C H A P T E R I

Introduction

CHAPTER I

INTRODUCTION

For a long time bone was described as a metabolically inert tissue which merely performed the mechanical functions of supporting the body and protecting vital organs. The evolution of more refined and powerful techniques in the fields of histochemistry and biochemistry in recent years has helped us to study bone at the cellular level. Investigations to date suggest that it is in a state of continuous metabolic activity.

Bone consists of inorganic as well as organic constituents (Eastoe, 1956). The major inorganic ions are calcium and phosphate while other ions such as carbonate, hydroxyl, sodium, potassium, magnesium and fluoride are present in small amounts. The major organic constituents are citrate, glycogen, mucopolysaccharides and collagen.

Many workers have estimated the calcium and phosphorus contents of bone of different species and the values reported for the calcium to phosphorus ratio ranged from 1.99 to 2.23 (Hammett, 1925; Shear and Kramer,

1928; Burns, 1929; Morgulis and Janecek, 1931; Leulier, Policard and Revol, 1941 a,b). Since the calcium to phosphorus ratio in $Ca_3 (PO_4)_2$ is 1.94, Burns and Henderson (1935) suggested that in addition to calcium phosphate there must be a small amount of calcium present as carbonate and hydroxide.

In order to understand the role of calcium and phosphorus in bone metabolism, isotopic studies have been carried out by many investigators. When Manly and Bale (1939) and Manly et al (1940) fed a single dose of $Na_2HP^*O_4$ containing radioactive phosphorus to rats, phosphorus from the blood was deposited rapidly in the bone. The epiphysis acquired twice as much of the labelled phosphorus per gram of tissue as the diaphysis on the first day, but the diaphysis retained more labelled phosphorus after radioactivity in the blood had fallen to a negligible amount. Therefore, they suggested that the epiphysis was more active in mineral metabolism than the diaphysis. Studies carried out by Armstrong and Barnum (1948) on turnover of radioactive calcium and phosphorus in different areas of the femur of rats showed that the relative specific activities of P^{32} and Ca^{45} were somewhat

higher for the epiphysis than for the diaphysis. Hence, these two parts of calcified tissue exchanged calcium and phosphorus at different rates.

Strobino and Farr (1949) observed that bones of cows and oxen of different ages were calcificied maximally at the longitudinal midpoint of the bone where the nitrogen content was low. Hence, they suggested that calcification and the formation of nitrogenous compounds were inversely proportional.They also reported that the degree of calcification increased with age.

Baker et al (1946) studied the calcium and nitrogen contents of cortical and cancellous areas of the human femur and found that the calcium to nitrogen ratio was greater in the cortex (5.8) than in the cancellous area (5.5). A similar conclusion is reached from the data of Rogers and his associates (Weidmann and Rogers, 1950, '58; Rogers et al, 1951, '52) who have reported that the calcium phosphorus contents of the cortical area from the femurs of cats, rabbits, rats oxen and humans were higher than those of the cancellous area whereas nitrogen content was less.

Whitehead and Weidmann (1957, '59a) separated the phosphorus compounds of ossifying cartilage in the long

bones of growing puppies and kittens into two fractions. One fraction, namely that soluble in 20% trichloroacetic acid (TCA), contained orthophosphate, adenosine triphosphate, adenosine diphosphate, and adenosine monophosphate. During the ossification of cartilage, the concentration of ATP in this fraction gradually diminished without any corresponding increase in ADP, or AMP content. The second fraction, which was insoluble in TCA, contained phospholipids, nucleic acids and unknown organic phosphates. While the calcium to phosphorus ratio of ashed cartilage increased from 1.1 to 1.8 with increasing age, the ratio in the TCA-soluble fraction was always 1.9 to 2.1 suggesting that tricalcium phosphate had been deposited even in the early phases of calcification.

