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The Uptake of -p3-s-p5-sSO-b-b4-s-p-- -sin the Proximal End of the Tibia in Normal and Rachitic Rats

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THE UPTAKE OF $^{35}SO_4^{--}$ IN THE PROXIMAL END OF THE TIBIA IN NORMAL AND RACHITIC RATS

by

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LIFE

PHILIP JOHN MILANOVICH was born in Butte, Montana on February 10, 1940. He was graduated from Butte High School, Butte, Montana in June, 1958. He entered Montana State College, Bozeman, Montana after high school and was graduated with a Bachelor of Science degree in December, 1963.

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He is married to the former Rosemary Mullen and has two children.

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CHAPTER I

INTRODUCTION

The rachitic rat shows a failure of mineralization of cartilage and bone. Sulfation of chondroitin is apparently an essential step in preparing cartilage and bone for mineralization. It is then possible that there is a failure of sulfation of chondroitin in rickets. To test this hypothesis, a measurement of the uptake of $^{35}SO_4^{--}$ in the proximal end of the tibia in normal and rachitic rats was undertaken.

This study deals with the incorporation of $^{35}SO_4^{--}$ into chondroitin sulfate and the recovery and measurement of the isotope.

When $^{35}SO_4^{--}$ is administered as the sulfate ion ($^{35}SO_4^{--}$) to be incorporated in a molecule by biosynthesis in young growing animals, the concentration of radiosulfur in the epiphyseal cartilage increases over a period of some 24 hours. This is attributed to the rapid utilization of the $^{35}SO_4^{--}$ by the cartilage for synthesis of chondroitin sulfate.

Chemical digestion is an apparent method for isotope recovery from bone and cartilage and was the procedure method employed.

The usefulness of a radioisotope as a tracer lies in its detection, recording, and measurement to provide a system for quantitative determination. Liquid Scintillation affords the highest possible sensitivity in detecting, recording and measurement.

CHAPTER II

REVIEW OF THE LITERATURE

Logan (1935) stressed the fact that calcification of cartilage is preceded by loss of organic sulphate. This relationship was later emphasized by Hass (1943), who suggested that the maintenance of a high level of chondroitin sulphate in cartilage is a device by which cartilage is protected against calcification.

Dziewiatkowski (1949) investigated the possible utilization of sulfate sulfur by the suckling rat for the synthesis of chondroitin sulfate as indicated by the use of radioactive sulfur. Observations on change in the concentration of ^{35}S with time in the cartilage of the joints after the injection showed that there is a progressive increase in the concentration of ^{35}S until the 24th hour. After the 24th hour ^{35}S appears to be slowly lost from the cartilage. This apparent decrease in ^{35}S concentration is primarily a result of dilution with newly formed sulfate in cartilage. Partial separation of chondroitin sulfate from the cartilage of the knee joint of the animals suggested that the major portion, if not all of the labeled sulfate sulfur retained by the articular cartilage, may be retained therein as chondroitin sulfate.

Dziewiatkowski (1957) examined the metabolism of ^{35}S and ^{45}Ca in the metaphysis of immature rats autoradiographicly. The deposition of ^{35}S in the long bones of suckling rats showed that the concentration of ^{35}S in the metaphysis increased as it decreased in the epiphyseal cartilage plates. This suggested that the ^{35}S which accumulated in the metaphysis was derived from the chondroitin sulfate of the epiphyseal cartilage

plates. In contrast with the chondroitin sulfate in the cartilage, the labeled material in the metaphysis was insoluble in formalin saturated with barium hydroxide, thus suggesting the likelihood that the ^{35}S in the metaphysis was in either inorganic sulfate or/and in some compound (or compounds) insoluble in the presence of barium ions at pH above 10.

This suggestion was supported by Engfeldt (1954) who concluded that 4 days after the administration of sodium sulfate ^{35}S to dogs, the isotope in bone was partitioned between inorganic sulfate and organic sulfate.

Kent (1956) separated the ^{35}S sulfate in rabbit bones into a form removed by decalcifying solutions and a form which was shown to be ^{35}S labeled chondroitin sulfate or a substance akin to it.

