

*From The Orthopaedic Department, Head: Prof. Sten Friberg, Karolinska Institutet, and
The Department of Developmental Physiology, Head: Dr. Trygve Gustafson,
The Wenner-Gren's Institute for Experimental Biology, Stockholm*

**THE EARLY STAGES OF
OSTEOGENESIS
IN TISSUE CULTURE**

A morphologic and biochemical study

By

John Sevastikoglou

EJNAR MUNKSGAARD

COPENHAGEN 1958

Almqvist & Wiksells

BOKTRYCKERI AKTIEBOLAG

UPPSALA 1958

ACKNOWLEDGEMENTS

This book is dedicated to Prof. Sten Friberg as a token of my gratitude to him for the privilege of being his pupil and for his invaluable help during the course of this work.

I also owe a great debt of gratitude to Prof. Erik Jorpes for placing at my disposal his wide experience and knowledge of biochemistry.

Dr. Tryggve Gustafsson, Assistant Professor of Zoophysiology, introduced me to the various methods of biologic investigation and sacrificed a good deal of his time in discussions and criticism. It is no exaggeration to say that this research would not have been accomplished but for his stimulating and practical guidance.

I should also like to thank Prof. Åke Wilton and Prof. Bengt Engfeldt for their expert criticism on matters concerning bone biology.

I wish to express my appreciation of the contribution of Dr. Harry Boström in the studies of radioactive sulphate, Dr. Diego Carlström in the X-ray crystallographic work and Dr. Sven Gardell in the studies of chondroitin sulphate.

It is a pleasure for me to acknowledge here the help of all the other persons concerned in various ways with this investigation, and in particular S. Bäckström, Fil. lic., for contributing the vitamin C determinations, Mrs. M.-B. Hjelte and Miss M. Maunsbach for technical assistance, and Miss K. Bergendahl, Mrs. K. Knutsson and Mrs. G. Nygren with respect to historadiographic, histochemical and histologic preparation, respectively.

The statistical analysis has been carried out by S. Ekblom, the photographs have been produced by S. Hedman, and the graphs drawn by S. E. Pettersson.

The manuscript has been revised from the linguistic aspect by Mr. Victor Braxton on behalf of the Academic Editorial Group.

The costs of this investigation have been partly defrayed by grants from the Swedish Medical Research Council, the funds of the Orthopaedic Clinic at the Caroline Institute, and by the Therese and Johan Andersson Foundation, Stockholm.

CONTENTS

Preface	5
Introduction	7
Culture methods	7
Material	11

PART ONE. MORPHOLOGIC STUDY

Methods	14
Results	16
Discussion	41

PART TWO. BIOCHEMICAL STUDY

Alkaline phosphatase activity	54
Experiments	55
Results	57
Discussion	62
Acid phosphatase activity	64
Experiments	65
Results	66
Discussion	69
Phenolsulphatase activity	70
Collagen	71
Experiments	72
Results	72
Discussion	73
Sulphomucopolysaccharides	75
Experiments	75
Results	77
Discussion	82
General discussion	84
Summary	88
References	90

PREFACE

At one time bone was considered as a biologically inert tissue, with one main physiologic function, the mechanical support of the body. During recent years the development of modern techniques of investigation such as tissue culture, enzymologic and microchemical methods, radioactive tracers, electron microscopy and X-ray crystallography, has resulted in a considerable addition to our fund of knowledge of the biology and biochemistry of bone, and it is now generally accepted that this tissue is a biologic entity *sui generis*. On the other hand, osteogenesis has been recognized as a highly complicated procedure. Several morphologic observations and studies on various biochemical reactions, enzymatic activities, and biophysical changes relating to osteogenesis have been published, but the intimate mechanism of this procedure still remains more or less unknown. One of the problems that has yet to be solved is the origin of the osteoblasts at the sites of endochondral osteogenesis. The principal opinions held on this subject may be summarized as follows: (a) Chondrocytes differentiate into embryonic tissue cells, which give rise to osteoblasts.^{132, 157, 133, 92} (b) The cartilaginous cells degenerate and disappear, and new bone is formed. Osteoblasts may develop either from the endothelial cells of the capillaries^{74, 75, 163, 164} or from the undifferentiated mesenchymal cells of the marrow tissue, and follow the capillaries.^{27, 129, 96, 58, 26} (c) Osteoblasts migrate to the site of endochondral osteogenesis along vessels that enter the cartilage from the periosteum.^{44, 68} (d) Osteoblasts are formed by direct metaplasia of certain liberated chondrocytes^{19, 91, 22, 67, 101} or fibroblasts.¹⁵³ (e) Osteoblasts are a temporary modification or modulation of series of mutually transformed connective tissue cells.¹²⁸ It is evident that the opinions held on the nature of the production of bone on cartilaginous basis are widely divergent, and, as often happens where knowledge is uncertain, these opinions tend to be expressed vigorously and categorically.

The object of the present investigation was to throw light on certain morphologic and biochemical aspects of the early stages of osteogenesis such as may occur in cultures of embryonic epiphyseal cartilage of the chick. This paper reports and discusses the results of this study. No attempt is made to advance new theses.

INTRODUCTION

The research is based on observations of cultured epiphyseal cartilage from the long bones of the embryonic chick, this species being chosen because, technically, the isolation of epiphyseal cartilage is easier to perform in chick than mammalian embryos. Furthermore, chick embryos of suitable age can be obtained as required—a major advantage when large serial examinations are involved—and strictly sterile conditions are easier to maintain in the case of the chick.

The epiphyseal cartilages to be explanted were generally obtained from 12-day-old chick embryos, at which age the long bones of the extremities are well developed. In earlier stages of incubation it is difficult to effect a satisfactory dissection of the bones and to free the explants from surrounding tissue. At later stages medullary tracts perforate the epiphyseal cartilage cones. Cartilage cones from the long bones of 11- to 12-day-old embryos were found to be suitable for the purpose of this study.

Skeletogenous material of the chick embryo has generally been chosen for earlier culture studies of osteogenesis; it is doubtful whether there are any essential differences in the process of osteogenesis in the various vertebrates. It is known, however, that there are certain morphologic differences in the skeletal development of the chick and mammals. In the former species, endochondral osteogenesis develops more slowly, and it is absent in all but the extremities of the diaphysis. As soon the formation of the periosteal bone is fairly well advanced, erosion of the enclosed cartilage begins, and it is not replaced by bony trabeculae as in mammals.⁴⁴ Moreover, there is no independent epiphyseal centre of ossification in the long bones of the fowl as there is in mammals. The ends of the bones remain cartilaginous for a while and provide for growth in length, but eventually they are ossified and united with the shaft, as its marrow cavity extends into them.⁸⁸

There are various accounts of the normal development of the skeleton in the chick embryo,^{77, 91, 88} the most detailed of which is by Fell.⁴⁴

CULTURE METHODS

The culture technique in the majority of the experimental series was that described by Fell and Robison.⁴⁶ The culture vessel was a watch-glass enclosed in a 70 mm Petri dish carpeted with a layer of cotton wool, in the centre of which a large round hole was cut for transillumination of the cultures during microscopic examination. Five to 7 millilitres of sterile distilled water was pipetted into each dish and absorbed by the cotton wool; this provided a very efficient moist chamber. The explants were laid on a plasma clot. This was prepared on a watch-glass, from plasma, embryo tissue extract and Tyrode's solution.

In some series, however, use was made of a modification of the above technique,²⁵ in which the medium was fluid. The composition of the fluid medium was the same as for the solid except that the homologous plasma was replaced by heterologous serum.

Blood plasma was obtained by bleeding to death from the carotid artery young adult roosters, which for 24 hours prior to operation had been fasted in order to reduce the amount of serum lipids. The blood was collected in well chilled centrifuge tubes that had been lined with a thin layer of paraffin to avoid coagulation, and was centrifuged immediately for 5 minutes at 3000 r.p.m. in an ordinary laboratory centrifuge. After centrifugation the clear supernatant plasma was withdrawn with a large bulb pipette, shared between paraffined test tubes and stored in a refrigerator at 0–4°C. Plasma collected in this way could be stored in fluid condition for several weeks without the addition of herapin or other anticoagulants. As a rule plasma was drawn two to four times a month to furnish the needs of the experiments.

Embryo tissue extract. The extract was either freshly prepared on the morning on which a culture or a subculture was to be made, or it was prepared in greater quantities, filtered and stored at about –15°C. In both cases 12-day-old chick embryos were used, with a few exceptions.

Gaillard⁵⁵ found that higher differentiation could be obtained if dynamic media were used, that is, if the subcultures were treated with extract obtained from embryos of increasing age. These observations have since been confirmed by other workers.^{105, 106} Notwithstanding these findings, static media were used exclusively in this investigation, as in the original method due to Fell.

(a) *Freshly prepared embryo extract.* Ten to 15 living chick embryos, removed from the eggs, were deposited on a Petri dish, washed with Tyrode's solution and transferred to a large watch-glass supported on a Petri dish. With a pair of curved scissors the embryos were finely chopped (about 100 to 150 scissor cuts per embryo). The tissue pulp was transferred to centrifuge tubes. An equal volume of Tyrode's solution was added and after mixing well with a glass rod the tubes were stoppered—first with a sterile cotton plug, which during centrifugation sank and swept away smaller tissue particles, and then with a cork stopper. The tubes were centrifuged at 4000 r.p.m. for 15 minutes in an ordinary laboratory centrifuge.

(b) *Filtered embryo extract* was used in the treatment of some of the series because it considerably facilitates the work, especially when large series of explants are to be treated, and in order to avoid any contamination of the explants by cells from the embryo extract.

Fifty to 100 embryos were ground in a motor-driven homogenizer. Tyrode's solution equal to the volume of the tissue pulp was added, and after mixing the pulp was centrifuged at 4000 r.p.m. for 10 minutes. The supernatant fluid was pipetted and ultracentrifuged for 30 minutes at 30,000 r.p.m. and an average field of 78,410 *g*. The centrifugate was filtered first through a one millimetre thick asbestos filter at a pressure of 9–10 atmospheres and then, for sterilization, through a glass filter (G5) with a pore diameter of 1.7–1.0 μ . The filtrate was stored in a freezing box at –15°C. This two-stage centrifugation and filtration procedures were found necessary because of the high viscosity of the initial centrifugate.

The embryo extract obtained by both methods was further diluted with equal parts of Tyrode's solution for use in the preparation of the culture media, or with twice the volume of solution for bathing the specimens before explantation or subculture (diluted embryo extract).

Blood serum. The serum was obtained from horse blood and stored in 50–100 ml tubes

in a freezing box at -15°C . When required for use the serum was thawed out and filtered to remove precipitates.

Tyrode's solution was used as a diluting medium. It was prepared according to the formula given by Parker¹²³: sodium chloride 7.7 Gm, potassium chloride 0.20, calcium chloride 0.20, magnesium chloride 0.10, sodium phosphate monobasic 0.05, sodium bicarbonate 1.00, glucose 4.00 Gm, made up to one litre with distilled water (normal Tyrode's solution).

In order to promote calcification a number of series were treated with Tyrode's solution enriched with calcium. Calcium glycerophosphate was added to the above formula and the acid phosphate was omitted to avoid precipitation⁵² (calcium-enriched Tyrode's solution).

The pH of the solution ranged from 7.4 to 7.8, which is usually considered optimal for the study of osteogenesis in tissue culture. Yoshitomi,¹⁶⁷ however, states that the optimal pH for the calcification of femoral bones of the 5- to 6-day-old chick embryos is between 7.2 and 7.4. Moreover, Paff,¹²² working with femurs of 7-day chick embryos, observed that more bone was formed and more calcium deposited at the low pH 7-7.3 than at 7.8-8.

