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Studies on the chick embryonic tibiae
cultivated in a chemically defined medium

CHAPTER II

STUDIES ON THE CHICK EMBRYONIC TIBIAE CULTIVATED

IN A CHEMICALLY DEFINED MEDIUM

Early studies on the cultivation of embryonic bone
rudiments were carried out using a natural medium
containing serum or plasma and extracts of chick embryos.
Miszurski (1938) demonstrated that an extract prepared
from 13-day-old chick embryos improved growth and
development of chick embryonic rudiments. Paff (1948)
observed that more calcium was deposited in chick
embryonic femora when the pH of the cultivation medium
was maintained at 7.0 - 7.3 than when maintained at pH
7.8 - 8.0. Carpenter (1950) reported that the long bone
rudiments of embryonic chick grew and developed in
reconstituted plasma plus fresh embryo extract, although
the cultures were slightly inferior to controls cultivated
in fresh plasma and fresh embryo extract. Quantitative
data have been reported by Miyazaki et al (1957) and
Saito (1959) who cultivated femora from 7 to 9-day chick
embryos in a culture medium consisting of 20% horse serum,
10% chick embryo extract and 70% Gey's saline solution.
When they renewed the medium at interval of 2 days, they

added an extract prepared from embryos corresponding in age to the cultivated tissue as suggested earlier by Gillard (1935). They found that calcium deposition increased when Ca-ATP was included at concentrations of 3×10^{-4} and 3×10^{-5}M.

Endo (1960) observed histochemically that physiological calcification of the femora of embryonic chick occurred when the medium contained 10% chick embryo extract, 20% horse serum and 70% Gey's saline solution. Ito et al (1963) have recently shown that the amount of inorganic phosphorus and hydroxyproline increased more rapidly in chick embryonic femora cultivated in the medium containing chick embryo extract, horse serum and Gey's saline solution in proportion of 1:5:4 than when the medium contained these in the proportions 1:2:7.

A chemically defined medium, if it permitted physiological calcification of embryonic bones, would be immensely useful in studying the nutritional requirements for development of bone free from host influences. Since synthesis of normal components of bone, viz., hexosamine, collagen, and nucleic acids have been demonstrated by Webb and Biggers (1961) and Lucy, Webb and Biggers (1961)

in chemically defined media, it seemed feasible to
study quantitative aspects of calcification accompanying
growth in vitro. Therefore, 10-day-old chick embryonic
tibiae were cultivated in the chemically defined medium
858 which was devised by Healy et al (1955) and which
had supported considerable growth in terms of increase in
length, wet weight and total nitrogen according to Biggers
et al (1957). Length and weight of tibiae plus their
composition with respect to calcium, phosphorus, nitrogen,
total hexosamine and citric acid were measured after
different periods of cultivation. The details of these
investigations are reported in this chapter.

<div align="center">MATERIALS AND METHODS</div>

Chemicals :-

The following chemicals were used in the experiments;
they were of reagent grade purity and were obtained from
the sources indicated:- l-arginine hydrochloride,
l-histidine hydrochloride, l-lysine hydrochloride,
dl-phenylalanine, dl-serine, dl-threonine, dl-isoleucine,
dl-valine, dl-aspartic acid, l-proline, l-hydroxyproline,
sodium acetate, phenol red, l-glutamine, potassium chloride,

magnesium sulfate, sodium dihydrogen phosphate, sodium

bicarbonate, d-glucose, Tween 80, ferric nitrate, sodium

carbonate, sodium tungstate, cupric sulfate, p-hydroxy-

diphenyl and dinitrophenyl hydrazine from the British

Drug Houses Ltd.; dl-tryptophane, dl-methionine, l-leucine,

l-glutamic acid, dl-alanine, glycine, l-cysteine

hydrochloride, ascorbic acid, l-tyrosine, l-cystine,

sodium chloride, calcium chloride, pyridoxine hydrochloride,

pyridoxal hydrochloride, p-aminobenzoic acid, choline

chloride, d-biotin, cholesterol, menadione, α-tocopherol

phosphate, calciferol, vitamin A, Norite, p-dimethyl-

aminobenzaldehyde, and ethyl acetate from E.Merck Co.;

glutathione, adenine deoxyriboside, guanine deoxyriboside,

cytosine deoxyriboside, thymidine, sodium glucuronate,

nicotinamide adenine dinucleotide, nicotinamide adenine

dinucleotide phosphate, coenzyme A, cocarboxylase, flavin

adenine dinucleotide, uridine triphosphate, glucosamine

hydrochloride and sodium pyruvate from Sigma Chemical Co.;

meso-inositol and folic acid from Hoffmann-La Roche Co.;

acetyl acetone, tricalcium phosphate, and anthrone from

Riedel-De Haen Ag.; streptomycin sulphate and sodium

penicillin G from Glaxo Laboratories; and lithium lactate

from Junsei Pure Chemicals Co.; Japan.

Glassware :-

Pyrex petri dishes, 7 cm in diameter, and watch
glasses, 6 cm in diameter, were cleaned and sterilized
before the bones were placed in them.

The watch glasses and petri dishes were cleaned
by soaking overnight in chromic acid, washing free of
acid with running tap water, then scrubbing with Fluft
(manufactured by Imperial Chemical Industries Private
Limited) and washing successively with tap water,
ordinary distilled water and glass-distilled water.
After draining, they were heated in an oven at $140^{\circ}C$ for
3 hours, wrapped in kraft paper and autoclaved at 15
p.s.i., for 15 minutes. The autoclaved packets were
dried in an oven at $80^{\circ}C$.

A similar procedure was used for cleaning and
sterilizing all other glassware such as pipettes, flasks,
glass filters etc. which were used in the experiments.

Cultures :-

Freshly-laid eggs of White Leghorn hens were obtained
from a local poultry farm within 18 hours after they were
laid and incubated at $38^{\circ}C$ in an atmosphere humidified by
evaporation of water kept in a tray in the incubator. The

eggs were turned once each day. At the end of 10 days, eggs with healthy embryos were selected by candling. Dissection was carried out in a sterile room by the investigator who wore a sterilized apron, cap and mouth cover. His hands were cleaned well with phenolic soap and water, then wiped with 75% alcohol. The surgical instruments had been sterilized by boiling them in a covered tray for at least 20 minutes. To ensure the continued sterility of the instruments during dissection the instruments were frequently dipped in alcohol for few moments and then dried in air.

Tibiae from the embryos were removed using the following procedure.

First, the egg was wiped with 75% alcohol, allowed to dry, then cracked and the embryo transferred to a sterile dry petri dish with the help of sterile forceps. The legs were separated and transferred to a second sterile petri dish containing Earle's (1943) balanced salt solution (BSS). The tibia from each leg was isolated from soft adherent tissues, fibula and other bones by rolling the leg gently, using forceps with pointed tips, on a filter

paper wetted with physiological saline solution. The rudiments were collected in BSS in a third petri dish.

Culture medium (M858) :-

BSS was prepared as described by Earle (1943) and was sterilized by passage through a sterilized glass filter (type: 25G 5m, Jenaer Glasswerk Schott & Gen., Mainz). Culture medium M858 was prepared according to Healy et al (1955) with the following modifications:-

(1) The concentration of glucose was increased from 1000 mg to 5000 mg.per litre because Biggers (1960a) had observed maximum growth of embryonic chick tibiae when cultivated in vitro was obtained when glucose concentration was between 4.0 - 8.0 mg/ml in the medium BL_1.

(2) 5-methyldeoxycytidine and n-butylparahydroxybenzoate were not added to the medium because they were not available.

(3) Since 'l' forms of the following amino acids were not available, 'dl' forms were used in double the prescribed concentrations:

Tryptophan, methionine, phenylalanine, isoleucine, alanine, serine, valine, aspartic acid and threonine.

(4)   Streptomycin sulphate was used instead of dihydro-
streptomycin sulphate because the latter was not readily
available.

Double glass-distilled water was used for the
preparation of the stock solutions and the working
culture medium.

All the stock solutions except Nos. 1 and 6 were
stored at $4^{o}C$ without filtration for periods not exceeding
30 days.   Solution 1 was prepared fresh on the day it was
used.   Solution 6 was stored at $-15^{o}C$.