A comparative study was conducted on the ^{35}S labeled materials in the metaphysis of normal rats and estradiol-treated rats (estrogen inhibit resorption of metaphyseal bone in immature rats; as a consequence, the spongiosa may be many times longer than that of normal rats.) One could more easily harvest a sufficient amount of metaphyseal bone from them for the determination of the nature of the ^{35}S labeled material shown by autoradiograms to be deposited in the metaphysis.

Determination by extraction with a 10 per cent sodium hydroxide solution showed 44 and 63 per cent of the ^{35}S was removed from the distal metaphysis of the femurs from the estradiol-treated and control rats, respectively. Of the extractable ^{35}S, 75 and 86 per cent, respectively, did not pass through a cellophane membrane when dialyzed against water.

Chromatography on an anion exchange resin, dowex 2, showed that 9 per cent of the ^{35}S in the extract of the bone from the control rats was in the form of inorganic sulfate; 22 per cent of the ^{35}S in the extract of the bone from the estradiol-treated rats was accounted for in this form. The remainder of the ^{35}S in both extracts behaved chromatographically as does the ^{35}S in a similar extract of epiphyses removed from 8-day old rats 24 hours after injecting with ^{35}S-sulfate. This latter observation strongly suggests that some of the ^{35}S in the metaphyses is present in material akin to chondroitin sulfate. If the extraction with sodium hydroxide solution is assumed to remove all of the material resembling chondroitin sulfate, but only a small fraction of the inorganic sulfate, then 33 to 54 per cent of the ^{35}S in the metaphyses is present in forms other than inorganic sulfate.

It is more likely that about 80 per cent of the ^{35}S in the distal metaphyses of the femurs is present in compounds other than inorganic sulfate. This higher value is indicated by the experiments in which the pulverized metaphyseal bone was dialyzed against a solution of sodium versenate and then against water. Dialysis removed 6 per cent of the ^{35}S from the control sample of bone and 21 per cent of the ^{35}S from the experimental bone. These values for dialyzable ^{35}S and the values for ^{35}S as inorganic sulfate, as determined by chromatography of extracts on dowex 2, suggest that relatively more of the ^{35}S was in the form of inorganic sulfate in the metaphyses of the estradiol-treated rats than in the metaphyses of the control rats. Why this should be is not apparent.

Of the ^{35}S which did not pass through the cellophane membrane, 83 to 87 per cent was precipitable by cetyltrimethylammonium bromide and was subsequently soluble in 0.05 N sodium hydroxide. In the latter solutions, in addition to ^{35}S sulfate, uronic acid and hexosamine were present. Indeed, when these solutions were analyzed by paper chromatography and paper electrophoresis, material was found which behaved as did purified chondroitin sulfate, isolated from bovine nasal septa by the procedure of Bostrom and Mansson (Dziewiatkowski-1957). The materials extracted from the bones were metachromatic in the same way as the purified chondroitin sulfate. Moreover, the areas with radioactivity on the papers, as determined by autoradiography, coincided with the areas of metachromasia.

D'Abramo and Lipmann (1957) synthesized adenosine-3-phosphate -5-phosphosulfate in particle-free extracts of chick embryo cartilage and converted this biological sulfate carrier, "active sulfate", into chondroitin sulfate. Adenosine-3-phosphate -5-phosphosulfate is enzymically formed in a two-step reaction: Step (1) ATP + S $\rightleftarrows$ APS + PP

Step (2) APS + ATP $\rightarrow$ PAPS +ADP

PAPS acts as a sulfate donor in the various sulfate acceptor systems. In the transfer of sulfate from PAP ^{35}S to chondroitin sulfuric acid, the addition of ATP stimulates greatly this incorporation into the polysaccharide. UTP gave a slightly higher incorporation than ATP.