Not only the actual contents of calcium and phosphorus in the diet but also their presence in the favourable ratio are important for the deposition of these minerals during the calcification of bone. McCollum et al (1921) and Steenbock and Black (1925) reported that an inadequate dietary supply of calcium and phosphorus or an unfavourable calcium to phosphorus ratio or a deficiency of vitamin D in the diet resulted in inadequate mineralization of

cartilage matrix. Later, Shohl (1936) demonstrated that a diet containing 1% calcium and 0.5% phosphorus did not produce rickets in rats whereas one containing 0.25% calcium and 0.12% phosphorus produced rickets although the calcium to phosphorus ratio was 2:1 in both cases. Similarly, a diet containing 1% calcium and 2% phosphorus did not produce rickets whereas one containing 0.06% calcium and 0.12% phosphorus did, although the ratio was 1:2 in both cases.For rats in these experiments, the best ratio of calcium to phosphorus in the diet for good mineralization was 1 or 2:1 whereas Bethke et al (1929) has reported that 3 or 4:1 was best for chicks. When Murao (1938) analysed the femur of white rats fed either a diet containing vitamin D and calcium and phosphorus in the ratio of 1:0.73, or the same diet without vitamin D, or a diet deficient in both vitamin D and phosphorus, he found the calcium to phosphorus ratios in the bones were 1.36, 1.30 and 1.25 respectively. Rickets appeared earlier on the diet deficient in both vitamin D and phosphorus.

Krieger and Steenbock (1940) observed that in rats the availability of phytate phosphorus was markedly affected by calcium and vitamin D. Pileggi et al (1955)

found that the hydrolysis of phytate present in a calcium-free cereal diet was markedly decreased when 1% or more calcium carbonate was added to the diet. The rate of hydrolysis could be increased by incorporating vitamin D in the cereal diet containing 1% calcium carbonate. However, vitamin D did not show any beneficial effect on the rate of hydrolysis of phytate if calcium was not added to the diet. Somewhat earlier, Steenbock et al (1953) had shown that phytase activity of an extract of the intestinal wall of rats and chicks kept on a cereal, rachitogenic diet was increased when the diet was supplemented with vitamin D. This effect of vitamin D was not limited to a cereal, rachitogenic diet because a similar increase in phytase activity was observed in animals fed a noncereal, nonrachitogenic diet. Considering their own data as well as that of Steenbock et al (1953), Pileggi et al (1955) suggested that the increased rate of hydrolysis when vitamin D was added to the calcium-free, cereal diet plus 1% calcium carbonate might be due to either increased absorption of calcium or increased intestinal phytase activity or both.

An inadequate supply of either calcium or phosphorus

in the diet has been reported to reduce the concentration of the corresponding element in the serum. Kramer and Howland (1922) produced rickets in rats by lowering the amounts of calcium and phosphorus in the diet, and observed a 50% decrease in the serum concentrations of both elements. This fall in serum concentration might account for rickets in part, becaused Shipley (1924) obtained calcification in cartilage slices from rachitic rats when they were incubated in serum or plasma obtained from normal animals.

The above studies suggest that for proper mineralization of cartilage, the levels of calcium and phosphorus in the serum of the animal are important and they, in turn, depend upon the concentrations of the same in the diet.

The organic matrix of bone is mainly collagen and mucopolysaccharides. Rogers et al (1951, '52) have reported that 90 to 96 % of the organic matrix of both cortical and cancellous areas of bones from several species consisted of collagen.

The amino acid composition of collagen obtained from bone is characterized by the presence of a large amount of hydroxyproline, glycine, alanine and glutamic

acid (Neuman, 1949 and Eastoe, 1955). To study the noncollagenous component of bone, Eastoe and Eastoe (1954) separated a mucopolysaccharide-protein complex from the organic matrix by extraction with lime water. The residue consisted mainly of collagen which was dissolved in hot water and converted into gelatin. A small amount of protein which resisted solution in hot water was called "resistant" protein. When Eastoe and Eastoe (1954) analyzed all the non-collagenous protein fractions including an "osseomucoid" fraction for amino acids, they found that they contained no hydroxyproline, lower amounts of glycine, alanine and proline, and higher amounts of leucine and tyrosine than collagen.

A non-collagenous protein with an extremely low hydroxyproline content was obtained from tibiae and femora of 7-day-old chick embryos and from the cones and shafts of tibiae of 13-to 14-day-old chick embryos after the removal of collagen, nucleic acids and hexosamine-containing substances, (Biggers et al, 1961).

"Osseomucoid" containing protein and carbohydrate moieties was isolated first by Hawk and Gies (1901). Later, Hisamura (1938a,b) obtained two fractions from

ox trachea cartilage; one contained chondroitin sulfate and the other was a protein contaminated with chondrosamine. Rogers (1949, '51) and Glegg and Eidinger (1955) found that the polysaccharide obtained from the demineralized epiphysis of a human femur contained galactosamine, glucuronic acid and sulfur.