The various stock solutions were prepared as
follows :-

Solution 1 :-

Chemicals	mg
l-arginine monohydrochloride	70
l-histidine monohydrochloride	20
l-lysine monohydrochloride	70
dl-tryptophane	20
dl-phenylalanine	50
dl-methionine	30

Chemicals	mg
dl-serine	50
dl-threonine	60
l-leucine	60
dl-isoleucine	40
dl-valine	50
l-glutamic acid	75
dl-aspartic acid	60
dl-α-alanine	50
l-proline	40
l-hydroxyproline	10
glycine	50
l-cysteine hydrochloride	260
glutathione	10
l-ascorbic acid	50
l-tyrosine	40
l-cystine	20
sodium acetate trihydrate	81.5
phenol red (water soluble)	20

The above chemicals were added together in 400 to 450 ml of water which had been heated to about 80°C and

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dissolved with continuous stirring. Then the solution
was cooled to room temperature and the following
chemicals added :

Chemicals	mg
l-glutamine	100
streptomycin sulfate B.P.	100
adenine deoxyriboside	10
guanine deoxyriboside	10
cytosine deoxyriboside	10
thymidine	10
sodium glucuronate (monohydrate)	4.2
sodium chloride*	6800
potassium chloride*	400
calcium chloride*	200
magnesium sulphate heptahydrate*	200**
sodium dihydrogen phosphate dihydrate*	160
sodium bicarbonate*	2200
d-glucose	5000

* Ingredients of balanced salt mixture (Earle, 1943)
  necessary for 1000 ml of culture medium.

** The prescribed concentration by Earle was 100 mg. In
   the present study 200 mg. were added as used by Healy
   et al (1955).

Finally, the volume of the solution was adjusted to 500 ml with water.

Stock solution 2 :-

Chemicals	mg/100 ml
pyridoxine hydrochloride	25
pyridoxal hydrochloride	25
m-inositol	50
p-aminobenzoic acid	50
choline chloride	500

This stock solution was diluted 1:50 and the diluted solution added to the culture medium.

Stock solution 3 :-

Chemicals	mg/100 ml
d-biotin	10
folic acid	10

These vitamins were dissolved by adding a few drops of 0.5N NaOH and the volume adjusted to 100 ml with Earle's balanced salt solution (BSS). The solution was diluted one hundred times and used for the preparation of the culture medium.

Stock solution 4 :-

The following solutions were prepared and then mixed in the proportions mentioned below :-

(a) Cholesterol   10 mg/ml of 95% alcohol

(b) menadione   10 mg/ml of 95% alcohol

(c) α-tocopherol phosphate   0.1 mg/ml of water

(d) calciferol   10 mg in 1 ml of (a)

(e) vitamin A   10 mg in 1 ml of (a)

(f) Tween 80   5% in water.

1.0 ml each of solutions (d) and (e) plus 0.1 ml of (b) were added to 10 ml of (f) in a 100 ml volumetric flask and made to volume with water. It was necessary to warm this solution in order to dissolve the cholesterol. Stock solution No.4 was prepared by taking 10 ml of this mixture and 1 ml of (c), and diluting them to 100 ml with water.

Stock solution 5 :-

Thirty-six milligrams of $Fe(NO_3)_3 \cdot 9H_2O$ were dissolved in water and the final volume made to 100 ml. One drop of concentrated nitric acid was added to prevent hydrolysis during storage.

Stock solution 6 :-

Chemicals	mg
nicotinamide adenine dinucleotide (90 - 95% pure)	14
nicotinamide adenine dinucleotide phosphate (80 - 95% pure)	2
coenzyme A (70 - 75% pure)	5
cocarboxylase (90% pure)	2
flavin adenine dinucleotide (90% pure)	2
uridine triphosphate (90% pure)	2

These chemicals were dissolved in 2 ml of water.
One milliliter of the stock solution was used immediately
to prepare 1 litre of working medium and the rest stored
in frozen state.

Preparation of the culture medium :-

To prepare one litre of the working culture medium
the stock solutions were combined in the following
proportions :

Solution 1	500 ml
Stock solution 2 (after diluting 50 times)	10 ml
Stock solution 3 (after diluting 100 times)	10 ml

Stock solution 4	10 ml
Stock solution 5	2 ml
Stock solution 6	1 ml

This mixture was diluted to 1000 ml with water, sterilized by passage through a sterilized glass filter and stored at 0 - 5°C. Just before use 1 μg/ml of sodium penicillin G was added.

Culture technique :-

The floating lens paper technique described by Chen (1954b) was employed for the cultivation of bone rudiments. Two milliliters of sterile medium were placed in a watch glass. A piece of sterile tea-bag paper (grade 10-V-7-¼, gifts from Prof. J.D. Biggers, King Ranch Laboratory of Reproductive Physiology, The Wistar Institute of Anatomy and Biology, Philadelphia 4, Pennsylvania; and C.H. Dexter and Sons, Inc., Windsor Locks, Connecticut, U.S.A.) approximately 1.0 inch square and previously washed with ether, alcohol and glass-distilled water was placed on the surface of the medium. Eight bone rudiments were accommodated on one floating piece of paper and the watch glass was placed on moist cotton wool in a petri dish as shown in Fig. 1. Four culture
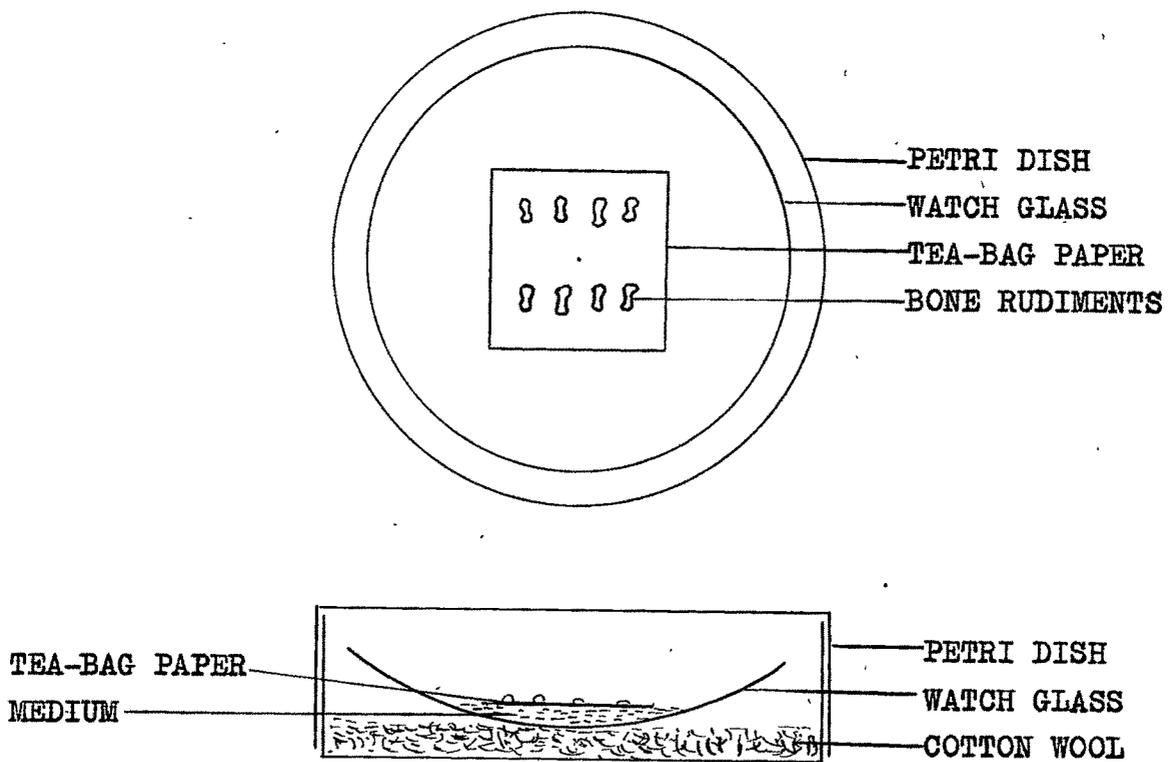
Fig. 1.   Diagram of culture vessel.   The watch
glass is enclosed in a petri dish kept
on cotton wool.   A piece of tea-bag-paper
is kept floating on the medium over which
8 bones were placed.

vessels were kept in a plastic container previously
sterilized with alcohol and ultraviolet irradiation.
The plastic containers with culture vessels were then
incubated at 37°C. The medium was renewed every second
day by transferring the floating paper with sterile
forceps to another watch glass containing fresh medium.
The bone rudiments were cultivated for different
number: of days.