Campo and Dziewiatkowski (1962) suggested that an understanding of the interrelationship between cells and the intercellular substance of cartilage may help resolve the question of why some cartilages ossify and

others do not. Thus far in autoradiograms of growing bones, ^{35}S sulfate has been seen concentrated initially in chondrocytes, from which it is extruded into the matrix, and thence, a position of the label persists in the bone that replaces the cartilage. Chondroitin sulfate labeled in the ester sulfate group has been isolated from the cartilage either after the administration of ^{35}S sulfate to animals or after incubation of slices of cartilage in buffered solutions of salts containing ^{35}S sulfate. Nearly all, if not all, of the chondroitin sulfate is present in the cartilage as part of a more complex macromolecules which have been termed chondromucoproteins, Malawista and Schubert (1958), protein-polysaccharides, Gerber, Franklin and Schubert(1960) or protein-polysaccharide complex, Gross, Mathews and Dorfman (1960). In view of this, it was of interest to determine whether the polysaccharide moiety and protein moiety were synthesized simultaneously by chondrocytes. It has been reported (Gross, Mathew and Dorfman-1960) that in costal cartilage of rats, the two moieties are catabolized at similar rates.

The resultant study by Campo and Dziewiatkowski showed that the protein, as well as the polysaccharide moieties of protein-polysaccharides, are synthesized simultaneously and intracellularly. Furthermore, it has been found that the turnover rates of chondroitin sulfate are similar when ^{14}C- labeled precursors or ^{35}S sulfate are used. It has been concluded, therefore, that ^{35}S sulfate does, indeed, serve as a convenient and appropriate marker in turnover studies of chondroitin sulfate.

Guri (1965) incorporated ^{35}S into protein-polysaccharides in vitro. The experiment indicated, not only rapid incorporation of labeled sulfate

into the isolated sulfated glycosamino-glycans but very little incorporation into the glycosamino-glycans remained in the residual matrix. Ninety per cent of the radioactivity incorporated was observed in the protein-polysaccharides fractions.

DeLuca and Silbert (1968) studied the biosynthesis of chondroitin sulfate by incorporation of sulfate ^{35}S into microsomal chondroitin sulfate. A microsomal preparation from chick embryo cartilage was shown to catalyze the incorporation of sulfate ^{35}S from 3' phosphoadenosise 5 phosphosulfate - ^{35}S into microsomal glycosaminoglycan. This enzyme preparation has previously been shown by the investigator to catalyze the formation of chondroitin (nonsulfated) from sugar nucleotides precursors. DeLuca and Silbert reported the microsomal sulfate acceptor to be of high sulfate content and appeared to be similar to chondroitin sulfate found in a supernatant preparation from chick embryo cartilage. The presence in the microsomal fraction of both sulfotransferase activity and glycosamenoglycan polymerizing activity, as well as chondroitin sulfate, suggest the sulfation in the cell may take place in close proximity to the location of glycosaminoglycan polymerization.

Robinson and Dorfman (1969) with the use of purified chondroitinase of "Proteus vulgaris" demonstrated a method for the determination of relative amounts of chondroitin, chondroitin -4-SO_4, and chondroitin -6-SO_4 in cartilage. The method was applied to the analysis of embryonic chick epiphyseal cartilage. With the use of $^{35}SO_4$ and ^{14}C- acetate, it was possible to study the relationship of embryo age to rate of formation of chondroitin -4-SO_4, and chondroitin -6-SO_4. Between 10 and 19 days, there

was a progressive decrease in synthesis of chondroitin and chondroitin -4-SO_4 and a progressive increase in synthesis of chondroitin-6-SO_4.

Rickets appeared when people began to live in cities. The first descriptions are attributed to Whistler and to Glisson, though there are earlier ones (Hess 1930, Findlay 1934, Clark 1962). Daniel Whistler, a student of Merton College in Oxford, wrote on rickets for his thesis for the doctorate of Leyden University in 1645; it was entitled Inaugural Medical Disputation on the Disease of English Children which is popularly termed Rickets (Smerdon 1950). Glisson wrote his treatise 'De Rachitide sive Morbo Puerili qui vulgo The Rickets dicitur' for a committee of the Royal College of Physicians in 1650. In 1666, John Locke reported a post-mortem examination of a child with rickets who died with pneumonia. It is clear that these men were writing about a disease which was already common and familiar. On the Continent, it came to be known as the English disease. The origin of the English word rickets is obscure; it may be derived from an Old English word 'wrick' meaning to twist, from 'wry-gates' meaning crooked gait, or from a Norman word for a hunchback, riquets. Rachitis, on the other hand, is presumably a professional term derived from the Greek word for the spine, rachis.

