissues with no significant differences between that of nuclei and mitochondria (106).

ISOZYMES OF ALKALINE PHOSPHATASE

The greatly varying alkaline phosphatase content of different tissues and organs of various species indicate that the content of isolated organs of one species are not directly comparable with those of another (92). Significant differences occur in concentration, composition and metabolic behavior of circulating alkaline phosphatase, both in different animal species and in different organs of the same species.

There is positive evidence for the existence of organ and tissue specific isozymes of alkaline phosphatase. These isozymes differ in electrophoretic mobility, heat stability, sensitivity to inhibition by amino acids, Km values and immunologic reactivity (100).

Alkaline phosphatase has been used extensively in the diagnosis of various disease states in the last three decades. It was formerly thought to originate primarily from the osteoblasts of the bone and be excreted in the bile. However, recent work has shown that many other tissues contribute to the circulating levels of the enzyme. There is strong evidence that the liver may be the main

contributing organ, but there is disagreement concerning the true nature of the components of human and animal alkaline phosphatase. Recent recommendations of the Enzyme Commission state that the enzyme derived from different origins should be regarded as isozymes (183).

Since alkaline phosphatase may be supplied to the blood by a number of organs, causes of elevated isozyme levels include skeletal disease, hepatic disorders, intestinal disease, hormonal imbalance, tumors, pregnancy, thyrotoxicosis, the injection of anti-convulsant drugs and rheumatoid arthritis (107). It is supposed that normal serum levels of alkaline phosphatase are a result of two factors: the rate of enzyme release from the tissues, and release and deposition in the bones. The circulating levels are also affected by the rate of enzyme inactivation in the serum and body protein pool. In osteoblastic bone disease the elevated levels of the enzyme in serum are due to the rate of release which exceeds the rate of inactivation. This does not reflect an inability of the liver to excrete the enzyme via the biliary duct. In liver disease the increase in serum levels result from increased liberation from the sinusoidal surface of the liver cell and regurgitation of biliary isozyme back to the serum (73).

Serum alkaline phosphatase in a normal human is assumed

to be regulated primarily by output from the liver and osteoblastic activity in the bone. The enzyme in circulation is metabolically inert (90). Enzyme levels are fairly stable in normal individuals and the fractions due to different organs vary little. The urinary removal is moderate compared to circulating amounts and there is apparently no correlation between circulating enzyme and the activity excreted in the urine. Urinary alkaline phosphatase migrates at a much faster rate in an electrophoretic field than kidney, liver, bone, small intestine or serum fractions and may consist of kidney enzyme which has been altered after its release into the urine (183).

There are two theories explaining the origins of circulating alkaline phosphatase isozymes: the "retention theory" that explains elevations as failure of excretion through the biliary tract of enzyme that has been produced in the bone, and the "regurgitation theory" which explains increased serum levels as due to circulation from bile by way of communication between bile caniculi and the sinusoids (189).

In 1962 studies were conducted on the sera of subjects with liver and bone disease by means of starch gel electrophoresis. Although different bands were attributed to the diseases, it was thought that the liver excreted alkaline

phosphatase derived from bone origins (28). In 1963, three alkaline phosphatase isozymes were demonstrated - bone, spleen and liver. Ultracentrifugation techniques showed that heterogenous fractions have similar molecular weights (19). Using paper and starch block electrophoresis, serum alkaline phosphatase was localized in zones corresponding to alpha-1, alpha-2 and beta-globulins (166).

The bone isozyme is associated histochemically with the osteoblasts. In analysis of 2000 sera, increased bone isozyme levels were found accompanying skeletal disease (189). Studies by Kaplan and Rogers, using polyacrylamide gel showed the separation of four bands of the enzyme: bone which is the slowest moving band and which is more diffuse than the others, liver and the rapidly-moving intestinal isozyme. A fourth band remained at the origin (87). The origin of liver or bone disease was predicted with 90 per cent accuracy by this method. Using polyacrylamide gel electrophoresis it was demonstrated that neither liver or bone isozyme is inhibited by the presence of fluoride at physiological levels (6). One other isozyme has been demonstrated in the rat, where injections of isoprenaline gave a rapid rise of alkaline phosphatase in the right atrium. This isozyme has a low thermostability and resembles that derived from bone (109).

Immunologically there are three antigenic classes of alkaline phosphatase. The first class includes isozymes of liver, bone, spleen and kidney. The second class includes the intestinal isozyme and the third includes the placental isozyme. The second and third classes appear to be partially cross-reactive. There are probably three genetic loci operating in the production of human alkaline phosphatase isozymes (177). Studies by immunological techniques are based on the ability of specific enzyme-antienzyme precipitates to catalyze chromogenic substances to demonstrate the presence of cross-reacting enzymes (115).

Thermostability differences in various subcellular structures indicate that the isozymes of alkaline phosphatase are localized in different compartments. Variations in the stability of the enzyme may be due to its being bound more or less firmly to the cell membrane. Studies indicating the presence of more than one isozyme in serum are based on the demonstration of a biphasic inactivation curve at higher than physiological temperatures. If under conditions of elevated temperature, a remaining enzyme activity is stable, the presence of at least two enzyme types is established. This does not rule out the possibility of unknown factors which may protect the alkaline phosphatase activity or enhance the inhibitory effects of heating.

These factors may be removed by gravitational effects during centrifugation (145).

The different isozymes have varying inactivation rates in response to heat. Bone is more heat labile than the other fractions and can be differentiated by preincubation of serum at 56 degrees centigrade for 10 minutes before assay. Various studies differ in the amount of inactivation observed. Bone isozyme inactivated under the above conditions was found to retain from two to 20 per cent of its original activity and liver isozyme retained from 20 to 40 per cent (165). After inactivation for 10 minutes at 56 degrees other investigators found that bone isozyme was inactivated to two-thirds of normal activity while liver was stable and not inactivated (99). There has been wide disagreement among investigators on heat denaturation methods.

In thermostability studies comparing alkaline phosphatase levels for subjects including normals, and those with bone and hepatobiliary disease, it was found that in normal subjects the thermostability was 30.7 per cent of orignal activity. In hepatobiliary disease the thermostability was 30.4 per cent and in bone disease it was 21.8 per cent. It was concluded that total alkaline phosphatase levels could be within normal range while masking an elevation or decrease in isozyme levels (147).

Alkaline phosphatase is inactivated, *in vitro*, by a low pH. There is first a configurational change to a different but still active form and then further changes to an inactive form. This change may be due to zinc loss and dissociation into subunits. These subunits are unstable and liable to irreversible denaturation. When not undergoing irreversible change, the subunits reassociate to the inactive form when pH is raised and slowly change back to the active form. This reaction proceeds more rapidly at greater pH levels (150).

Different inhibitors act on the various isozymes of alkaline phosphatase. L-homoarginine inhibits liver isozyme but not bone, placenta or the intestinal fraction. L-histidine inhibits liver isozyme preferentially and L-tryptophan and L-phenylalanine inhibit placental and intestinal isozyme (53). The latter amino acids have similar action on the isozymes and are stereospecific. The inhibitory action is independent of pH but dependent on substrate concentration up to 5.0 millimolar after which concentration increases have little effect. The number of inhibitor molecules combined with one active site of alkaline phosphatase is 1.02 to 1.04 for bone and 0.90 to 0.94 for liver. A possible hydrophobic binding action with uncompetitive and organ specific inhibition is suggested (97).

Alkaline phosphatase, particularly the bone isozyme, is inhibited by urea. Urea has two effects, reversible and instantaneous inhibition and irreversible time dependent inhibition. The latter inhibition occurs at higher concentrations and is probably due to protein denaturation. The reversible inhibition is noncompetitive. The inhibition of serum alkaline phosphatase resembles that of the tissue of origin (13). Urea is known to rupture the hydrogen bonds which cause the polypeptide chains to unfold and results in deformation of the molecule. At higher concentrations there is more complete unfolding and greater loss of activity (8).

SUBSTRATE SPECIFICITY

A number of substrates are used in vitro to measure the activity of alkaline phosphatase and substrate requirements are relatively nonspecific. Among the substrates used are beta-glycerophosphate, para-nitrophenylphosphate, phenylphosphate, indoxylphosphate, beta-napthylphosphate, phenolthaleinphosphate and methyl umbelliferylphosphate. The substrate specificities are similar but not identical for the isozymes and there are differences in the Michaelis constants among them (120).

Nucleotide phosphates are hydrolyzed by only certain fractions of alkaline phosphatase and 5' nucleotidase has been recommended as a means for differentiating between

liver and bone isozyme since elevated activities occur in liver disease but serum nucleotidase remains normal in bone disease. Liver enzyme has a marked preference for AMP, especially in the presence of added substrate. It has been demonstrated that the minor bands of phosphatase activity of liver, intestine and kidney are the same as those of major electrophoretic bands indicating that each organ may elaborate a single alkaline phosphatase which subsequently may undergo structural change and have substrate preference (183).

Homogenates of intestine and hypodermis hydrolyzed sodium para-nitrophenylphosphate twice as fast as they did beta-glycerophosphate. The difference when compared with other substrates indicate that para-nitrophenylphosphate is the substrate of choice in the analysis of the enzyme (22).

EFFECT OF DIET

Reduction of calcium or phosphorus in the diet lowers total alkaline phosphatase activity in the serum. Primarily affected is the bone isozyme, with little effect noted on either liver or intestinal isozyme (164).

A correlation was found, in rats, between an increased level of alkaline phosphatase in the serum and high dietary lipid intake. Increased dietary fat primarily affected the intestinal isozyme. It could not be determined whether the

enzyme levels were raised in the presence of highly absorbable fat due to increased requirements for alkaline phosphatase in the resynthesis of triglycerides in the intestinal mucosa, the increased rate of transport by micelles through the intestinal epithelial cells or the increase in the secretion of bile salts as a result of stimulation by fat (164).

Using two mutants of E. coli lacking the ability to synthesize oleate from acetate, it was demonstrated that oleate must be supplied during alkaline phosphatase induction. This permits the formation of the active dimer in the periplasmic space where the dimer is formed by association of monomers. Monomers released from the polyribosomes accumulated temporarily in the cytoplasm before they moved across the cell membrane. It is suggested that vesicles of the membrane may carry the monomers outside the cell membrane. The cessation of alkaline phosphatase formation is probably not due to loss of protein synthesizing activity but to enzymes caused by unsaturated fat deficiency. De nova synthesis of lipid is involved in the development of enzymic activity. It is not known whether alkaline phosphatase accumulates during starvation (80).

Regular application of vitamin A to the tail skin of mice alters epidermal keratinization and causes strong

alkaline phosphatase activity to appear in the peripheral cytoplasm of basal and prickle cells. Similarly stimulated human epidermis shows changes in keratization but no increase in alkaline phosphatase. Vitamin A could stimulate synthesis or may be a cofactor causing aggregation of inactive subunits or reduction of enzymic breakdown. Mouse studies indicate that vitamin A specifically induces DNA-directed synthesis of alkaline phosphatase. Vitamin A stimulated increases of RNA synthesis and might be expected in cells which synthesize large amounts of the enzyme. Some of the effects of vitamin A in bone formation, lipid deposition and transport might be mediated through alkaline phosphatase since it is required for phosphate transfer. Alkaline phosphatase also is increased by vitamin A in certain protein secreting cells having membrane-bound ribosomes and may be concerned with membrane phospholipid metabolism. There is an increase in the enzyme levels in hypervitaminosis A in tissues other than the epidermis (142).

Intestinal alkaline phosphatase from rats fed a rachitic diet showed different electrophoretic mobilities, Km for para-nitrophenylphosphate at pH 10.0, curve of inactivation by magnesium and sensitivity to fluoride. Additions of vitamin D_3 did not prevent this alteration. These rats were fed wheat germ which had been extracted with ethanol

and it appeared that the deficiency was due to some factor in wheat germ which was lost after extraction and which was responsible for enzyme alteration. The enzyme from rachitic animals showed a higher affinity for substrate than normals. When unextracted wheat germ was used, enzyme behavior was normal (105).

Zinc-induced deficiency in rats led to a reduction in kidney and intestinal isozymes while addition of zinc in the form of zinc acetate increased enzymic activity. In the presence of zinc acetate all intestinal alkaline phosphatase reached the same level in deficient rats and pair-fed controls. The zinc deficient rats produced the apoenzyme of alkaline phosphatase which was activated with the addition of the cation to the diet (64). There are contradictory reports on the effects of zinc deficiency on alkaline phosphatase. When severe zinc deficiency was induced and enzyme levels measured in the stomach, kidney and intestine it was concluded that the primary action of zinc is its effect on turnover in the target organ (77).

CHAPTER II

PLAN OF PROCEDURE

INTRODUCTION

The present study was concerned with alkaline phosphatase activity, particularly the bone isozyme, in sera as effected by inorganic fluoride. In addition, the direct response of bone to various levels of inorganic fluoride was studied by tissue culture methods, as a means of confirming the direct relationship between alkaline phosphatase and bone.

This study is part of continuing research at Texas Woman's University on the mineralization of bone as effected by various dietary supplements. The participants in the study were residents of Travis State School, an institution for the mentally retarded located in Austin, Texas.

SUBJECTS

Screening procedures were carried out for six months prior to the beginning of the study and 300 individuals were screened initially for suitability to the needs of the study. Subjects were divided into groups of approximately 40 individuals each for a control group and those to be used for experimental therapy. The subjects were selected

on the basis of low bone density together with overall physical condition. Individuals with overt disease were excluded from the study. The subjects were between the ages of 50 and 80 years.

The subjects used in the present study were divided into two groups: Group I received sodium fluoride (Davies-Rose-Hoyt 100-OSTEO (F) TROLCAPS) in the following dosages:

Body weight less than 135 lbs-- 20 mg fluoride ion/day
Body weight 135-175 lbs -- 30 mg fluoride ion/day
Body weight more than 175 lbs-- 40 mg fluoride ion/day

Group II received one teaspoon of a placebo (Emerson Laboratories Syrpalta) daily (137). Possible side effects of the inorganic fluoride administered were monitered by periodic urinalyses and serum fluoride analyses.

The bone used in tissue cultures was obtained from two subjects during autopsy. One sample was obtained from a 22 hour old premature infant with respiratory failure but otherwise in normal condition. The other sample was from a 29 day old infant which had expired due to complications of Herpes simplex.

DIETARY ASSESSMENT

A record of the menus in use during the study was obtained from the state nutritionist. Size of servings and plate wastes were observed and recorded. The menus for Travis State School are planned in three month cycles by

a Therapeutic Dietitian and evaluated for nutrient content according to the Recommended Dietary Allowances of the Food and Nutrition Board of the National Research Council. Menus for all state institutions are centrally planned and distributed to the Food Service Managers in each facility for use in food purchasing. Provisions are made to adapt the menu for special diets. A Food Service Supervisor is assigned to each dining hall to encourage quality food control and quality patient care.