Collection of culture fluid :-

After each transfer, the spent medium was collected
in a sterilized centrifuge tube and centrifuged at 2000
x g. in a refrigerated centrifuge at 0 to 1°C for 20
minutes. The resulting supernatant, if not analysed
immediately, was frozen and stored at -15°C. Medium
incubated without bones served as a control.

Collection of explants :-

At intervals of 2,4,8 and 12 days, 2 cultures each
of 8 bones were removed. The bones in each culture were
washed thrice with 0.9% physiological saline solution
and used for analysis.

Measurement of growth :-

    Length :- The length of 6 straight bones from each
culture (at least 6 out of eight bones in each culture
were straight) was measured under the microscope using
a magnification of 12 x. A thin cross wire was placed
in the eye piece as an index marker. By adjusting the
platform of the microscope one end of the bone was
brought under the eye piece so that the cross wire
coincided with it. The reading of a vernier scale
fixed to the platform was recorded. Once again the
platform was adjusted by sliding it sideways so that
the other end of the bone coincided with the cross wire.
A second reading of the vernier scale was recorded and
the difference between the initial and final readings
gave the measure in millimeters of the length of the
bone. Each bone was measured individually two or three
times and an average length calculated.

Wet weight of the bones :-

    Six bones from each culture vessel were used. The
cultures were carefully rolled once on moist Whatman
filter paper, grade 1, as described by Biggers (1960b)

and quickly weighed on a Mettler semimicrobalance kept
in a humidified, and temperature controlled room. The
average wet weight per bone was calculated.

Dry weight of the bones :-

Six bones from each culture vessel were weighed
and placed on an aluminium pan, dried in an oven at
$100^{o}C$ for 24 hours (constant weight was obtained within
this period of time), cooled in a desiccator and weighed
quickly on a Mettler semimicrobalance. Average dry
weight per bone was calculated.

Nitrogen content of the bones :-

Six dried bones from each culture vessel were pooled
for estimation of nitrogen by the microkjeldahl method
(Hawk, Oser, and Summerson, 1954) and the average nitrogen
content per bone calculated.

Calcium and phosphorus content of the bones :-

Six dried bones from one culture vessel were pooled
and digested with concentrated nitric acid in pyrex test
tubes (size 155 x 27 mm) on an electrically heated coil
until a colourless solution was obtained. Heating was
continued to eliminate all moisture and acid. The dry
residue was cooled and dissolved in 0.2 ml concentrated

nitric acid and diluted to 3 ml with water. This
solution was used for the estimation of both calcium
and phosphorus. Calcium was estimated by the flame
photometeric method of Bauer (1954) using an EEL (Evans
Electroselenium Ltd.) flame photometer with calcium
filter. Sufficient sample was used to give a scale
reading of atleast 10. The standard reference was
$Ca_3 (PO_4)_2$ (4 meq/litre) which was dissolved in water
with the aid of few drops of concentrated nitric acid.
The average calcium content per bone was calculated.

Phosphorus was estimated by colorimetric method
of Fiske and SubbaRow (1925) and the average content
per bone calculated.

Total hexosamine content of the bones :-

A separate experiment was carried out for the studies
on total hexosamine content of bone cultivated in vitro.
The cultures were removed, one each day, on the 2nd, 4th,
8th and 12th day of cultivation, six out of the eight
bones in each culture were washed with 0.9% saline,
measured for length, weighed and dried. The dried bones
were pooled and hydrolysed by the method of Cipera,
Migicovsky and Belanger (1960) with 2 ml of 4 N hydro-
chloric acid in a sealed glass tube at $100^{\circ}C$ for 12 hours.

The hydrolysate was dried over phosphorus pentoxide in a vacuum desiccator. The dried residue was dissolved in 2 ml. of water and approximately 20 mg of Norite added. The mixture was stirred for 2 minutes, then centrifuged at 2000 x g. at room temperature (about $30^{o}C$) for 10 minutes. Total hexosamine content of the supernatant was estimated by the method of Rondle and Morgan (1955) and the average content per bone calculated.

Citric acid content of the bones :-

For the studies on citric acid content of bone, 3 separate experiments were carried out. The cultures were removed, one each day, on the 2nd and 4th day of cultivation. The bones were washed with 0.9% saline, measured, weighed and dried.

The preparation of the sample for the estimation of citric acid was similar to that described by Krishna Rao and Patwardhan (1960).

Six bones from each culture were dried to constant weight, and then ground in a small mortar with 0.25 ml of 10% sodium tungstate and 0.25 ml of 2/3 N sulfuric acid for 15 minutes. Two milliliters of water were added and the suspension transferred to a centrifuge tube. After

centrifugation at 2000 x g. at room temperature (about 30°C) for 10 minutes, citric acid in the supernatant was estimated by the method of Natelson et al (1948) and the average content per bone calculated.

Glucose, lactic acid and keto acid estimations in the culture fluids :-

Glucose content of the medium was determined by the method of Gothoskar, Ratnam and Ramakrishnan (1959), lactic acid by the method of Barker and Summerson (1941), and total keto acids by the method of Friedmann and Haugen (1943). From the data obtained the utilization of glucose and the formation of lactic acid and keto acids during cultivation were calculated.

RESULTS AND DISCUSSION

The data on the effect of period of cultivation on the growth and chemical composition of tibia are given in Tables 1 to 3 and graphically represented in Figs. 2,3 and 4.

The data on the percentage increase in different constituents over zero day of cultivation are given in Table 4.

Table 1

Growth and chemical composition of embryonic chick tibiae cultivated

in vitro in medium 858*

Period of cultivation (days)	Length (mm)	Wet weight (mg)	Dry weight (mg)	$\log_{10}T\%$**	Calcium (µg)	Phosphorus (µg)	Nitrogen (µg)	Ca/P	Ca/N
0	7.5 (7.1–7.8)	3.6 (3.2–4.0)	0.40 (0.33–0.45)	1.046	9.8 (9.5–10.5)	7.7 (6.9–8.5)	11.7 (10.5–12.7)	1.28 (1.24–1.37)	0.85 (0.81–0.90)
2	8.8 (8.3–8.9)	5.0 (4.6–5.8)	0.55 (0.46–0.60)	1.041	16.7 (15.5–18.0)	10.3 (9.3–11.1)	15.4 (14.0–16.3)	1.63 (1.56–1.67)	1.08 (1.04–1.11)
4	10.5 (10.7–10.9)	6.8 (6.4–7.2)	0.72 (0.65–0.78)	1.025	22.5 (21.0–24.0)	12.6 (11.5–13.5)	20.9 (18.7–22.1)	1.79 (1.74–1.83)	1.11 (1.09–1.13)
8	12.3 (11.9–12.6)	8.8 (8.0–9.4)	0.84 (0.76–0.90)	1.032	34.7 (34.0–35.0)	15.2 (13.1–16.4)	27.9 (25.6–30.3)	2.19 (2.05–2.31)	1.24 (1.17–1.32)
12	13.2 (12.8–13.7)	10.7 (10.0–11.8)	0.95 (0.83–1.08)	0.948	35.4 (33.0–37.0)	18.7 (16.9–20.0)	32.1 (30.3–34.9)	1.89 (1.85–1.95)	1.10 (1.06–1.15)

* Results are expressed per tibia. Mean values of four separate experiments are given. Values in parentheses are the range of the means of the four experiments. In each experiment, two cultures, each containing 8 bones, were removed. Six straight bones from each culture were measured for length. The bones of two groups each containing six bones were weighed and dried. Six dried bones from one group were used for calcium and phosphorus determinations and six dried bones from other group were used for nitrogen determinations.

** $\log_{10}T\%$ was calculated using the formula: log dry weight—log wet weight+2 where dry and wet weights are expressed in micrograms. Results were calculated using only the mean values for wet weight and dry weight.