The distinctive histological features of rickets and osteomalacia are the persistence of an excess of uncalcified bone, which Virchow (1953) named osteoid, and the numerous osteoblasts. Normally new bone is calcified as it is formed; only a thin layer of osteoid can be detected covering the trabeculae, and osteoblasts are scanty. Pommer (1885) was the first to recognize that osteoid is new bone which has failed to calcify,

and not, as von Recklinghausen believed, tissue from which calcium has been removed (Fourman and Royer - 1968).

In rickets the process by which bone is formed in cartilage is modified in a characteristic way. Normally, the cartilage cells proliferate in columns and calcium is laid down along the oldest parts of the columns. From the metaphysis blood vessels invade this provisional zone of calcification, which is then reabsorbed by osteoclasts and replaced by bone. In rickets, the calcification along the columns of cartilage cells is defective, the invasion by blood vessels is disorderly, and the zone of provisional calcification is not reabsorbed. The cartilage cells continue to proliferate and the zone of provisional calcification, therefore, becomes wider than normal (Follis 1958). It should be noted that there is not any hypertrophy of the cartilage, but a failure of the normal process of maturation which ultimately leads to the removal of cartilage.

Engfeldt and Zetterstrom (1955) elaborated these findings and their illustrations of micro-radiograms and of photo-micrographs by polarized and natural light were consulted. They made the new observation that the arrangement of the collagen in compact bone is abnormal in rickets: the bundles of fibres run perpendicularly to the Haversian canal, instead of parallel to it (Engfeldt and Hjertquist-1960). Sheldon and Robinson (1961) examined the bone of rats with low phosphate rickets by electron-microscopy. They found no unusual features in the collagen fibrils of the uncalcified osteoid, except for variations in their diameter and array. The cells appeared normal, but there was an abnormal afibrillar material near

many osteoblasts. This material may accumulate as a result of a block in the maturation of collagen.

Howel (1965) analyzed costal tissues biochemically in respect to a profile of sulfated mucopolysaccharides from normal and rachitic calves which provide large samples of anatomically identifiable regions for biochemical analysis. Histological distributions of sulfur compounds were obtained through the use of x-ray elemental analysis for sulfur. Distinctive difference between polysaccharide content of normal and rachitic tissue were found. Total sulfated mucopolysaccharide content was higher in demineralized osteoid and epiphyseal plate cartilage of normal calves than in comparable rachitic calf osteoid and hypertrophic cartilage. Qualitative differences in polysaccharide composition between mineralizing and non-mineralizing regions were not detected. Measurements of S content by microscopic x-ray analysis of the untreated rachitic cartilage - osteoid fraction indicated most of the sulfated mucopolysaccharides were in the cartilaginous regions rather than osteoid.

CHAPTER III

MATERIALS AND METHODS

Twenty rats of the Sprague-Dowley Strain at the age of 21 days were separated without regard to sex into an experimental group of 10 animals and a control group of 10 animals. The experimental group was placed on a Vitamin "D" deficiency diet obtained from Grand Island Biological Supply Company. The control group was maintained on a Purini Labbox diet. The animals were weighed every 4th day. A rachitic condition of sufficient intensity was thought induced when an experimental animal weight of 60% that of the control animal was achieved. Verification of rickets was made by examination of routine H & E histological sections and autoradiograms. The autoradiograms were prepared using liquid emulsion Kodak NTB3. The sections were exposed for one week. The autoradiograms were then stained with nuclear fast red and counterstained with indigo carmine.

$Na_2\ ^{35}SO_4$, 1.3 c/millimole specific activity was injected intraperitoneally at the rate of 4 uc per gram of animal weight. The animals were sacrificed after approximately two hours and the proximal end of the tibia removed containing the articular cartilage, epiphyseal plate, and the metaphysis. The samples were dried at 50°C for 24 hours, and brought to a constant weight (mg.).