The complete dietary served during the period of this study was assessed and allowances were made for trimming and cooking losses. Protein, calories, vitamin A, thiamine, riboflavin, ascorbic acid, vitamin D, calcium and iron were recorded on a quarterly basis and daily averages were calculated from these totals. Meats were divided as to source, vegetables as to green or yellow and fruits as to citrus, dried or other type.

PROCEDURE

COLLECTION OF SAMPLES

At intervals of approximately monthly, blood samples were collected by venipuncture and stored in tubes which were immediately placed in an ice bath and maintained between zero and five degrees centigrade until the completion of centrifugation. Sera were separated and stored

in a freezer until the samples were thawed for analysis. Samples which had been treated in this manner and analyzed for alkaline phosphatase activity indicated no loss in activity (137).

Samples for bone tissue culture were collected on autopsy immediately after death. Dissection of the rib cage was performed by a qualified pathologist and a section of excised rib placed in a sterile container. The rib section was placed in a hood previously exposed to ultra-violet light and cut into appropriate slices by means of a bone knife which had been sterilized by five applications of absolute ethanol. Bone slices were placed in vessels with Basal Salt Solution containing 100 µg Neomycin sulphate per milliliter, which had been previously sterilized. The samples were washed free of adhering tissue and transferred to sterile culture bottles.

ANALYTICAL TECHNIQUES

Alkaline Phosphatase-General Procedure

Total alkaline phosphatase (orthophosphoric monoester phosphohydrolase) in serum was measured by the established procedure involving incubation of the sample containing the enzyme with para-nitrophenyl phosphate as the sbustrate and the subsequent measurement of p-nitrophenol released (154). The general reaction is:

$$\text{p-nitrophenyl phosphate} + \text{water} \xrightarrow[\text{Alkaline phosphatase}]{\text{pH } 10.5} \text{p-nitrophenol} + H_3PO_4$$

The compound p-nitrophenyl phosphate is colorless but upon hydrolysis of the phosphate group, the yellow salt of p-nitrophenol is liberated and the product is yellow in a basic medium. The intensity of this product can be determined spectrophotometrically. The amount of product formed in a given time is indicative of the rate of hydrolysis and is a measure of alkaline phosphatase activity. Studies using various substrates in the measurement of alkaline phosphatase have indicated that p-nitrophenyl phosphate is the substrate of choice (176).

The stored samples were thawed. A 0.5 ml aliquot of alkaline substrate buffer (glycine-0.1 M, magnesium chloride-0.001M, chloroform to saturate, adjusted to pH 10.5) and 0.5 ml p-nitrophenyl substrate were pipetted into tubes and allowed to equilibrate in a water bath set at 37°C for five minutes. A 0.1 ml aliquot of serum was added to the sample tubes and a blank was prepared with 0.1 ml distilled water. Incubation at 37° C was allowed to proceed in the water bath for 30 minutes. Exactly 30 minutes after the addition of the serum, 10 ml of 0.02 N sodium hydroxide was added to each tube to stop the color reaction and the

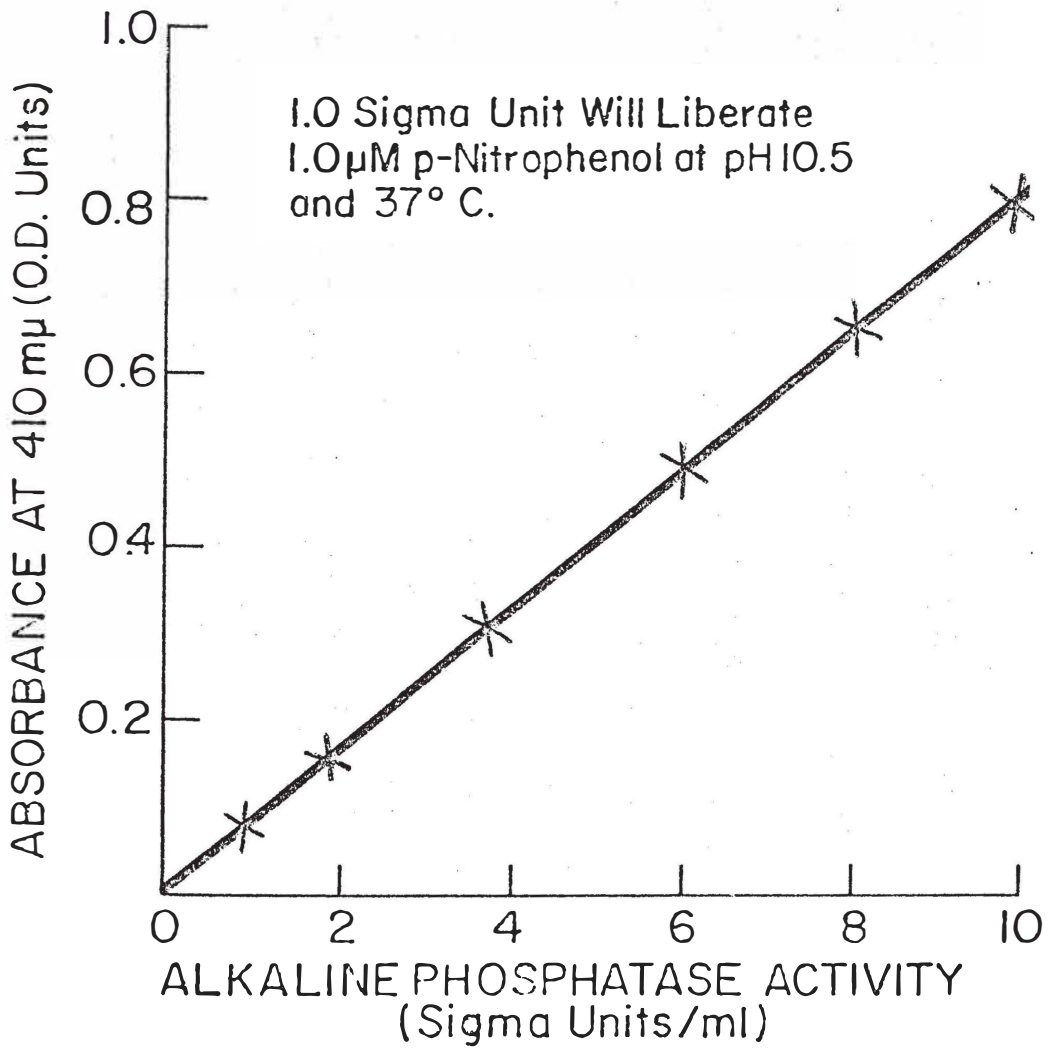
tubes were mixed thoroughly by inversion. The absorbance, expressed in optical density units, was read on a Gilford 300-N spectrophotometer set at 410 mu and recorded. After all the samples were read, 0.1 ml of concentrated hydrochloric acid was added to each tube to remove the color due to the p-nitrophenol and the samples were re-read at 410 mμ to determine the amount of color due to the sample. The second reading was subtracted from the first and the resulting optical density units were compared with a standard curve.

The standard curve was prepared using a stock standard solution of p-nitrophenol containing 100 millimoles per liter. A 0.5 ml aliquot of the stock standard solution was pipetted into a 100 ml volumetric flask and diluted to 100 ml with 0.02 N sodium hydroxide. The contents were mixed thoroughly and six aliquots of 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 mls were pipetted into test tubes. Each of these tubes was brought to a total volume of 10 ml by the addition of 0.02 N sodium hydroxide. These samples were equivalent to 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 Sigma units per ml of alkaline phosphatase. The samples were then read at 410 mμ against an 0.02 N sodium hydroxide blank and the results recorded (Figure 1).

Reagents were obtained from Sigma Chemical Company

FIGURE 1

ALKALINE PHOSPHATASE ACTIVITY
IN SIGMA UNITS PER MILLILITER



and the activities were recorded in Sigma units per milliliter. One sigma unit of alkaline phosphatase will liberate one micromole of p-nitrophenol per hour under the specified conditions (1 μM = 0.1391 mg). The range of alkaline phosphatase activity in the healthy adult, as determined by the above procedure, is between 0.8 and 2.3 Sigma units per milliliter.

Separation of Isozymes

The existence of more than one form of alkaline phosphatase was first demonstrated by paper electrophoresis (9). After separation the paper was cut into strips and incubated in a suitable buffer-substrate mixture. The isozymes were found to migrate with the α_2 globulins. With this technique, the number and intensity of the bands varied according to the method of extraction from the tissues.

Various other electrophoretic techniques were employed using other supporting media including starch block, starch gel, agar gel, agarose gel, cellulose acetate strips, Sephadex gel and Pevikin C-870 which is a copolymer of polyvinyl chloride and polyvinyl acetate (175).

On starch-block support medium the migration of alkaline phosphatase resembles that on paper, migrating in the zone of α_2-globulin. An additional band is observed in the region of β-globulin. Agar gel electrophoresis

also resembles that on paper except there is the separation of three bands of activity. One band is associated with alkaline phosphatase of bone origin while the other two are derived from the liver.

With the development of starch-gel electrophoresis there was better resolution of the isozyme bands and alterations in staining intensity. These bands could be correlated with certain diseases of either liver or bone origin.

Separation of the isozymes of alkaline phosphatase of E. coli has been demonstrated using ion-exchange chromatography with DEAE cellulose columns. Sephadex-100 has been used as a medium for the separation of human serum and tissue alkaline phosphatases. The above methods all varied with the extraction procedures and were subject to differences in interpretation. Until polyacrylamide gel came into general use, cellulose acetate electrophoresis gave the best resolution for isozyme separation (183). Cellulose acetate permits a rapid analysis which gives a sharp separation of liver, bone, and intestinal isozymes (54).

Polyacrylamide gel is the method of choice in the separation of the isozymes of alkaline phosphatase because it achieves a sharper separation between bands and permits the use of smaller samples than other techniques. Resol-

ution by this method produces a greater degree of reproducibility and more sharply defined bands (85). The use of this technique should be suitable for work in clinical laboratories for the diagnosis of disease states and for accurate evaluation of the physiological condition of bone (32).

Alkaline phosphatase isozymes in the present study were separated using the EC 250 Gel Column Electrophoresis System. The standard unit accepts from one to 12 columns with standard columns of 5 mm internal diameter and 80 mm in length. The apparatus consists of two tanks. The outer tank forms the lower buffer compartment and has a capacity of from 700 to 1200 ml. The larger amount of buffer is used when heat labile samples are being separated. The inner buffer tank has a self supporting center post, 12 grommeted holes for the support of the gel columns and the platinum electrodes for electrophoresis. This inner tank has an approximate capacity of 350 ml. A safety interlock insures that the lid must be in place before the power supply can be connected and activated. The electric field varies from two to five milliamperes per tube depending on the complexity of the analysis (43).

Preliminary studies were conducted to determine the amount of sample required, suitable power setting and

appropriate concentration of gel. Various concentrations of gel have been used in different studies ranging from five per cent to eight per cent (23, 36, 179).

Samples were thawed immediately prior to use and were mixed with equal volumes of 40 per cent sucrose solution to give a density which allowed the sample to rest immediately on top of the gel column. A Tris-Maleic acid buffer was prepared (Appendix A) and stored at 4^{o} C.

A 5.0 per cent Cyanogum-41 gel was made up in the Tris buffer with 0.1 ml of N,N,N,N-Tetramethylethylene-diamine (TMED) added for each 100 ml of gel solution. Just before the gels were poured into the columns, 100 mg of ammonium persulfate was added for each 100 ml of solution to initiate the solidification of the gel.

Electrophoretic columns were placed in a rack with the bottom end of each wrapped securely with Parafilm to prevent leakage. Gels were transferred by means of a disposable pipette and were poured to within one centimeter from the top of the column. Approximately 20 minutes were required for the gel to solidify at which time the Parafilm was removed from the bottom of the column and the columns were placed in the buffer rack. Two drops of distilled water were placed on top of each column. Samples were then applied in the amount of 20 μl directly on top

of the gel and underneath the water layer. Columns were "topped" with cold buffer to prevent air being trapped between the sample and buffer. The lower buffer tank was filled with 1200 ml of Tris buffer, the column holder placed in the tank and the top tank filled with enough buffer to completely cover the tops of the columns. The power source was connected and an initial setting of one milliampere per tube was maintained for five minutes. The setting was then increased to three milliamperes per tube for two hours.

The gel column was removed by injecting distilled water down the side of the column and catching the gel in a test tube. The gel column was then placed in a 10 ml test tube with the top of the gel up and 10 ml of the incubation mixture was added to each tube (Appendix B). The incubation mixture was made up immediately prior to use.

Gel columns were allowed to incubate in a water bath at 37^{o} C for one hour. After incubation they were removed and washed repeatedly with five per cent acetic acid to remove the color due to the incubation mixture. The resulting column exhibited brown bands corresponding to the alkaline phosphatase isozyme fractions. At this time the columns could be stored in a tightly closed container in five per cent acetic acid solution.

A standard alkaline phosphatase Type I (Sigma Chemical Company) was run for a marker as well as for quantitation. The alkaline phosphatase standard was obtained from calf mucosa and had an activity of 1.0 unit/mg (1 unit will hydrolyze 1.0 μm of p-Nitrophenyl phosphate per minute at pH 10.4 and 37° C). All samples were run in duplicate.

In preliminary studies the isozyme fractions were eluted from the gel column for analysis. The objection to this method was that the bands cannot be seen until after incubation and when bands are eluted from unstained columns part of a band or additional bands would be missed. For this reason a Photovolt Densitometer was adapted for reading the isozyme bands present on the gel columns as a unit. A carrier was made which would ride directly beneath the slit and the density of the bands was determined colorimetrically using a 505 mμ filter. The densitometer was equipped with an integrator unit and areas were determined for each peak on the column. When present, the liver isozyme and intestinal isozyme was recorded in addition to that from bone. By this method, highly reproducible results were obtained.

Heat Denaturation Technique

Heat denaturation was carried out on samples from both the control and fluoride treated group. This was

done for the purpose of measuring residual activities in the samples and comparing these activities with bone isozyme levels and related parameters. Heat denaturation has been the major technique by which the isozymes of alkaline phosphatase have been identified for diagnosis of related diseases and it was thought to be of interest to compare the accuracy of this method with that of actual isozyme determinations.

Initially pooled serum from each group was incubated at various temperatures and times of incubation and after response was noted individual samples were denatured and residual activity of alkaline phosphatase measured. Denaturation was carried out in a carefully controlled water bath and alkaline phosphatase activity determined according to the procedure outlined above. The residual activity was calculated for samples from both groups.

