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Cell Density and Labelling Index of the Periosteum of Rat Skull Treated with Tissue Culture Medium

Ruben Bayardo
Loyola University Chicago

CELL DENSITY AND LABELLING INDEX OF THE PERIOSTEUM OF RAT SKULL TREATED WITH TISSUE CULTURE MEDIUM

by

Ruben Eduardo Bayardo

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LIFE OF AUTHOR

Ruben Eduardo Bayardo Casillas, the last of eight siblings, was born in Guadalajara Jal., Mexico, on November 6, 1952.

His secondary education was received at Instituto de Ciencias in Guadalajara from 1964 to 1967, while his college education was obtained at Escuela Vocacional Universidad de Guadalajara from 1967 to 1969.

In September 1969 he began dental studies at Facultad de Odontologia, Universidad de Guadalajara. In 1973 He was elected Vice-president of the alumni society, and in 1974 elected President of the student group of the Sociedad Odontologica Jalisciense. He graduated in 1974 and after one year of social service He received his D.D.S. Degree in 1975.

In September 1975 He began a two year graduate program at Loyola University School of Dentistry, Chicago Ill., leading to a Master of Science in Oral Biology and a Postgraduate Certificate in Pedodontics.

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CHAPTER I

INTRODUCTION

Initially, tissue culture methods were designed to study cell anatomy. Later, they were used to determine the essential nutrients required for growth and development of the cells.

The application of this area of scientific effort became valuable with the observations of the effect of drugs and materials on the living cells in vitro. This was followed by the wide use of natural and synthetic media promoting and supporting the stimulation of cell proliferation in vitro, but rarely used for research in vivo.

The purpose of this study is to determine the in vivo effects of the local infiltration of artificial tissue culture medium on the growth of the periosteal cell population of the interparietal suture demonstrated by autoradiographic and cell density analysis.

CHAPTER II

REVIEW OF LITERATURE

GROWTH OF BONE TISSUE OF THE SKULL

The human skull, as a whole, depends for its over-all dimensions on the growth of the cartilages at the base of the skull, connective tissue at the sutures in other places, and of course the simultaneous replacement of these growing tissues by bone. Sicher[1], and Weinman and Sicher[2] have stated that with the exception of the cranial base, the growth of the cranium occurs by sutural growth which is initiated by a proliferation of the connective tissue in the suture and simultaneous apposition of bone.

In the growing suture, Enlow[3] has distinguished three distinct layers which have been variously named. In general a sutural membrane has a separating "capsular" layer with two "cambial" zones, one on either side of it adjacent to the two bone surfaces. The capsular zone is composed largely of coarse bundles of mature, thick collagenous fibers and blood vessels. It is a dense, regular connective tissue layer which is directly continuous with the dense fibrous portion of the periosteum. When active growth and remodeling changes cease, the entire suture becomes essentially a single capsular zone. The cambial layer is much looser in texture and more cellular. With differential staining methods, this zone is seen to be composed largely of inmature collagenous fibrils. In structure and function, it is comparable with the intermediate zone of the periodontal membrane. The fibrils may be

appropiately termed linkage fibrils. They thicken to become mature, coarse collagenous fibers and enter the bone in this form to serve as attachment fibers (Sharpey's fibers).

Like the periodontal and sutural membranes, the periosteum is composed of several distinct structural and functional layers. The dense fibrous zone of the periosteum is similar to the capsular zone in the suture and is continuous to it, showing a dense arrangement of collagenous fibers and rich in blood vessels and nerves. A border zone of coarse, mature collagenous fibers is present in the periosteum and adjacent to the bone surface. This is continuous with a similar layer in the suture, and both are comparable with the outer surface of the periodontal membrane. Also an intermediate zone is present in the periosteum which is comparable with the same layer in the other membrane types. It is composed of delicate precollagenous, linkage fibrils with a scattering of coarser, mature fibers. The fibers and fibrils are continuous through these three zones. Sharpey's fibers extend directly from the border zone into the cortex as attachment fibers. They are arranged in coarse, paralel bundles and enter the bone at various angles depending upon the direction of periosteal tension at the time of bone deposition[3].

FACTS AND INVESTIGATIONS IN THE RAT

The rat as an experimental animal continues to hold its preeminent position for use in laboratory investigation, since its characteristics make

it suitable for research intended for application to human biology. The accepted view that the similarities between mammals having the same food habits tend to be close, and that in some instances at least, by the use of equivalent ages, the results obtained with one form can be very precisely transferred to the other. If the life span of three years in the rat is taken as equivalent to 90 years in man, it is found that the growth changes in the nervous system occur within the same fraction of life span and also probably true for the other systems and this makes possible the cross reference of the results from the two species, with a high degree of precision.[4,5]

Studies of growth pattern of the cranial vault in albino rats made by Massler and Schour[6] *in 1951, found that the sutures are the most prolific sites of post-natal growth, and that active bone deposition upon all bony surfaces was characteristic in the period from birth to 60 days of age. This investigation pointed out that the growth of the cranial vault is intimately connected with the growth of the brain and follows the same type of neural growth curve.*

Research on growth of the calvaria of the rat was also made by Moss[7] *in 1954 by means of extirpation of the sutures singly or severally, and periosteal scraping and burnishing. He concluded that the suture is not the primary, active site of growth of the calvaria. Specifically, the growth of the neurocranium is not accomplished by means of interstitial expansive force originating within the soft tissues of the suture, but the periosteal osteogenic tissues, ectocranial as well as endocranial, appear to be the primary*

growth sites.