Sixteen experimental bone samples and fifteen control bone samples were selected and ground into powder by placing each sample in liquid nitrogen to cool, then pulverized with a mortar and pestle. The bone powder of each sample was divided approximately into thirds (in order to

to obtain a more desirable weight of bone for digestion) and placed into scintillation vials. To each scintillation vial was added 0.2 ml. of 70% $HCLO_4$ (lead free) and 0.4 ml. of 30% H_2O_2 (fresh); the caps were tightly replaced and the vials swirled. The scintillation vials were placed in a water bath at 50°C for 30 minutes. The vials were then removed and bench cooled. The samples were prepared for counting by addition of 15 cc of scintillation fluor (PPO 8 gm/leter POPOP 0.2 gm/leter in toluene trade name "Scintisol" Beckman Corporation, Fullerton, California) to each vial.

A standard was established by the addition of 1.26×10^5 dpm/100λ $Na_2{}^{35}SO_4$ to 0.2 ml 70% $HCLO_4$ (lead free) + 0.4 ml. 30% H_2O_2 (fresh) + 15 cc of scintillation fluor. A blank of 15 cc of scintillation fluor was also prepared.

All radioactive fractions were counted in a Beckman direct data Readout liquid scintillation system (Beckman Corporation, Fullerton, California) with all channels open and radioactivity recorded in counts per minute per sample, and expressed as uc $\times 10^{-3}$/mg. tibia.

Quenching was experimentally established and found to be non-significant as summarized in Table V.

The tibial specimens were decalcified in formic acid and sodium citrate and were cut at 5u and autoradiograms were prepared and stained with indigo carmine and nuclear fast red.

Light transmission measurements were made of the autoradiograms of

the epiphyseal cartilagenous plates of the tibia taken from both experimental and control animals.

The sections were examined at 450 magnifications using the cytophotometer, Zeiss Sandritter model (Brinkmann Instruments Inc., Westburg, New York). Five selected areas of grains in the autoradiograms of each epiphyseal plate were selected and direct per cent light transmissions were indicated on a spectrophotometer. Such readings were recorded and are summarized in Table VI.

Tibial bone samples of the experimental and control animals were analyzed for ash content by oxygen combustion in a "Thomas OGG safety oxygen flask igniter (A.H. Thomas Company, Philadelphia, Pennsylvania) and expressed in per cent ash per mg. Table VII.

CHAPTER IV

RESULTS

Observations of the measurement of the specific activity of $^{35}SO_4^{--}$ in the proximal end of the rat tibia after intraperitoneal administration of labeled sodium sulfate to normal and rachitic rats are summarized in Table IV. The observations indicate clearly that there is a higher specific activity per mg. of tibia sample in the rachitic animal.

Light transmission measurements made of autoradiograms of the epiphyseal cartilagenous plates of the tibia from control and experimental animals suggests that the radioactive sulfate is more intensely localized within the control cartilage as compared with the experimental cartilage as summarized in Table VI.

Examination of the ash content of tibial bone samples revealed 43.8% ash content per mg. for the control animals as opposed to 30.3% ash content per mg. of tibia bone for the experimental animals suggesting a greater amount of mineralization in the control tibia and a greater amount of cartilage in the experimental tibia.

Examination of histologic sections (H & E and autoradiograms) indicate clearly that there is visibly more cartilage in the experimental rat tibia. The plates are thicker and the columns of cells are strikingly longer than normal.

The radioactive labeling of the chondrocytes is characterized by larger numbers of grains over the proximal chondrocytes in the columns. In the normal epiphyseal plate, the sulfation as indicated by the density

of grains appears rapidly to diminish when the cartilage calcifies. In the experimental animal, epiphyseal plate similarly shows greater labeling in the proximal cell, but one can see some labeling even in the distal cells of the lengthened columns.

CHAPTER V

DISCUSSION

A higher specific activity of $^{35}SO_4^{--}$ was found in the proximal ends of the tibia of the rachitic rats than in the proximal end of the tibia of the control rats. This observation was due in part to the ratio of specific activity to the weight of rachitic cartilage and to the weight of control cartilage. This ratio was greater for samples of cartilage from the rachitic rats than for samples from the control rats. If now the concentration of $^{35}SO_4^{--}$ in cartilage is greater than in bone, as suggested by Dziewiatkowski and others, one should find more radioactive sulfate per milligram of sample from the tibia of the rachitic rats than in samples from the tibia of the control rats. Indeed, the proximal end from the tibia of the experimental animals contained more radioactive sulfate per milligram of sample than the comparable tibial ends of the control rats.