Transaminase

Serum glutamic-oxaloacetic transaminase (SGO-T) was determined on samples from both groups. The procedure used was a spectrophotometric method involving the rate of conversion of reduced nicotinamide adenine dinucleotide to the oxidized form (155).

Bone Density Measurements

In the radiology unit of Travis State School, X-rays

were taken of the os calcis and phalanx 5-2 of the subjects under controlled exposure conditions. All procedures were conducted by registered X-ray technicians who are supervised by a board of certified Radiologists. Quantitative analysis of bone mineralization was determined by use of a photometric computing apparatus consisting of a microphotometer, recorder, digital computer, analog-to-digital converter, and teletype for output of data.

Tissue Culture Method

Preliminary investigation in the present study was conducted on the femurs of two 90 day old male albino rats. The purpose of this preliminary work was to develop satisfactory techniques leading to a viable sample.

The development of sterile techniques and the choice of media are the two most important factors in cell and tissue culture. All glassware and equipment used in the experiment including the millipore apparatus used for sterilization of the media was autoclaved in a Castle unit after being thoroughly washed and rinsed in triple distilled water. Sterilization was achieved by using a pressure of 12 psi and temperature of 250° C for 15 minutes. Temperature sensitive tape was used and all openings securely wrapped and taped to insure sterilization.

Serum and synthetic media cannot be autoclaved due

to their thermolabile properties and must be sterilized by filtration. Protein solutions are best filtered using positive pressure since vacuum pressure can cause foaming and result in denaturation. Positive pressure was obtained using a mechanical pressure pump.

The previously sterilized millipore filter was used to filter all solutions except those which could be autoclaved such as the water. Millipore filters are manufactured from cellulose esters and have the advantage of not altering the pH of the filtrate. A 0.2 millipore filter was used which removes all contaminants except the nonfiltrable viruses.

In preliminary studies on rat femurs, Eagles Essential Medium was used (Appendix C). While it was demonstrated that the bones could be cultured in this medium, a different synthetic media was considered necessary for maximum length of bone life *in vitro*. The media of choice for the rib section cultures was McCoy's 5A Medium, described in Appendix D. The differences in the media are mainly in the addition of nutrients in McCoy's 5A Medium. Amino acids contained in McCoy's 5A Medium that are not included in Eagles Minimum Essential Medium are L-alanine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glycine, L-Isoleucine, L-serine, L-hydroxyproline, L-proline.

Other additions to McCoy's 5A Medium are p-amino-benzoic acid, ascorbic acid, D-biotin, vitamin B_{12}, and glutathione bacto-peptone.

Concentration of the media was 12 grams per liter and it was made up in a balanced salt solution specifically formulated for mammalian cell lines. Earle's Balanced Salt Solution (Appendix E) was used to provide the proper pH, osmotic pressure and nutrients for the survival of the culture.

Tissue culture is sensitive to a variety of environmental factors including temperature, osmotic pressure, hydrogen ion concentration, other inorganic ions and essential nutrients including carbohydrates, amino acids, vitamins, proteins, and dissolved gases.

Mammalian cells are rapidly destroyed by temperatures in excess of physiological level which is between 37 and 38.5^{o} C. If the temperature is raised by eight degrees, the tissue culture will die within an hour (117). It is important that the media be kept at this physiological temperature. Cooling has much less effect than heating and a drop of as much as 15 degrees may produce only a deccelerated rate.

Osmotic pressure of the medium is critical and for mammalian cells the normal osmotic pressure at 37^{o} C is

approximately 7.6 atmospheres. Cells will withstand a variation of no more than 10 per cent. The pH of biological fluids has to be near neutrality to permit survival of the tissue. The media in the present study was adjusted to pH 7.4 and monitored daily. On standing the pH of the media will decrease and fresh media must be added to the culture.

In most animal cells the inorganic ions which are required are sodium, potassium, calcium, magnesium, carbonate, phosphate and probably sulfate. The function of some of these ions is obscure.

A source of carbohydrate is essential to cultured cells and the most commonly used source is glucose. Most animal cells have a specific requirement for 12 amino acids--arginine, cystine, histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, tyrosine, lysine, and valine. In the intact animal, cystine and methionine can replace each other and cystine is not considered necessary, but in cell cultures cystine is required for growth irrespective of methionine levels. Similarly phenylalanine is not transformed to tyrosine and therefore the latter must be added to the medium. Most cells have a high requirement for glutamine and as culture medium ages this may have to be added as a supplement. Several vitamins

are necessary for cell growth, most of them are involved in metabolism in the form of coenzymes.

Both oxygen and carbon dioxide are essential for cell survival. These gases are usually supplied to tissue culture in the amount of five per cent carbon dioxide with the balance made up of oxygen.

Besides the synthetic medium, cell and tissue cultures require the addition of a natural fluid. Bovine serum was added to media in the present study in the amount of 15 per cent of the total volume. Serum was thawed at room temperature and filtered through the sterilized millipore filter. Prior to filtration the serum was placed in a 56^{o} C water bath for five minutes to inactivate the alkaline phosphatase activity present which might mask the activity due to the cell cultures.

After the medium was filtered, penicillin-streptomycin and neomycin sulfate were added at concentrations of 100 µg/ml and 160 µg/ml respectively. This antibiotic concentration effectively inhibits bacterial growth (180).

After the components of the culture medium were well mixed, the solution was transferred in 200 ml aliquots by sterilized teflon tubing into previously autoclaved Erlenmeyer flasks. One flask of media was used as a control and varying amounts of inorganic fluoride were added to

the others. Fluoride ion levels were : 1.0, 10.0, 20.0 and 50.0 parts per million. Besides the bone cultures incubated without addition of fluoride, a media sample was incubated by itself and alkaline phosphatase activity measured.

All materials were prepared in advance and media was stored in the refrigerator. Sterilized equipment was kept tightly wrapped under a hood equipped with an ultraviolet light. The bone knife was kept immersed in last absolute ethanol wash until just prior to use. Sterile surgical gloves and mask were worn at all times when handling the bone slices or media.

After the rib section was excised during autopsy it was immediately placed in a sterile container. As this bone section was essentially non-sterile and contamination was expected to be heavy, a method of rendering the tissue sterile was necessary. The bones were first placed in Earle's Basal Salt Solution, which had been sterilized and contained 100 μg neomycin sulfate per milliliter, and washed free of adhering tissue. The bone was then washed three times in McCoy's 5A Medium containing 15 per cent bovine serum all being previously sterilized and antibiotics were added as described in Appendix E. The tissue was then immersed in fresh sterile media, transferred to a sterile container and was ready for sectioning. The bone was cut

into sections with the bone knife and weighed in pre-weighed sterile containers on a Mettler balance. These sections were immediately transferred by means of sterile forceps to 30 ml culture bottles containing 10 ml of the appropriate media. Two sections each were placed in the control media (0 ppm) in separate bottles and two in each of the media containing the four levels of fluoride.

Screw caps were placed on the culture bottles very loosely and the bottles were placed in an incubator which was electrically heated with oxygen and carbon dioxide circulation with the chamber. The bottles were placed at angles of four degrees to allow the sample to be bathed in media without drying.

Temperature was checked several times a day and the incubator variation was ± 0.25 degrees. Media was checked regularly to detect a decrease in pH.

Media was changed by means of extraction of the used media with a previously sterilized disposable pipette with a cotton filter. New media was added in the same manner. The media which was removed was placed in test tubes and stored in the freezer for later alkaline phosphatase and isozyme analysis which was accomplished by the method previously outlined using 20 µl aliquots of the media.

At the end of the culture period bone sections were

removed from the culture bottles. One section of each fluoride level and control was saved for alkaline phosphatase analysis and the other used for histological staining.

The bone section used for alkaline phosphatase determination was homogenized with 20 ml of cold glycine buffer (see alkaline phosphatase-general procedure), two ml of p-nitrophenyl phosphate was added to a 2 ml aliquot of the homogenate and the reaction mixture was incubated for 30 minutes at 37° C. A 0.2 ml aliquot of the incubation mixture was added to 3.0 ml of 0.02 N sodium hydroxide and read on a Gilford spectrophotometer at 410 mμ (169).

The remaining bone section was fixed in absolute ethanol for 24 hours. It was then placed in distilled water for one minute, and then placed in a five per cent solution of silver nitrate for two minutes. The samples were rinsed with distilled water for one minute and exposed to ultraviolet light for approximately 10 minutes. When blackening occurred the samples were treated with a five per cent sodium thiosulfate solution for one minute and rinsed with distilled water. Dehydration was accomplished by successive immersion in 70, 95, and 100 per cent ethanol. The sample was then cleared in xylol and photographed.

Serum Fluoride Determination

Serum samples were diluted 2:1 with a high ionic

concentration buffer to produce a constant degree of ionization. Serum fluoride concentration was measured by means of a special ion activity electrode (Orion Model 90-09) with a single junction reference electrode. The readout was displayed on a digital pH/millivolt meter. The electrode measures activity which is then converted into concentration units. Calibration curves were made from standard solutions of sodium fluoride and glass distilled water. The same buffer was used for serum dilution and standard preparation.

CHAPTER III

PRESENTATION OF DATA

Previous studies have demonstrated that the administration of inorganic fluoride results in increased alkaline phosphatase levels in the sera (37, 64, 104, 118, 182). The present study was conducted to investigate the nature of this relationship. The purpose of this study, specifically, was to measure the effects of inorganic fluoride on the metabolic activity of bone by total alkaline phosphatase and bone isozyme in the sera, and to observe the response of bone to inorganic fluoride *in vitro*.

The samples include sera from geriatric subjects who were divided among two groups of approximately 40 each: a control group which received a daily placebo and a fluoride treated group receiving between 20 and 40 milligrams of fluoride ion per day depending on body weight.

The *in vitro* studies consist of two sets of tissue cultures of rib sections: one from a 22 hour old infant and the other from a 29 day old infant. The bones were treated with varying levels of inorganic fluoride in the culture media.

Statistical data are presented comparing total alkaline phosphatase and bone isozyme response to inorganic fluoride

in vivo and *in vitro*. Comparisons are made to related parameters such as serum fluoride levels and bone density as measured by aluminum equivalency. Total serum alkaline phosphatase levels and bone isozyme levels are compared, by weeks, in both groups when the fluoride treated group was on therapy, after discontinuation of therapy and when treatment was resumed. Heat denaturation results are presented. In addition, the liver isozyme activity of alkaline phosphatase is compared between control and the fluoride treated groups to rule out the possibility that liver disease or liver damage could be a contributing factor to elevated total alkaline phosphatase levels.

EFFECT OF FLUORIDE ON TOTAL ALKALINE PHOSPHATASE IN SERA

Total alkaline phosphatase activity in the sera of individuals in the control and fluoride treated groups at specific intervals during the study are presented in Tables 1 and 2, respectively. Weeks for which levels are reported are week 95, 107, 120, 130 and 143. These weeks correspond to the following:

Week 95 - subjects had received fluoride therapy since week 1.

Week 107- subjects had received fluoride therapy since week 1 and fluoride was discontinued.

Week 120- fluoride had been discontinued for 13 weeks.

TABLE 1

TOTAL ALKALINE PHOSPHATASE* IN THE SERA OF GERIATRIC MALES RECEIVING DAILY PLACEBO ADMINISTRATION (CONTROL GROUP)

Subject Number	Week of Study				
	95	107	120	130	143
1	1.92	1.43	1.74	1.85	1.62
2	1.62	1.82	2.22	2.00	1.85
4	2.63	2.06	2.18	2.20	2.00
9	2.18	2.00	1.55	2.09	2.31
15	2.93	3.25	2.83	2.96	3.53
16	2.19	2.29	1.50	1.94	1.68
19	3.62	2.29	3.18	--	4.41
20	2.93	--	--	--	--
21	2.28	1.98	4.09	1.94	--
26	--	--	2.16	1.99	1.70
31	1.35	1.31	1.26	1.14	2.12
34	2.66	2.36	2.72	2.43	2.14
35	2.79	3.29	3.20	2.52	0.99
60	2.31	1.82	1.74	2.46	2.16
63	2.16	1.75	1.98	2.30	1.83
65	2.28	2.05	2.39	2.19	1.95
66	2.40	1.49	1.72	2.23	2.08
68	2.01	2.29	2.11	1.49	1.74
85	2.60	3.05	2.35	2.72	2.77
92	--	2.09	2.07	2.34	1.66
96	2.03	1.91	1.81	2.16	1.70
103	--	1.94	1.61	1.65	1.47
112	2.53	--	1.41	2.60	2.25
113	2.07	2.29	2.37	1.62	0.50
116	2.21	2.16	2.59	2.29	1.89
118	2.49	2.05	2.11	2.41	1.55
125	1.65	1.63	1.33	1.33	1.95
131	2.45	2.18	3.55	3.05	2.46
142	--	--	--	3.20	2.90
144	2.72	2.70	3.64	2.51	3.00
146	1.67	1.67	1.42	1.54	1.24
148	3.04	3.02	2.83	2.63	2.92
161	3.01	3.60	3.26	2.37	3.07

TABLE 1 - Continued

Subject Number	Week of Study				
	95	107	120	130	143
162	2.62	4.45	2.44	2.54	2.88
163	2.63	3.07	1.98	2.05	1.45
164	3.56	3.86	4.13	4.37	--
167	2.87	2.58	2.39	2.60	2.63
168	2.12	2.32	2.59	2.43	2.71
171	3.04	3.07	2.61	2.62	3.26
173	2.56	--	2.74	2.64	2.73
183	--	--	1.37	1.87	1.20

* Activity expressed in Sigma units per milliliter (one Sigma unit will liberate 1.0 μM of p-nitrophenol/hour at pH 10.5 and 37^{o} C.)