A longitudinal study of growth changes in the rat cranio-facial complex was reported by Cleall, Jacobson, and Berker[8] in 1971, finding that the sutures were most active and varied greatly in the amount of growth demonstrated by the bones at each side of the suture. The cranial vault enlarged in the vertical and lateral dimensions by appositional growth in both the intracranial and extracranial surfaces. Compensation for increase in intracranial volume was made at the sutures and by differential growth at the centers and edges of the bone to reduce curvature.

Examination of rat's blood for 100 grs. show: Uric acid 2mg; Non-protein nitrogen 38mg, and Urea 2mg. The proportional amount of serum proteins in 60 day old albino rats is 5.3 percent total proteins; 3.0 albumins and 2.3 globulins. The percentage of water in the entire blood of 60 day old rats is approximately 80 percent.[5]

The summary of known dietary requirements of the rats is reported in the Memoir #6 of the Wistar Institute of Anatomy and Biology[5] as follows:
Calcium and Phosphorus.- 0.5 to 0.6% calcium and the same or slightly lower concentration of phosphorus. This corresponds to an average daily intake of about 40-50mg and 35-45mg of phosphorus.
Potasium.- males require a minimum of 15mg and females 8mg per day.
Sodium.- 0.5% appear to be the most satisfactiry.
Chlorine.- about 5mg per day are sufficient.

Copper.- 0.1mg is more nearly the optimum intake.

Iodine.- diets supplying about 2mg per day are minimal.

Magnesium.- at least 4 mg. are required per kilo of body weight per day.

Manganese.- about 0.5mg per day is minimal.

Zinc.- Normal growth is possible on diets supplying 40 micrograms per day.

Protein.- optimum growth occur on diets containing 25 to 30% aminoacids. The minimum amount is: Lysine 1%, Tryptophane 0.2%, Histidine 0.4%, Phenylalanine 0.7%, Leucine 0.9%, Isoleucine 0.5%, Thereonine 0.6%, Methionine 0.6%, Valine 0.7%, and Arginine 0.2%.

Fatty acids.- 25mg of methyl linolate are sufficient.

Vitamin A.- about 4 micrograms of vitamin A or 15 to 20 micrograms of carotene per kilo of body weight are satisfactory.

Thiamin.- 10 micrograms are sufficient.

Riboflamin.- 40 micrograms are satisfactory.

Pyridoxine.- 10 micrograms are sufficient.

Vitamin D.- apparently vitamin D is not required if the Ca:P ratio is between 1:1 and 2:1 and about 0.5% phosphorus is present on the diet.

Vitamin E.- about 1mg of alpha-tocopherol per day is sufficient for normal growth.

TISSUE CULTURE MEDIUM SUPPORTING GROWTH IN VITRO AND IN VIVO

Animal tissue culture is generally considered to have begun in the early 20th century with the pioneer experiments of Harrison[9] in 1906. The first tissue culture methods were designed primarily for studies of cell

structure, morphology and development. They were based on the observation that many tissues could be propagated almost indefinitely in media derived from the animal body. Tissue culture became recognized as a basic research technique with the advent of the antibiotics and the use of bacteriological principles.

The first dental study in organ culture was introduced by Glasstone[10] *in 1935. She cultived tooth germs from rat embryos, corfirming that ameloblasts are necessary for differentiation of odontoblasts but not for dentine formation.*

Tissue culture medium composed of equal parts of plasma, calcium Ringer, feeding solution containing cysteine and thyroxine, and 2.10% glycine solution, was used by Vogelaar[11] *in 1936 to culture human fibroblasts in vitro. He showed that the toxical or beneficial influence of aminoacids is not primarily dependant upon their concentration, but mainly upon chemical composition of the medium of which they form part.*

Morgan[12] *in 1958 stated that modern tissue culture provides a method through which the morphology, biochemistry, metabolism, survival, and growth of individual mammalian cells, the progeny of these cells, or mixed cell population may be studied simultaneously under precisely controled conditions.*

Das[13] *in 1967 described the reaction of human dental pulp cells cultured in vitro to certain endodontic materials. The nutrient medium that was used consisted of ten percent of tissue culture medium T199. She declared*

that this was the first time the human dental pulp cells were cultured succesfully in continuous propagations.

Toto, Black, and Sawinski[14] *in 1968 used tissue culture medium T199 to stimulate the proliferation of subcutaneous connective tissue in the mouse. They found that T199 substrate enriched the environment of the competent cells stimulating the connective tissue to begin the synthesis of DNA in numbers significantly greater than which normally occurs, this finding suggested that it is enterely probable that enriched substrate alone could have this growth-stimulating effect in vivo, as is known to occur in the in vitro system free of homeostatic regulation.*

The effect of hyperalimentation by means of parenteral administrations of hypertonic solutions containing protein hydrolysate, glucose, vitamins, minerals and small amounts of plasma, permit human and dogs to synthezise proteins and fat, and promote skeletal growth. It has been proven by Dudrick[15] *in 1968 and Nardi*[16] *in 1972. They found it to maintain normal growth, development, and a positive nitrogen balance for extended periods of many months.*

AUTORADIOGRAPHY IN BIOLOGIC STUDIES

Autoradiography is the term used for the method of photographic emulsions to study the occurrence and distribution of radioactive substances. Autoradiogram is referred to the resulting picture.[17]

Autoradiography has made possible the localization of labeled substances in microscopic structures, and has been used effectively by biologists working on the field of genetics, botany, biochemestry, physiology, pharmacology, etc. The technique has proved invaluable for visualizing the location of compounds known from other evidence to be firmly bound or inmobilized, such as thymidine incorpored into the DNA molecule.