One may now tend to conclude that, if as recorded by Campo, Dziewiatkowski, Malawista, Schubert and others, that the majority of the labeled $^{35}SO_4^{--}$ present in cartilage and bone is present in the form of chondroitin sulfate or a substance akin to it, e.g. protein-polysaccharide, then the mechanism of sulfation of chondroitin in rachitic animals is apparently as efficient, if not more so, than that of normal animals.

Examination of histologic sections (H & E and autoradiograms) indicate clearly that there is visibly more cartilage in the experimental

rat tibia. The columns of cartilage in the experimental animal are longer than those of the control animal. This is typical of the histologic finding seen in the epiphyseal plate of rachitic rats.

If the condrocyte of the experimental animal functions through a greater period of time, as reflected by the longer columns of cartilage, then the condrocytes should continue to contribute to the production of chondroitin sulfate resulting in the higher specific activity of sulfate in the experimental rat tibia.

However, examination of light transmission measurements of autoradiograms of epiphyseal cartilagenous plates suggests that the radioactive sulfate is more intensely localized within the control cartilage as compared with the experimental cartilage.

The loss of inroganic sulfate in the autoradiogram may be attributed to the decalcification process.

The reduced ash content exemplifies the smaller amount of calcified cartilage and bone in the proximal tibia of the experimental animal as opposed to control. This is, of course, a classic observation in rickets, which is a failure of cartilage and bone to mature.

The reduced ash content, coupled with the increase sulfation in the rachitic cartilage suggests an inverse relation. Hass has interpreted the relation of sulfate to calcification as inhibitory. This cannot be entirely supported by the findings as the distal part of the long columns of cartilage in the rachitic animal shows only slight sul-

fation. The sulfation proceeds from the proximal to the distal in both normal and rachitic cartilage. The longer columns of the rachitic cartilage probably only represent mature sulfated cartilage. Only a failure of maturation can be interpreted.

The representative figures in Table IV reflect only an approximate two-hour segment of the actual biosynthetic mechanism of $Na_2\ ^{35}SO_4$ utilization and not the maximum saturated 24 hour end point. Experimental evidence suggests that the uptake of $^{35}SO_4^{--}$ in cartilage increases for a period of 24 hours after the intraperitoneal administration of $Na_2\ ^{35}SO_4$.

CHAPTER VI

SUMMARY

Twenty rats of the Sprague-Dawley Strain at the age of 21 days were separated without regard to sex into an experimental group of 10 animals and a control group of 10 animals. The experimental animals were placed on a Vitamin "D" deficiency diet.

Experimental and control animals were given intraperitoneal injections of $Na_2{}^{35}SO_4$, 1.3 c/millimole specific activity at the rate of 4 µc per gram of animal weight. The animals were sacrificed after approximately two hours and the proximal end of the tibia removed containing the articular cartilage, epiphyseal plate and metaphysis.

Liquid scintillation counting evaluations were made of the proximal ends of the rat tibia. The experimental rat tibia showed a higher specific activity per mg. of tibia than that of the control (Table IV).

Light transmission measurements were made of the autoradiograms of the epiphyseal cartilagenous plates of the control and experimental animals. The light transmission measurements suggest that the radioactive sulfate is more intensely localized within the control cartilage as compared with the experimental.

Examination of the ash content of the experimental and control animal revealed a greater amount of ash per mg. of tibia sample for the control animal as compared with the experimental animal.

CHAPTER VII

CONCLUSION

Experimental evidence suggest that the metabolism of $^{35}SO_4^{--}$ in the proximal end of the tibia, of the rachitic rat is as efficient, if not more so, than that of the normal animal.