TABLE 2

TOTAL ALKALINE PHOSPHATASE* IN THE SERA OF GERIATRIC MALES RECEIVING DAILY ADMINISTRATION OF INORGANIC FLUORIDE

Subject Number	Week of Study				
	95	107	120	130	143
3	1.75	1.72	1.17	2.02	1.60
11	1.80	2.00	1.63	2.33	--
22	2.77	2.62	2.09	2.72	2.71
28	1.94	1.72	1.55	2.11	1.93
29	2.93	2.34	2.05	1.81	2.42
37	4.60	3.82	3.35	2.50	3.26
38	5.29	5.36	5.88	--	6.55
39	3.49	2.57	3.03	--	--
44	2.84	2.60	2.76	2.72	3.40
46	2.07	2.24	2.11	2.57	3.28
47	2.40	2.72	2.15	2.11	6.72
50	3.23	3.72	2.79	2.98	--
54	6.18	7.26	5.22	6.14	1.89
57	3.31	3.25	2.24	2.09	1.68
67	1.11	1.26	0.68	1.42	1.22
69	2.12	2.63	1.87	2.63	2.73
70	2.36	2.39	2.59	2.37	4.52
75	--	--	1.89	1.92	--
83	3.50	3.60	3.27	2.72	3.19
84	6.31	8.58	4.98	--	--
87	2.74	--	--	--	--
89	2.83	4.40	2.57	2.63	2.90
93	2.72	3.24	1.67	3.15	3.32
95	2.69	2.83	2.28	2.68	3.97
97	2.87	3.02	2.46	3.07	--
99	2.86	2.66	2.15	2.07	2.50
107	2.19	2.60	2.39	2.52	1.91
109	4.04	4.18	2.83	2.55	2.79
110	2.45	2.45	3.24	2.50	3.19
117	4.87	3.65	--	--	--
119	3.55	3.97	2.78	3.70	3.74
122	2.42	2.72	1.81	2.78	2.86
124	2.68	2.72	2.61	1.85	2.46
129	2.80	2.90	3.16	2.41	--
137	5.40	5.60	5.85	5.18	6.20

TABLE 2 - Continued

Subject Number	Week of Study				
	95	107	120	130	143
149	--	3.07	2.79	2.83	3.89
150	2.07	--	2.53	2.28	2.50
153	1.62	2.18	1.78	2.66	2.60
156	--	3.16	3.29	3.83	--
157	4.84	4.79	4.37	4.57	5.46
170	--	2.03	2.15	2.61	3.36
174	2.71	3.36	4.48	3.48	2.75
177	3.44	3.00	3.20	3.39	3.72
178	2.74	3.46	2.31	2.87	3.23

* Activity expressed in Sigma units per milliliter (one Sigma unit will liberate 1.0 μM of p-nitrophenol at pH 10.5 and 37° C.)

Week 130- fluoride therapy resumed.

Week 143- fluoride therapy resumed for 13 weeks.

Control subjects received a daily placebo during the entire period (week 1 to week 143). Previous studies have shown that there is a high retention of fluoride when supplement is administered. After cessation of therapy, the fluoride is not released to any appreciable extent (159). However, when fluoride therapy is discontinued it is reasonable to suppose that resorption would increase without further deposition of fluoride and that bone would gradually demineralize.

Periodic comparisons of total alkaline phosphatase activities within groups (Table 3) show that in control subjects there is no significant difference between the mean enzyme levels of the different weeks of measurement. Variations in individual subjects may be accounted for by natural fluctuations in day to day enzyme levels.

When total alkaline phosphatase activities are compared by weeks in the fluoride treated group (Table 4), it is observed that there is no significant differences between the means, however standard deviation is greater in the fluoride group. This observation may be due to variation in response to treatment between individuals, while in the control group lower standard deviation reflects no treatment.

TABLE 3

STATISTICAL EVALUATION OF MEAN SERUM TOTAL ALKALINE PHOSPHATASE ACTIVITIES* OF SUBJECTS IN THE CONTROL GROUP THROUGH 143 WEEKS OF STUDY

Week of Study	Mean Alkaline Phosphatase ($\bar{x}$)	Comparison with Week	t	p
95	2.45 ± 0.51 (n=36)	107	0.52	NS
107	2.37 ± 0.72 (n=35)	120	0.23	NS
120	2.34 ± 0.75 (n=38)	95	0.76	NS
130	2.29 ± 0.57 (n=39)	95	1.30	NS (0.10)
		107	0.55	NS
		120	0.33	NS
143	2.18 ± 0.76 (n=39)	95	1.79	NS (0.10)
		107	1.13	NS
		120	0.93	NS
		130	0.72	NS

* Activity expressed in Sigma units per milliliter (one Sigma unit liberates 1.0 μM p-nitrophenol/hr at pH 10.5 and 37° C.)

TABLE 4

STATISTICAL EVALUATION OF MEAN SERUM TOTAL ALKALINE PHOSPHATASE ACTIVITIES* OF SUBJECTS IN THE FLUORIDE TREATED GROUP THROUGH 143 WEEKS OF STUDY

Week of Study	Mean Alk. P'tase ($\bar{x}$)	Comparison with week No.	t	p
95 (on F^- 95 wks)	3.11 ± 1.22 (n=40)	107	0.57	NS
107 (on F^- 107 wks)	3.28 ± 1.42 (n=41)	120	1.83	NS(>.05)
120 (off F^- 13 wks)	2.76 ± 1.16 (n=42)	95	1.35	NS(0.10)
130 (off F^- 23 wks)	2.79 ± 0.91 (n=39)	95 107 120	1.37 1.87 0.11	NS(0.10) NS(>.05) NS
143 (on F^- 13 wks)	3.25 ± 1.33 (n=34)	95 107 120 130	0.54 0.06 1.68 1.69	NS NS NS(0.10) NS(0.10)

* Activity expressed in Sigma units per milliliter (one Sigma unit liberates 1.0 μM of p-nitrophenol/hr at pH 10.5 and 37° C.)

Comparisons reveal that in terms of total alkaline phosphatase in the sera, there are significant differences between the means of the control versus the fluoride treated group (Table 5). These differences are consistent with those noted throughout the 143 weeks of the study. Examination of data on total alkaline phosphatase levels of the groups from week 0 (Appendix G) show that the group means at week 0 were 2.00 sigma units for the control group and 2.55 for the group to be treated with fluoride. In the control group the total alkaline phosphatase levels vary from week to week but never rise above 2.44 sigma units. When the other group began to receive fluoride therapy there was a consistent rise in the group mean of total alkaline phosphatase activity in the sera exceeding 3.00 sigma units for each week that measurements were taken. The exception to this elevation was in those weeks when fluoride therapy was discontinued during this study. The least difference between the group means was shown at 120 and 130 weeks, after the discontinuation of fluoride therapy. Figure 2 shows the frequency distribution between groups for weeks 107, 120, and 143. The distribution frequency for weeks 107 and 143 show slightly more spread than for week 120 which may indicate a higher frequency of subjects with increased enzyme activity in the fluoride treated group. At week

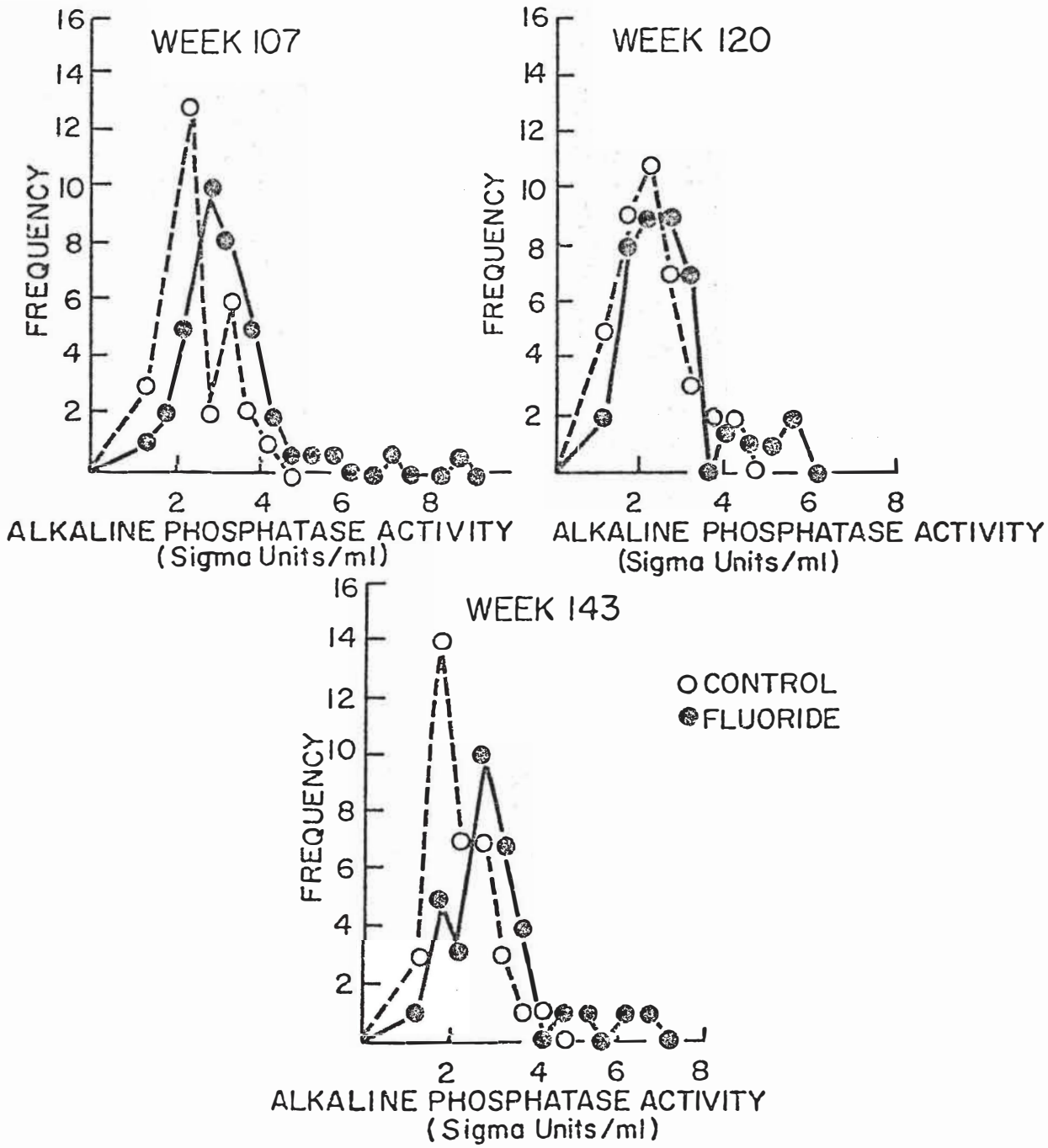
TABLE 5

EFFECT OF FLUORIDE SUPPLEMENTATION ON TOTAL SERUM ALKALINE PHOSPHATASE ACTIVITY* OF GERIATRIC MALES

Week	Mean Total Alkaline P'tase Activity			
	Control	Fluoride	t value	p value
95	2.45	3.11	3.21	>0.01
107	2.37	3.28	3.65	>0.01
120	2.34	2.76	2.00	>0.05
130	2.29	2.79	2.94	>0.01
143	2.18	3.25	4.14	>0.01

* Activity expressed in Sigma units per milliliter (one Sigma unit will liberate 1.0 µM p-nitrophenol/hr at pH 10.5 and 37° C.)

FIGURE 2
FREQUENCY DISTRIBUTION OF
TOTAL ALKALINE PHOSPHATASE
IN SERA OF CONTROL AND FLOURIDE GROUPS



120 the frequency of occurrence is more nearly equal as demonstrated by the superimposed curves. These observations may indicate a slight effect of fluoride on total alkaline phosphatase and could reflect the same trend as that shown between the total enzyme levels (Table 4). These are not significant differences and may be due to diurnal fluctuations.

When subjects are paired with reference to age and total surface area, the fluoride group mean is significantly higher than that of the control group (Table 6). This difference is more pronounced at week 143 after fluoride therapy has been discontinued and then resumed.

Tables 7 and 8 show the correlation between total alkaline phosphatase and serum fluoride levels. In the control group (Table 7) there are no significant correlations between the measurements except at week 120 ($p = 0.02$). Table 8 shows a similar trend for the fluoride treated group.

Previous studies have indicated that there is an inverse relationship between the amount of serum fluoride and total alkaline phosphatase levels (138). However, it has been observed that halide ions causing liminal inhibition of activity is an *in vitro* rather than an *in vivo* effect (29, 44). It is questionable whether fluoride inhibits alkaline phosphatase activity in the body. Human blood normally

TABLE 6

STATISTICAL ANALYSIS OF TOTAL ALKALINE PHOSPHATASE ACTIVITIES IN SERA OF PAIRED SUBJECTS*: CONTROLS VERSUS FLUORIDE TREATED GROUP

Week	Fluoride $\bar{x}_1$	Control $\bar{x}_2$	$(x_1 - x_2)$	S.E.	t	p
107	3.12	2.36	0.76	0.36	2.11	0.05
120	2.85	2.16	0.69	0.34	2.05	0.05
143	3.59	2.02	1.57	0.48	3.28	0.01

* N = 18

TABLE 7

CORRELATION BETWEEN TOTAL ALKALINE PHOSPHATASE ACTIVITY* AND FLUORIDE LEVELS** IN SERA OF CONTROL GROUP

Week	Mean			
	Fluoride	Total Alk. P'tase	r	p
107	2.5	2.37	0.221	NS
120	2.4	2.34	0.399	>0.02
130	2.1	2.29	0.295	NS
143	2.2	2.18	0.216	NS

* Expressed in Sigma units per milliliter (one Sigma unit liberates 1.0 μM of p-nitrophenol/hr at pH 10.5 and 37°C.)

**Expressed in micromoles

TABLE 8

CORRELATION BETWEEN TOTAL ALKALINE PHOSPHATASE ACTIVITY* AND FLUORIDE LEVELS** IN SERA OF FLUORIDE TREATED GROUP

Week	Mean			
	Fluoride	Total Alk. P'tase	r	p
107	9.0	3.28	0.097	NS
120	2.5	2.76	0.483	<0.01
130	2.3	2.79	0.268	NS
143	8.2	3.25	0.092	NS

* Expressed in Sigma units per milliliter (one Sigma unit liberates 1.0 µM of p-nitrophenol/hr at pH 10.5 and 37° C.)

** Expressed in micromoles

contains both free and bound fluoride, with 80 to 90 per cent being in the bound form, probably fixed to serum protein. At this level of fixed fluoride, assuming the serum contains 0.1 to 0.2 parts per million of fluoride, only 0.02 to 0.04 parts per million are available for exchange and this is below reported inhibition levels (118).

Table 9 presents the comparisons of serum fluoride between controls and fluoride treated groups by weeks. Weeks 107 and 143, when fluoride was being administered, show highly significant differences between group means. Week 120, during the discontinuation of therapy showed no significant difference between group means.

The correlation between total alkaline phosphatase activities in sera and X-ray aluminum equivalency which measures millimeters of an aluminum standard per millimeter of bone is presented in Table 10. The control group showed no significant correlation between the two parameters. Previous studies have confirmed that X-ray absorption by bone increases after the administration of fluoride (14). If alkaline phosphatase levels reflect bone metabolism, the slight trend toward correlation ($p = 0.02$) at week 143 could be due to a decline in mineralization of bone with time. In the fluoride treated group, there are significant correlations between alkaline phosphatase activity and X-ray

TABLE 9

COMPARISON BETWEEN FLUORIDE LEVELS* IN THE SERA OF CONTROL AND FLUORIDE TREATED GROUPS

Week	Mean			
	Control	F^- Group	t	p
107	2.5	9.0	13.05	<0.001
120	2.4	2.5	0.67	NS
143	2.2	8.2	17.30	<0.001

* Activity expressed in μM

TABLE 10

CORRELATION BETWEEN TOTAL ALKALINE PHOSPHATASE ACTIVITY* IN SERA AND X-RAY ALUMINUM EQUIVALENCY**

Group	Week	Mean			
		Alk. P'tase	Al Eq.	r	p
Control	107	2.37	0.207	0.129	NS
	120	2.34	0.208	0.128	NS
	143	2.18	0.210	-0.438	>0.02
Fluoride	107	3.12	0.204	0.474	>0.01
	120	2.85	0.206	0.525	>0.01
	143	3.59	0.207	0.282	NS

* Expressed in Sigma units per milliliter (one Sigma unit liberates 1.0 μM of p-nitrophenol/hr at pH 10.5 and 37° C.)