Table 2

Growth and citric acid content of embryonic chick tibiae cultivated in vitro in medium 858*

Period of cultivation (days)	Length (mm)	Wet weight (mg)	Dry weight (mg)	Citric acid (µg)
0	7.7 (7.0 – 8.3)	3.7 (3.3 – 3.9)	0.37 (0.34 – 0.39)	1.06 (0.84 – 1.24)
2	8.8 (8.5 – 9.5)	5.4 (5.2 – 5.7)	0.53 (0.50 – 0.57)	0.00
4	10.6 (10.3 – 11.1)	6.6 (6.4 – 6.9)	0.68 (0.65 – 0.70)	0.00
8	12.2 (11.8 – 12.9)	9.0 (8.6 – 9.4)	0.82 (0.80 – 0.85)	N.E.

N.E. = Not estimated.

* Results are expressed per tibia. Mean of 3 separate experiments. In each experiment 8 bones were cultured. The length of six bones was measured individually. Wet weight, dry weight and citric acid determinations were made on a group of six bones and the mean value was calculated. The mean values in each experiment fall in the range given in parentheses.
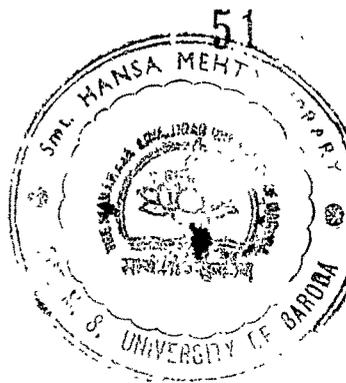
Table 3

Growth and hexosamine content of embryonic chick tibiae cultivated

in vitro in medium 858*

Period of cultivation (days)	Length (mm)	Wet weight (mg)	Dry weight (mg)	Hexosamine (µg)
0	7.5 (7.0 – 8.0)	3.4	0.38	12.0
2	8.6 (8.3 – 8.9)	5.2	0.51	16.2
4	10.6 (10.3 – 11.0)	6.4	0.69	21.8
8	12.0 (11.8 – 12.4)	8.6	0.81	23.4
12	12.9 (12.6 – 13.4)	10.2	0.97	26.8

* Results are expressed per tibia. Eight bones were cultured. Six bones were measured for length individually. Wet weight, dry weight and hexosamine determinations were made on a group of six bones and the mean was calculated per bone. The length of all bones fall within the range given in parentheses.

Table 4

Changes in length and chemical composition of embryonic chick tibiae cultivated in vitro with period of cultivation*

(expressed as per cent increase over zero day)

Period of cultivation (days)	Length	Wet weight	Dry weight	Calcium	Phosphorus	Nitrogen	Citrate	Hexo-samine
2	13	39	37	70	34	32	0	35
4	40	89	80	130	64	79	0	82
8	64	144	110	256	99	138	0	95
12	76	197	138	261	143	174	0	123