Preparation of the explants. The epiphyseal cartilage explants used throughout were obtained from the long bones of the lower extremities of living chick embryos. 12-day embryos were used almost exclusively (p. 11). The whole limb, including the soft tissues, was separated at the hip joint and placed on a watch glass containing diluted embryo extract, this then being enclosed in a Petri dish. The soft parts were removed with fine catarract knives and the femur and tibia transferred to a cover slip on a Petri dish. The lid of the dish was lifted and the dissection performed under a dust shelter. A dissecting microscope ($\times 30$) was used. By gentle manipulation with the two knives the periosteum was stripped from the diaphysis to the epiphysis and removed with the perichondrium, the articular cartilage and a part of the epiphyseal cartilage. The thin perichondral bone cylinder enclosing the cartilaginous cone of the epiphysis was opened by means of longitudinal incision, the bony layer removed by gentle scraping with the knife in the direction from epiphysis to diaphysis, and the cartilaginous cone freed. The diaphyseal end, which was generally perforated by one or more medullary tracts, was cut away. The remaining part of the cone usually had the form of a flattened cylinder, 2-4 mm long and up to 2 mm thick. When lower tibial or lower femoral epiphyses were used the explant had a longitudinal depression in one surface, corresponding to the intercondyloid fossa. The dissected explant was then transferred to a new watch-glass containing dilute embryo extract and placed in a Petri dish. Before explantation the specimens were freed from particles of soft tissue by repeated vigorous irrigation with a pipette and changes of the bathing medium.

Preparation of the media. Solid medium: Nine drops of plasma for the original culture and 12 for the subcultures were deposited on the watch-glass with a pipette. An equal amount of dilute embryo extract was added and thoroughly mixed with a fine glass rod. The plasma coagulated within few minutes and a semisolid, clear translucent jelly was obtained.

The fluid medium was composed of a mixture of equal parts of horse serum and chick embryo extract. One millilitre of the medium was pipetted on each watch-glass and an octagonal piece of lens paper, treated as described by Chen,²⁵ was carefully placed on the surface of the medium.

Explantation and subculture. Four of 5 explants were laid on the plasma clot or the floating lens paper in dishes prepared as described above. Each dish was covered with its lid, marked for identification and placed in an incubator at 37°C . Explantations were

TABLE I. *Analysis of*

Experimental groups	No. of series per group	No. of cultures per group	No. of cultures from				No. of cultures excluded owing to		
			L.T.	U.T.	L.F.	Mx.	infection	accident	poor growth
XVI, 1-9	10	102	—	—	—	102	50	14	8
22-30	9	120	50	40	30	—	11	2	11
31-33	3	36	12	12	12	—	8	1	6
36-38	3	46	—	46	—	—	—	3	—
39-45	7	123	35	35	35	18	35	24	—
46-53	8	141	—	—	—	141	38	23	9
54-77	24	363	123	124	101	15	77	18	15
78-102	25	437	120	122	118	77	89	11	1
103-133	31	318	106	106	106	—	54	18	103
134-220	87	847	334	335	178	—	207	24	119
221-297	72 ^a	671	264	243	182	—	10	7	347
298-385	79 ^a	797	283	282	232	—	99	1	206
386-397	12	114	37	39	38	—	—	—	—
398-423	26	261	—	—	—	261	—	—	—
Diaphyseal series	14	147	28	28	8	83	1	2	16
TOTAL	410	4523	1386	1411	1029	697	679	148	841

^a Diaphyseal series are not included.

performed every week so that at the end of 10-12 week period of observation a complete range of cultures at all stages was available for biochemical examination. With very few exceptions, cultures were transferred to fresh medium every third day when solid medium was used, and every fourth day in the case of fluid medium.

For subculture from solid medium the cultures were carefully dissected from the network of the wandering cells under a dissecting microscope with a pair of catarract knives. They were laid for about 5 minutes on a drop of diluted embryo extract on a microscope slide in a dish, the drop of bathing medium was changed twice and the explant again transferred to the surface of a freshly prepared plasma clot.

For subculture of explants grown in fluid medium the octagonal lens paper bearing the explants was carefully lifted from the medium with a pair of forceps and placed on another watch-glass in a Petri dish containing 5 ml of diluted extract. The dish was carefully agitated for a few seconds. After about 5 minutes the lens paper was transferred with the cultures to freshly prepared medium. Five papers were usually bathed on the same medium.

the material.

MEDIUM VARIANTS							
Basal medium			Tyrode's solution			Embryo extract	
homologous (solid)	homol. + heterol.	heterol. (fluid)	normal	normal + calcium	calcium- enriched	fresh	filtered
102	—	—	102	—	—	102	—
120	—	—	120	—	—	120	—
36	—	—	—	36	—	36	—
46	—	—	46	—	—	46	—
—	—	123	123	—	—	123	—
—	—	141	—	—	141	141	—
—	363	—	—	—	363	—	363
—	—	437	—	—	437	—	437
318	—	—	318	—	—	—	318
847	—	—	847	—	—	847	—
671	—	—	671	—	—	671	—
797	—	—	797	—	—	797	—
114	—	—	114	—	—	114	—
261	—	—	261	—	—	261	—
147	—	—	147	—	—	147	—
3459	363	701	3546	36	941	3405	1118

MATERIAL

The study is based on observations made on a total of 4523 cultures divided into 15 experimental groups, or 410 series each of 5–20 cultures. 1386 explants obtained from the lower tibial (L.T.), 1411 from the upper tibial (U.T.), and 1029 from the lower femoral (L.F.) epiphysis of both legs of chick embryos were cultivated in separate series. A further 697 explants obtained from the same epiphyses were cultivated in mixed series (Mx.). All but 184 explants were provided by 12-day-old chick embryos, 57 (series 7–9 and 386–8) by 10-day-old embryos, 49 (series 5, 6 and 389–91) by 11-day-old embryos, 39 (series 3 and 392–4) by 13-day-old embryos and 39 (series 2 and 395–7) by 14-day-old embryos.

3459 explants were cultivated in solid—homologous—and 701 in fluid—heterologous—medium, while 363 were first cultivated in solid medium for a period of about two weeks and thereafter transferred to fluid medium.

3546 cultures were treated with normal Tyrode's solution and 941 with calcium-enriched Tyrode's solution. 36 explants were cultivated for 2 weeks in normal Tyrode's solution and thereafter in calcium-enriched solution.

TABLE II. *Distribution of culture groups according to medium variants.*

Culture variants	Culture groups						
	I	II	III	IV	V	VI	VII
Homologous medium	x	x	x				
Heterologous medium				x	x	x	
Homologous + heterologous medium . .							x
Normal Tyrode's solution	x	x		x			
Calcium-enriched Tyrode's solution . .					x	x	x
Normal + calcium-enriched Tyrode's solution			x				
Fresh embryo extract	x		x	x	x		
Filtered embryo extract		x				x	x
Total number of cultures in each group	3105	318	36	123	141	437	363

3405 explants were treated with freshly prepared embryo extract, and 1118 with filtered extract.

Of the 4523 explants 685 were rejected because of infection and 148 were lost through accidents. A further 841 cultures were rejected after at least 2 weeks of cultivation, owing to "poor growth"; cultures were so designated when there was nei-

TABLE III. *Distribution of material according to period of cultivation.*

Series	Period of cultivation (days)											Total
	0-7	8-14	15-21	22-28	29-35	36-42	43-49	50-56	57-63	64-70	< 70	
XVI, 1-9	28	37	9	25	1	2	—	—	—	—	—	102
22-30	11	21	38	10	20	10	7	3	—	—	—	120
31-33	2	2	14	1	8	9	—	—	—	—	—	36
36-38	24	22	—	—	—	—	—	—	—	—	—	46
39-45	17	33	23	5	16	14	—	15	—	—	—	123
46-53	32	13	14	19	29	6	8	8	—	2	10	141
54-77	23	50	49	14	37	48	25	62	26	9	20	363
78-102	43	123	99	75	63	18	13	3	—	—	—	437
103-133	11	8	28	72	45	61	50	18	9	3	13	318
134-220	123	140	227	97	92	50	66	40	12	—	—	847
221-297	52	113	210	179	87	30	—	—	—	—	—	671
298-385	99	116	141	95	101	159	29	28	16	13	—	797
386-397	—	—	29	85	—	—	—	—	—	—	—	114
398-423	261	—	—	—	—	—	—	—	—	—	—	261
Diaphyseal series	9	35	23	27	38	—	15	—	—	—	—	147
TOTAL	735	713	904	704	537	407	213	177	63	27	43	4523

ther migratory activity nor increase in area. This selection was, however, highly subjective.

A detailed analysis of the material is given in Table I.

The material was divided into two main groups according to whether the cultures were grown on homologous or heterologous medium. These groups were subdivided into three according to the medium variants. A seventh group contained cultures grown 2 weeks in homologous medium and thereafter in heterologous medium (Table II).

A grouping of the material into diaphyseal and epiphyseal cultures was made. The diaphyseal group was composed of 14 series—a total of 147 cultures. These cultures were grown in homologous medium and treated with normal Tyrode's solution and fresh embryo extract. The explants of this group were obtained from lower and upper tibial and lower femoral epiphyses dissected as above, with the difference that the diaphyseal part of the epiphyseal cartilaginous cones, together with the medullary tracts perforating them, were used. Only that part of the epiphyseal cones covered by the periosteal bone cylinder was used, as this is composed almost solely of hypertrophied and degenerate chondrocytes, with practically no flattened chondrocytes. This group of cultures was used as controls.

The distribution of the material according to the period of cultivation is shown in Table III. Generally speaking, the number of cultures decreases with the time of cultivation.

PART ONE

MORPHOLOGIC STUDY

The osteogenetic capacity of embryonic skeletogenous materials of various origins has been widely studied by the tissue culture technique,^{21, 185, 124} or similar methods, such as implantation in the chorio-allantoic membrane,^{114, 150} and grafting.¹⁴⁹ However, H. B. Fell,⁴⁵ with her systematic investigations on tissue culture, opened a new epoque in the history of bone physiology. Since then several morphologic reports have been published on the osteogenetic capacity of embryonic skeletogenous tissues studied by tissue culture. Convincing morphologic evidence of the differentiation and formation of bone was provided by work on undifferentiated limb-buds,^{45, 81} cartilaginous extremities of long bones of 8- to 8½-day chick embryos,^{45, 34} rudimentary periosteum of 6-day chick embryos,^{48, 49, 149} endosteal bone,⁵⁰ and human phalangeal and metacarpal bones.¹⁶⁸ Epiphyseal cartilage has rarely been used in this connection.^{21, 45}

METHODS

The results obtained in these series of experiments were studied from a morphologic standpoint. The following methods were used.

Living cultures. (i) Observations of macroscopic changes occurring during cultivation. (ii) Observations of the migratory activity of the cells. (iii) Recording of changes in the initial area of the cultures.

Changes in the fine structure of the cultures could not be followed, as the culture method used permitted observations only at low magnification.

The migratory activity of the cells was studied and recorded every third day in the cultures grown in solid medium, as a rule in connection with the subculture. In fluid medium observations of this kind were precluded by the opacity of the paper. The observations were performed under the dissection microscope ($\times 30$).

The results were recorded by the following notation: no signs of wandering cells (-), few cells and cell debris found in the clot-bed beneath the culture ($\pm$), definite signs of wandering cells moving radially, and limited to a small peripheral area (+), radial migration of wandering cells from the greater part of the periphery (+ +), formation of a typical halo of wandering cells (+ + +).

This subjective estimate of the activity of the wandering cells is clearly of value only in this context.

The areal growth was followed by projective microscopy ($\times 27$), the projected outline of the cultures being traced at regular intervals by the method due to Ebeling.⁴¹ The total area was determined with a planimeter (Maho-35) and the increase expressed as a percentage of the initial area. Before each measurement, uniformity of the micropro-

jection was ensured by means of an ocular micrometer. Measurements were performed at intervals of about one week, and where possible shortly after making a subculture. Records of the areal growth were made for 278 series (Nos. 1-9, 22-33, and 36-297), or 3188 cultures.

Observations on sectioned material. After fixation in a 10 per cent solution of formaldehyde neutralized with magnesium carbonate, the explants were imbedded in paraffin and 5 μ sections were cut. Eosin-haematoxylin stain was used throughout. To detect calcium salts alizarin red and von Kossa's silver stains were used in certain cultures. The location of the regions of calcification was studied also by microradiography. In one case the deposited calcium salts were studied by X-ray diffraction.^{42a}

Control sectioned specimens. Thirty-seven epiphyseal cartilages obtained from 12-day chick embryos were dissected in the same manner as the explants and 5 sections were cut as controls. Twenty-one of these controls (KA 1-21) were sectioned perpendicularly and 16 (KB 1-16) transversely, all of them being stained with haematoxylin-eosin. Six more specimens were provided as controls for the diaphyseal series (KC 1-6); these were sectioned perpendicularly and treated with haematoxylin-eosin, von Kossa's and alizarin red stains.