** Expressed in millimeters aluminum/millimeters bone

aluminum equivalency for weeks 107 and 120 with no significant correlation for week 143, perhaps indicating a response lag after resumption of therapy.

BONE ISOZYME OF ALKALINE PHOSPHATASE

Alkaline phosphatase isozymes of bone, liver and sometimes intestine are normally present in the sera. Studies have shown that none of these isozymes are normally elevated in geriatric patients (23). Data concerning the effect of inorganic fluoride on bone isozyme in the present study are presented in Tables 11 and 12. The statistical evaluation of these effects are shown in Tables 13 through 21.

Tables 13 and 14 show the effects of fluoride on bone isozyme in fluoride treated and control subjects when these subjects are paired. The means and net changes are given in densitometer units. Preliminary studies using bovine intestinal isozyme of alkaline phosphatase have demonstrated that 1.0 uM of alkaline phosphatase activity using p-nitrophenyl phosphate as substrate at 37^{o} C. and pH 10.5 for 30 minutes result in 150 densitometer units. Comparisons of differences in units show that between weeks 107 and 120 in the control group there is a change of + 13 units (Table 13). In the fluoride treated group (Table 14) the corresponding change is - 238 units, reflecting the period when fluoride therapy was discontinued. Between weeks

TABLE 11

BONE ISOZYME OF ALKALINE PHOSPHATASE* IN THE SERA OF GERIATRIC MALES RECEIVING DAILY ADMINISTRATION OF A PLACEBO

Subject Number	Week		
	107	120	143
1	24	28	15
2	19	29	41
4	34	40	23
9	38	42	52
15	37	39	32
16	--	--	27
19	5	10	17
21	14	6	--
26	--	--	33
31	0	8	4
34	33	34	49
35	20	14	26
60	3	12	11
63	10	15	--
65	20	30	--
66	39	--	28
68	37	42	32
85	42	33	38
92	22	30	27
96	--	--	34
103	31	20	19
112	--	--	10
113	27	20	25
116	13	--	26
118	17	10	26
125	11	20	29
131	3	8	43
142	--	--	22
144	14	13	48
146	6	10	10
148	26	12	32
161	18	15	25
162	20	23	13

TABLE 11 - Continued

Subject Number	Week		
	107	120	143
163	18	22	24
164	8	14	--
167	19	22	14
168	19	30	23
171	0	--	14
183	--	--	7

* Activity expressed in densitometer units

TABLE 12

BONE ISOZYME OF ALKALINE PHOSPHATASE* IN THE SERA OF GERIATRIC MALES RECEIVING DAILY ADMINISTRATION OF INORGANIC FLUORIDE

Subject Number	Week		
	107	120	143
3	31	30	39
11	39	24	37
22	44	19	34
28	114	32	64
29	52	45	54
37	78	124	158
38	62	23	77
39	20	28	--
44	28	23	51
46	27	22	39
50	36	18	--
54	51	16	67
57	26	24	47
67	36	40	58
69	67	27	115
70	36	30	89
83	33	6	94
89	30	10	38
93	80	74	68
95	38	--	179
97	30	20	--
99	30	26	32
107	53	26	67
109	60	46	66
110	35	16	58
117	30	--	--
119	33	--	14
122	31	33	82
124	35	17	39
129	25	15	--
137	39	18	45
149	27	20	10

TABLE 12 - Continued

Subject Number	Week		
	107	120	143
150	--	--	38
153	40	20	66
156	40	27	--
157	37	21	33
170	30	25	44
174	33	22	38
177	49	32	57
178	50	27	43

* Activity expressed in densitometer units

TABLE 13

EFFECT OF FLUORIDE ON THE ACTIVITY* OF BONE ALKALINE PHOSPHATASE IN SERAS OF CONTROL SUBJECTS**

Subject Number	Week			Week		
	107	120	change	143	change	Net change
15	37	39	+ 2	32	- 7	- 5
85	42	33	- 9	38	+ 5	- 4
103	21	20	- 1	19	- 1	- 2
31	0	8	+ 8	4	- 4	+ 4
167	19	22	+ 3	14	- 8	- 5
113	27	20	- 7	25	+ 5	- 2
118	17	10	- 7	26	+16	+ 9
9	38	42	+ 4	52	+10	+14
125	11	20	+ 9	29	+ 9	+18
4	34	40	+ 6	23	-17	-11
35	20	14	- 6	26	+12	+ 6
1	24	28	+ 4	15	-13	- 9
162	20	23	+ 3	12	-10	- 7
92	22	30	+ 8	27	- 3	+ 5
103	21	20	- 1	19	- 1	- 2
34	33	34	+ 1	49	+15	+16
148	26	12	-14	32	+20	+ 6
2	19	29	+10	41	+12	+22
Change	24	25	+13	27	+40	+53

* Expressed in Densitometer units

** Paired with fluoride treated subjects (Table 13)

TABLE 14

EFFECT OF FLUORIDE ON THE ACTIVITY* OF BONE ALKALINE PHOSPHATASE IN SERAS OF FLUORIDE TREATED GROUP**

Subject Number	Week			Week		
	107	120	change	143	change	Net change
3	31	30	- 1	39	+ 9	+ 8
29	52	45	- 7	54	+ 9	+ 2
38	62	23	-39	77	+54	+15
47	19	21	+ 2	40	+19	+21
57	26	24	- 2	47	+23	+21
69	67	27	-40	115	+88	+48
70	36	30	- 6	89	+59	+53
99	30	26	- 4	32	+ 6	+ 2
107	53	26	-27	67	+41	+14
109	60	46	-14	66	+20	+ 6
110	35	16	-19	58	+42	+23
122	31	33	+ 2	82	+49	+51
124	35	17	-17	39	+22	+ 4
137	39	18	-21	45	+27	+ 6
149	27	20	- 7	10	+10	+ 3
157	37	21	-16	33	+12	- 4
170	30	25	- 5	44	+19	+14
177	49	32	-17	57	+25	+ 7
Change	40	27	-238	55	+534	+274

* Expressed in Densitometer units

** Paired with control subjects (Table 14)

120 and 143 there was a change of + 40 units for the control group, and a corresponding change of + 534 for the fluoride group when therapy was resumed. These results may be interpreted as indicating that the administration of inorganic fluoride produces greater effects on the bone isozyme of alkaline phosphatase than can be accounted for by day to day fluctuations.

Figure 3 shows the frequency distribution of bone isozyme by weeks. As previously illustrated with total alkaline phosphatase there are greater frequencies of higher isozyme levels at weeks 107 and 143 in the fluoride group. At week 120, during the discontinuation of fluoride therapy, the frequency distributions are more nearly equal between the groups. However, the bone isozyme distributions show greater separation than those of total alkaline phosphatase. This difference in separation could be due to the presence of other isozymes in the total alkaline phosphatase that are not effected by fluoride.

Table 15 gives weekly comparisons of the bone isozyme levels in control subjects. There are no significant differences between the group means at any week for which sample values are reported. The comparisons between weeks for fluoride group are shown in Table 16. There are significant differences between the means of each week reported.

FIGURE 3
FREQUENCY DISTRIBUTION OF
BONE ISOZYME OF ALKALINE PHOSPHATASE
IN SERA OF CONTROL AND FLUORIDE GROUPS

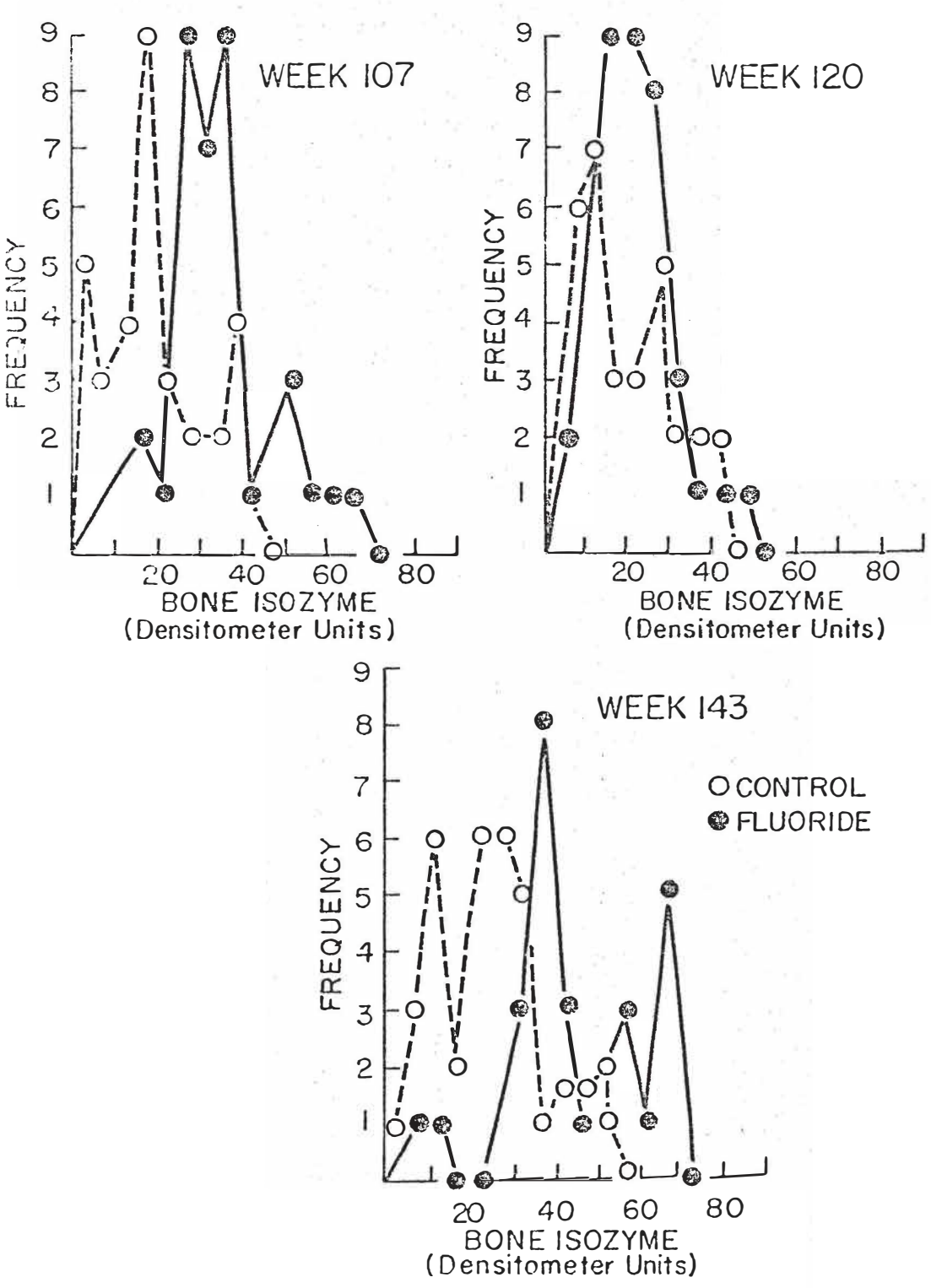


TABLE 15

STATISTICAL ANALYSIS OF MEAN ACTIVITY* OF THE BONE ISOZYME OF ALKALINE PHOSPHATASE IN CONTROL GROUP DURING 143 WEEKS OF STUDY

Week	Mean of Bone Isozyme	Comparison with Week	t	p
107	19.3 (n=26)	120	0.83	NS
120	21.7 (n=26)	143	1.27	NS (0.10)
143	25.4 (n=26)	107	2.09	<0.05

* Activity expressed in densitometer units

TABLE 16

STATISTICAL ANALYSIS OF MEAN ACTIVITIES* OF THE BONE ISOZYME OF ALKALINE PHOSPHATASE IN FLUORIDE GROUP DURING 143 WEEKS OF STUDY

Week	Mean of Bone Isozyme	Comparison with Week	t	p
107 on F^- 107 Wks	41.3 (n=32)	120	3.00	>0.01
120 off F^- 13 wks	28.1 (n=32)	143	4.62	>0.01
143 on F^- 13 wks	59.4 (n=32)	107	2.75	>0.01

* Activity expressed in densitometer units

Week 107 versus 120 shows the differences during the time period corresponding to the discontinuation of fluoride therapy. Week 107 and 143 also demonstrate a significant difference between means, possibly due to the rate of remineralization after therapy. Week 120 is also significantly different than week 143 reflecting the discontinuation and resumption of therapy.

Table 17 illustrates comparisons between means of bone isozymes in the sera of controls and the fluoride treated group. There were statistically significant differences between the groups for weeks 107 and 143 when the fluoride was being given and no difference at week 120 when no fluoride was being administered. The subjects were paired (Table 18), and the same differences were noted.

Correlation between bone isozyme levels and fluoride concentration in the sera showed no significance in either group (Tables 19 and 20). This could indicate no inhibiting effect on alkaline phosphatase activity, or if there is inhibition, it is not reflected in the bone isozyme.