* Based on data of Tables 1, 2 and 3.
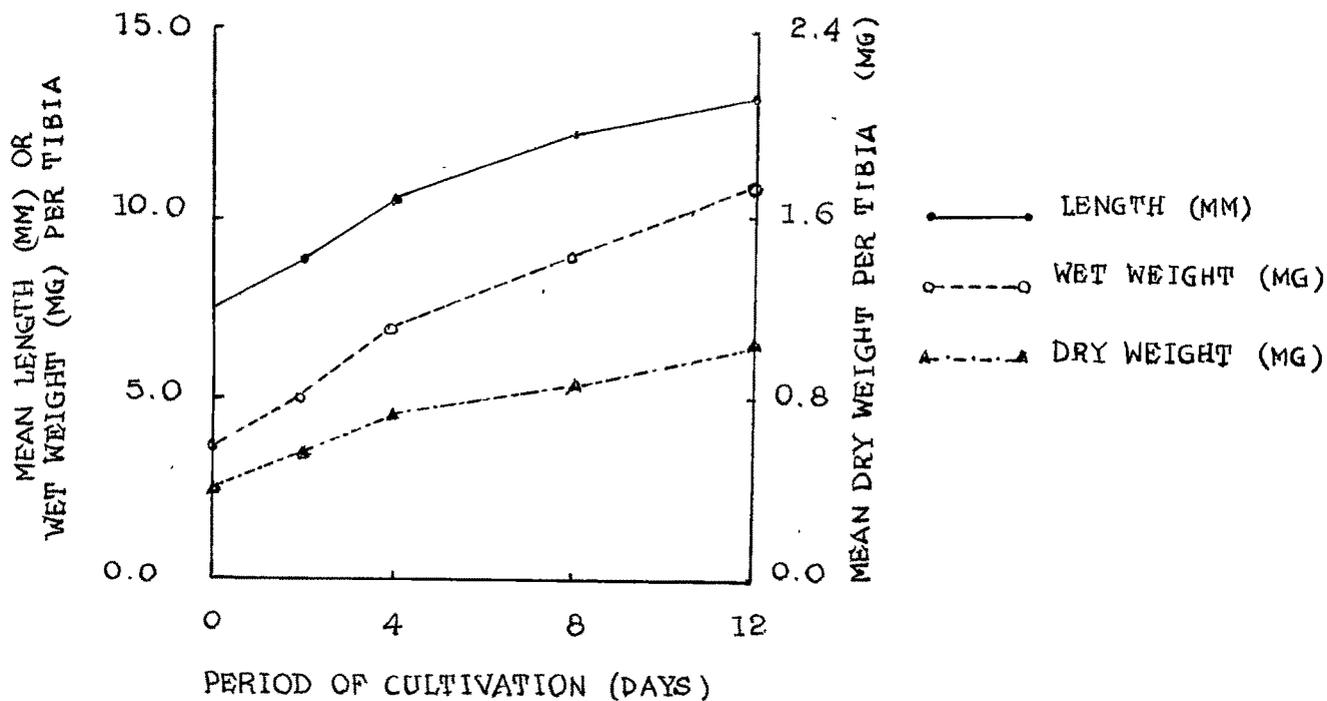
Fig. 2. Length, wet weight and dry weight of embryonic chick tibia cultivated in vitro.
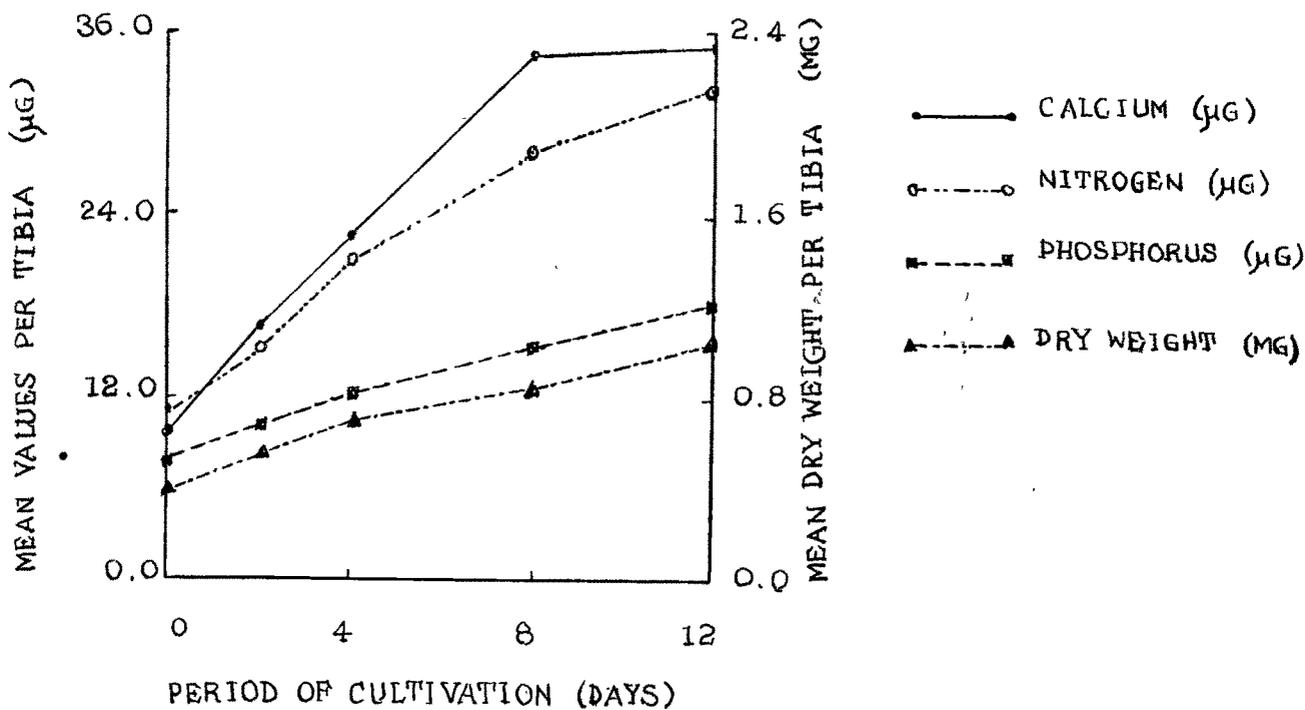
Fig. 3. Calcium, phosphorus, nitrogen and dry weight contents of embryonic chick tibia cultivated in vitro.
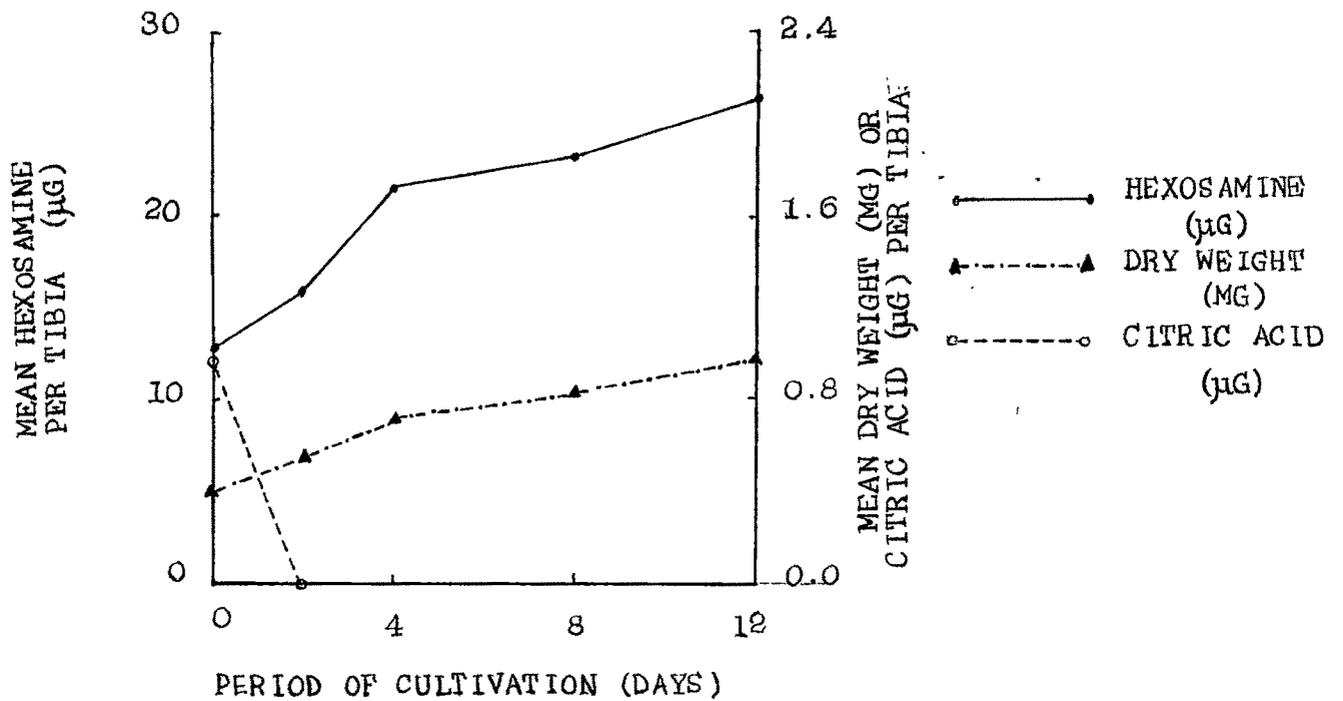
Fig. 4. Dry weight, hexosamine and citric acid
         contents of embryonic chick tibia
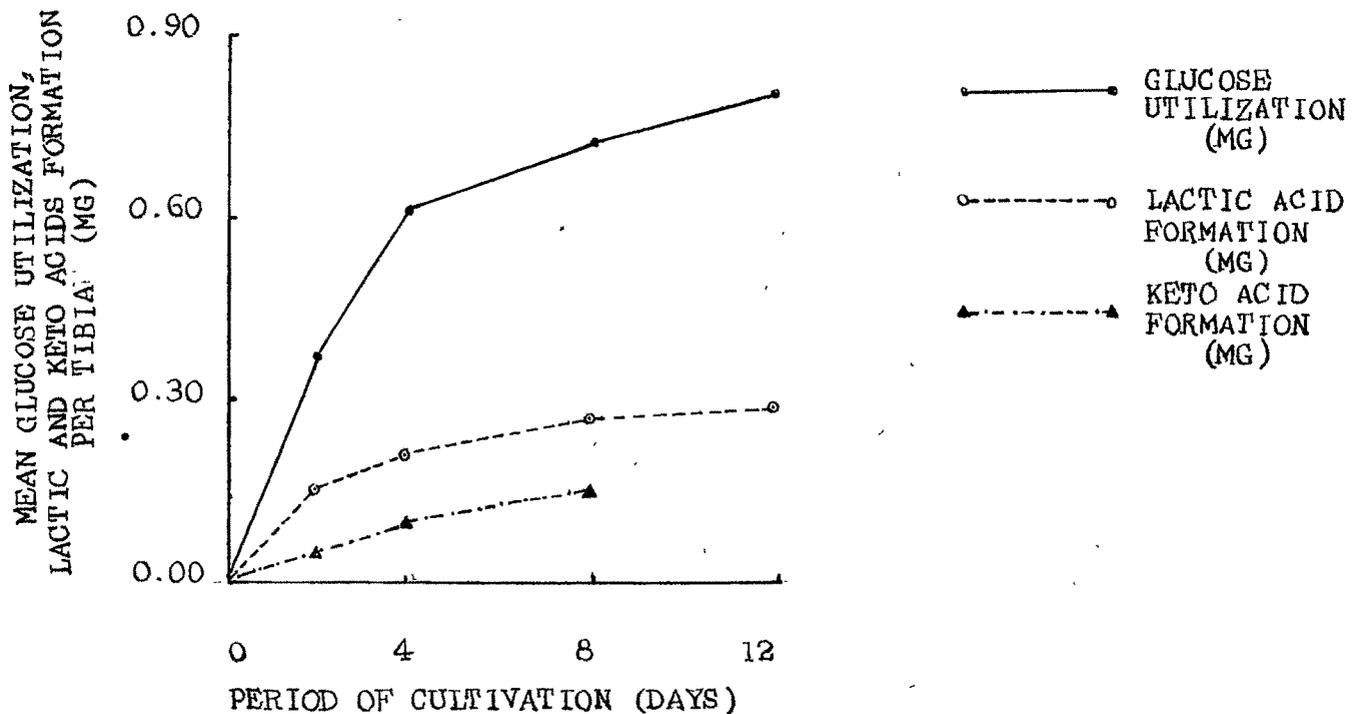   cultivated _in vitro_.

Fig. 5. Glucose utilized, lactic and keto acid produced
         by embryonic chick tibia cultivated _in vitro_.

Plate 1 is a photograph of bones cultivated for
0, 1, 2, 3, and 4 days and plate 2 shows bone sections
prepared from tibiae stained for calcium.

Table 5 summarizes the results of the composition
of embryonic bone during cultivation expressed as
percentage of the dry weight. The composition of the
bone from the embryos of successive ages and the new
born chick is shown in Tables 6 and 7.