Meyer et al (1958) isolated a polysaccharide from the costal cartilage of newborn infants and identified it as chondroitin sulfate A, whereas that from adults was identified as chondroitin sulfate C. Subsequently, Kaplan and Meyer (1959) reported that keratosulfate (polygalactosido-N-acetylglucosamine) was negligible or absent in the bones of the human newborn child and that chondroitin sulfate decreased and keratosulfate increased with age. On the other hand, Lash and Whitehouse (1960) and Biggers et al (1961) found that keratosulfate was present even in the embryonic bone of chicks. Galactosamine was found to decrease with age in cartilage of man and rabbits whereas glucosamine increased (Kuhn and Leppelmann, 1957, '58; and Bertolin and Matucci, 1962).

The hexosamine content of epiphyseal cartilage of three-week old rachitic chicks increased when Cipera et al

(1960) gave vitamin D_3 orally. On the other hand, Dikshit (1959) has reported that the hexosamine content of epiphyseal cartilage of rachitic rats decreased after the administration of vitamin D. These conflicting data show that the exact role of vitamin D in hexosamine metabolism has not been elucidated.

Recently, Dingle et al (1961) studied the effect of excess vitamin A on the metabolism and composition of 7-day-old chick embryonic femora and tibiae cultivated in vitro. They found that the total aminosugar decreased by 50% in six days when bones were cultivated in the presence of excess vitamin A.

Castellani and Zambotti (1956) have reported that the enzyme synthesizing hexosamine from glutamine and glucose-6-phosphate was present in the epiphyseal cartilage of growing rabbits. Studies by Dikshit (1959) showed that, although the hexosamine content of bone from rachitic rats was higher than that of rats treated with vitamin D, the activity of hexosamine synthese in epiphyseal cartilage was lower for rachitic rats and was restored when the animals were fed vitamin D. Cipera and Willmer (1962,'63) have observed a similar decrease in the activity of the

hexosamine synthesizing enzyme in the epiphyseal cartilage from growing rachitic chicks and a reversal of this decrease by administration of vitamin D_3.

The addition of glutamine to the incubating medium for cartilage slices stimulated incorporation of radioactive sulfur into chondroitin sulfate according to Bostram, Roden and Vestermark (1955). Recently D'Abramo and Lipmann (1957) showed that $S^{35}O_4$ was incorporated into adenosine-3-phosphate-5'-phosphosulfate (PAPS) both by slices and by extracts of the cartilage of chicks in the presence of ATP and magnesium ions. Thus, the bone is a site of active sulphate metabolism, although it is not known at which stage the sulfation occurs in the biosynthesis of chondroitin sulfate. Meyer et al (1956) have suggested that chondroitin was synthesized first and then sulfated whereas Adams (1959) demonstrated that chondroitin was not the immediate precursor of chondroitin sulfate.

Of other organic compounds present in the bone, citric acid is present in considerable amounts. In the mouse, 70% of the total body citrate was found in the skeletal tissue (Dickens, 1941). Analysing the bones of different species, Thunberg (1948) obtained 5% citric acid in the dry, fat-free bone of the herring whereas

in dry, fat-free human bones citric acid ranged from 0.71 to 1.88 %. In birds, citric acid content varied from 0.60 to 2.67 % on dry, fat-free basis. Long bones of rabbits of various ages were analysed by Cartier (1949). He found that citric acid content was nearly constant at approximately 0.9%. Dixon and Perkins (1952) noted that the diaphysis contained more calcium and also more citrate than the epiphysis. To them, this suggested that citrate might be involved in the calcification of bone.

Sodium citrate has been effective as an antirachitic factor under certain conditions. For example, Hamilton and Dewar (1937) and Shohl (1937) showed that addition of sodium citrate to a high calcium, cereal diet could prevent the development of rickets as well as cure previously developed rickets. Day (1940) reported that this effect was found with a ration high in calcium but not with a ration low in calcium whether or not cereal was present.