Table 21 presents correlations between bone isozyme and X-ray aluminum equivalency. There is no significant correlation between these parameters at any week in either group. This result could be due to an increase in bone density during a period of high mineralization which had

TABLE 17

STATISTICAL ANALYSIS OF MEAN ACTIVITIES* OF BONE ISOZYME OF ALKALINE PHOSPHATASE IN SERA OF CONTROL VERSUS FLUORIDE TREATED GROUP

Week	Mean			
	Control	Fluoride	t	p
107	19.3	41.3	6.12	<0.01
120	21.7	28.2	1.67	0.10 NS
143	25.4	59.4	5.46	<0.01

* Activity expressed in densitometer units

TABLE 18

STATISTICAL ANALYSIS OF MEAN ACTIVITIES* OF BONE ISOZYME OF ALKALINE PHOSPHATASE IN SERA OF PAIRED SUBJECTS**

Week	Fluoride $\bar{x}_1$	Control $\bar{x}_2$	$(x_1 - x_2)$	S.E.	t	p
107	39.9	23.9	16.0	3.54	4.58	>0.01
120	24.7	26.7	2.0	2.33	0.86	NS
143	55.2	26.8	28.4	6.91	4.11	>0.01

* Activity expressed in densitometer units

** N = 18

TABLE 19

CORRELATION BETWEEN BONE ISOZYME* OF ALKALINE PHOSPHATASE AND FLUORIDE LEVELS** IN SERA OF CONTROL GROUP

Week	Mean			
	Bone Isozyme	F^-	r	p
107	19.3	2.5	0.228	NS
120	21.7	2.4	0.432	>0.05 NS
143	25.4	2.2	0.038	NS

* Activity expressed in densitometer units

** Activity in μM

TABLE 20

CORRELATION BETWEEN BONE ISOZYME* OF ALKALINE PHOSPHATASE AND FLUORIDE LEVELS** IN SERA OF FLUORIDE TREATED GROUP

Week	Mean			
	Bone Isozyme	F^-	r	p
107	41.3	9.0	0.130	NS
120	28.1	2.5	0.212	NS
143	59.4	8.2	0.215	NS

* Activity expressed in densitometer units

** Activity in μM

TABLE 21

CORRELATION BETWEEN BONE ISOZYME* OF ALKALINE PHOSPHATASE IN SERA AND X-RAY ALUMINUM EQUIVALENCY**

Group	Week	Mean			
		Bone Iso	Al Eq.	t	p
Control	107	19.3	0.207	0.210	NS
	120	21.7	0.208	0.180	NS
	143	25.4	0.210	0.130	NS
Fluoride	107	41.3	0.204	0.237	NS
	120	28.1	0.206	0.257	NS
	143	59.4	0.207	0.203	NS

* Activity expressed in densitometer units

** millimeter aluminum/millimeter bone

passed by the time samples were taken.

Comparisons between heat denaturation techniques and bone isozyme analysis by gel electrophoresis as an indicator of the amount of total bone isozyme were made. Previous studies have produced varying results. Residual activity reported has been 0 to 10.0 per cent (163), 2.0 per cent (48), 4.0 to 23.0 per cent (95), 20.0 per cent (141), 8.0 to 20.0 per cent (137), and 33.0 per cent (99). Table 22 gives the results of preliminary study on heat denaturation. There is a high rate of inactivation for the first three minutes at higher temperatures which gradually decreases with time. Optimum inactivation time is between three and six minutes as the rate of decline is not as rapid as during the first three minutes but there is still enough activity remaining for accurate analysis. Figure 4 illustrates the difference in residual activity between the groups after heat inactivation. As expected the fluoride group containing the highest amount of bone isozyme per total serum alkaline phosphatase is inactivated at a more rapid rate than samples from the control group.

Table 23 gives the residual activity of individual samples after heat denaturation in control and fluoride treated subjects. These data demonstrate residual activities of 12.9 per cent for the control group and 5.1 per

TABLE 22

EFFECT OF FLUORIDE ON THE THERMOLABILE FRACTION OF POOLED SERUM ALKALINE PHOSPHATASE*

Incubation Temperature	Group	Time (minutes)				
		0	3	6	9	12
56° C	Control	0.406	0.378	0.353	0.291	0.252
	Fluoride	0.499	0.367	0.265	0.152	0.139
45° C	Control	0.422	0.389	0.372	0.319	0.299
	Fluoride	0.501	0.403	0.326	0.278	0.232
37° C	Control	0.412	0.402	0.385	0.353	0.342
	Fluoride	0.503	0.463	0.436	0.417	0.399

* Activity in Sigma Units (one Sigma unit will liberate one μM of p-nitrophenol/hour at pH 10.5 and 37° C.)

FIGURE 4

THERMOLABILITY OF SERUM ALKALINE PHOSPHATASE AT 56° C

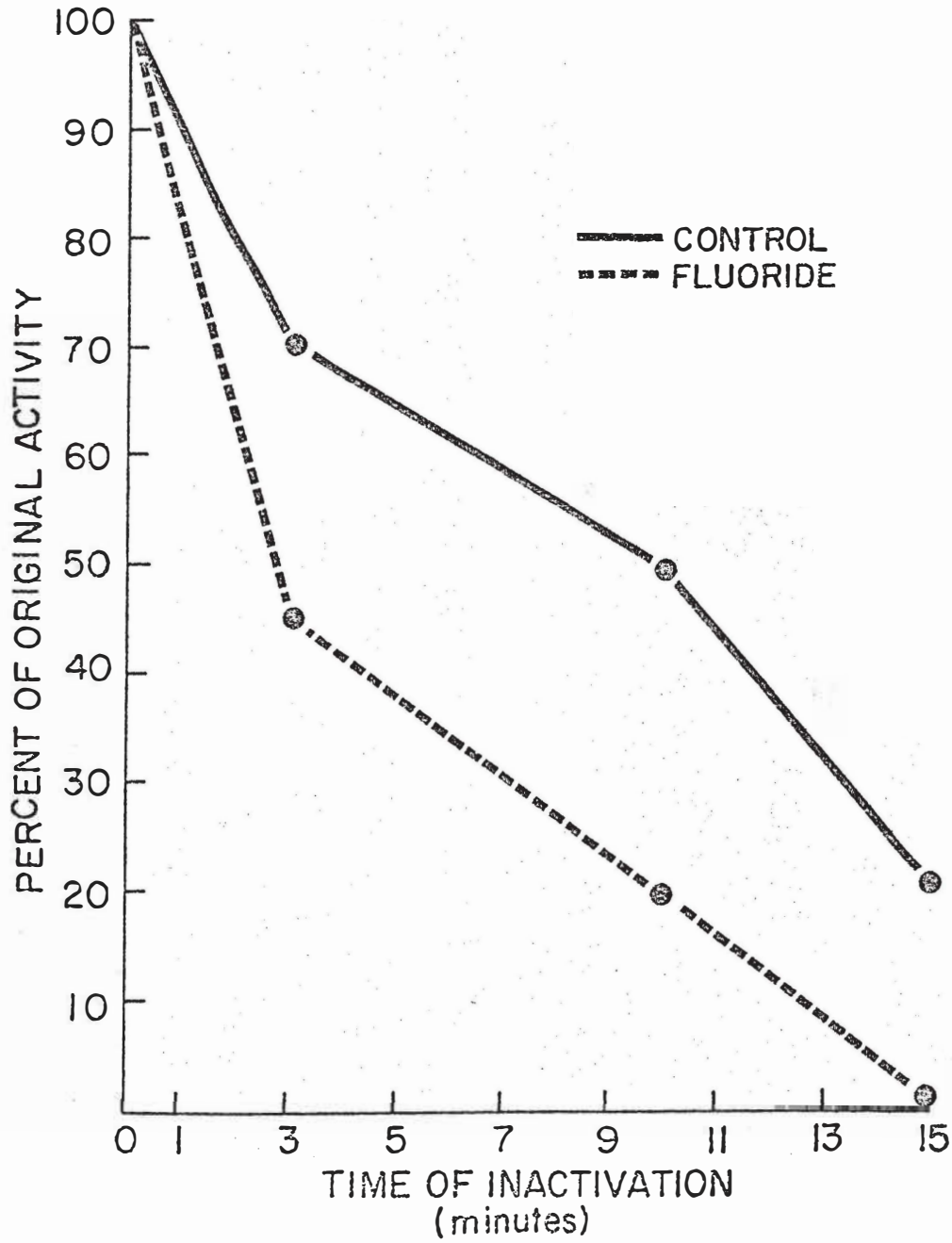


TABLE 23

RESIDUAL ACTIVITY* OF ALKALINE PHOSPHATASE IN SERA AFTER HEAT DENATURATION**

Control (Subject Number)	Residual Activity	Fluoride Group (Subject Number)	Residual Activity
		3	8.7
		22	7.9
		28	0.8
		29	6.2
1	9.6	37	4.3
2	11.3	44	9.1
4	10.3	46	10.7
9	3.1	47	12.5
31	25.0	54	6.8
35	9.0	57	2.1
60	22.8	67	14.5
68	4.8	69	5.9
85	3.2	89	1.7
103	8.7	99	4.3
118	7.9	107	3.2
125	15.9	109	1.5
131	18.7	110	3.7
144	16.7	122	2.2
146	21.4	124	2.8
163	12.6	137	3.2
167	17.2	153	1.1
168	13.1	157	5.2
		170	4.0
		174	1.9
		177	4.4
		178	3.9
Mean per cent	12.85		5.1

* Activity expressed in amount of total remaining after heat denaturation

** 56^{o} C. for five minutes

cent for the fluoride group. Table 24 gives comparisons between residual activity for the groups. There are significant differences between group means which may be due to differences in the ratio of bone to non-bone isozyme in the sera.

Table 25 presents the results of correlations between residual activity in the sera and serum fluoride, bone isozyme, total alkaline phosphatase, and aluminum equivalency. In the control group there was no significant correlation between serum fluoride levels and the residual activity, while in the fluoride treated group there was a significant correlation ($p = 0.001$). Previous studies have reported decreased residual activity with increased serum fluoride levels (167).

The control group demonstrated a correlation between residual activity and bone isozyme levels, while the fluoride group showed no correlation. This effect could be due to the greater concentrations of bone isozyme in the sera of the fluoride treated group which would show a high residual activity even after heat inactivation. Total alkaline phosphatase showed no correlation with residual activity in either group, possibly due to the presence of isozymes other than bone. Aluminum equivalency showed no correlation with residual activity in either group. In

TABLE 24

COMPARISON OF RESIDUAL ACTIVITY* OF TOTAL ALKALINE PHOSPHATASE REMAINING IN SERA AFTER HEAT DENATURATION IN CONTROL VERSUS FLUORIDE TREATED GROUPS

Group	$\bar{X}$	$(\bar{x}_1 - \bar{x}_2)$	S.E.	t	p
Control (n=18)	5.10	7.75	1.54	4.93	<0.001
Fluoride (n=26)	12.85		0.70		

* Expressed as per cent of original activity remaining after denaturation at 56^{o} C. for five minutes.

TABLE 25

CORRELATIONS BETWEEN RESIDUAL ACTIVITY* OF ALKALINE PHOSPHATASE IN SERA AFTER HEAT DENATURATION AND RELATED PARAMETERS

Group	Residual Act. Compared with	r	p
Control	Serum fluoride	0.152	NS
	Bone isozyme	0.907	0.001
	Total Alk. P'tase	0.372	NS (0.10)
	Aluminum Equivalency	0.007	NS
Fluoride	Serum fluoride	0.592	0.001
	Bone isozyme	0.325	NS (0.10)
	Total Alk. P'tase	0.235	NS
	Aluminum Equivalency	0.391	0.05

* Expressed as per cent of original activity remaining after denaturation at 56° C. for five minutes

view of the disparity of previously reported results and variability between samples the determination of isozymes by heat denaturation seems unreliable.

COMPARISON BETWEEN TOTAL ALKALINE PHOSPHATASE AND BONE ISOZYME AS AN IN VIVO MEASUREMENT OF BONE ACTIVITY

Bone isozyme of alkaline phosphatase appears to be a more accurate measurement of the state of bone metabolism than is total alkaline phosphatase. The validity of this observation is demonstrated by comparisons with related parameters. Data previously presented (Table 26) indicates there was no significant correlation between total alkaline phosphatase activity and bone isozyme. Comparing correlations between controls and fluoride treated subjects it is observed that there is greater correlation to fluoride therapy in the bone isozyme. This observation is confirmed in paired subjects.

ALKALINE PHOSPHATASE IN VITRO

Previous studies have demonstrated that the fluoride ion *in vitro* has a greater inhibitory effect than *in vivo* (81). It has been suggested that loss of alkaline phosphatase activity is due to the formation of a fluoride-magnesium complex which may also be formed, to a lesser extent, *in vivo* (138).

When investigators cultured five day old mouse calvaris

TABLE 26

CORRELATION BETWEEN TOTAL ALKALINE PHOSPHATASE ACTIVITY* AND BONE ISOZYME** IN SERA OF CONTROLS AND FLUORIDE TREATED GROUPS

Group	Week	Total Alk. P'tase ($\bar{x}$)	Bone Isozyme ($\bar{x}$)	r	p
Control	107	2.37	19.3	0.089	NS
	120	2.34	21.7	0.288	NS
	143	2.18	25.4	0.083	NS
Fluoride	107	3.28	41.3	0.080	NS
	120	2.76	28.2	0.143	NS
	143	3.25	59.4	0.018	NS

* Activity expressed in Sigma units per milliliter (one Sigma unit liberates 1.0 μM of p-nitrophenol/hr at pH 10.5 and 37° C.)

** Activity expressed in densitometer units

in the presence of 1.9 parts per million of fluoride, bone resorption was inhibited. Adding 0.019 parts per million of fluoride to the culture media was not as effective and 0.011 parts per million did not affect resorption. Toxic effects were demonstrated with the addition of 19 parts per million and levels of 915 parts per million did not exhibit toxic effects but neither was there formation of new bone (59).

Table 27 illustrates the amount of total alkaline phosphatase activity in bone homogenates after growth in culture containing varying levels of fluoride. Activity is greatest at levels of 1 part per million and least at 50 parts per million. This apparently represents a stimulation at relatively low fluoride levels and inhibition which increases as concentration increases. Both samples show approximately the same effect at the various levels of fluoride in the culture medium.

Tables 28 and 29 show the total alkaline phosphatase in Samples I and II by days. In each sample there is an increase in total alkaline phosphatase with 1 part per million of fluoride followed by a decrease at higher levels. These decreases give total alkaline phosphatase activities that are less than control levels (0 parts per million), indicating inhibitory effect operating at fluoride levels

TABLE 27

TOTAL ALKALINE PHOSPHATASE ACTIVITY* IN RIB SECTION GROWN IN VITRO IN THE PRESENCE OF VARYING LEVELS OF FLUORIDE

Sample	Fluoride Concentration (ppm)	Alkaline P'tase Activity (Sigma units/100 mg bone)
I*	0	27.54
	1	71.97
	10	14.35
	20	6.14
	50	0.74
II**	0	22.06
	1	105.37
	10	18.91
	20	14.73
	50	1.02

* 22 hour old bone

** 29 day old bone

TABLE 28

TOTAL ALKALINE PHOSPHATASE ACTIVITY* OF A 22 HOUR OLD BONE GROWN IN VITRO IN MEDIA WITH VARYING LEVELS OF INORGANIC FLUORIDE

Fluoride Level	Age of Culture (Days)			
	1	2	3	5
0 ppm-1**	2.45	2.66	3.04	1.22
2	2.70	3.00	1.90	1.03
1 ppm-1	8.71	7.94	6.63	3.76
2	7.34	7.08	5.75	5.07
10 ppm-1	1.49	1.22	0.77	0.68
2	1.43	0.76	0.70	0.34
20 ppm-1	0.74	0.55	0.17	0.10
2	0.80	1.09	0.26	0.15
50 ppm-1	0.17	0.04	0.04	0
2	0.38	0.15	0.08	0

* Activity in Sigma units per 100 milligram bone
** Duplicate samples of media

TABLE 29

TOTAL ALKALINE PHOSPHATASE ACTIVITY* OF A 29 DAY OLD BONE GROWN IN VITRO IN MEDIA WITH VARYING LEVELS OF INORGANIC FLUORIDE

Fluoride Level	Age of Culture (Days)				
	1	2	3	4	5
0 ppm-1**	4.48	5.10	4.98	4.08	3.23
2	5.01	5.33	5.01	4.16	3.77
1 ppm-1	12.86	13.46	14.76	11.89	8.36
2	9.99	11.11	10.96	9.21	6.27
10 ppm-1	2.78	2.96	2.64	1.68	0.72
2	3.23	3.07	2.91	2.35	1.00
20 ppm-1	1.34	1.29	1.00	0.55	0.29
2	1.03	1.16	0.89	0.89	0.38
50 ppm-1	0.84	0.74	0.71	0.16	0.05
2	0.79	0.69	0.61	0.35	0

* Activity in Sigma units per 100 milligram bone
** Duplicate samples of media

somewhere between 1.0 and 10.0 parts per million.