The data presented in Tables 1, 2 and 3 suggest
that bones grow and calcify in a chemically defined
medium as indicated by the increase in length, wet
weight, dry weight, the contents (per bone) of calcium,
phosphorus, nitrogen and hexosamine. The absolute
length, wet weight, dry weight, calcium, phosphorus,
nitrogen and hexosamine increased linearly during four
days of cultivation in vitro (Figs. 2, 3 and 4). However,
the concentrations of calcium, phosphorus, nitrogen and
hexosamine, when considered as percentage of the dry
weight of the bone (Table 5) changed very little in vitro,
whereas the concentration of calcium and phosphorus
increased steadily in vivo and that of nitrogen increased
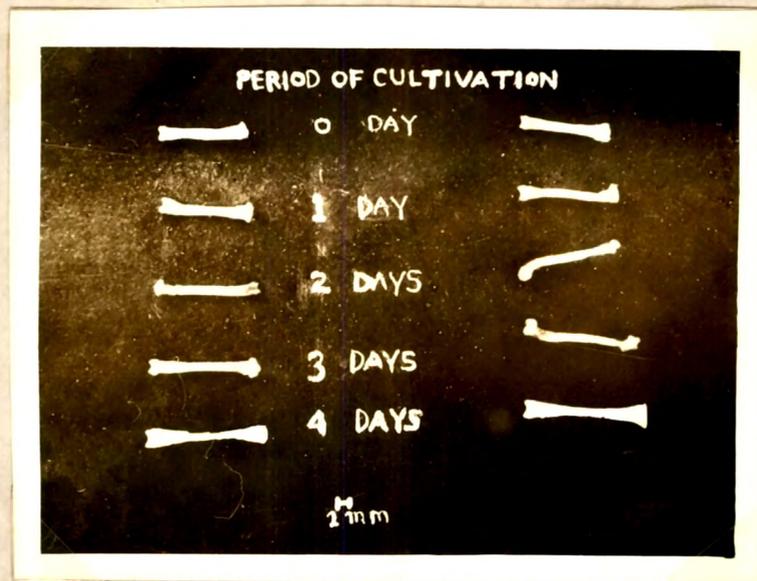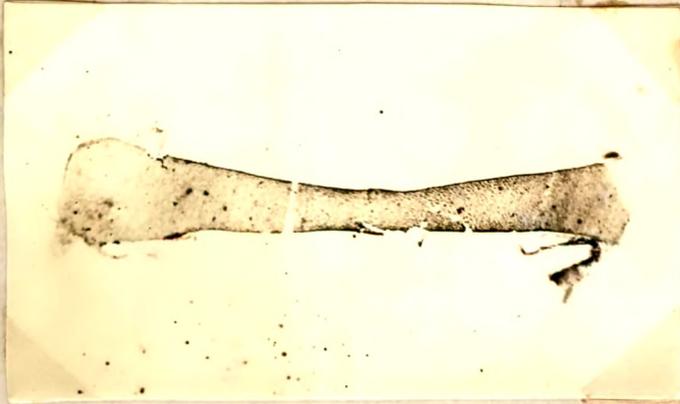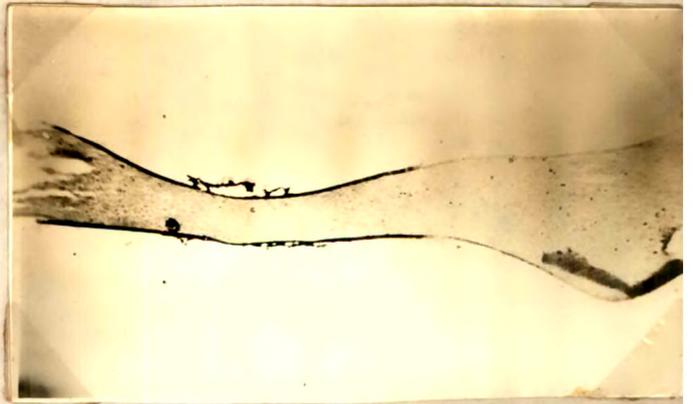from 2.92 to 6.83% from the 10th to the 14th day after

PLATE 1.

PLATE 1. 10-day-old embryonic chick tibiae and the
same after cultivation in medium 858 for
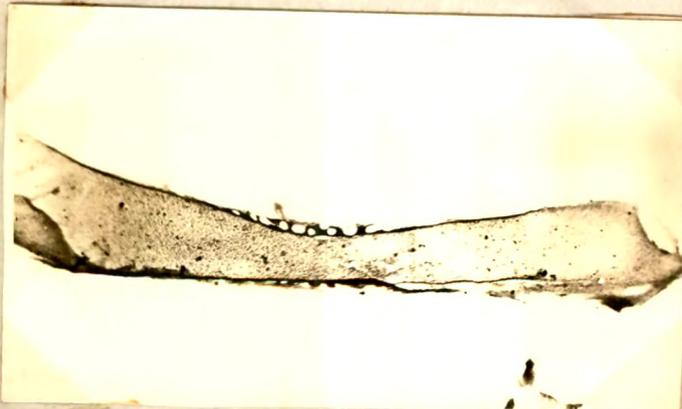1,2,3 and 4 days.

PLATE 2.

2(a)

2(b)

2(c)
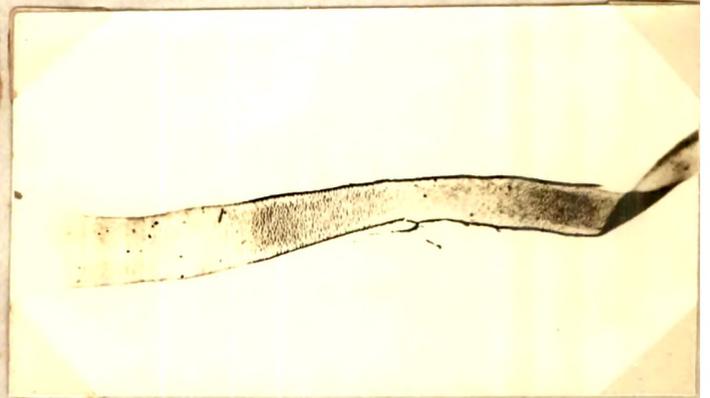
2(d)

PLATE 2. Frozen section of 10-day-old embryonic chick
tibiae cultivated on medium 858 for the period
of 0,2,4 and 6 days. The sections cut were of
10 to 15 u thickness and were stained for
calcium phosphate by the method of Von Kossa
(1901) and counterstained with safranine
(magnification, x 11).

2(a) 10-day-old chick embryonic tibia.
2(b) The same after cultivation for 2 days.
2(c) The same after cultivation for 4 days.
2(d) The same after cultivation for 6 days.

Table 5

Composition of embryonic chick tibiae cultivated in vitro in medium 858*

(results are expressed as per cent of dry weight of the bone)

Period of cultivation (days)	Calcium	Phosphorus	Nitrogen	Citric acid	Hexosamine
0	2.6 (2.3 – 2.9)	2.0 (1.9 – 2.1)	2.8 (2.6 – 3.0)	0.29 (0.25 – 0.32)	3.2
2	3.1 (3.0 – 3.4)	2.0 (1.9 – 2.0)	2.8 (2.7 – 2.8)	0.00	3.2
4	3.2 (3.2 – 3.3)	1.8 (1.7 – 1.8)	2.8 (2.7 – 2.8)	0.00	3.2
8	4.0 (3.9 – 4.2)	1.8 (1.6 – 1.9)	3.4 (3.4 – 3.5)	N.E.	2.9
12	3.8 (3.7 – 3.9)	2.0 (1.9 – 2.1)	3.4 (3.3 – 3.4)	N.E.	2.8

N.E. = Not estimated

* Results are calculated from the data presented in Tables 1, 2 and 3.

TABLE - 6

Composition of tibiae from chick embryos of different ages and newly hatched chick

(The results are expressed per tibia.)