Although both citrate and vitamin D increased the availability of phytate phosphorus when added to a high calcium, phytate diet, citrate was more effective in increasing the rate of hydrolysis of phytic acid than

vitamin D (Pileggi et al, 1956). Citrate probably also favoured the absorption of inorganic phosphorus since the formation of calcium citrate would be expected to reduce the fecal loss of phosphorus as calcium phosphate. However, when vitamin D was added to the high calcium, phytate diet, the phytase activity of an intestinal extract was increased but it was decreased when citrate was added. This may be due to the fact that citrate reduced the amount of calcium, an ion which inhibits hydrolysis of phytic acid. They also reported that with a mildly rachitogenic, noncereal diet, citrate was antirachitic when the diet contained 1% calcium and 0.2% inorganic phosphorus, but it was not antirachitic when the diet contained 0.5% calcium and 0.015% phosphorus.

Citrate has been shown to be rachitogenic under certain conditions by Cramer et al (1956). They reported that absorption of calcium from a vitamin D-free, noncereal diet containing 0.5% calcium and 0.015% phosphorus was greatly reduced by the incorporation of citrate in the diet. When vitamin D was added, the absorption of calcium was increased whether citrate was present or not, but the absorption of calcium was less

when citrate was added than when it was not. This seems to be in disagreement with the observation made by Heinz, Muller and Rominger (1948) who suggested that formation of calcium citrate in the intestine facilitated the absorption of calcium.

The concentration of citrate in the blood is dependent on absorption of dietary citrate and on citrate released from tissues. In cartilage, citrate is obtained from blood, but its concentration would depend also on synthesis and oxidation of citrate within the tissue itself. Several workers have reported that citrate in the bone was decreased in rickets and that it increased on administration of vitamin D. (Nicolaysen and Nordbo, 1943; Steenbock and Bellin, 1953; Dikshit, Joshi and Patwardhan, 1956; Dikshit and Sriramachari, 1961).

The presence in the bone of citrate which is an intermediate of the respiratory cycle raised the question whether or not it has a role in respiration. Several attempts which have been made to detect glycolysis and respiration in bone tissue are reviewed below.

After Robison (1923) identified an alkaline phosphatase at the site of calcification in the slices

of cartilage of rats, Kay and Robison (1924) postulated that this enzyme brought about calcification in cartilage because it hydrolysed phosphoric acid esters of monosaccharides, thereby liberating phosphate ions. Harris (1932) demonstrated that glycogen was present in the cells of cartilage, and studies by Bywaters (1936), Hills (1940) and Lutwak-Mann (1940) suggested that an active glycolysis was operating in the cartilage of rats, horses and calves. Additional proof for glycolysis was obtained when phosphorylase, phosphoglucomutase etc., phosphorylated intermediates and adenosine triphosphate were detected in bone slices (Gutman and Gutman, 1941; Gutman, Warrick and Gutman, 1942; Gutman and Yu, 1950; Marks and Shorr, 1950; Roche et al, 1951; Albaum, Hirschfeld and Sobel, 1952a,b).

Aerobic oxidation in the cartilage of embryonic chick was demonstrated by Boyd and Neuman (1954), while Whitehead and Weidmann (1959b) observed that utilization of oxygen by cartilage slices of growing kittens was increased when citrate, 2-oxoglutarate, succinate, malate or pyruvate was added to the incubating mixture. The latter workers also demonstrated by histochemical

methods that cytochrome oxidase and dehydrogenases were present in cartilage slices. Dixon and Perkins (1952), Hiatt and Shorr (1953) and Follis and Melanotte (1956) confirmed histochemically the presence of dehydrogenases while Boyd and Neuman (1954) and Castellani and Zambotti (1954) identified succinic dehydrogenase in cartilage by manometric and colorimetric procedures respectively. These findings indicated that the tricarboxylic acid cycle was operating. Further, Cartier (1950) and Cartier and Picard (1955) observed that adenosine triphosphate was important for formation of the primary seed that initiated calcification after which bone salt was deposited independently of cellular metabolism. On the other hand, Whitehead and Wiedmann (1959b) have suggested that the respiratory cycle produced energy required both to initiate and continue calcification.

Several people have tried to correlate glycolysis and respiration with the formation of citric acid. For example, Dixon and Perkins (1952) found a greater activity of citrogenase and aconitase and a lower activity of isocitric dehydrogenase in the epiphyseal cartilage plate than in the diaphyseal area of the rabbit bone. They

suggested that the increased citrate in the epiphysis was coprecipitated with bone salt. Citrogenase activity in the bone was greater in young rats than in old ones (Perkins and Dixon, 1953). Because the isocitric dehydrogenase and aconitase activities in highly calcified cortical bone of rabbits and dogs were low, Van Reen and Losee (1958) and Van Reen (1959) suggested that they may regulate citrate metabolism during calcification.