Methods of statistical analysis

In the statistical analysis of the material only standard methods have been used.³² Thus, the mean $\bar{x}$ of n observations and its standard error ε were calculated according to the formulae:

$$\bar{x} = \frac{\sum x}{n} \quad (\text{I})$$

and
$$\varepsilon = \frac{s}{\sqrt{n}}, \quad (\text{II})$$

where the standard deviation $s = \sqrt{\sum (x - \bar{x})^2 / (n - 1)}$. The standard error of the percentage $p = 100 f/n$ was calculated according to

$$\varepsilon_p = \sqrt{\frac{pq}{n}}, \quad (\text{III})$$

where $q = 100 - p$.

The 95 per cent confidence limits of a percentage were found from the tables of Mainland.⁹⁴

The error of the method for determining the areal growth was calculated from the formula:

$$\sqrt{\frac{\sum d^2}{2n}}, \quad (\text{IV})$$

where d is the difference between the duplicate measurements made on the same culture.

In comparing two age values in the same series, the difference d between the two values was calculated for each culture. The mean difference, $\bar{d}$, was examined by Student's t -test:

$$t = \frac{\bar{d}}{s_d/\sqrt{n}}, \quad (\text{V})$$

where s_d is the standard deviation of the differences and t has $n - 1$ degrees of freedom. The significance of the values of t was obtained from a table of distribution of t .

The difference between two means, $\bar{x}$ and $\bar{y}$, of two independent groups of observations was tested by the expression

$$t = (\bar{x} - \bar{y}) \sqrt{\frac{(n_1 + n_2 - 2) n_1 n_2}{(n_1 + n_2) [\sum (x - \bar{x})^2 + (y - \bar{y})^2]}}. \quad (\text{VI})$$

This value of t has $n_1 + n_2 - 2$ degrees of freedom.

When more than two series of observations were compared an analysis of variance was performed. In this method the variance between the series (S_1^2) is compared with the variance within the series (s_2^2) by means of the F -test:

$$F = \frac{s_1^2}{s_2^2}. \quad (\text{VII})$$

The significance of the value was found in a table for the distribution of F .³²
The levels of significance were

$p > 5$	per cent	not significant
1 per cent $< p < 5$	per cent	almost significant
0.1 per cent $< p < 1$	per cent	significant
$p < 0.1$	per cent	highly significant,

where p is the probability of the difference being due to chance.

RESULTS

Macroscopic observations

Observations were made on the shape, colour and consistency of the cultures, the relationship of the cultures to the medium, and changes occurring in the medium during the course of cultivation.

Shape. During the first days of cultivation the cultures usually retained their original shape, and their contours were as sharp as at the time of explantation. Between the 1st and 2nd week there was a distinct increase in the area at the expense of thickness, although the cultures retained the principal features of their initial shape. At the same time their contours became smoother and rounded. Moreover, many explants, especially the longer ones, became curved along their longitudinal axis, the central part thus losing contact with the surface of the medium.

Colour. During the first stages of cultivation the cultures usually retained their initial, translucent, bluish colour. However, between the 2nd and 4th week, they

turned whitish and their translucency decreased, this change becoming more marked over the following two weeks, at the end of which the cultures were opaque, occasionally with regions of intense white, which were generally located at their diaphyseal pole.

Consistency. Initially, the explants were soft and elastic, like hyaline cartilage, but they gradually became harder, until they had the consistency of fibrous cartilage. These changes usually accompanied the development of the opaque whitish colour.

Culture-medium relationship. During the first week of cultivation, the cultures were usually lying free on the surface of the medium, but as the migratory activity of the cells developed the cultures became anchored to the medium. This difference was especially noticeable on removal from solid medium for subculture. As long as the cultures were lying free on the solid medium practically no changes in this were noticeable, except for a slight depression beneath the culture and the formation of an imprint on the plasma clot, as if from the weight of the specimen. No liquefaction could usually be observed as evidence of the proteolytic action on the clot. On removal of the explant for subculture as the migratory activity began, a more or less irregular groove was left on the clot, at the bottom of which there was a very small amount of fluid. At later stages of cultivation there was extensive liquefaction of the plasma clot around the culture, which was thus bathed in a large drop of fluid.

These changes were commonly observed in the case of cultures grown in both solid and fluid media. However, it was much easier to follow them in the former case, as the translucency of the plasma clot provided better illumination. The cultures that failed to display any migratory activity of the cells and that as a rule underwent no significant alteration in area did not exhibit any change in the above properties. Such cultures were considered as inactive. They always lay free on the media; they preserved the sharpness of their contours and their initial colour, and only at later stages of cultivation did white areas appear—usually at the diaphyseal pole. The majority of the cultures of the diaphyseal series belonged to this category.

Migratory activity of the cells

The migratory activity of the cells appeared much later in cultures of cartilage than in those of other tissues. As a rule very weak signs of activity appeared on the 5th to 6th day of cultivation. During this stage a few free cells and cell debris could usually be observed on the clot bed beneath the cultures. On about the 5th day much elongated cells appeared as streams radiating from points on the long sides of the culture, usually near its epiphyseal pole, and never at the diaphyseal, cut surface of the explant. After a while the radially migrating cells extended to most of the periphery of the culture, and finally they formed a halo. A typical halo was very seldom formed before the 2nd week of cultivation. Once the migration of the cells was established, it proceeded uninfluenced by manipulation of the explant

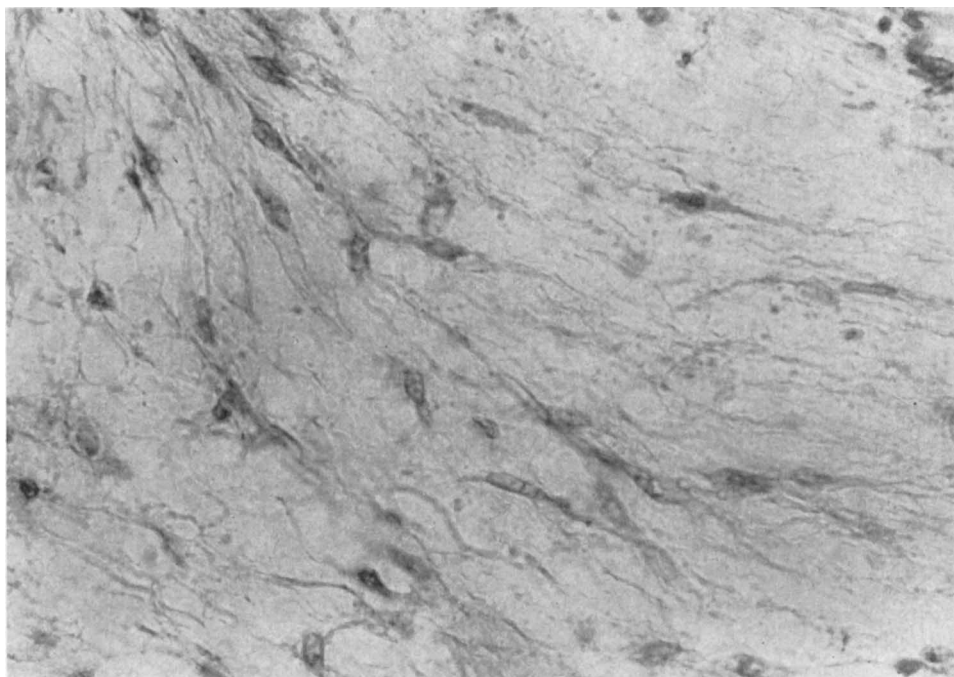


FIG. 1. Wandering cells filling the intercondyloid fossa of culture 350e grown for 29 days in homologous medium with fresh embryo extract and normal Tyrode's solution. Haematoxylin-eosin. $\times 480$.

TABLE IV. *Migratory activity of the cells.*

Series	—	±	+	++	+++	Total
XVI, 1-9	41	—	12	9	25	87
22-30	18	12	15	11	63	119
31-33	—	—	6	1	27	34
36-38	—	9	19	4	1	33
54-77	14	50	182	110	5	361
103-133	145	94	44	30	5	318
134-220	243	177	93	76	176	765
221-297	119	379	60	42	77	677
298-385	46	252	159	100	180	737
386-397	3	7	27	46	31	114
398-423	39	24	—	—	—	63
Diaphyseal series	109	15	6	4	1	135
TOTAL	777	1019	623	433	591	3443
PER CENT	22.5	29.5	18.0	12.5	17.1	
	± 0.71	± 0.77	± 0.65	± 0.57	± 0.64	

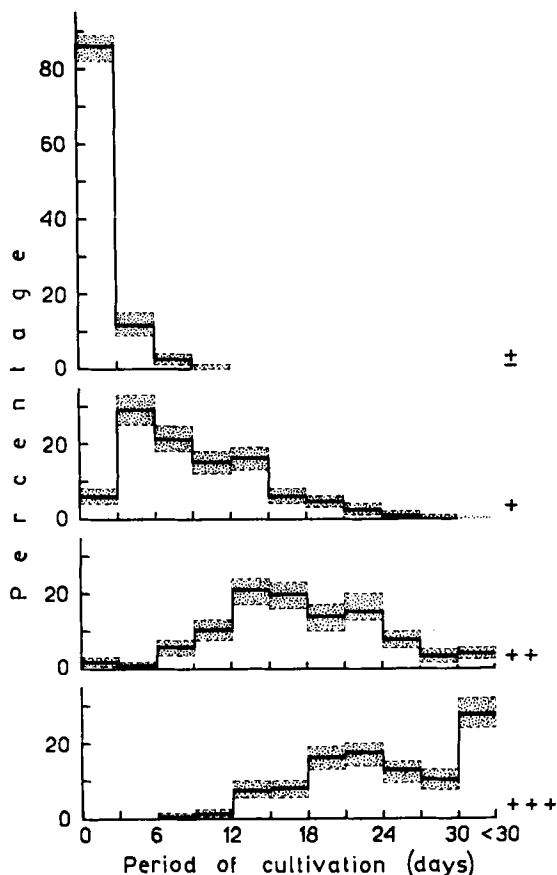


FIG. 2. The percentage frequency of the various degrees of migratory activity of cells at the various stages of cultivation in cultures grown in homologous medium with fresh embryo extract and normal Tyrode's solution. Shaded areas denote the confidence intervals.

during a subculture, and if a halo had formed, it was usually found to have developed again between two subcultures within 3 days. Seen under high magnification ($\times 80-720$) the wandering cells were spindle-shaped and resembled fibroblasts (Fig. 1); they lay with their long axes radially disposed. This orientation was well defined at the periphery of the zone of outgrowth, while near the culture cells formed a network. The nuclei of these cells were elongated and were as a rule located centrally. If kept at room temperature they lost their characteristic spindle shape in few hours and become rounded. Signs of degeneration then appeared; this was characterized by the presence of vacuoles and fat droplets in the cytoplasm.

As has been mentioned earlier, the activity could be followed in cultures grown on solid medium exclusively or for a short period only.

The extent of the migration of the cells in the various culture groups is surveyed in Table IV. It is seen that in about 50 per cent of the cultures there was no such

activity at all. However, as a large number of the cultures had grown for too short a period to manifest any migratory tendency, the percentage showing no signs of migration cannot be considered as representative. Thus, if the 735 cultures grown for a period of no more than 7 days are excluded owing to the usual absence of migratory activity during this period, the percentage showing no such activity is reduced to 30.8 ± 0.079 . The variation of the migratory activity with respect to the time of cultivation is demonstrated in Fig. 2, which is based on the chronologic appearance of the various degrees of migratory activity in 534 cultures presenting a halo during the period of cultivation.

Areal growth

Measurements performed at short intervals during the first 3 days of cultivation on explants from the epiphyseal part of the cartilaginous cones, cultured in homologous medium and treated with normal Tyrode's solution and fresh embryo extract revealed a marked increase in the initial area during the 1st hour of cultivation. No increase was found during the 2nd hour but between 2nd and 24th hour there was a progressive expansion. From the 24th hour there was no further increase until the beginning of the 3rd day of cultivation (Fig. 3).

Measurements performed at weekly intervals gave a curve of areal growth that rose steeply in the different culture groups during the first three weeks of cultivation, depending on the culture medium used in the groups. There followed a slight increase, or even a decrease, in area.