Age of Embryos Days	Length (mm)	Wet weight (mg)	Dry weight (mg)	Log$_{10}$ m%	Calcium (µg)	Phosphorus (µg)	Nitrogen (µg)	Citric acid (µg)	Total hexosamine (µg)	Ca/P***	Ca/N***
10	7.5 (7.1-7.8)	5.6 (3.2-4.0)	0.40 (0.33-0.45)	1.046	9.8 (9.5-10.5)	7.7 (6.9-8.5)	11.7 (10.5-12.7)	1.06 (0.84-1.24)	12.0	1.28	0.85
12	10.9* (10.6-11.3)	12.2** (11.7-12.5)	1.47 (1.37-1.54)	1.081	57.4 (55.2-61.5)	34.8 (31.6-37.3)	88.0 (81.5-91.5)	3.23 (2.97-3.72)	N.E.	1.65	0.65
14	15.1 (14.8-15.5)	31.3 (30.2-31.9)	3.54 (3.12-3.97)	1.054	210.4 (202.4-211.2)	132.6 (126.4-137.1)	240 (230-247)	10.8 (10.0-11.8)	197 (192-201)	1.65	0.88
16	19.4 (18.8-19.8)	67.0 (62.0-73.0)	9.0 (8.4-9.6)	1.128	884 (870-912)	594.2 (586.4-602.6)	624 (606-642)	24.87 (24.20-25.61)	N.E.	1.49	1.42
Newly hatched chick	30.3 (29.6-30.9)	212 (206-217)	57.0 (52.8-61.6)	1.430	7920 (7735-8120)	5347 (5012-5830)	3914 (3722-4108)	160.5 (151.4-165.9)	N.E.	1.48	2.02

N.E. = not estimated.

*   Three separate experiments were carried out and mean values calculated.

**   Values in each experiment fall in the range given in parentheses.

*** Ratios calculated from the mean values of calcium, phosphorus and nitrogen.

Table 7

Composition of tibiae from chick embryos of different ages and newly hatched chick*

(expressed as per cent of dry weight of the bone)

Age of embryo (days)	Calcium	Phosphorus	Nitrogen	Citric acid	Hexosamine
10	2.45	1.93	2.92	0.27	3.0
12	3.89	2.36	5.97	0.22	N.E.
14	5.99	3.81	6.83	0.30	5.56
16	9.85	6.60	6.93	0.28	N.E.
Newly hatched chick	13.90	9.38	6.86	0.28	N.E.

N.E. = Not estimated

* Values are for embryonic tibiae calculated from the mean value given in Table 6.

which it appeared to remain constant (Tables 6 and 7).
The amounts of wet weight, dry weight, calcium,
phosphorus, nitrogen and citric acid increased
logarithmically and at similar rates during the
development of bones in vivo until the time of hatching
(Fig. 6). The increase in length was much less than
that for the other parameters, suggesting that the bone
was also increasing in volume. Hence, the rate of
growth and composition of the bone cultivated in vitro
was less than that of the bone developed in vivo for
the same periods of time.

The increase in the values for Ca/N ratio from 0
to 8 days of cultivation suggests that calcium salts
were being deposited in the matrix synthesized by the
bone. The nitrogen content of the bone increased as
did one of the constituents of the matrix, namely,
hexosamine. The value for Ca/P ratio in the bones
developed in vivo was slightly increased from 1.28 on the
10th day to 1.65 on the 14th day and decreased from 1.65
to 1.48 thereafter; the Ca/N ratio decreased from 0.85 on
10th day to 0.65 on the 12th day and increased from 0.88
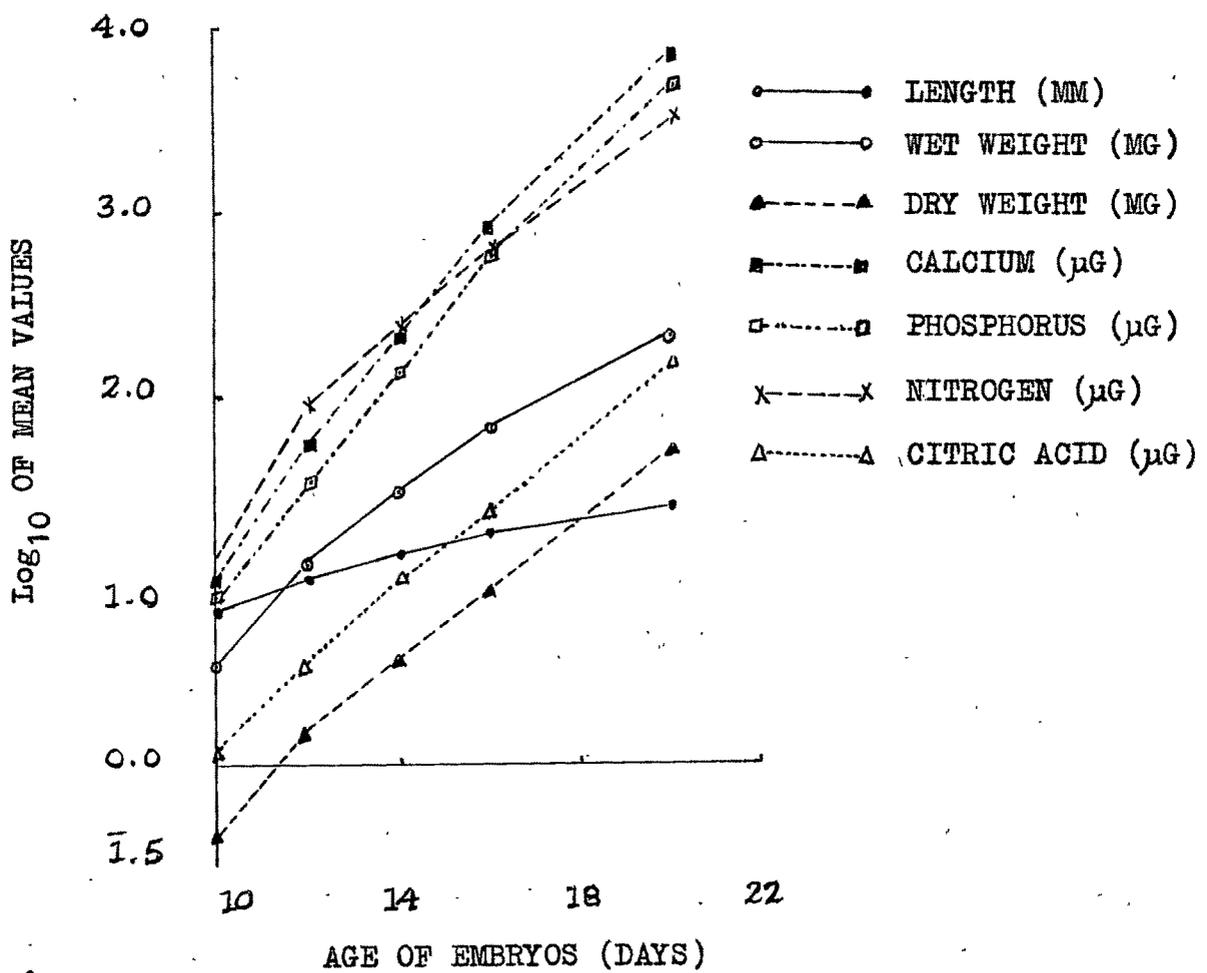on the 14th day to 2.02 thereafter.

Fig. 6. Logarithm of length, wet weight, dry weight, calcium, phosphorus, nitrogen and citric acid content of tibia from chick embryos of different ages.