TABLE I

Weight of Rats Expressed in Grams

	Control	Experimental
1)	84.1	75.6
2)	107.2	85.8
3)	133.1	97.6
4)	143.1	98.4
5)	148.8	99.3
6)	149.2	99.6
7)	149.4	101.7
8)	149.9	103.4
9)	159.6	107.5
10)	162.6	107.9
	Mean = 138.7	Mean = 97.7
	Range = 84.1-162.6	Range = 75.6-107.9
	Variance = 548.4	Variance = 88.5
	Sigma = 23.8	Sigma = 9.4
	t = 4.874	

TABLE II

CONTROL RAT TIBIA DATA

Rat Tibia	Wt. Dried Tibia mg.	Total cpm/tibia	Total dpm/tibia	dpm/mg. Tibia
1	90.3	839,019	902,170	9,991
2	94.8	654,435	703,690	7,422
3	96.0	994,215	1,069,040	11,135
4	103.0	1,126,800	1,211,600	11,763
5	101.0	1,083,728	1,165,200	11,536
6	94.0	1,016,089	1,092,500	11,622
7	119.0	1,089,475	1,171,400	9,843
8	96.0	676,547	727,460	7,577
9	93.0	972,937	1,046,160	11,249
10	108.0	1,023,437	1,100,400	10,188
11	107.0	1,086,797	1,116,850	10,437
12	92.0	974,208	1,047,530	11,386
13	98.0	1,029,092	1,106,500	11,290
14	96.0	765,067	822,650	8,569
15	109.0	1,015,255	1,091,600	10,014

(1) Tibia dried in drying oven @ 50°C for 24 hours and samples brought to constant weight (mg.)

(2) Background 60 ± 5 cpm

(3) Efficiency calculation $\text{eff} = \frac{\text{cpm}}{\text{dpm}} = \frac{11,718}{1.26 \times 10^5} = 93\%$

(4) Sulfur $^{-35}$ standard 1.26×10^5 dpm/100λ standard value in cpm expressed as average of six values $\pm$ 1.0%.

(5) Standard = 0.2 ml. $HClO_4$ 70% (lead free) + 0.4 ml. H_2O_2 30% (fresh) + 15 cc scintillation fluor (PPO 8 gm/leter + POPOP 0.2 gm/leter in toluene trade name "Scintisol" Beckman Corp., Fullerton, California) + 1.26×10^5 dpm/100λ Sulfur -35.

TABLE III

EXPERIMENTAL RAT TIBIA DATA

Rat Tibia	Wt. Dried Tibia mg.	Total cpm/tibia	Total dpm/tibia	dpm/mg. tibia
1	55.9	563,627	606,050	10,841
2	58.7	620,444	667,140	11,365
3	54.3	783,028	841,960	15,505
4	70.0	1,018,619	1,095,200	15,645
5	56.0	1,067,227	1,147,500	20,491
6	70.0	917,543	986,600	14,092
7	68.0	684,679	736,210	10,826
8	62.0	859,587	924,280	14,907
9	65.0	882,536	948,960	14,599
10	60.0	973,378	1,046,640	17,444
11	74.0	637,124	685,070	9,257
12	64.0	1,026,745	1,104,000	17,250
13	60.0	731,373	786,420	13,107
14	62.0	872,837	938,530	15,137
15	53.0	685,027	736,580	13,897
16	62.0	1,019,178	1,095,800	17,674

(1) Tibia dried in drying oven @ 50°C for 24 hours and samples brought to constant weight (mg.)

(2) Background 60 ± 5 cpm

(3) Efficiency calculation $\text{eff} = \frac{\text{cpm}}{\text{dpm}} = \frac{11,718}{1.26 \times 10^5} = 93\%$

(4) Sulfur $^{-35}$ standard 1.26×10^5 dpm/100λ standard value in cpm expressed as average of six values $\pm$ 1.0%.

(5) Standard = 0.2 ml. $HClO_4$ 70% (lead free) + 0.4 ml. H_2O_2 30% (fresh) + 15 cc scintillation fluor (PPO 8 gm/leter + POPOP 0.2 gm/leter in toluene trade name "Scintisol" Beckman Corp., Fullerton, California) + 1.26×10^5 dpm/100λ Sulfur -35.