The percentage of labeled cells (labelling index) is frequently counted in experiments on DNA synthesis using tritiated thymidine. 3H-thymidine has proved to be a very good test substance in experiments for establishing if a method is reliable and yields genuine "autoradiographic silver grains". Thymidine is a pyrimidine derivative linked to deoxyribose. As a nucleoside, it is the starting material in DNA synthesis and, in connection with mitosis, is predominantly incorpored into cell nuclei. Thymidine has thus proved its value not only in cytological studies but also in connection with electron microscopic technique.[17,19]

Several studies of growth of connective tissue have been possible by the use of autoradiography.[14,20] *Investigations of bone formation in vitro and in vivo detected by radioactive substances such as Thymidine 2-*^{14}C *and* Ca^{45} *respectively, have been reported by Dooner and Porter*[21]*, and Jowsey, Rowland, Marshall, and McLean.*[22]

In a study reported by Nichols[23] *in 1967, the osteogenic cells of the periosteum were labeled by injecting tritiated thymidine intraperitoneally in albino rats, determining the proliferative cell rate in normal and hypophy-*

sectomized rats. It was the feeling of the author that the purpose of his study was succesful in demonstrating autoradiographically such proliferative rates of the periosteum.

Recently, Reardon[24] in 1976 presented one study about the reaction of the intermaxillary suture, the maxillopalatine suture, and the periodontal membrane to the palatal expansion procedures. This research was performed on young rhesus monkeys with the help of tritiated thymidine administered intravenously on the tenth day of the experiment. The evaluation of the histological changes was mainly judged by means of cell density and labelling index differences.

In view of the foregoing literature reviewed in this work, it seems relevant to study the periosteal growth on the sagital suture in the calvarium of the rat. This study reports upon the influence of tissue culture medium, T199, on sutural growth as measured by cell density and tritiated thymidine labeled cells.

CHAPTER III

MATERIAL AND METHODS

Twenty albino rats* 45 days old, each averaging 60grs in weight, were divided into two groups of ten each. Three cubic centimeters of air was subcutaneously injected on the periosteal surface of the interparietal suture area of each rat to create a pouch. After 24 hours the control group was injected with 1cc of buffered physiologic saline solution** into the little bubble created by the previously injected air, repeating each procedure every third day for two weeks (Table I). The experimental group was treated as the control group but instead of saline solution, was administered 1cc of an artificial isotonic tissue culture medium T199*** in a concentration of 11.1 grs/liter (TableII). The preparation of the air pouches and the injection of saline solution or T199 were done with great care in order to minimize the injurious effect of such administrations.

Approximately 8 hours after the last injection, the animals were weighted and 1μci of tritiated thymidine**** per gram of body weight was administered intraperitoneally to each one of the rats in both groups. One

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* Abrams Animal Supply
** Fisher Scientific Company. Fair Lawn, New Jersey.
*** T199. Grand Island Biological Supply. Grand Island, N.Y.
**** New England Nuclear

hour post-injection, the animals were sacrificed using chloroform, and 1 X 1 cm block of the interparietal suture area was excised. The blocks were fixed in cold neutral formalin and then prepared for microscopic examination by cutting sections of 6μ thick after they were imbeded in parafin. Autoradiograms of the sections were prepared with NTB3*. After 5 week exposure at 4°C, the sections were developed, washed, fixed, and stained with nuclear fast red and indigo carmine dyes.

The slides were placed under a binocular microscope with reticular of $100\mu^2$ **, and the nuclei counts were determined utilizing a blood cell counter***. The nuclei of the interparietal suture area of randomly selected preparations were counted in both controls and experimental groups to a total of $600\mu^2$ for each animal. The mean and standard deviation of cell density per $100\mu^2$ were calculated as well as the "t" test for significant differences between the means.

The labeled cells of the interparietal suture area of the previously randomly selected preparations were counted in both control and experimental groups using the same number of reticulars for each animal. The mean and standard deviation of labeled cell per $100\mu^2$ were calculated as well as the "t" test for significant differences between the means.

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* NTB3, Eastman Kodak Company. Rochester N.Y.
** American Optical Corporation. Buffalo N.Y.
*** Clay Adams. Parsippany, N.J.

TABLE I

SCHEDULE OF FREQUENCY OF ADMINISTRATION OF SALINE AND T199 IN 45 DAYS OLD ALBINO RATS

DATE	CONTROL	EXPERIMENTAL
February 12, 1976	3cc Air	3cc Air
February 13, 1976	1cc Saline Sol.	1cc T199
February 15, 1976	1cc Saline Sol.	1cc T199
February 17, 1976	1cc Saline Sol.	1cc T199
February 19, 1976	1cc Saline Sol.	1cc T199
February 21, 1976	1cc Saline Sol.	1cc T199
February 23, 1976	1cc Saline Sol.	1cc T199

TABLE II

COMPOSITION OF THE TISSUE CULTURE MEDIUM

T199

COMPONENT	mg/L
INORGANIC SALTS	
$CaCl_2$ (anhyd)	140.00
$Fe(NO_3)_3 \cdot 9H_2O$	0.72
KCl	400.00
KH_2PO_4	60.00
$MgSO_4 \cdot 7H_2O$ (anhyd)	97.72
$NaCl$	8000.00
$Na_2HPO_4 \cdot 7H_2O$ (anhyd)	47.70
OTHER COMPONENTS	mg/L
Adenin Sulfate	10.00
Adenylic Acid	0.20
Adenosinetriphosphate	1.00
Alpha Tocopherol Phosphate	0.01
Cholesterol	0.20
Deoxyribose	0.50
Glucose	1000.00
Glutathione	0.05
Guanine HCl (Free base)	0.30
Hypoxanthine Na salt	0.35
Phenol Red	20.00
Ribose	0.50
Sodium Acetate	50.00
Thymine	0.30
Tween 80 (TM)	20.00
Uracil	0.30
Xanthine Na salt	0.34