Tables 30 and 31 give bone isozyme levels for Samples I and II. These levels follow the same pattern as the total alkaline phosphatase activity. These results would be expected as the major portion of isozyme in samples would be that of bone.

Figure 5 illustrates the response of total alkaline phosphatase and bone isozyme to increasing levels of fluoride in Samples I and II. Similar response is observed in both samples. Figure 6 shows the response by days of total alkaline phosphatase and bone isozyme to fluoride. The gradual decline in both samples as tissue culture ages is probably due to gradual necrosis of bone forming cells. At 50 parts per million both total alkaline phosphatase and bone enzyme are almost completely inhibited.

Table 32 shows correlations between alkaline phosphatase and bone isozyme in tissue cultures. As noted above these high correlations are as expected.

Figures 7 through 11 illustrate the effect of various levels of fluoride on bone (Sample II). The darkened areas represent sites of active bone while resorption areas are clear. Figure 7, which presents bone culture used as the control (0 ppm fluoride), shows less active bone than does that cultured in the presence of 1.0 ppm fluoride (Figure 8).

TABLE 30

BONE ISOZYME OF ALKALINE PHOSPHATASE* IN 22 HOUR OLD BONE GROWN IN VITRO IN VARYING LEVELS OF INORGANIC FLUORIDE

Fluoride Level	Age of Culture (Days)			
	1	2	3	5
0 ppm-1**	272	288	262	135
2	210	241	220	137
1 ppm-1	571	573	562	476
2	576	588	541	511
10 ppm-1	143	119	88	76
2	136	103	94	45
20 ppm-1	53	49	22	14
2	31	32	29	9
50 ppm-1	27	7	5	0
2	39	8	5	0

* Activity in densitometer units per 100 milligram bone
** Duplicate samples of media

TABLE 31

BONE ISOZYME OF ALKALINE PHOSPHATASE* IN 29 DAY OLD BONE GROWN IN VITRO IN VARYING LEVELS OF INORGANIC FLUORIDE

Fluoride Level	Age of Culture (Days)				
	1	2	3	4	5
0 ppm-1**	397	425	388	303	276
2	458	484	473	461	352
1 ppm-1	983	999	1010	871	728
2	756	837	821	695	602
10 ppm-1	195	201	181	109	57
2	260	253	249	134	68
20 ppm-1	107	97	75	41	15
2	93	89	84	72	23
50 ppm-1	62	57	46	9	0
2	57	50	37	17	0

* Activity in densitometer units per 100 milligram bone
** Duplicate samples of media

FIGURE 5
TOTAL ALKALINE PHOSPHATASE AND BONE ISOZYME IN BONE CULTURE MEDIA IN RESPONSE TO VARIOUS LEVELS OF FLUORIDE

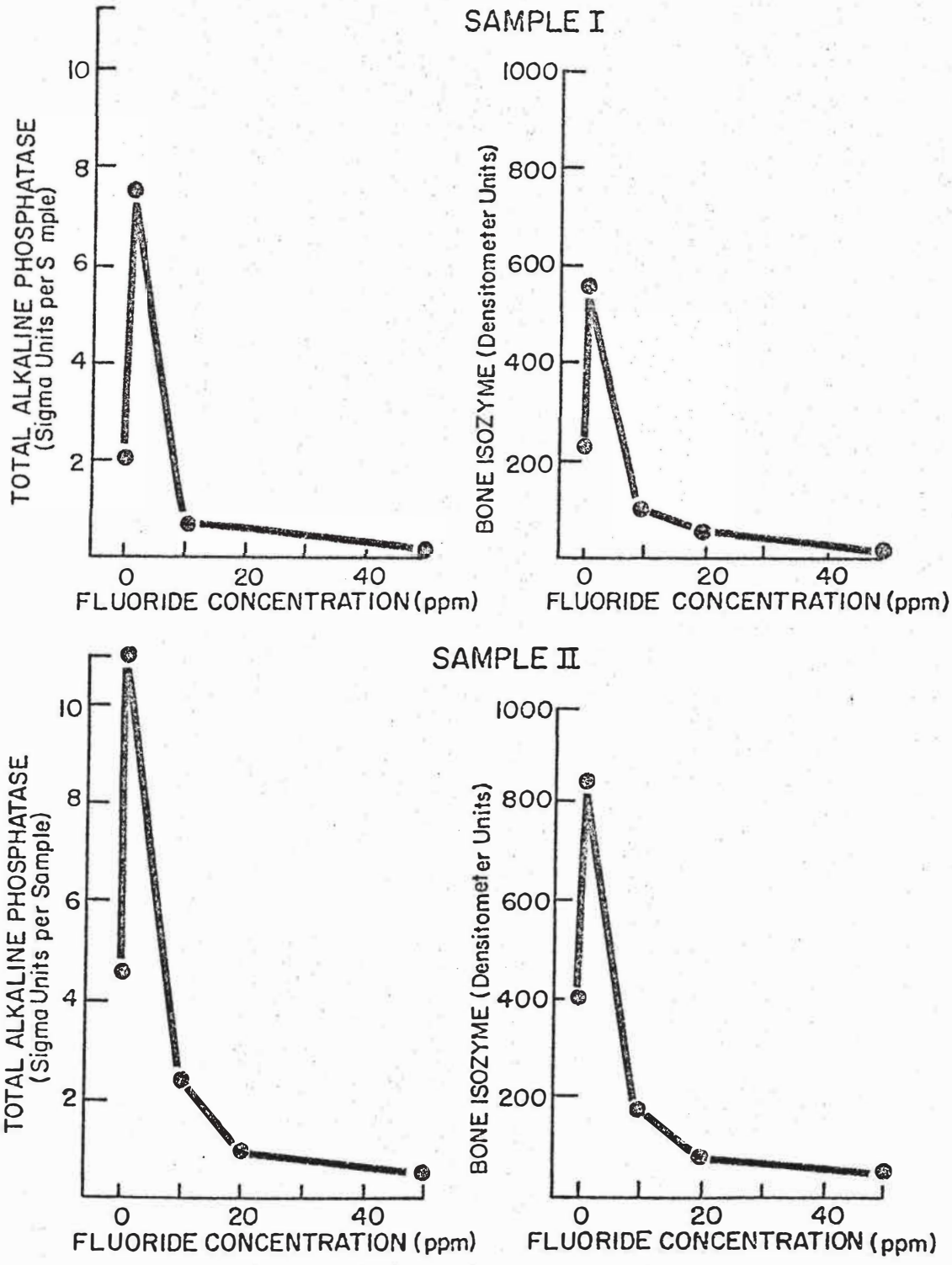


FIGURE 6

RESPONSE OF TOTAL ALKALINE PHOSPHATASE AND BONE ISOZYME TO AGE OF BONE CULTURE IN MEDIA CONTAINING VARIOUS CONCENTRATIONS OF FLUORIDE

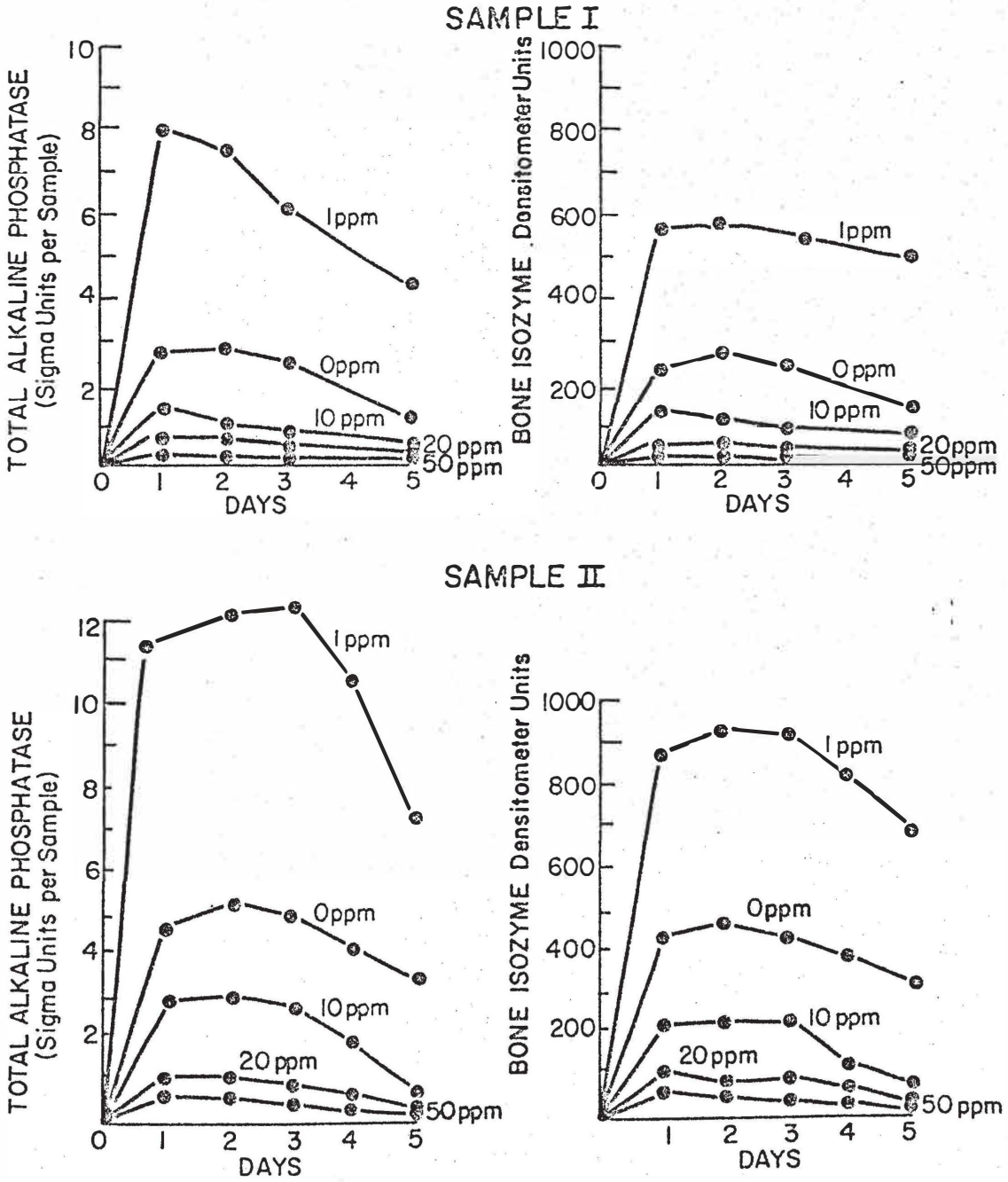


TABLE 32

CORRELATION BETWEEN TOTAL ALKALINE PHOSPHATASE ACTIVITY AND BONE ISOZYME CULTURED IN THE PRESENCE OF VARYING LEVELS OF FLUORIDE

Sample	Fluoride Level (ppm)	r	p
I*	0	0.853	<0.01
	1	0.903	<0.001
	10	0.964	<0.001
	20	0.621	<0.05
	50	0.938	<0.001
II**	0	0.810	<0.001
	1	0.975	<0.001
	10	0.956	<0.001
	20	0.976	<0.001
	50	0.989	<0.001

* 22 hour old bone

** 29 day old bone

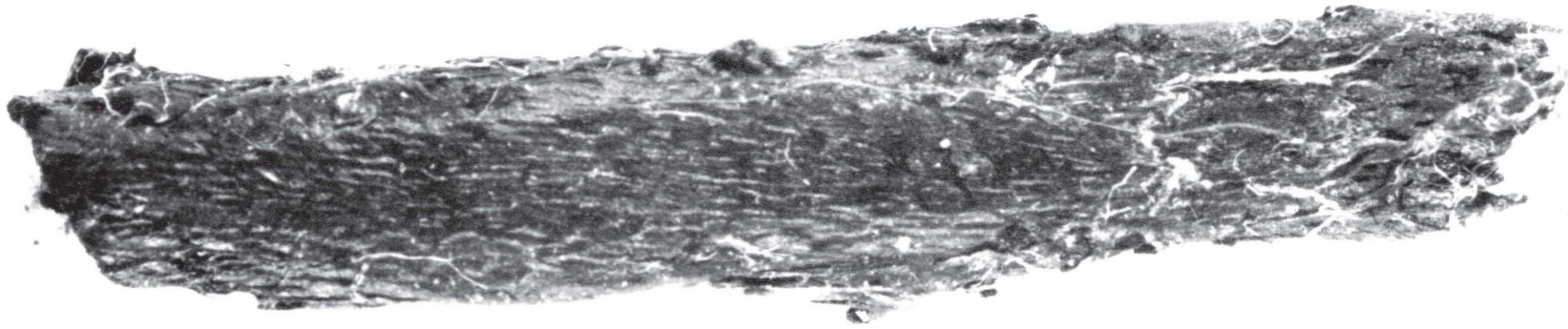


Figure 7. Bone resorption as measured by uptake of silver nitrate, control - 0 ppm fluoride (12x).

Figure 8. Effect of fluoride on bone resorption as measured by uptake of silver nitrate - 1.0 ppm (12x).