It has been shown that in the cartilaginous extremities of the long bones of 8-8½ day chick embryos growth is most rapid during the first 10 days *in vitro*, after which it slows down.⁴⁵

Analysis of the results revealed rather large, statistically significant variations in the pattern of areal growth for the various experimental groups, these being related principally to the histologic composition of the explants; they depended on, for instance, whether or not the explants contained flattened chondrocytes. Moreover, variations associated with the composition of the medium were also observed. There were, however, no statistically significant differences in the pattern of the areal growth of explants derived from different epiphyses—for instance the lower tibial, the upper tibial and the lower femoral—nor between explants of different initial areas.

THE HISTOLOGIC COMPOSITION OF THE EXPLANTS

Diaphyseal series. Explants belonging to the diaphyseal series were obtained from the diaphyseal part of the cartilaginous cones and were composed almost exclusively of hypertrophied and degenerated chondrocytes, while flattened chondrocytes were absent. Such cultures were grown on homologous medium and were treated with normal Tyrode's solution and freshly prepared embryo extract. Up to the 6th week

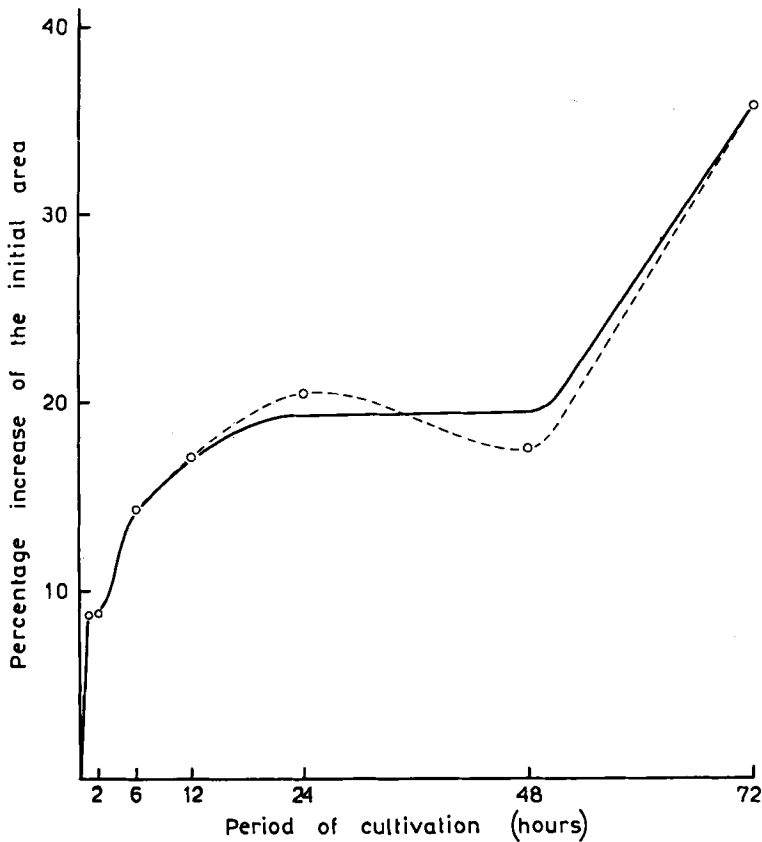


FIG. 3. Areal growth during the first 3 days of cultivation in cultures grown in homologous medium with fresh embryo extract and normal Tyrode's solution.*

of cultivation these explants showed no change in their initial area. By the 35th day there was a moderate decrease but by the 40th day the area was back at about its original value (Fig. 5 d).

Epiphyseal series. Explants obtained from the epiphyseal part of the cartilaginous cones were composed of all three groups of chondrocytes—flattened, hypertrophied and degenerated. The initial area in those cultures showed highly significant variations during cultivation.

COMPOSITION OF THE MEDIUM

Cultures composed of all three types of chondrocytes displayed large variations in the pattern of the areal growth according to the composition of the medium in each group.

* The broken line is drawn through the averages of the experimental values; the continuous line represents the assumed development. The values were too few for statistical analysis.

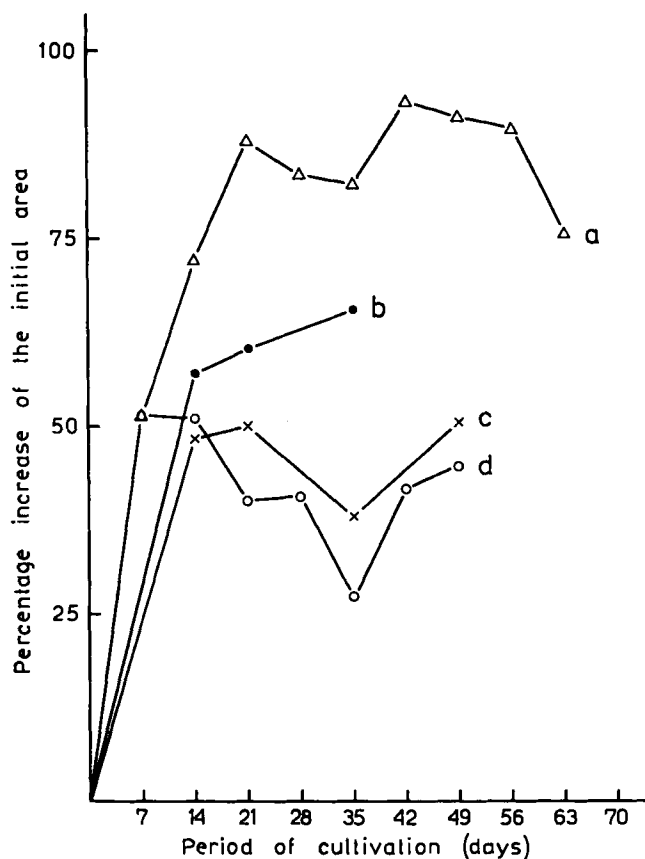


FIG. 4. Areal growth in epi-physeal cultures grown for a period, or exclusively, in heterologous medium. (a) Series grown in homologous medium for 2 weeks and thereafter in heterologous medium. Filtered embryo extract and calcium-enriched Tyrode's solution were used throughout. (b) Heterologous medium with fresh embryo extract and calcium-enriched Tyrode's solution. (c) Heterologous medium with fresh embryo extract and normal Tyrode's solution. (d) Heterologous medium with filtered embryo extract and calcium-enriched Tyrode's solution.

Heterologous medium. In all the cases the curve for the areal growth in cultures grown in heterologous medium showed a steep rise during the first week of cultivation. There followed a slow increase up to the 5th week in cultures treated with calcium-enriched Tyrode's solution and freshly prepared embryo extract (Fig. 4 b). If, however, the explants were treated either with normal Tyrode solution and freshly prepared extract (Fig. 4 c) or calcium-enriched Tyrode's solution and filtered extract (Fig. 4 d) the areal growth decreased after the 1st week and reached a minimum at the end of the 5th. A slight increase was observed between the 5th and 7th weeks of cultivation. The difference between the values of the areal growth for each group at the end of the 3rd week is statistically significant. There was an almost significant difference at the end of the 5th week, while at the end of the 2nd and 7th weeks the difference was not significant.

Homologous medium. Explants grown on homologous medium also showed a marked increase in areal growth during the early stage of cultivation, which for this

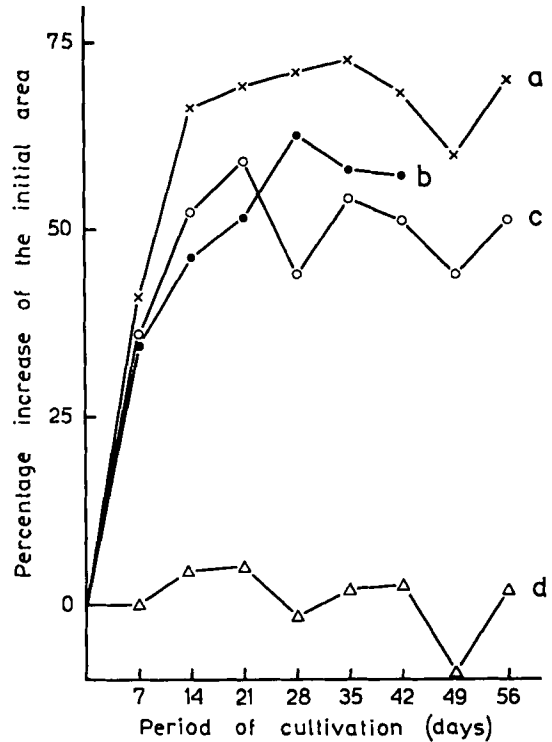


FIG. 5. Areal growth in cultures grown in homologous medium. (a) Epiphyseal series; fresh embryo extract and normal Tyrode's solution. (b) Epiphyseal series; fresh embryo extract and calcium-enriched Tyrode's solution. (c) Epiphyseal series; filtered embryo extract and normal Tyrode's solution. (d) Diaphyseal series; fresh embryo extract and normal Tyrode's solution.

group was prolonged up to about the end of the 2nd week, after which there followed a slower increase up to the 5th week. Thereafter, a decrease in the areal growth was recorded, the minimum being reached at the end of the 7th week. Between the 7th and 8th weeks of cultivation the rise was small. This evolution was rather similar in explants treated with normal or calcium-enriched Tyrode's solution and freshly prepared embryo extract (Figs. 5a and b). However, explants treated with normal Tyrode's solution and filtered extract (Fig. 5c) showed a progressive decrease in areal growth from as early as the beginning of the 3rd week of cultivation. In this case, too, minima were observed at the end of the 7th week. Here the areal growth curve was more irregular than in the other two cases. The statistical analysis of the results showed that between the values for the various groups a significant difference existed at the end of the 4th week of cultivation. At the end of the 2nd, 5th, 6th and 7th weeks the differences were almost significant; at the end of the 1st, 3rd and 8th weeks they were not significant.

Homologous and heterologous media. The last group consisted of cultures grown in the homologous medium for the first two weeks and subsequently in the heterologous medium. All the cultures were treated with calcium-enriched Tyrode's solution and freshly prepared embryo extract. This group exhibited the greatest areal growth.

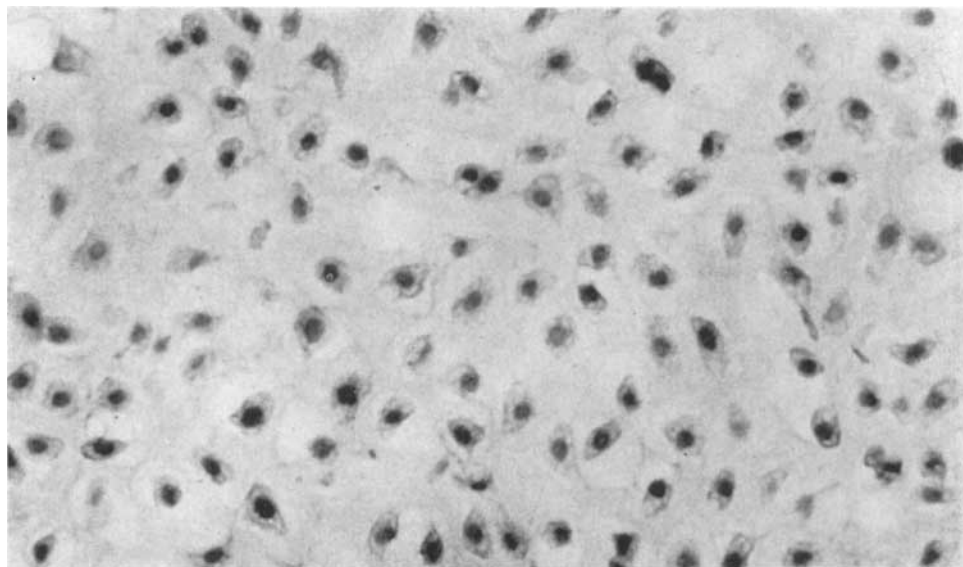


FIG. 6 a. Normal chondrocytes.