Dikshit, Joshi and Patwardhan (1956) and Krishna Rao and Patwardhan (1960) found that citrogenase activity in the slices of cartilage tissue of rachitic rats was low and that it could be raised by the administration of vitamin D. Not only synthesis but also oxidation of citrate was lower in the cartilage of rachitic rats than in that of rats given vitamin D, according to Meyer et al (1959).

Ramalingaswami et al (1954) reported that glycogen breakdown in the hypertrophic zone of cartilage of rachitic rats was reduced and could be restored by administration of vitamin D. On the other hand, Tulpule and Patwardhan (1954) did not find any change in anaerobic glycolysis during rickets. Instead, they found a decrease in pyruvic oxidase in rachitic rat cartilage. Krishna Rao

and Patwardhan (1961) reported that enzymes forming acetyl lipoate and acetyl coenzyme A were also reduced in rickets.

In summary, the above studies suggest that the formation of citrate via the tricarboxylic acid cycle was impaired in rachitic tissue.

Citrate has been located at the crystal surface of apatite in the bone. There it would be in dynamic equilibrium with citrate of body fluids (DeLuca et al, 1956). When DeLuca and Steenbock (1957) and DeLuca, Gran and Steenbock (1957) added vitamin D to the diet of rachitic rats, they found a decrease in the rate of oxidation of citrate and isocitrate but not of other tricarboxylic acid cycle intermediates in kidney homogenates and mitochondria. Electron microscopic studies carried out by DeLuca et al (1960) on isolated rat kidney mitochondria showed that dietary vitamin D protected markedly the structural integrity of these particles. Hence, diminished oxidation of citrate in the presence of vitamin D could be due to physical inhibition of citrate penetration into the mitochondria.

Acetate-1-C^{14} was incorporated to a greater extent into citric acid in different areas of the bone of rachitic

rats given vitamin D than in bones of rats fed a rachitogenic diet only (Norman and DeLuca,1964). This suggested an increased synthesis of citric acid. At the same time, treatment with vitamin D resulted in reduced amounts of labelled CO_2, suggesting that oxidation of citric acid was decreased.

Several studies have reported that parathyroid hormone plays a role in the formation and accumulation of citrate in bone tissue. Laskin and Engel (1956) observed that when weanling rabbits were treated with parathyroid extract, the respiration of slices from their bones was reduced largely as the result of depression of the succinoxidase system. Parathormone has been shown to stimulate the formation of lactic and citric acids from pyruvic acid in bone and kidney tissues (Firschen et al, 1958 and Martin et al, 1958). Hekkelman (1961) reported that isocitric dehydrogenase activity in bone extract of normal rabbits treated with parathormone was significantly decreased whereas no change in aconitase activity was observed. Histochemical studies carried out by Walker (1961) showed that the osteoclasts of the bone of 1- to 3-day-old rats treated with parathormone did not have isocitric

dehydrogenase activity. Earlier, it had been reported by Neuman et al (1956) that parathormone could destroy the chromophoric group of reduced NADP in vitro rendering it inactive thus blocking NADP-linked reactions of glucose metabolism and causing the accumulation of citric acid.

Injection of parathormone produced bone resorption as a result of increased solubilization of bone minerals McLean (1956). Whether the increased solubilization of bone minerals was due to the fall in pH caused by formation of either citric acid or lactic acid or both was not known. Studies carried out on metaphyseal slices of long bones of normal adult mice by Borle et al (1960a) established the metabolic pattern as chiefly anaerobic and even in the presence of oxygen, 84% of glucose was metabolized to form lactic acid and only 16% was metabolized through the TCA cycle. Borle et al (1960b) also reported that treatment with parathormone surficient to raise the concentration of plasma calcium by 30% increased lactic acid production of metaphysical slices of long bone by 34%, but no significant change in citrate production occurred. To them, mobilization of minerals by parathormone seemed more likely the result of fall in pH due to increased

lactic acid formation than the increased citrate formation reported by others (Neuman et al, 1956).

In later studies, Vaes and Nichols (1961) demonstrated that the cells of metaphyseal bone from normal mice were able to oxidize large amounts of citrate, although they could form only a small quantity of citrate when incubated in vitro with oxaloacetate and pyruvate or acetate. They also observed that under parathormone treatment the formation of citrate was increased without affecting its oxidation rate. This finding could account for the increased citrate in venous blood observed by Neuman et al (1956).