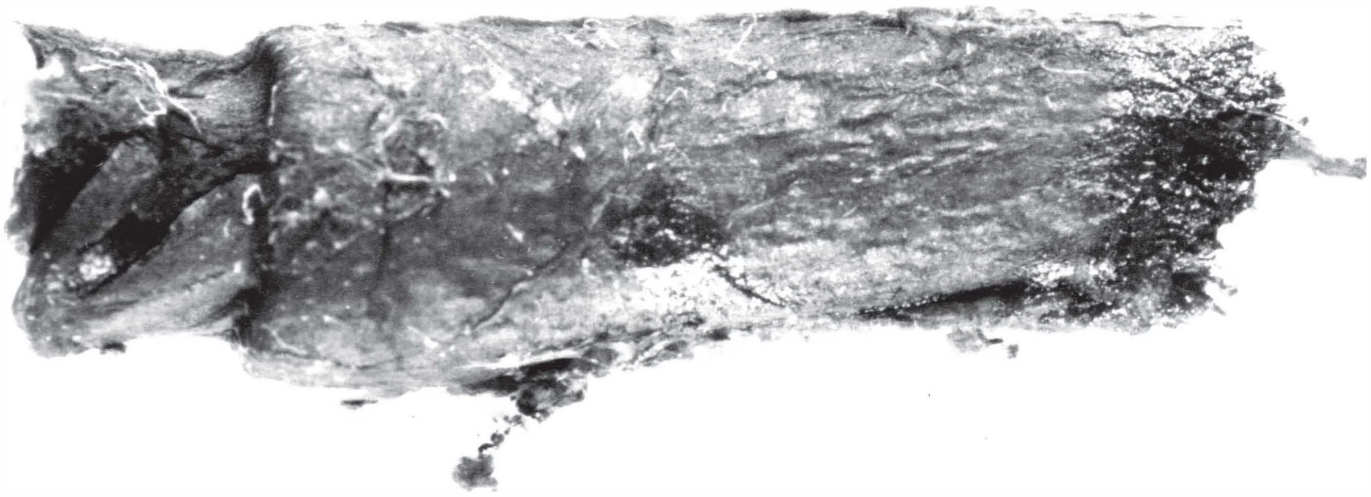


Figure 9. Effect of fluoride on bone resorption as measured by uptake of silver nitrate - 10.0 ppm (12x).

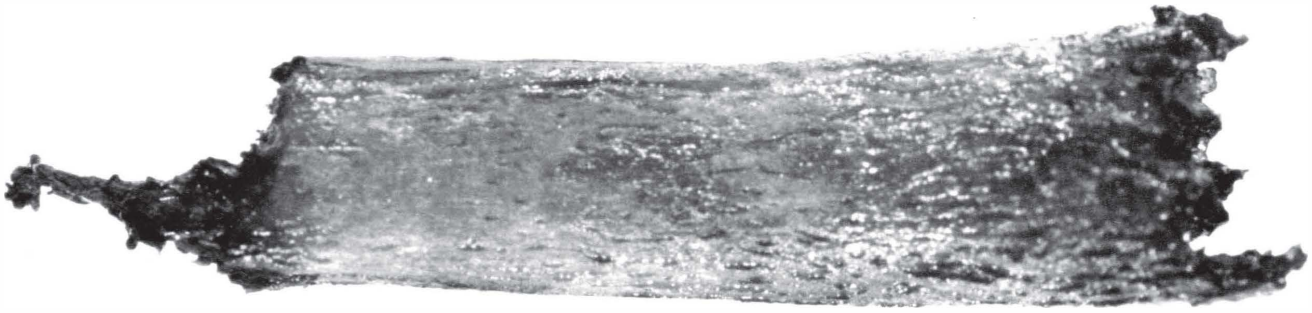


Figure 10. Effect of fluoride on bone resorption as measured by uptake of silver nitrate - 20.0 ppm (12x).

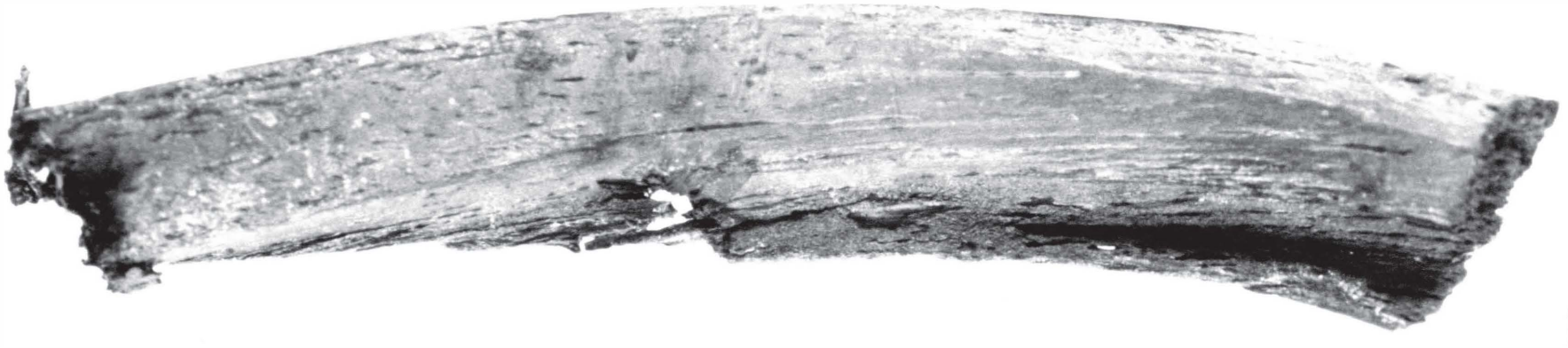


Figure 11. Effect of fluoride on bone resorption as measured by uptake of silver nitrate - 50.0 ppm (12x).

Figures 9, 10, and 11 represent cultures containing 10, 20 and 50 parts per million of fluoride respectively. These bone sections show the effects of increased bone resorption and loss of viability as fluoride levels increase. At 50 ppm there is almost total loss of active bone and necrosis. These results tend to confirm the relationship between alkaline phosphatase and actively metabolizing bone.

LIVER ISOZYME OF ALKALINE PHOSPHATASE

Comparisons between weeks at which samples were collected for the liver isozyme of alkaline phosphatase were made. The purpose of this measurement was to rule out the possibility that elevations in alkaline phosphatase could be due to liver isozyme resulting from liver damage or disease. Table 33 presents the results of these comparisons. There were no significant differences between control and fluoride group means.

TABLE 33

COMPARISON OF LIVER ISOZYME OF ALKALINE PHOSPHATASE* BETWEEN CONTROL AND FLUORIDE GROUPS

Week	Liver Isozyme Levels				
	Control** $\bar{x}_1$	F^-*** $\bar{x}_2$	(x_1-x_2)	t	p
107	42.4	38.3	4.1	0.81	NS
120	49.9	44.7	5.2	0.89	NS
143	43.2	43.5	0.3	0.05	NS

* Activity expressed in densitometer units
** n=26
*** n=32

CHAPTER IV

CONCLUSIONS AND RECOMMENDATIONS

The purpose of this study was to determine the response of the bone isozyme of alkaline phosphatase to changes in bone as influenced by inorganic fluoride therapy. Specifically, the practicality of using the bone isozyme as a clinical determinant of bone metabolism was studied. Both *in vivo* and *in vitro* total alkaline phosphatase activity and bone isozyme concentrations were measured and compared.

A simple, comparatively rapid method of isozyme analysis by gel electrophoresis is described in Chapter II. The advantages of this method are reproducibility and the use of small samples. A ten microliter aliquot is adequate, in the presence of normal enzyme levels, and produces good resolution of the isozyme bands. The separated bands may be related to a standard by means of a simple adaptation of a densitometer with an attached recorder. This method is preferable to heat denaturation methods now in general clinical use. These methods vary greatly in the amount of residual activity remaining and the response of the various isozymes present in the sera.

Using the Student's "t" test for a two tailed sample, statistical significance level was established at less than $p = 0.05$. By this test comparisons were made between total alkaline phosphatase activity and bone isozyme levels in the sera at different weeks when fluoride was being administered and when it had been discontinued. There were no differences between the group means in total alkaline phosphatase activity in either group. However, the bone isozyme concentrations demonstrated significant differences at each interval with subjects receiving fluoride therapy. These differences were observed when the subjects had received fluoride therapy for 107 weeks, when therapy was discontinued for 13 weeks and when it was resumed. The control group levels of bone isozyme were not significantly different during the same interval. These observations based on the direct increase and decrease of isozyme with fluoride therapy indicate that the bone isozyme of alkaline phosphatase is affected in some manner by the administration of fluoride.

There were differences in the group means between the control and fluoride groups in both total alkaline phosphatase and the bone isozyme. This difference was more pronounced in bone isozyme levels and was demonstrated at intervals corresponding to the administration of fluoride

therapy. When fluoride was discontinued there was no difference between the control and fluoride group means. This effect was also observed in total alkaline phosphatase levels, but to a lesser degree. The control group exhibited some periodic variations in alkaline phosphatase activity but never reached either the total levels or the periodic increase in levels that is shown by the treated group. These observations also tend to confirm an effect of fluoride therapy on bone as reflected by alkaline phosphatase levels. It was concluded from these studies that the bone isozyme is a more responsive measurement of the metabolic state of bone than total enzyme levels. Since the sera of humans contains several isozymes of alkaline phosphatase it is reasonable to assume that if the enzyme does respond to changes in the bone state, concentrations of the isozyme derived from bone would be a more accurate measurement of metabolism.

Pearson's Correlation Coefficient was applied to parameters such as serum fluoride levels and X-ray equivalency. Little correlation was found with either total alkaline phosphatase or the bone isozyme.

The results obtained *in vivo* were applied to an *in vitro* situation using bone tissue culture techniques. A pronounced response of alkaline phosphatase to inorganic

fluoride was observed. At concentrations lower than inhibition levels the inorganic fluoride in the culture media had a stimulatory effect on both total alkaline phosphatase and the bone isozyme. One part per million of inorganic fluoride produced a much greater response than that observed in controls (0 parts per million). Levels of ten parts per million or more produced inhibition. These *in vitro* studies confirmed the observations made *in vivo* on the stimulatory effect of fluoride on bone as reflected by alkaline phosphatase.

It can be concluded that within the limitations imposed by the study, that the state of bone metabolism can be measured in terms of alkaline phosphatase activity in the serum, specifically that of the bone isozyme which exhibits a more pronounced response. It can also be concluded that measurement of the bone isozyme by the above method could be utilized for the determination of the state of bone metabolism or the detection of disease, and the results of therapy on a clinical basis.

Further studies should be conducted to establish normal levels for the bone isozyme at different age levels. In addition, the activity of the isozyme should be observed with other bone affecting treatments including those that alter the mineralization directly and those, such as hor-

mones, which may have an indirect effect. In further studies on the effect of fluoride administration on bone, the inhibition level, which in this study was established as somewhere between 1.0 and 10.0 parts per million, should be determined. Studies on the effect of levels of fluoride between these concentrations could be observed by additional *in vitro* cultures.

A P P E N D I X

A

TRIS-MALEIC ACID BUFFER

Compound	Amount
Tris *	137 grams
Maleic acid	9 grams **
Water to	4 liters

* Tris (Hydroxymethyl) Amino-Methane

** Tris was dissolved in 3.75 liters of water and maleic acid added to adjust to pH 9.0

B

ALKALINE PHOSPHATASE INCUBATION MIXTURE

Compound	Amount
Sodium- -napthyl acid phosphate	40 mg
Diazonium salt	100 mg
Magnesium sulfate, hydrate	120 mg
Maleic acid	60 mg
Tris	6 gm
Water	200 ml

C

EAGLES MINIMUM ESSENTIAL MEDIUM

	mg/liter
L-Arginine HCl	126.4
L-Cystine	24.0
L-Glutamine	292.0
L-Histidine HCl·H_2O	41.9
L-Isoleucine	52.5
L-Leucine	52.4
L-Lysine HCl	73.1
L-Methionine	14.9
L-Phenylalanine	33.0
L-Threonine	47.6
L-Tryptophan	10.2
L-Tyrosine	36.2
L-Valine	46.8
D-Calcium-Pantothenate	1.0
Choline chloride	1.0
Folic acid	1.0
i-Inositol	2.0
Nicotinamide	1.0
Pyridoxal HCl	1.0
Riboflavin	0.1
Thiamine HCl	1.0

D

McCOY'S 5A MEDIUM

	mg/liter
L-Alanine	13.0
L-Arginine HCl	42.0
L-Asparagine	45.0
L-Aspartic acid	20.0
L-Cysteine	32.0
L-Glutamic acid	22.0
L-Glutamine	219.0
L-Gylcine	8.0
L-Histidine HCl·H_2O	21.0
L-Tryptophan	3.0
L-Phenylalanine	17.0
L-Tyrosine	18.0
L-Lysine HCl	37.0
L-Methionine	15.0
L-Isoleucine	39.0
L-Leucine	39.0
L-Valine	18.0
L-Threonine	18.0
L-Serine	26.0
L-Hydroxyproline	20.0
L-Proline	17.0
p-Aminobenzoic acid	1.0
Ascorbic acid	0.5
D-Biotin	0.2
D-Calcium-pantothenate	0.2
Choline chloride	5.0
Folic acid	10.0
i-Inositol	36.0
Nicotinamide	0.5
Nicotinic acid	0.5
Pyridoxal HCl	0.5
Pyridoxine HCl	0.5
Riboflavin	0.2
Thiamine	0.2
Vitamin B_{12}	2.0
Glutathione Bacto-peptone	600.0

E

EARLES BALANCED SALT SOLUTION

	gms/liter
NaCl	6.80
KCl	0.40
$CaCl_2$	0.20
$MgSO_4 \cdot 7\ H_2O$	0.10
$NaH_2PO_4 \cdot H_2O$	0.125
Glucose	1.00
Phenol red	0.05
$NaHCO_3$	2.20
Gas phase	5.0 per cent CO_2 in oxygen

F

ANTIBIOTICS USED IN CULTURE MEDIUM

Streptomycin sulphate	100 µg/ml
Penicillin	100 µg/ml
Neomycin sulphate	160 µg/ml
Polymyxin B sulphate	50 µg/ml
Fungizone-amphotericin	20 µg/ml

G

EFFECT OF TREATMENT WITH INORGANIC FLUORIDE ON SERUM ALKALINE PHOSPHATASE* OF GERIATRIC MALES BY WEEKS

Week	Control		Fluoride	
	Mean	SD	Mean	SD
0	2.00	± 0.58	2.55	± 0.96
33	2.09	0.88	3.38	1.25
52	1.86	0.52	3.48	1.51
73	2.30	0.67	3.28	1.69
82	2.19	0.57	3.06	1.29
95	2.45	0.51	3.09	1.19
107	2.37	0.72	3.28	1.42
120	2.34	0.75	2.76	1.16
130	2.29	0.57	2.79	0.91
143	2.18	0.76	3.25	1.33

* Activity expressed in Sigma units/ml (1 Sigma unit will liberate 1 uM of p-nitrophenol per hour

Normal range: 0.8 to 2.3 Sigma units/ml

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