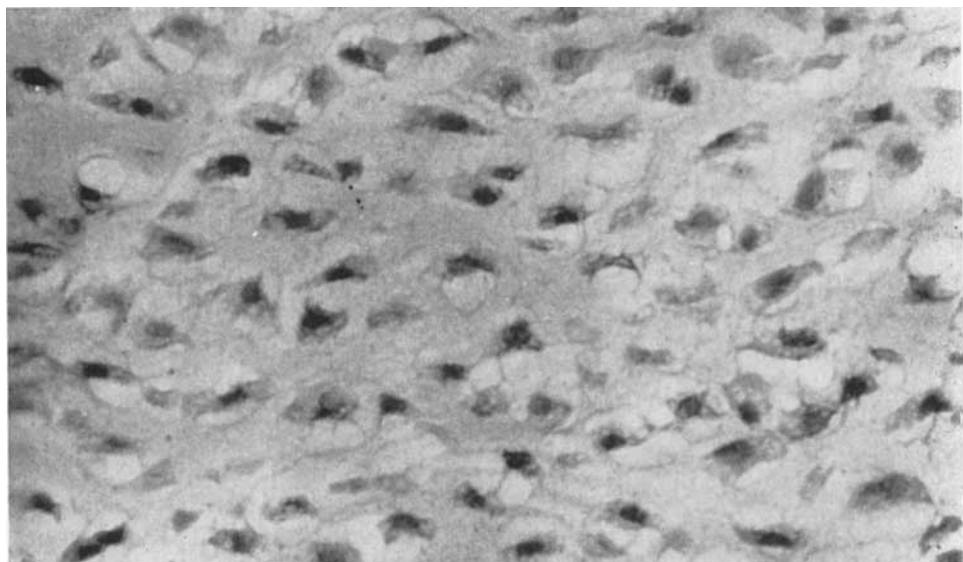


FIG. 6 b. Flattened chondrocytes.

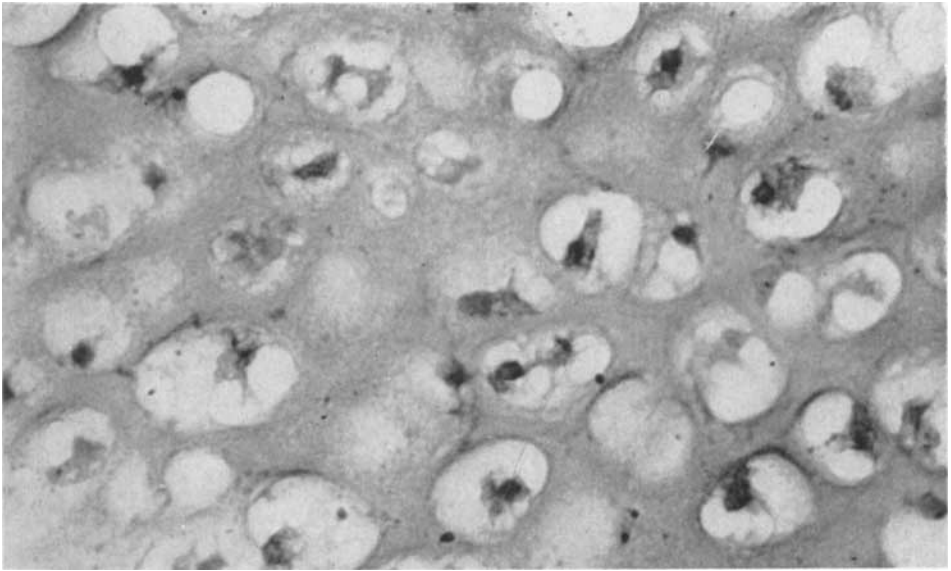


FIG. 6 c. Hypertrophied and degenerating chondrocytes.

FIG. 6. The 4 types of chondrocytes from the upper tibial epiphysis of a 12-day chick embryo. Haematoxylin-eosin. $\times 620$.

The curve rose steeply during the first 3 weeks and then fell slightly until the 5th week, when it began to rise again, the maximum value being attained at the end of the 6th week. There followed a gradual decrease to the end of the 9th week (Fig. 4 a).

The differences between the patterns of areal growth for the 4 main groups of cultures were highly significant throughout the period of observation. The individual patterns of areal growth of the cultures displayed marked differences. In some cases the maximum increase in the initial area was more than 125 per cent during the period of observation, while in others the increase was less pronounced.

Observations on sectioned material

In order to follow the development of morphologic changes, cultures at different stages of cultivation and control specimens were examined histologically.

CONTROL SPECIMENS

The study of the 37 controls (KA and KB) showed them to be composed of well differentiated cartilage tissue. Three main types of chondrocytes could be identified, these corresponding to the ones described by Fell.⁴⁴ At the upper—that is, the epiphyseal—pole of the specimen, flattened chondrocytes with small nuclei and dense matrix were visible, with their long axes approximately perpendicular to the

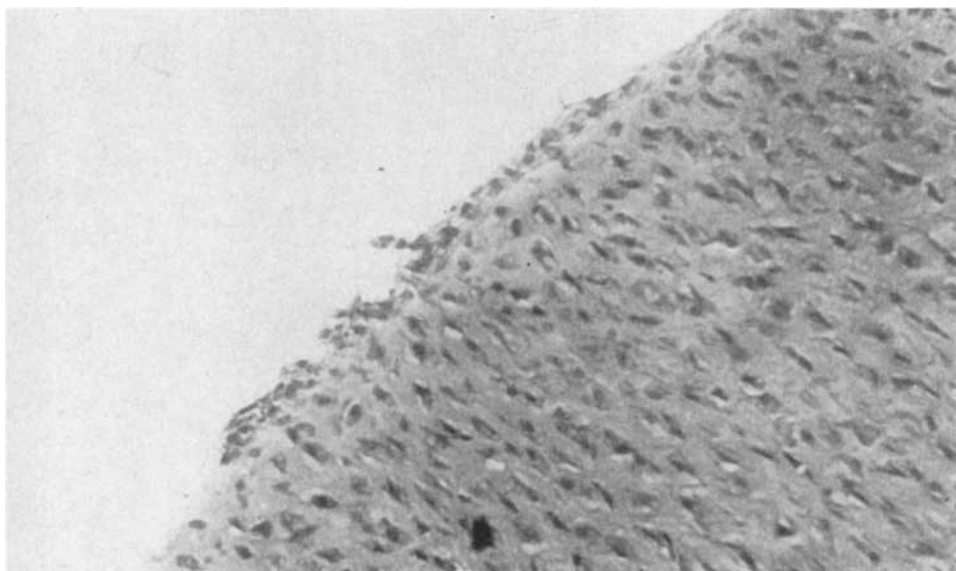


FIG. 7. Perichondral remnants adherent to the surface of control specimen KB₅. They are visible at the boundary of the region of flattened chondrocytes. Haematoxylin-eosin. $\times 290$.

long axis of the specimen (Fig. 6b). Immediately underneath, but with no sharp line of demarcation, these chondrocytes had hypertrophied. They had lost their compressed appearance and become spherical and vacuolated. Their nuclei were considerably enlarged and the amount of matrix had obviously decreased. The hypertrophied chondrocytes were gradually transformed into degenerating chondrocytes at the diaphyseal pole (Fig. 6c).

As a rule the specimens contained no small-celled articular cartilage, nor the part of the epiphysis composed of normal hyaline cartilage (Fig. 6a). These elements of the epiphysis adhering to the perichondrium were usually removed during dissection. In some cases, however, extremely rare areas of normal hyaline cartilage were visible at the epiphyseal pole. Under the microscope most of these controls (94.6 ± 3.72 per cent) were found to carry remnants of perichondrium (Figs. 7 and 8). In 12 specimens (32.4 ± 7.70 per cent) remnants of medullary tract were found, 9 of them empty and 3 containing medullary remnants and a few erythrocytes. A few red corpuscles were found in a further 3 cases, but here they were located on the surface of the specimen. No remnants of bony tissue could be identified in the controls. The perichondral remnants were in all the cases located on the surface of the specimens and near the epiphyseal pole, adjacent to the region of the flattened chondrocytes—sometimes so close that it was difficult to distinguish the regions of the perichondrium and flattened-celled cartilage. In this area the periosteum of the diaphysis merged with the perichondrium of the epiphysis, which is here usually very strongly attached

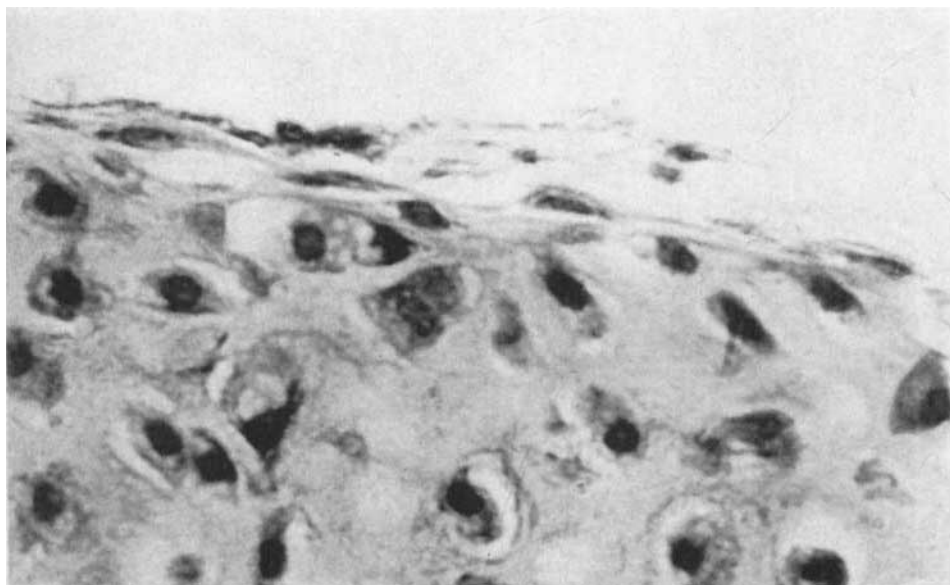


FIG. 8. Perichondral remnants in control specimen KB₆ under high magnification. Haematoxylin-eosin. $\times 960$.

to the cartilage.^{132, 44, 27, 126} In some specimens metachromatic staining was suspected in narrow regions usually located between cartilage and perichondral remnants. In no case, however, was such staining identified with certainty.

On the other hand in all the specimens sectioned for use as controls in the diaphyseal series (KC) a medullary tract containing marrow substance was present, while in one there was also a very small quantity of perichondral remnants. At their epiphyseal end these specimens were almost exclusively composed of hypertrophied cells, which were gradually transformed into degenerating chondrocytes. In the above case in which a small quantity of perichondral remnants was present flattened chondrocytes could also be detected.

HOMOLOGOUS MEDIUM

During the early stage of cultivation—at about 6 days—no essential morphologic changes in the composition of the cultures could usually be found. Thereafter, however, the following changes were observed. A few fibroblast-like, oblong cells with small nuclei, and bearing a close morphologic resemblance to wandering cells usually appeared. They were located at the periphery of the culture; and mostly on the long sides, with their long axes parallel to the surface of the culture. These cells were abundant in the intercondyloid fossa in the cultures from the lower tibial and lower femoral epiphyses. They also filled any gaps caused in the surface of the explants during dissection.

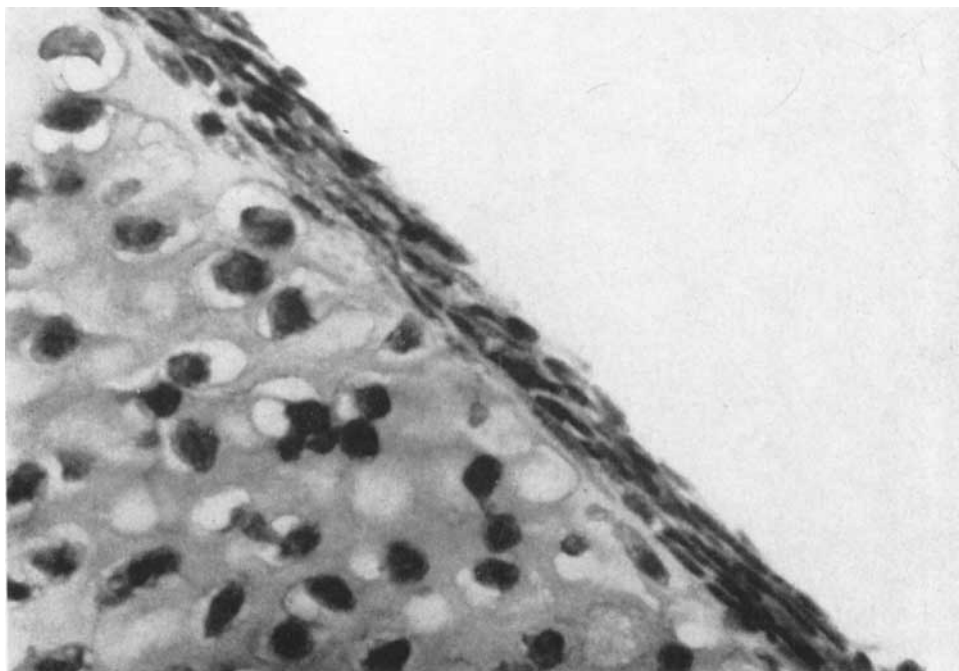


FIG. 9. Normal perichondrium of a phalanx from a 10-day chick embryo. Osteoblasts differentiating from the inner layer of the perichondrium are visible on the diaphyseal side (*upper left*), adjacent to hypertrophied chondrocytes. Haematoxylin-eosin. $\times 620$.