COMPONENT	
AMINOACIDS	
DL-Alpha-Alanine	50.000
L-Arginine HCl	70.000
DL-Aspartic acid	60.000
L-Cysteine HCl-H_2O	0.110
L-Cysteine 2HCl	26.000
DL-Glutamic acid · H_2O	150.000
L-Glutamine	100.000
Glycine	50.000
L-Histidine HCl·H_2O	21.880
L-Hydroxyproline	10.000
DL-Isoleucine	40.000
DL-Leucine	120.000
L-Lysine HCl	70.000
DL-Methionine	30.000
DL-Phenylalanine	50.000
L-Proline	40.000
DL-Serine	50.000
DL-Thereonine	60.000
DL-Tryptophan	20.000
L-Tyrosine	57.880
DL-Valine	50.000
VITAMINS	
Vitamin A (acetate)	0.140
Ascorbic acid	0.050
d-Biotin	0.010
Calciferol	0.100
Ca Pantothenate	0.010
Chlorine Chloride	0.500
Folic acid	0.010
i-Inositol	0.050
Menadione	0.010
Niacin	0.025
Niacinamide	0.025
Para-aminobenzoic acid	0.050
Pyridoxal HCl	0.025
Riboflavin	0.010

CHAPTER IV

RESULTS

The animals survived and appeared healthy during the experimental period of two weeks.

The histological sections show a frontal view of the parietal bones separated by the suture (Figures 1 to 6). The bone is composed of an inner and outer plate of compact bone containing in between a small amount of spongy bone and associated marrow.

The suture presents in its border several thick collagenous fibrils approaching the sutural bone surface. The intermediate zone is much looser in texture and is the most cellular. The separating capsular layer is composed largely of coarse bundles of mature thick collagenous fibers. Many blood vessels are seen in this area.

The periosteum is an irregular connective tissue membrane containing bundles of collagenous fibers, elastic fibers and fibroblasts. The periosteum continues from the surface of the outer plate of the parietal bones with the connective tissue of the suture, and from this membrane, this tissue continues with the endosteum on the inner endosteal surface of the parietal bone. On the surface of the periosteum, flattened fibroblasts are easily seen. The middle layer shows indifferent, osteoprogenitor cells. The innermost layer is composed mainly of osteoblasts. In general the periosteum is richly vas-

cularized with many small capillaries.

Near the junction of the sutural mambrane with the periosteal surface of the bone, the slender precollagenous linkage fibrils are particularly abundant, representing an active location of bone growth.

Examples of the difference in cell density of the sections from the control and experimental group are presented in Figures 1,2 and 3,4 respectively. Examples of differences in number of labelled cells are presented in Figure 5 for the control group and Figure 6 for the experimental.

The cell density in the sagital suture area in albino rats 56 days old treated with saline solution or tissue culture medium T199, appear in the Tables III and IV. The mean control group of cell density was found to be $\bar{x} = 120.7/100\mu^2$, *while that of the experimental group was* $\bar{x} = 151/100\mu^2$. *There was a significant difference in such densities measured by the "t" test;* $t = 5.90$, *statistically significant at* $P = .01$ *(Table V).*

The labeled cell index of the sagital suture area in albino rats 56 days old treated with saline solution or tissue culture medium T199, appear in the Tables VI and VII. The mean control group of labeled cells was found to be $\bar{x} = 1/100\mu^2$, *while that of the experimental group was* $\bar{x} = 1.38/100\mu^2$. *There was a slight but significant difference in the indexes as measured by the "t" test. However, at* $P = .1$, $t = 1.72$ *is considered statistically significant (Table VIII).*

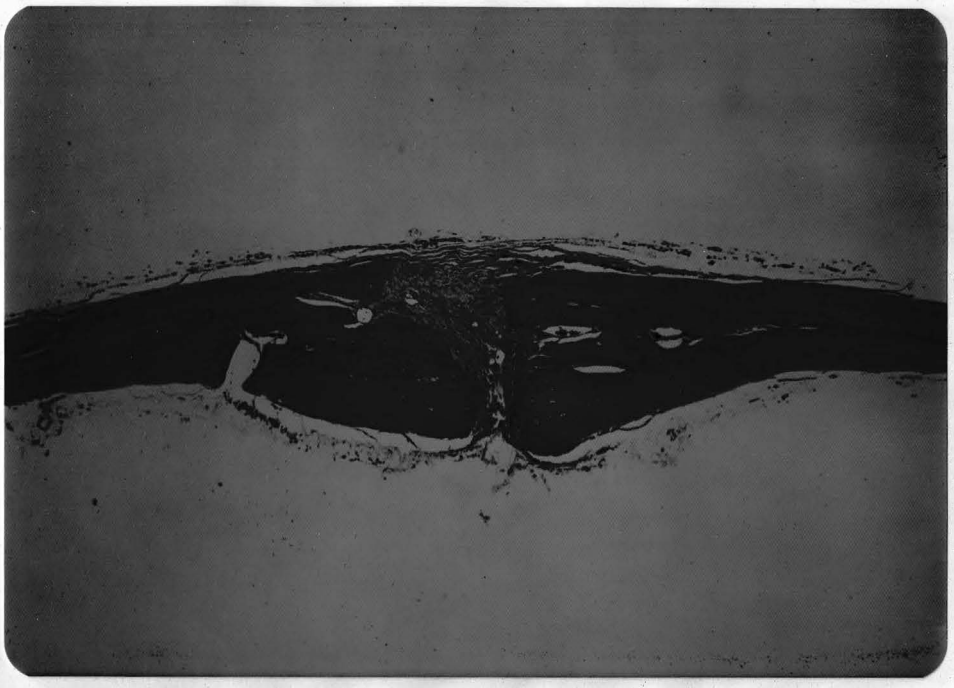

FIGURE 1.- Representative example of a frontal view of the interparietal suture of albino rat 56 days old treated with saline solution. Hematoxylin eosin. Low magnification.