TABLE IV

Summary of the analysis of $^{35}SO_4^{--}$ in rat tibia.

Values are expressed in u ci x 10^{-3}/mg. of tibia.

Control		Experimental	
Tibia		Tibia	
(1)	3.34	(1)	4.17
(2)	3.41	(2)	4.87
(3)	3.86	(3)	4.88
(4)	4.43	(4)	5.12
(5)	4.50	(5)	5.90
(6)	4.51	(6)	6.26
(7)	4.58	(7)	6.34
(8)	4.70	(8)	6.58
(9)	5.01	(9)	6.71
(10)	5.06	(10)	6.82
(11)	5.08	(11)	6.98
(12)	5.12	(12)	7.04
(13)	5.19	(13)	7.77
(14)	5.23	(14)	7.86
(15)	5.29	(15)	7.96
		(16)	9.23
MEAN	4.6207	MEAN	6.5306
RANGE	3.34-5.29	RANGE	4.17-9.23
VARIANCE	4.0742E-01	VARIANCE	1.7843
SIGMA	6.383E-01	SIGMA	1.3358
"t" FACTOR	4.888		

TABLE V

QUENCHING EFFECT OF TIBIA

ON $^{35}SO_4^{--}$ COUNTING EFFICIENCY

Tibia Sample	Wt. Dried Tibia mg.	Total cpm/tibia	Per Cent Recovery
1	10	176,307	99.3
2	10	177,450	99.9
3	50	176,590	99.4
4	50	177,240	99.8
5	100	175,650	98.9
6	100	174,470	98.2
Standard		177,595	

(1) Tibia dried in drying oven @ 50°C for 24 hours and samples brought to constant weight (mg.)

(2) Background 60 ± 5 cpm

(3) Efficiency calculation $\text{eff} = \frac{\text{cpm}}{\text{dpm}} = \frac{177,595}{190,960} = 93\%$

(4) Each tibia sample spiked with sulfur $^{-35}$ standard 190,960 dpm/100λ

(5) Standard value in cpm expressed as average of four values $\pm$ 1.0%

(6) Standard = 0.2 ml. $HClO_4$ 70% (lead free) + 0.4 ml. H_2O_2 30% (fresh) + 15cc scintillation fluor (PPO 8 gm/leter + POPOP 0.2 gm/leter in toluene trade name "scintisol" Beckman Corp., Fullerton, California) + 190,960 dpm/100λ Sulfur $^{-35}$

TABLE VI

Light Transmission of autoradiograms of rat epiphyseal cartilagenous plates expressed in per cent.

	Control					Experimental				
	1	2	3	4	5	1	2	3	4	5
(1)	15	20	15	20	15	15	20	20	20	20
(2)	20	20	25	25	25	20	20	20	10	15
(3)	20	25	20	15	20	20	20	35	20	20
(4)	10	10	10	10	10	30	30	30	35	25
(5)	10	10	15	15	15	20	20	25	20	30
(6)	35	30	35	25	30	35	30	35	40	40
(7)	25	30	20	35	20	50	50	45	35	40
(8)	20	20	20	20	25	40	25	30	30	30
(9)	25	20	20	20	25	30	25	25	20	20
(10)	25	30	40	25	25	30	30	25	30	30
(11)	40	40	45	45	45	35	35	40	30	35

MEAN	23.1	MEAN	28.1
RANGE	10-45	RANGE	10-50
VARIANCE	86.49	VARIANCE	96.04
SIGMA	9.3	SIGMA	9.8
t	2.612		

TABLE VII

Summary of the analysis of ash content of rat tibia. Values are expressed in per cent ash/mg. of tibia.

Experimental Tibia	Control Tibia
30.3%	43.8%

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APPROVAL SHEET

The thesis submitted by Dr. Philip John Milanovich has been read and approved by three members of the faculty of the Graduate School of Loyola University.

The final copy has been examined by the director of the thesis, and the signature which appears below verifies the fact that any necessary changes have been incorporated into the thesis, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis, therefore, is accepted as partial fulfillment of the requirements for the Degree of Master of Science.

May 21 1970
Date

Signature of Advisor