The number of these cells gradually increased so that after long cultivation a layer had formed that had the morphologic properties of fibrous tissue. At first this layer was usually located at places on the long sides of the cultures, and later extended towards the ends of the epiphysis and diaphysis. In many cases it covered the surface of the epiphysis but rarely that of the diaphysis. At about the end of the 3rd week of cultivation a thick membrane of dense fibrous tissue had formed in a high percentage of the cultures; this enclosed the whole culture like a capsule, which showed a remarkable morphologic similarity to the perichondrium of normal bone (Fig. 9).

At first this perichondral capsule had a homogeneous structure, being composed of fibroblast-like cells with elongated nuclei and little intercellular substance. However, as the capsule developed further, two layers of different morphologic structure were formed, the outer one resembling the original capsule, which itself was similar to embryonic perichondrium, while the inner layer contained more rounded cells with nuclei larger than those of the cells of the outer layer, and resembling osteoblasts (Figs. 10 and 11). The inner layer of the capsule was found to be similar to the osteoblastic layer of the periosteum, while the outer layer had characteristics of the fibroblastic coat. There was no sharp demarcation of the two layers.

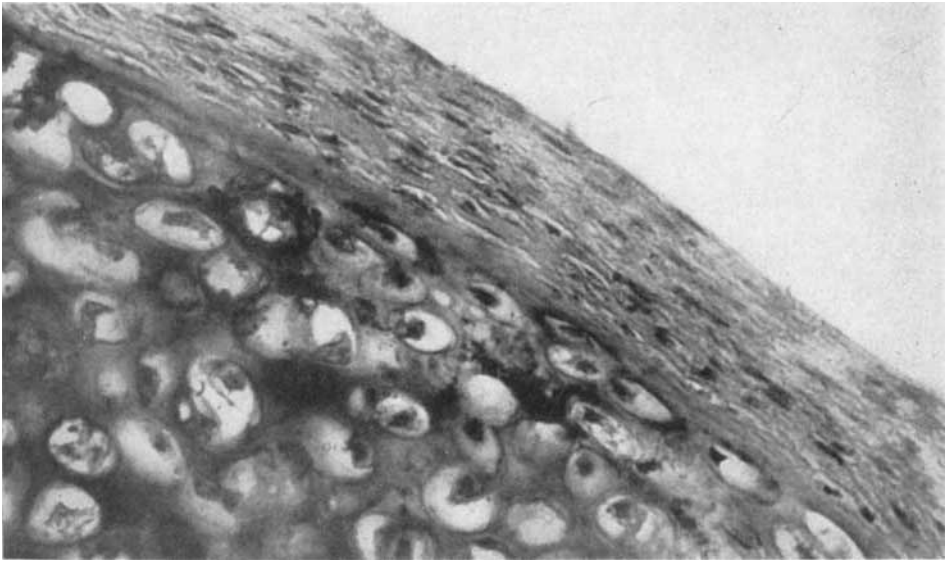


FIG. 10. Culture 162*e* grown for 56 days in homologous medium with fresh embryo extract and normal Tyrode's solution. The two layers of the perichondral capsule are clearly discernible. Note the marginal structureless zone between the perichondral capsule and the cartilage. Haematoxylin-eosin. $\times 480$.

Almost simultaneously with the formation of the capsule, metachromatic haematoxylin-eosin staining of the cartilage in contact with it produced a light to dark rose colour along a narrow marginal zone—usually on the long sides of the explant, more seldom at the epiphyseal pole, and almost never at the diaphyseal pole. In 24-hour fixed cultures of cartilaginous extremities of the long bones of 8–8½ day chick embryos the cartilage matrix is reported as displaying an intense metachromatic purplish coloration, except near the cut surface, which was colourless.⁴⁵ In the sectioned material of the present study, however, metachromatic staining could be observed in a few cases very early, but in the majority of the cases it did not appear until after the second week of cultivation. Metachromatic staining was almost always observed where the formation of the capsule had started or was fully developed.

As soon the formation of the perichondral capsule was complete and its two layers had become discernible a narrow structureless zone appeared at places on the border between the marginal chondrocytes and the inner layer of the capsule (Fig. 10). This zone displayed intense metachromatic staining. Subsequently a tissue of new morphologic appearance developed and filled this region. This tissue was thus located between the marginal chondrocytes of the culture and the osteoblastic layer of the perichondral capsule; it was always found on the long sides of the cultures, but not

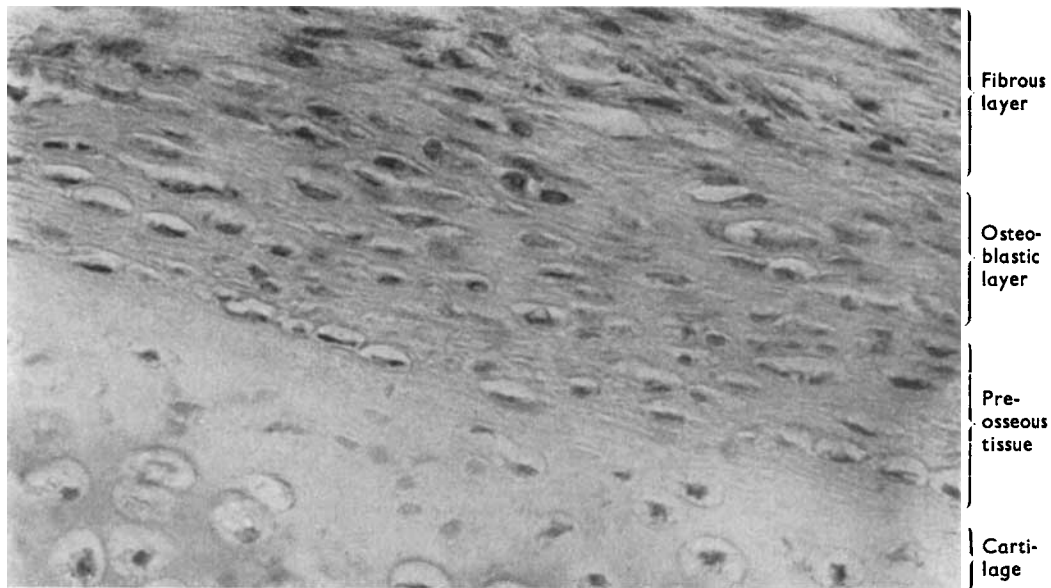


FIG. 11. Culture 30*m* grown for 40 days in homologous medium with fresh embryo extract and normal Tyrode's solution. The two layers of the perichondral capsule, the pre-osseous tissue and the cartilage are visible. Haematoxylin-eosin. $\times 480$.

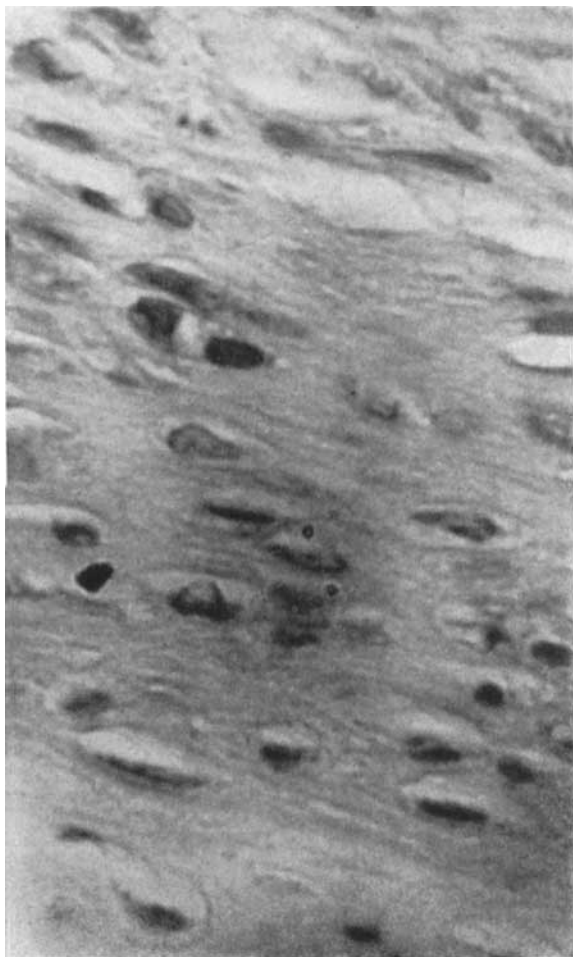
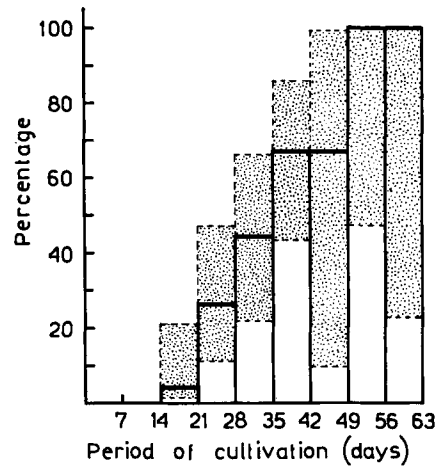


FIG. 12. Same culture as Fig. 11. The pre-osseous tissue seen under high magnification. Note the fibrillar structure of the intervertebral substance. $\times 960$.

Fig. 13. The percentage frequency of pre-osseous tissue at the various stages of cultivation in sectioned cultures grown in homologous medium with fresh embryo extract and normal Tyrode's solution. Shaded areas denote the confidence intervals.



necessarily adjacent to flattened chondrocytes. The cells of the newly formed tissue were slightly rounder than those of the inner layer of the perichondral capsule, and contained a more or less flattened nucleus. There was also an abundant, dense intercellular substance, displaying intense metachromatic staining, and at later stages of cultivation showing a fibrillar structure (Figs. 11 and 12). The cells of the new tissue merged imperceptibly with those of the inner layer of the capsule, and had morphologic properties of osteoblasts. This tissue was recognized as pre-osseous tissue, the chronologic development of which is illustrated in Fig. 13.

While the above changes were taking place, the chondrocytes of the explants displayed no evident morphologic changes. In some cases rupture of the cell capsules and formation of small cavities containing cell debris were observed in the region of degenerating chondrocytes and usually at the diaphyseal pole. In these areas there was often a deposition of calcium salts. Otherwise, the chondrocytes of the cultures appeared quite healthy even after long periods of growth.

However, it was evident that in cultures in which there was development of a capsule and formation of pre-osseous tissue the flattened chondrocytes hypertrophied throughout the period of cultivation, a fact supported by the presence in older such cultures of hypertrophied and degenerating chondrocytes, to the virtual exclusion of flattened chondrocytes.