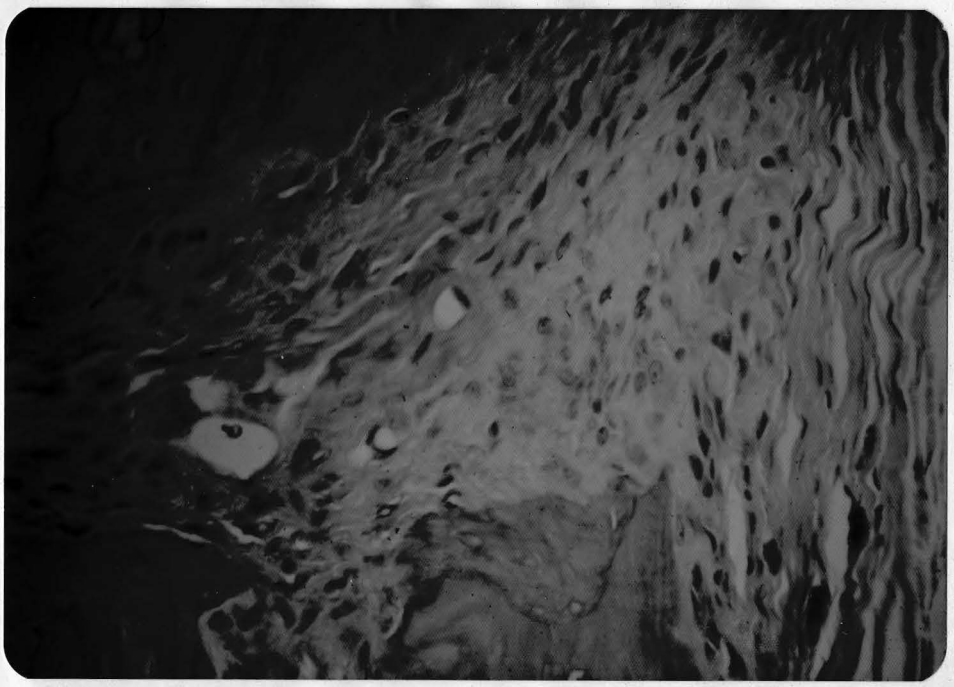

FIGURE 2.- Representative example of a frontal view of the interparietal suture of albino rat 56 day old treated with saline solution. Hematoxylin eosin. High magnification.

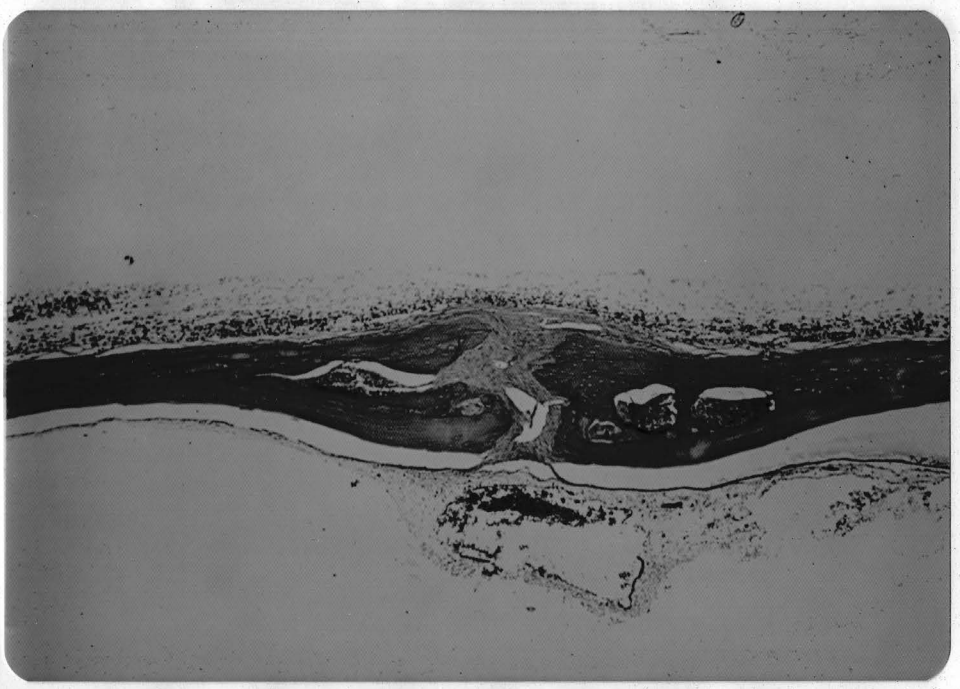

FIGURE 3.- Representative example of a frontal view of the interparietal suture of albino rat 56 days old treated with T199. Hematoxylin eosin. Low magnification.