Dickerson (1962a, b) analysed epiphyseal and cortical areas of femur and humerus as well as the whole bone from pig, rat, fowl and human and reported that, in the human femur, calcium salts began to be deposited in the cartilage at about 8 weeks' gestation. His data on the whole bone, viz., human femur, pig humerus, fowl femur and rat femur are presented in Table 8 together with the present data on the composition of whole tibia at various stages of development of chick embryos and in the newly hatched chick. The constituents of bone salt and the nitrogen content of human femur and pig humerus increased from early stages of gestation to the time of birth and thereafter. Similar increases occurred in the bone of the developing chick embryos in the present study. The composition of the tibiae of the newly hatched chicks and the Ca/N ratio were similar to that of the human femur between 12 - 14 weeks gestation and birth, and to the pig humerus between 46 and 90 days of gestation. The Ca/P ratio of the newly hatched chick was considerably lower than that reported for femurs of other species. Compared with the composition of newly-hatched fowl femur reported by Dickerson (1962a), both the

Table 8

Comparative data on the chemical composition

of the bone in different species

Bone	Calcium	Phosphorus	Nitrogen	Ca/P	Ca/N	Moisture
Human femur*						
12-14 weeks gestation	2.42	1.50	1.61	1.61	1.50	77.8
Newborn	6.06	2.84	2.71	2.13	2.24	63.9
11-12 years	13.80	6.25	3.82	2.21	3.62	27.0
Pig humerus*						
46 days gestation	1.88	0.94	1.29	2.00	1.39	80.5
90 days gestation	6.89	3.04	2.24	2.27	3.06	65.5
Newborn	5.99	2.60	2.33	2.30	2.57	63.7
1 year old	14.80	6.75	3.53	2.19	4.32	36.2
Fowl femur*						
Newly hatched	2.90	1.41	2.20	2.05	1.37	72.3
Rat femur*						
Newborn	2.24	1.39	2.56	1.61	0.88	71.0
Chick tibiae embryonic**						
10-day-old	0.27	0.21	0.33	1.27	0.84	89.9
12-day-old	0.47	0.28	0.72	1.65	0.65	88.0
14-day-old	0.67	0.42	0.77	1.65	0.88	88.7
16-day-old	1.32	0.88	0.93	1.49	1.42	86.6
Newly hatched chick	3.73	2.51	1.37	1.48	2.02	73.1

* Based on data reported by Dickerson (1962b and 1962a) and expressed as gm per 100 gm of fat free bone.

** Results obtained in the present investigation. Values calculated from the mean values given in Table 6 expressed as gm per 100 gm of fresh bone assuming fat content is negligible.

calcium and phosphorus contents of chick tibiae were higher and the nitrogen content lower, thus causing the ratios of Ca/P and Ca/N to differ considerably from those for femur. The femur of the newborn rat had less calcium, the same amount of phosphorus and more nitrogen than fowl femur. This was also reflected in the values for Ca/P and Ca/N ratios. Whether or not these differences are due to species and strain variations requires further investigation.

The dry weight/wet weight ratio of the embryonic tibia (expressed as $\log_{10}$ dry weight/wet weight x 100) increased as the development of embryo progressed in vivo. (Biggers et al, 1961). However, when the bone rudiments were cultivated in vitro, either in a chemically defined medium or in a natural medium, the dry weight/wet weight ratio decreased on the second day of cultivation, increasing thereafter up to the fourth day in the chemically defined medium and up to the sixth day in the natural medium. Subsequently, the dry weight/wet weight ratio gradually declined (Biggers, 1960a, b; Biggers, Gwatkin and Heyner, 1961).

In the present studies, the logarithm percentage dry weight/wet weight ratio of the bone increased as it developed _in vivo_ from the 10th day till the time of hatching but with a slight fall on 14th day (Table 6). This ratio for bone cultivated in medium 858 decreased during the first four days of cultivation, then increased on the 8th day of cultivation and again decreased thereafter (Table 1). A possible explanation for the initial fall in dry weight/wet weight ratio after culturing bone has been given by Biggers (1961a) who suggested that it might be due to adjustments in the water balance of the bone in the new environment. Biggers and Gwatkin (1961) suggested that it might also be due to the hypertrophy of the cells of diaphysis since one of the earliest signs of cellular degeneration is the uptake of water, possibly caused by impairment of the cell membrane.

In the present studies the bone grew in the chemically defined medium, but the values obtained for length, wet and dry weights were lower than those obtained by Biggers (1960a). During the cultivation period of four days, the percentage increase over zero day in length of

the bone obtained by Biggers was approximately 80%
whereas it was 40% in the present studies. The
percentage increase over zero day in wet and dry
weights of bone during cultivation obtained by Biggers
was approximately 150 and 136% respectively, whereas
the increases in present studies were 89 and 80%
respectively.

The absolute gains in elongation (3.0 mm), wet
weight (3.2 mg) and dry weight (0.32 mg) obtained during
cultivation of tibiae for the first four days in present
investigations were similar to those obtained by Biggers
(1960a) during cultivations from the 2nd to the 6th day,
i.e. 2.4 mm, 3.5 mg. and 0.31 mg for increase in length,
wet weight and dry weight respectively. Since Biggers
used 6½ to 7 day old embryos, the percentage increase
over zero day of cultivation was greater than in the
present study presumably due to the fact that the initial
values for length, wet and dry weights of bones used by
Biggers were much lower than those of the bones used in
the present studies. Another major difference between
Biggers' experiments and the present studies was in the
preparation of the bone rudiments for the experiments

reported here. Periosteum was partly damaged* while

trying to remove as much adhering tissue as possible

whereas this does not appear to have been the case in

Biggers' studies.

Nonetheless, the rates of gain from 0 to 2 and

from 2 to 4 days of cultivation were similar; for

instance, length increased by 1.3 and 1.7 mm, wet weight

by 1.4 and 1.8 mg and dry weight by 0.15 and 0.17 mg per

bone between 0 and 2 and between 2 and 4 days respectively.

The data on utilization of glucose and formation of

lactic acid and keto acid by the tibia cultivated in

chemically defined medium are given in Table 9 and

graphically in Fig. 5. The rate of utilization of glucose

by the tibiae was highest, 0.37 mg/bone, during the first

two days of cultivation and decreased to 0.24 mg/bone

during the next two days, and further decreased to 0.12

mg/bone during cultivation from 4th to 8th day. The

slightly increased rate, 0.15 mg/bone from the 8th to 12th

day may not be significant.

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* Prof. H.B.Fell, when she visited the laboratory where this
  investigation was carried out, pointed out that the
  periosteum was not likely to have been intact in the bones
  used in the present investigation.

TABLE 9

Glucose utilization and lactic and keto acid formation by embryonic
chick tibiae during cultivation in vitro in medium 858

Period of cultivation Days	Glucose utilization mg/bone	Lactic acid formed mg/bone	Keto acid formed mg/bone	Lactic acid produced / Glucose utilized	Keto acid produced / Glucose utilized	Lactic acid produced / Keto acid produced
0-2	0.37* ** (0.33-0.41)	0.15 (0.14-0.17)	0.005 (0.004-0.007)	0.42	0.015	30.7
2-4	0.24 (0.20-0.27)	0.06 (0.05-0.07)	0.005 (0.003-0.007)	0.25	0.021	12.0
4-8	0.12 (0.10-0.13)	0.06 (0.05-0.07)	0.005 (0.004-0.007)	0.50	0.042	12.0
8-12	0.15 (0.14-0.17)	0.03 (0.02-0.03)	N.E.	0.20	—	—

N.E. = not estimated.

* Mean value of four separate experiments. In each experiment the medium from two cultures containing 8 bones each were pooled together for estimations.

** Values in each experiment fall in the range given in parentheses.

The rate of formation of lactic acid during the
first two days of cultivation was 0.15 mg/bone, a
higher value than during successive periods of
cultivation. In the first four days, 0.21 mg/bone
was formed whereas in the second four days 0.06 mg/bone
was formed.

The rate of formation of keto acid was somewhat
higher during first four days of cultivation than later;
0.01 mg/bone was formed in first four days whereas
0.005 mg/bone was formed in second four days. It should
be noted that keto acid formation was small at all times.
The ratios of lactic acid formed to glucose utilized and
of keto acid formed to glucose utilized as well as the
rates of lactic acid formed to the keto acid formed suggest
that glycolysis predominated.

Citric acid was reduced to zero by the second day
of cultivation (Table 2). This may be due either to
the predominantly anaerobic condition which would have
prevented much synthesis of citrate or to the fact that
whatever citrate present in the bone, or formed by the
bone was oxidized rapidly. Also, it may be possible that
citrate was not coprecipitated along with bone salts
because no citrate was added to the medium. It would be

worthwhile to carry out further work to assess the
effect of citrate addition to the medium since there
has been much speculation about the role of citrate in
calcification.

SUMMARY

10-day-old embryonic chick tibiae were cultivated
in a chemically defined medium and their growth, chemical
composition and glucose metabolism were studied on
different days of cultivation. The bones increased in
length and weight and calcified. Glucose was utilized
glycolytically. In vitro growth in length occurred linearly
with time and calcification took place inspite of the fact
that some of the periosteum was removed. Tibiae developed
in vivo grew much more rapidly than tibiae cultured
in vitro.