The chronologic evolution of these changes could not be defined with any degree of accuracy as they displayed fairly large variations from one culture to another and as the various processes developed more or less simultaneously in different parts of the same culture. However, it is of interest to note that most of the sectioned cultures in which pre-osseous tissue had developed had grown for more than 28 days (Fig. 13). Once the pre-osseous tissue had been formed, no other morphologic changes

TABLE V. *Results of microradiographic examination of calcium-salt deposit.*

Culture no.	Culture variant group	Period of cultivation (days)	Formation of capsule	Pre-osseous tissue	Deposition of calcium
28 o	I	48	+	+	-
30 p		30	+	+	-
349 d		49	-	-	±
349 e		49	+	+	-
358 d		42	-	-	-
358 e		42	+	+	-
31 j	III	21	+	+	-
32 c		39	+	+	-
32 f		39	+	+	±
33 b		39	+	±	-
111 d	II	87	-	-	±
118 m		47	+	-	-
118 o		80	+	±	-
119 m		80	+	-	-
120 a		41	+	±	-
120 f		47	-	-	-
50 a	V	6	-	-	+
80 k	VI	49	-	-	-
80 m		49	-	-	+
81 b		49	+	-	+
85 s		35	-	-	+
86 b		35	-	-	+
87 f		35	-	-	+
57 e	VII	25	-	-	+
59 e		20	-	-	+
60 f		40	-	-	+
61 m		50	-	-	+
65 r		49	-	-	+
68 j		60	-	-	+
69 f		60	-	-	+
71 f		15	-	-	+
72 a		< 70	-	-	+
74 g		15	-	-	+

could be found in the cultures, even after cultivation for longer periods. Nor were there signs of a further increase in the amount of the pre-osseous tissue beyond a certain stage of cultivation.

In some cases calcification of the cultures could be identified by microradiography, and by alizarin red and von Kossa's staining. Altogether 12 cultures of this group

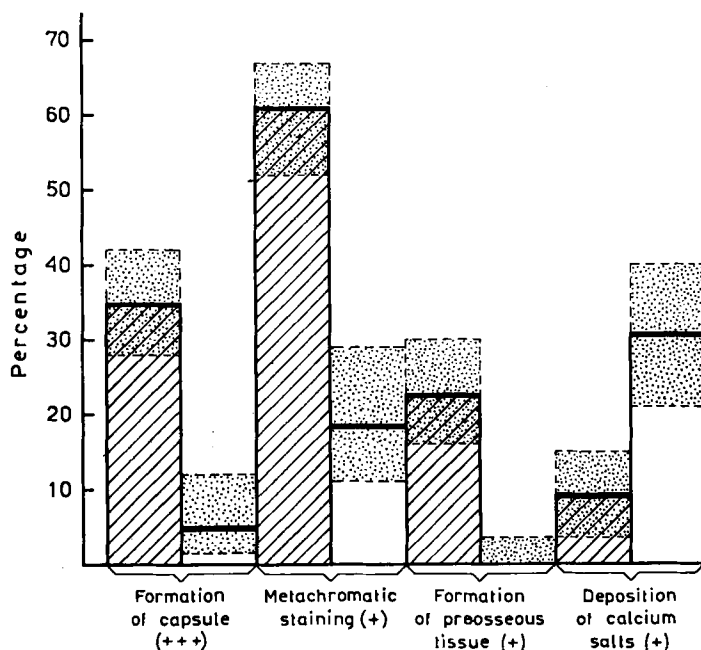


FIG. 14. The percentage frequency of the various morphologic changes in sectioned cultures grown in homologous medium (hatched columns) or in heterologous medium (unhatched columns). Shaded areas denote the confidence intervals.

were studied by microradiography; the results are presented in Table V. The deposition of the calcium salts was almost exclusively located in the region of the degenerating chondrocytes at the diaphyseal pole of the culture. This was especially the case where there was extensive degeneration of chondrocytes, with destruction of the structure of the cartilage and formation of cavities. Calcification was as a rule restricted to the nuclei and the protoplasm of the cells, but there was also evidence of calcium deposition in the intercellular substance. In no case was there any sign of calcification of the perichondral capsule or mineralization of the pre-osseous tissue.

Table VI presents a detailed analysis of the sectioned material with respect to the period of cultivation, the culture medium used in each group and the principal observations.

The values relating to the morphologic changes in the cultures grown on homologous and heterologous media may be compared in Fig. 14. However, these values cannot be regarded as representative owing to the influence of various factors associated with the material. The real values must be put considerably higher since, for instance, 10 of the sectioned cultures of this group were cultivated for less than 7 days, whereas the formation of the capsule usually

TABLE VI. *Analysis of sectioned material and*

Series	Culture variant group	Period of cultivation (days)											Total
		0-1	8-14	15-21	22-28	29-35	36-42	43-49	50-56	57-63	64-70	< 70	
XVI, 1-30	I	10	19	11	11	6	4	1	1	—	—	—	63
31-33	III	—	—	4	1	5	4	—	—	—	—	—	14
39-45	IV	3	10	5	3	—	1	—	—	—	—	—	22
46-53	V	—	—	2	1	5	—	5	—	—	2	—	15
54-77	VII	—	3	3	—	3	—	—	—	—	—	6	15
78-102	VI	3	10	6	2	3	2	3	—	—	—	—	29
103-133	II	—	—	2	3	4	11	9	1	—	—	9	39
134-423	I	—	1	10	15	10	13	2	3	2	—	—	50
Diaphyseal series	I	—	6	4	4	3	4	3	1	—	—	—	25
TOTAL		16	49	47	40	39	39	23	6	2	2	15	278

did not commence until after this period. However, in some cultures there was evidence of limited formation of the capsule as early as the 4th day. The same factor of unreliability pertains to the metachromatic staining, the formation of pre-osseous tissue and deposition of calcium salts, so that the values in the figure must be considered as unrepresentative.

The observations on sectioned cultures grown in homologous medium may be summarized as follows. After an initial period—usually the first week of cultivation—during which no essential morphologic changes could be observed, certain cells bearing a morphologic resemblance to wandering cells appeared at the surface of the cultures. By intense proliferation these cells soon formed a perichondral capsule, which enclosed the culture. Later on the capsule developed as two layers. Inter-cellular substance was evidently produced by the cells of the inner layer of the perichondral capsule and a new tissue was formed that exhibited close morphologic similarity to immature bone. The newly formed pre-osseous tissue originated from the cells of the inner layer of the perichondral capsule, which thus functioned as a periosteum.

In general the main changes observed in cultures grown in homologous medium were (a) an increase in the initial area of the explants, (b) the presence of wandering cells, with consequent formation of a perichondral capsule around the cultures and the differentiation of osteoblasts from its inner layer; and (c) production of intercellular substance by the osteoblasts and formation of pre-osseous tissue. There were no further changes even after prolonged cultivation.

principal microscopic observations.

Migratory activity of the cells					Formation of capsule					Metachromatic staining			Pre-osseous tissue			Deposition of calcium		
-	±	+	++	+++	-	±	+	++	+++	-	±	+	-	±	+	-	±	+
13	1	9	17	23	4	9	14	16	20	23	19	21	42	10	11			
—	—	—	1	13	—	1	1	2	10	1	2	11	3	3	8			
					—	8	7	6	1	22	—	—	22	—	—	22	—	—
					1	2	3	8	1	5	9	1	13	2	—	10	2	3
					2	7	1	3	2	9	—	6	13	2	—	7	2	6
					5	12	9	3	—	20	1	8	28	—	—	24	—	5
15	5	5	13	1	5	12	5	12	5	1	7	31	32	7	—	34	4	1
—	8	8	10	30	4	5	9	14	24	5	9	42	18	18	20	31	18	7
19	—	2	3	1	22	1	—	1	1	16	5	4	24	—	1	24	—	1
47	14	24	44	68	43	57	49	65	64	102	52	124	196	42	40	196	26	23

HETEROLOGOUS MEDIUM

In sectioned cultures grown in heterologous medium changes could be observed that resembled those occurring in cultures grown in homologous medium. In the former group of cultures, however, the evolution of the morphologic changes was in many respects different. The perichondral capsule could be recognized at almost the same stage of cultivation in both groups. In heterologous medium development of the capsule progressed more slowly, and complete encapsulation of the culture was achieved less frequently. There were, however, no morphologic differences in the structure of the perichondral capsule between the two groups. In almost no case did the capsule display a tendency to develop as two layers. Metachromatic staining was also observed in cultures grown in heterologous medium, but here, too, it occurred in fewer cases, and as a rule appeared later. The metachromatic staining in this group of explants was characterized by a lighter colouring—light rose—and involved almost only the perichondral capsule.

Extensive degenerative changes of the cartilage tissue of the cultures were in some cases observed to result in the formation of central cavities which later were filled with a fibrous-like tissue, the cells of which bore a morphologic similarity to wandering cells.

The structureless marginal zone observed in cultures grown in homologous medium could not be found here, nor could the formation of pre-osseous tissue be detected. However, there was evidence of small narrow areas of such tissue beneath the perichondral capsule.

The deposition of calcium salts in cultures grown in heterologous medium was studied by microradiography and staining with alizarin, 17 cultures of this group

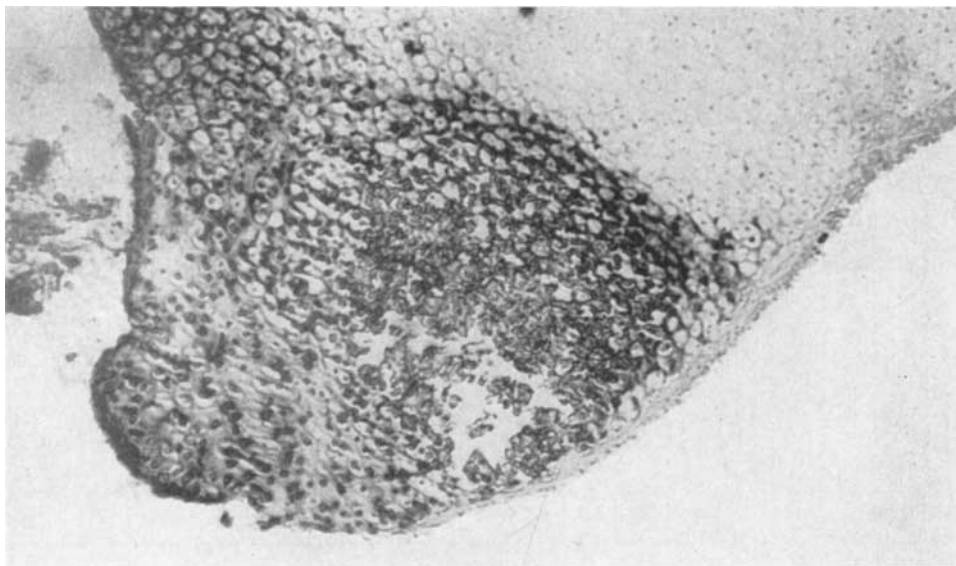


FIG. 15. Deposition of calcium salts in culture 50*p* grown for 70 days in heterologous medium with fresh embryo extract and calcium-enriched Tyrode's solution. Note the cavity filled with debris of degenerated chondrocytes (*lower centre*). No perichondral capsule or pre-osseous tissue was formed in this culture. Alizarin. $\times 90$.

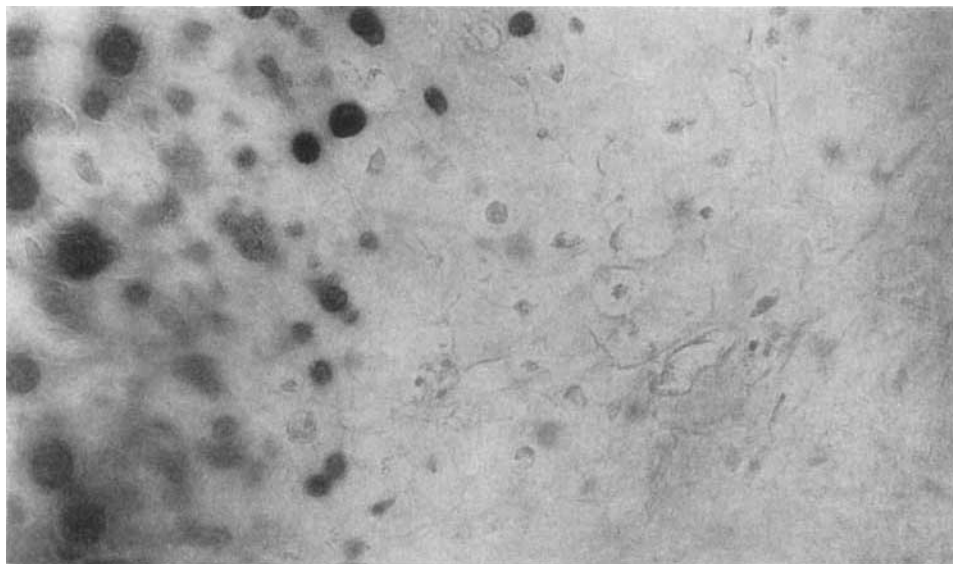


FIG. 16. Deposition of calcium salts in culture 69*e* grown for 71 days, first in homologous medium for 2 weeks and thereafter in heterologous medium. Filtered embryo extract and calcium-enriched Tyrode's solution were used throughout. Note the highly calcified cells. The intercellular substance is not calcified. Alizarin. $\times 280$.