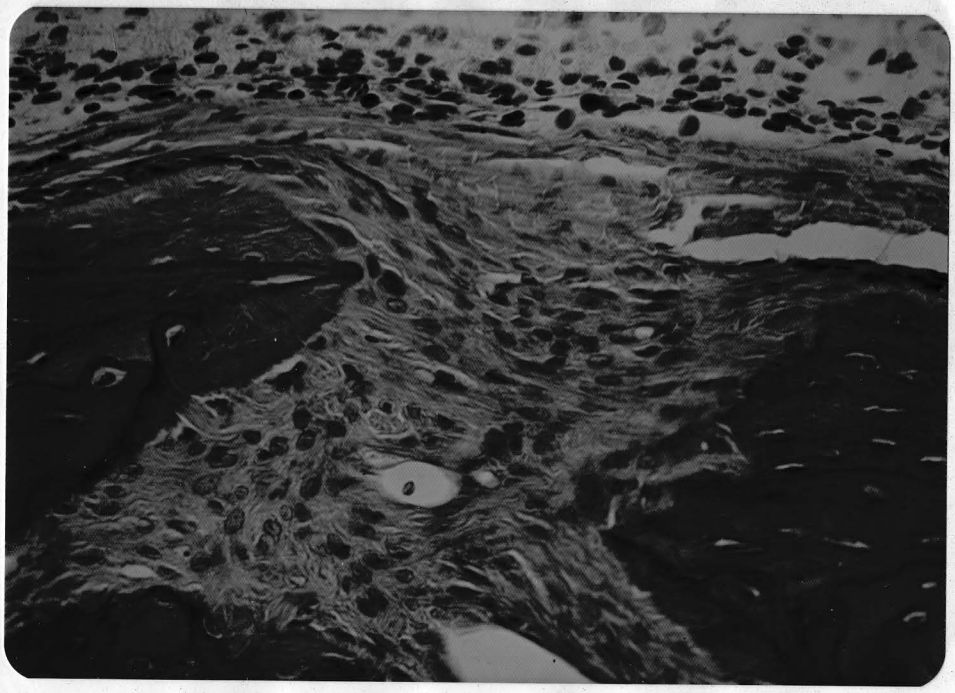

FIGURE 4.- Representative example of a frontal view of the interparietal suture of albino rat 56 days old treated with T199. Hematoxylin eosin. High magnification.

FIGURE 5.- Representative example of an autoradiogram of the interparietal suture of albino rat 56 days old treated with saline solution. High magnification.

FIGURE 6.- Representative example of an autoradiogram of the interparietal suture of albino rat 56 days old treated with T199. High magnification.

TABLE III

CELL DENSITY IN THE SAGITAL SUTURE OF 56 DAYS OLD ALBINO RATS TREATED WITH SALINE SOLUTION

ANIMAL NUMBER	SUTURE NUMBER	RETICULAR NUMBER	NUMBER OF NUCLEI
1	2	1	151
1	2	2	124
1	2	3	154
1	8	4	130
1	8	5	119
1	8	6	142
2	8	7	131
2	8	8	104
2	8	9	93
2	12	10	128
2	12	11	103
2	12	12	90
3	8	13	150
3	8	14	119
3	8	15	91
3	11	16	132
3	11	17	149
3	11	18	96
4	3	18	125
4	3	20	112
4	3	21	99
4	8	22	106
4	8	23	134
4	8	24	97
5	7	25	118
5	7	26	81
5	7	27	78
5	8	28	137
5	8	29	83
5	8	30	125
6	11	31	118
6	11	32	71
6	11	33	123
6	12	34	128
6	12	35	168
6	12	36	146

TABLE III (cont)

ANIMAL NUMBER	SUTURE NUMBER	RETICULAR NUMBER	NUMBER OF NUCLEI
7	1	37	155
7	1	38	132
7	1	39	173
7	5	40	131
7	5	41	127
7	5	42	124
8	5	43	152
8	5	44	143
8	5	45	118
8	9	46	133
8	9	47	121
8	9	48	128
9	5	49	126
9	5	50	137
9	5	51	107
9	7	52	120
9	7	53	105
9	7	54	117
10	11	55	100
10	11	56	134
10	11	57	76
10	14	58	124
10	14	59	96
10	14	60	105

TABLE IV

CELL DENSITY IN THE SAGITAL SUTURE OF 56 DAYS OLD ALBINO RATS

TREATED WITH TISSUE CULTURE MEDIUM T199

ANIMAL NUMBER	SUTURE NUMBER	RETICULAR NUMBER	NUMBER OF NUCLEI
1	5	1	215
1	5	2	129
1	5	3	154
1	6	4	216
1	6	5	148
1	6	6	139
2	10	7	192
2	10	8	159
2	10	9	133
2	16	10	187
2	16	11	117
2	16	12	129
3	5	13	198
3	5	14	131
3	5	15	122
3	12	16	224
3	12	17	138
3	12	18	152
4	18	19	225
4	18	20	131
4	18	21	125
4	23	22	180
4	23	23	120
4	23	24	190
5	5	25	208
5	5	26	201
5	5	27	186
5	11	28	181
5	11	29	181
5	11	30	171
6	19	31	173
6	19	32	105
6	19	33	133
6	5	34	174
6	5	35	122
6	5	36	142

TABLE IV (cont)

ANIMAL NUMBER	SUTURE NUMBER	RETICULAR NUMBER	NUMBER OF NUCLEI
7	19	37	162
7	19	38	119
7	19	39	111
7	21	40	125
7	21	41	124
7	21	42	124
8	7	43	197
8	7	44	181
8	7	45	102
8	11	46	144
8	11	47	159
8	11	48	100
9	19	49	135
9	19	50	103
9	19	51	101
9	22	52	128
9	22	53	108
9	22	54	148
10	9	55	141
10	9	56	168
10	9	57	121
10	13	58	149
10	13	59	137
10	13	60	145

TABLE V

COMPARISON OF TWO SAMPLE MEANS OF CELL DENSITY IN SALINE AND T199 TREATED ALBINO RATS, BY THE "t" TEST.