It has been reported that estradiol caused extensive new bone formation in the metaphysis of long bones (Urist et al,1950). Borle et al (1960b) showed that when metaphyseal slices of the long bone from adult mice injected with estradiol benzoate were incubated aerobically with glucose, lactate production was decreased by 19% compared with controls. In contrast to the controls, there was no increase in lactic acid production from glucose anaerobically by slices from estradiol benzoate treated mice. Therefore, they proposed that the lower lactate production in the presence of estradiol benzoate created a favourable condition by raising the pH locally

and permitting mineral deposition. Vaes and Nichols (1961) reported that estradiol treatment increased both the production of citrate and its rate of oxidation. Hence, required energy would be available for active anabolic processes in formation of new bone. An increased rate of oxidation of citrate would also provide an increased amount of α-ketoglutaric acid which is a key intermediate in the synthesis of new protein.

Studies mentioned in the previous paragraphs were mostly carried out in vivo so that many host factors can be expected to have influenced bone metabolism. Studies with tissue slices, on the other hand, must consider whether permeability has obscured the picture or not. Cultivation of bone in vitro would provide a means to exclude host influences in the study of bone metabolism. In addition, studies with cell-free extracts would provide a solution to the problem of permeability.

Limb buds of three-day old chick embryos were cultured successfully by Strangeways and Fell (1926) on the surface of a clot composed of chicken plasma and chick embryo extract. Calcification as well as anatomical differentiation were observed in embryonic bone tissues

cultivated in this manner (Fell, 1928, '32, '39; Fell and Robison, 1929, '30; Fell and Canti, 1934). Nutritional requirements for growth and calcification in embryonic rudiments have been defined by Gillard (1935), Miyazaki et al (1957), Saito (1959), Endo (1960) and Ito et al (1963). The effects of different factors such as hormones and vitamins on the growth and development of embryonic rudiments have also been studied (Chen, 1954a; Goyena, 1955; Ito, Takamura and Endo, 1959; Fell and Mellanby, 1952; Herbertson, 1955; Fell and Thomas, 1961; Dingle, Lucy and Fell, 1961; and Lawson, 1961).

These studies clearly showed that tissue culture of bone could be used for physiological and biochemical studies. However, for detailed nutritional and biochemical studies, a chemically defined medium was necessary rather than one consisting of plasma or serum and embryo extract. Wolff et al (1953), Biggers et al (1957), Biggers (1960a) and Biggers, Gwatkin and Heyner (1961) have cultivated embryonic bone in chemically defined media and have achieved elongation accompanied by maintenance of metabolism resembling that occurring in vivo.

Wolff et al (1953) devised a simple medium consisting

of amino acids, vitamins, glucose, salts and agar for cultivation of chick embryonic tibiae and observed that differentiation of cells occurred. Biggers et al (1957) and Heyner and Biggers (1958) cultivated chick and rat embryonic tibiae in a chemically defined medium (medium 858) originally designed by Healy et al (1955) for the culture of Earle's L-strain cells. The tibiae elongated considerably in this medium and histologically, they remained healthy and normal for six days. Biggers and Lucy (1960) devised a modified medium, BL_1, which was very similar in composition to medium 858. Two components, viz., 5-methyldeoxycytidine and penicillin G were omitted and Earle's physiological salt solution (1943) was replaced by that of Hanks and Wallace (1949). Biggers (1960a) observed that the increase in length, wet weight and dry weight, depended on the concentration of glucose in this medium. Medium BL_1 containing 4.0 mg glucose per ml was used by Biggers (1960b) to compare the growth of embryonic chick bone with that of bone cultivated in a natural medium consisting of adult chicken serum, chick embryo extract prepared from 13-day-old chick embryos, and 4.0 mg glucose per ml. Both wet and dry weights of the rudiments cultivated in medium BL_1 were

lower than those cultivated in the natural medium. Two modified media, BGJa and BGJb, were prepared by Biggers, Gwatkin and Heyner (1961). In these media many compounds which were present in medium BL_1 were omitted. They were : l-cystine, l-glutamic acid, l-aspartic acid, l-alanine, l-hydroxyproline, vitamin A, ascorbic acid, calciferol, vitamin K, nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, coenzyme A, cocarboxylase, flavin adenine dinucleotide, uridine triphosphate, glutathione, cholesterol, adenine deoxyriboside, guanine deoxyriboside cytosine deoxyriboside, thymidine, sodium acetate, d-glucuronic acid, solvents for fat soluble components viz., Tween 80 and ethanol, n-butylparahydroxybenzoate, ferric nitrate and disodium hydrogen phosphate. Pyridoxal hydrochloride and calcium chloride were replaced by pyridoxal phosphate and calcium lactate in BGJa and BGJb. Calcium pantothenate, nicotinamide, thiamine hydrochloride, riboflavin, vitamin B_{12} and penicillin G which were not present in medium BL_1 were added to both new media. Medium BGJa also contained glycine, which was absent from medium BGJb, and adenosine which was absent from media BGJb and BL_1. The concentration of glutamine was decreased from

35 mg/100 ml in BGJa to 20 mg/100 ml in BGJb. The concentrations of the ingredients common to media BGJa, BGJb and BL_1 were also different.

Elongation and increase in wet and dry weights of embryonic bone rudiments from chick, rat, mouse and turkey cultivated in these media, viz., BGJa, BGJb, and BL_1 were similar (Biggers, Gwatkin and Heyner 1961). These results showed that many substances, for example, aminoacids, such as serine, alanine, proline, hydroxyproline, aspartic acid, glutamic acid and cystine, were nonessential for growth of embryonic chick tibiae cultivated in a chemically defined medium. However, glutamine was found to be essential because when it was omitted, from medium BGJa embryonic bones of chick, rat and mouse did not grow. Their results failed to confirm the report of Kieny (1958) that glutamic acid and not glutamine was indispensable for prolonged growth of embryonic chick tibiae. Other studies of Biggers (1961a,b) confirmed the requirement for glutamine and suggested that glutamic acid could not serve as a precursor. Biggers (1961a) has suggested that the failure of glutamine to promote growth in Kieny's (1958) experiment was due to the fact that she used a concentration of 0.16 mg per 100 ml, a value

which was at the lower end of their concentration -response curve. Lawson and Lucy (1961) reported that threonine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine which were among the amino acids demonstrated to be essential by Biggers et al (1957) were removed from the medium by embryonic bones as they grew. Other amino acids present in the medium were also decreased. Methionine and tyrosine were utilized in relatively small quantities while glycine, leucine and proline were utilized in relatively large quantities. Alanine accumulated in the medium. Biggers and Heyner (1961) showed that histidine, arginine and tryptophan were also essential for growth of embryonic bones in a chemically defined medium in addition to threonine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine.

Using uniformly labelled glucose in medium 858, Webb and Biggers (1961) found that glutamic acid, aspartic acid, alanine, serine, glycine and proline were synthesized and incorporated into protein of embryonic chick bones which were cultivated for 24 hours. From data with D-(1^{14}C)-glucose and uniformly labelled l-aspartic acid and glycine, they concluded that adenine, guanine, cytosine, uracil and thymine were synthesized from glucose via the

amino acids glycine and aspartic acid and incorporated into nucleic acids. Similarly, isotopic studies carried out by Lucy, Webb and Biggers (1961) showed that amino sugars, viz., glucosamine and galactosamine, as well as ribose and deoxyribose, were synthesized from glucose when embryonic bone rudiments were cultivated in a medium containing labelled glucose.

These studies suggest that bone cells possess the machinery to synthesize many cell constituents from glucose when cultivated in a chemically defined medium. The detection of the necessary enzymes in cell-free extracts of bone cultivated in vitro would give additional proof for the capacity of the bone to synthesize its cellular constituents from glucose.

In order to add to the evidence that bone is metabolically active and that enzymic activities are maintained when embryonic bone rudiments are cultivated in vitro, the present studies were carried out on : (a) the chemical composition of 10-day-old chick emryonic tibiae cultivated in a chemically defined medium with regard to calcium, phosphorus, nitrogen, total hexosamine, citric acid and Ca/P and Ca/N ratios; (b) the utilization

of glucose and formation of lactic acid and keto acid during *in vitro* cultivation; (c) the activities of certain key enzymes in cell-free extracts of different areas of matured chick tibia and their relation to the chemical composition of these areas, (d) a comparision of the chemical composition and enzyme make up of tibiae cultivated *in vitro* with those of bones obtained *in vivo* from embryos of corresponding age.

Details of these studies are described in this thesis.