$$t = \frac{(\bar{x}_1 - \bar{x}_2) - 0}{\sqrt{s^2 \left(\frac{1}{n_1} + \frac{1}{n_2} \right)}}$$

$$s^2 = \frac{\sum (x_1 - \bar{x}_1)^2 + \sum (x_2 - \bar{x}_2)^2}{(n_1 - 1) + (n_2 - 1)} = \frac{30475 + 63573}{(60-1)+(60-1)} = \frac{94048}{118}$$

$$s^2 = 797.01694$$

$$t = \frac{(120.7 - 151) - 0}{\sqrt{797.01 \left(\frac{1}{60} + \frac{1}{60} \right)}} = \frac{30.30}{\sqrt{797.01 \ (.033)}} = \frac{30.30}{\sqrt{26.3015}}$$

$$t = \frac{30.30}{5.128} = 5.9087$$

The critical t value with 118 degrees of freedom for P = .01 is 2.61 so that at 5.9087 is judged to be statistically significant.

TABLE VI

LABELED CELLS IN THE SAGITAL SUTURE OF 56 DAYS OLD ALBINO RATS TREATED WITH SALINE SOLUTION

ANIMAL NUMBER	SUTURE NUMBER	RETICULAR NUMBER	NUMBER OF NUCLEI
1	2	1	3
1	2	2	1
1	2	3	2
1	8	4	2
1	8	5	1
1	8	6	0
2	8	7	2
2	8	8	2
2	8	9	0
2	12	10	1
2	12	11	3
2	12	12	1
3	8	13	2
3	8	14	1
3	8	15	0
3	11	16	0
3	11	17	1
3	11	18	0
4	3	19	3
4	3	20	2
4	3	21	0
4	8	22	3
4	8	23	1
4	8	24	0
5	7	25	1
5	7	26	0
5	7	27	0
5	8	28	0
5	8	29	1
5	8	30	0
6	11	31	0
6	11	32	0
6	11	33	0
6	12	34	2
6	12	35	0
6	12	36	0

TABLE VI (cont)

ANIMAL NUMBER	SUTURE NUMBER	RETICULAR NUMBER	NUMBER OF NUCLEI
7	1	37	1
7	1	38	2
7	1	39	1
7	5	40	2
7	5	41	0
7	5	42	2
8	5	43	1
8	5	44	3
8	5	45	0
8	9	46	0
8	9	47	0
8	9	48	4
9	5	49	0
9	5	50	0
9	5	51	0
9	7	52	2
9	7	53	4
9	7	54	1
10	11	55	0
10	11	56	0
10	11	57	1
10	14	58	0
10	14	59	1
10	14	60	0

TABLE VII

LABELED CELLS IN THE SAGITAL SUTURE OF 56 DAYS OLD ALBINO RATS TREATED WITH T199

ANIMAL NUMBER	SUTURE NUMBER	RETICULAR NUMBER	NUMBER OF NUCLEI
1	5	1	2
1	5	2	1
1	5	3	3
1	6	4	1
1	6	5	3
1	6	6	1
2	10	7	1
2	10	8	2
2	10	9	1
2	16	10	1
2	16	11	0
2	16	12	2
3	5	13	1
3	5	14	1
3	5	15	0
3	12	16	0
3	12	17	2
3	12	18	2
4	18	19	1
4	18	20	2
4	18	21	0
4	23	22	1
4	23	23	0
4	23	24	0
5	5	25	3
5	5	26	3
5	5	27	1
5	11	28	2
5	11	29	1
5	11	30	1
6	19	31	1
6	19	32	3
6	19	33	0
6	5	34	1
6	5	35	1
6	5	36	1

TABLE VII (cont)

ANIMAL NUMBER	SUTURE NUMBER	RETICULAR NUMBER	NUMBER OF NUCLEI
7	19	37	1
7	19	38	2
7	19	39	0
7	21	40	3
7	21	41	1
7	21	42	0
8	7	43	1
8	7	44	1
8	7	45	1
8	11	46	1
8	11	47	0
8	11	48	1
9	19	49	0
9	19	50	6
9	19	51	6
9	22	52	2
9	22	53	2
9	22	54	0
10	9	55	1
10	9	56	2
10	9	57	0
10	13	58	1
10	13	59	1
10	13	60	4

TABLE VIII

COMPARISON OF TWO SAMPLE MEANS OF LABELED CELLS IN SALINE AND T199 TREATED ALBINO RATS, BY THE "t" TEST.

$$t = \frac{(\bar{x}_1 - \bar{x}_2) - 0}{\sqrt{s^2 \left(\frac{1}{n_1} \quad \frac{1}{n_2}\right)}}$$

$$s^2 = \frac{\sum (x_1 - \bar{x}_1)^2 + \sum (x_2 - \bar{x}_2)^2}{(n_1 - 1) + (n_2 - 1)} = \frac{73.9617 + 98.072}{(60-1) + (60-1)} = \frac{172.03}{118}$$

$$s^2 = 1.4579$$

$$t = \frac{(1 - 1.38) - 0}{\sqrt{1.45 \left(\frac{1}{60} + \frac{1}{60}\right)}} = \frac{.38}{\sqrt{1.45\ (.0333)}} = \frac{.38}{\sqrt{.04854}}$$

$$t = \frac{.38}{.2203} = 1.7249$$

The critical t value with 118 degrees of freedom for P = .10 is 1.66 so that at 1.7249 is judged to be statistically significant.

CHAPTER V

DISCUSSION

The objective of early experiments in synthetic media for cell cultures was to devise an adequate medium to support unlimited cell survival and multiplication. At the present time the composition of the synthetic media most used, include as many as possible nutritional factors already shown to be necessary for man, animals, and bacteria.

The artificial medium T199 contains the essential elements required for cell growth and proliferation; therefore the injections of this substrate into the preformed air pouches reproduced to a great extent its in vitro and in vivo qualities reported by previous experiments of Morgan[12] in 1958 and Toto, Black, and Sawinski[14] in 1968.

This study was performed with the help of autoradiography which made possible the localization of labeled compounds in the microscopic structure.

Mitosis is preceded by synthesis of Deoxyribonucleic acid (DNA), and thymidine is a specific precursor of DNA, therefore when radioactive thymidine was administered in the rats, the nuclei became radioactive by the incorporation of thymidine to the DNA molecule. By this mechanism, the extent of DNA synthesis and mitosis occuring in the sagital suture area adjacent to the T199 and saline solution pouches was determined.

In regard to the histological morphology of the periosteal and sutural membranes, three layers were observed: a dense fibrous zone in the periosteum or a separating capsular layer in the suture composed largely of coarse bundles of mature thick collagenous fibers, the intermediate zone that is much looser in texture and is the most cellular, and the innermost layer or border zone composed mainly of osteoblasts and several thick collagenous fibers approaching the periosteal or the sutural bone surface. These three layers are consistent with the description done by Enlow[3], and definitively these membranes constitute a very important site of cell proliferation.

In this work, the labelling index represents the quantity of cells that are organizing for mitosis during the one hour period established after the injection of radioactive thymidine. The slight but significant difference found in the index represents the mitotic activity of the cells on the sutural area eight hours after the last injection of saline solution or tissue culture medium T199. The increased labelling index in the experimental group suggest that the artificial medium used in this study stimulates cell proliferation under the specified conditions.

The mesenchymal cells in the suture are competent with respect to mitotic division and growth when placed in tissue culture medium in vitro. It is evident that T199 in vivo also has a local growth promoting effect. Of course, as the sutural mesenchyme receives its nutrients via the local blood supply, the local administration of T199 paravascularly can be considered a nutritive-rich supplement enhancing local growth.

The high difference in cell density between the control and experimental group indicates the most probably result of the T199 given to support sutural cell growth during the total period of the experiment of two weeks. The larger number of cells in the sutural area of the experimental group, represents an evidence of increased mitotic activity as a function of the tissue culture medium T199 administered during the two week experiment period. Increased number of cells in the suture is evidence of increased sutural growth.

The mechanism of action of the T199 seems to be related to the enriching of the local environment of the competent cells and probably to the systemic intake of the growth promoting substances, like it has already proved in some studies by means of similar substances for the in vivo and in vitro cell culture, and also in the case of hyperalimentation. It is interesting to note that artificial synthetic nutrients used in hyperalimentation have a growth supporting and promoting effect, as has already proved by Dudrick[15] *and Nardi*[16]*. Thus it is strongly suggested that sutural growth may be modified by enhancement by local administration of enriched medium. However, it is difficult to define in this study which is the specific factor that propitiate these changes.*

The results of this investigation allows the consideration of the concept of sutural cell proliferation dependance upon a substrate, and agrees with the statement of Sicher[1] *who declared that sutural growth is a primary and active mechanism for the enlargement of the cranium. Such expansion, ini-*

tiated by proliferation of the connective tissue and followed by apposition of bone at the sutural borders, is belived by Sicher to be greater than it is necessary to accomodate the growing brain, and thus allows, at the same time for apposition of bone in the cerebral surfaces. These findings are inconsistent with Enlow[3] who considered that the sutural bone growth is a secondary response to other expansive forces of growth responsible for the actual displacement of the bones involved. Growth expansion within soft tissues associated with the bones such as the brain, for example, is belived by Enlow to represent the primary source of expansive force, since the enlarging brain produces a field of tension within the connective tissue of the suture, which in turn induces deposition of bone on the supporting bony margins.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Twenty young albino rats were divided into two groups of ten each. Three cubic centimeters of air was subcutaneously injected into the periosteal surface of the interparietal suture to create a pouch.

After 24 hours, one cubic centimeter of tissue culture medium T199 or saline solution was injected into the pouch of the experimental and control groups. The same procedure was repeated every third day for a total period of two weeks.

Eight hours after the last injection, the animals were intraperitoneally injected with tritiated thymidine at the rate of one microcurie per gram of body weight. One hour after the thymidine administration, all animals were sacrificed.

Histological sections of the interparietal suture areas were prepared for autoradiograms.

Autoradiographic analysis of the periosteal tissue of the interparietal suture showed a slight increase in labeled cells of animals receiving tissue culture medium T199 as compared to the saline control animals.

Cell density analysis of the interparietal suture area showed a high statistically significant increase in the number of cells of the experimen-

tal group as compared to their control.

The results of this study indicate that the artificial medium T199 has a stimulating effect on the growth of the periosteum of the sagital suture area on the albino rat.

The increased number of cells in the suture is evidence of increased sutural growth, and it is strongly suggested that the sutural growth may be modified by enhancement with local administration of enriched medium.

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APPROVAL SHEET

The thesis submitted by Ruben Eduardo Bayardo, has been read and approved by the following committe:

Dr. Patrick D. Toto, Director
Coordinator of Advanced Education,
Coordinator of Dental Research, Department
Chairman and Professor, Pathology, Loyola.

Dr. Eugene R. Grandel
Department Chairman and Professor, Pedodontics,
Loyola

Dr. Wayne E. Milos
Director of Postgraduate Course of Pedodontics,
Assistant Professor, Pedodontics, Loyola

Dr. Douglas C. Bowman
Associate Professor, Physiology/Pharmacology
Loyola

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

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

March 9, 1977 — Patrick D Toto

Date — *Director's Signature*