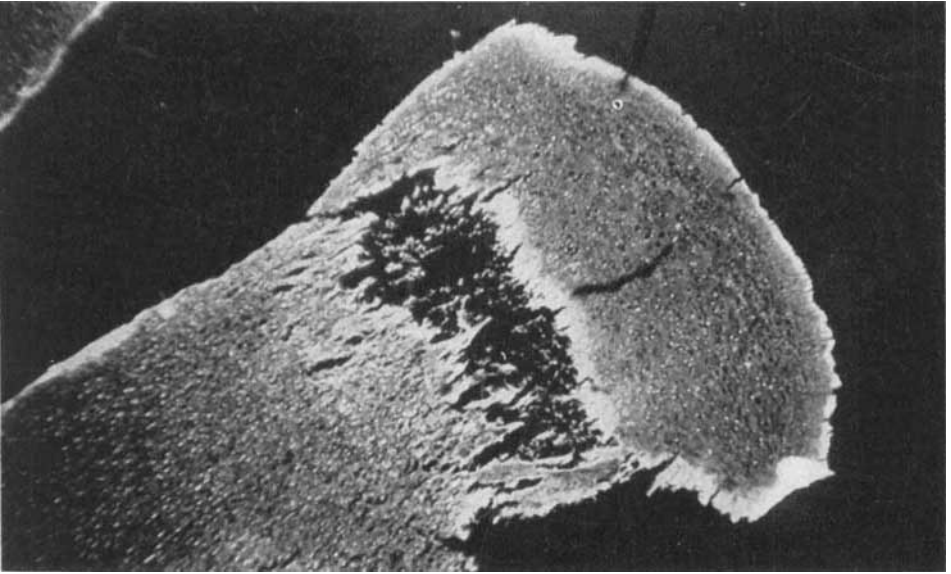


FIG. 17. Microradiogram of culture 50a grown for 6 days in heterologous medium with fresh embryo extract and calcium-enriched Tyrode's solution. Degeneration has resulted in the formation of a central cavity. $\times 40$.

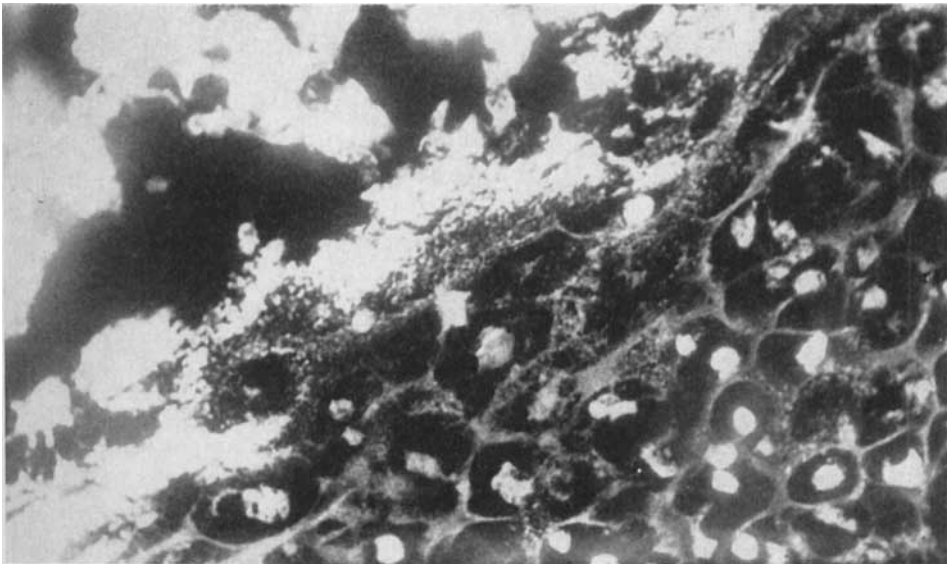


FIG. 18. Microradiogram of culture 69f grown for 60 days, first in homologous medium for 2 weeks and thereafter in heterologous medium. Filtered embryo extract and calcium-enriched Tyrode's solution were used throughout. Extensive degeneration has resulted in the formation of a cavity filled with highly calcified tissue debris (*upper left*). $\times 480$.

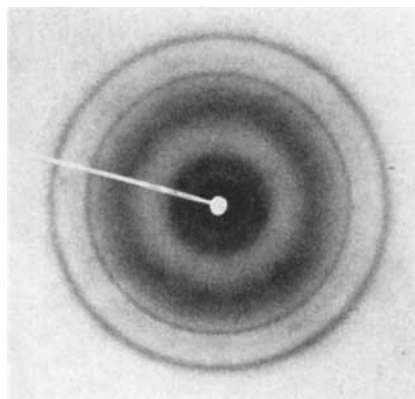


FIG. 19. X-ray diffraction pattern of culture 61 *m* grown for 50 days, first in homologous medium for 2 weeks and thereafter in heterologous medium. Filtered embryo extract and calcium-enriched Tyrode's solution were used throughout.

being examined by the former method (Table V). In some cases the methods disclosed early signs of calcification but generally there was no such indication until about the 30th day of cultivation.

The salt deposit was always diffuse and usually located in a central region of the diaphyseal pole of the culture, where extensive degenerative changes of the cartilage and formation of cavities could be seen (Figs. 15–18). In cases of early calcification, deposition of calcium salts was first located in the nuclei of the chondrocytes, but at later periods of cultivation the intercellular substance, too, displayed intensive calcification, this involving large areas of the older cultures.

In order to study the composition of the calcium salt deposits the X-ray diffraction pattern was recorded for one culture (no. 61 *m*) grown in homologous and heterologous media for 50 days. A few diffuse diffraction rings were observed which were probably due to the organic substance of the culture. Moreover, there were numerous rings characteristic of hydroxyapatite, $(\text{Ca}_{10}\text{PO}_4)_6 \cdot (\text{OH})_2$. Apatite was found in fairly large quantities, the crystals being the same size as in normal bone, but they were not oriented (Fig. 19).

The values relating to the morphologic changes observed in this group are given in Fig. 14. As in the case of cultures grown in homologous medium, these values, too, cannot be regarded as representative.

The observations on sectioned cultures grown in heterologous medium may be summarized as follows. As in the case of the homologous medium, no essential morphologic changes could be observed until the formation of the perichondral capsule was begun. Practically no osteogenetic layer developed in the capsule, nor was any pre-osseous tissue formed. Degenerative changes of the cartilage could be detected in some cases, and calcium deposition occurred in a higher percentage of these cultures than of those grown in homologous medium.

DIAPHYSEAL SERIES

The study of the 25 sectioned cultures of the diaphyseal series revealed one case of a fully developed perichondral capsule, one in which the capsule was moderately developed, and a third in which it had just begun to form. In the first 2 cases flattened cells could be identified, and in the second of these cases a thin layer of pre-osseous tissue was found below the perichondral capsule. Metachromatic staining was observed in 2 cases in addition to the 3 above-mentioned, and in another 5 cases it was suspected. In one case there was a positive calcium reaction to both alizarin and von Kossa's stain, but this was ascribed to a small remnant of periosteal bone. In the remaining cases the sectioned cultures were found to be composed of hypertrophied and degenerated chondrocytes, with none of the above, or other, changes. Remnants of medullary tracts were present in all but one of the sectioned explants of this group. In 6 cases the medullary tract was empty, while in the rest it contained medullary rests.

Practically no morphologic changes were evident in the sectioned explants of the diaphyseal series.

INFLUENCE OF CULTURE VARIANTS

An analysis of the results of the study of the sectioned material with respect to the type of medium used in each group suggested the following conclusions.

Media. A distinct difference existed between the results obtained from cultures grown in the homologous and those grown in the heterologous medium. The perichondral capsule formed earlier and its development was more marked in the former group than in the latter. The same difference was evident in respect of the metachromatic staining. No pre-osseous tissue at all was found in sectioned cultures grown on the heterologous media, while there was clear evidence of this tissue in 22.1 ± 3.16 per cent of the cultures grown in the homologous media. In contrast the deposition of calcium salts appeared earlier and developed more steadily in cultures grown in the heterologous media.

Tyrode's solution. Owing to the marked disparity in the size of the homologous and heterologous groups treated with calcium-enriched Tyrode's solution, it is difficult to ascertain whether the addition of calcium to the solution was a significant factor in the occurrence and development of the morphologic changes.

Embryo extract. Out of the 81 sectioned cultures grown exclusively in the heterologous medium, 37 were treated with fresh embryo extract while the remainder were treated with filtered extract.

No essential differences between these two groups could be found. However, a significant difference was observed in respect of the 172 cultures grown exclusively in the homologous medium, of which 39 were treated with filtered and the remainder with freshly prepared extract. It was found that those treated with filtered extract developed no pre-osseous tissue at all, whereas 29.3 ± 3.95 per cent of those cultures

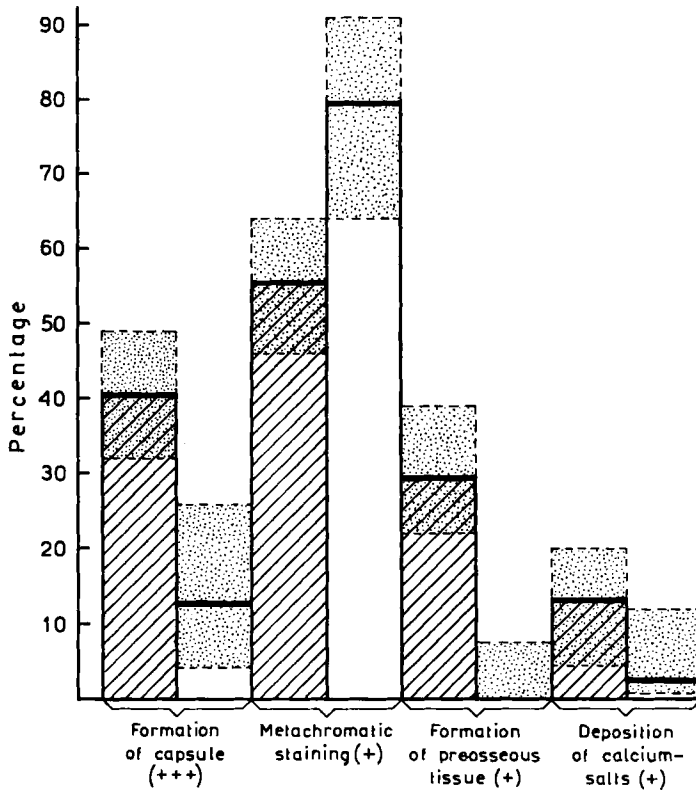


FIG. 20. The percentage frequency of the various morphologic changes in sectioned cultures grown in homologous medium either with fresh embryo extract (hatched columns) or with filtered embryo extract (unhatched columns). Shaded areas denote the confidence intervals.

treated with fresh extract did display evidence of such tissue. The development of the perichondral capsule and the deposition of calcium salts were also more pronounced in cultures treated with fresh extract. The metachromatic staining, however, was better in cultures treated with filtered extract. As the number of cultures grown in each medium variant was small only major differences in the observed morphologic changes have been discussed.

The values relating to the morphologic changes observed in cultures grown in homologous medium with either fresh or filtered embryo extract may be compared in Fig. 20.

To summarize, there was evidence that cultures grown in the homologous medium and treated with normal Tyrode's solution and freshly prepared extract showed a higher degree of morphologic differentiation and better formation of pre-osseous tissue than cultures grown on the same medium but treated with filtered embryo extract. Cultures of the latter group failed completely to form pre-osseous tissue. In

either medium, the morphologic changes described above could be observed only where there was a definite formation of perichondral capsule. This feature and the migratory activity of the cells were always coincident, in so far as they were observed in cultures in solid media. Thus, irrespective of the period of cultivation, cultures displaying no migratory activity of the cells usually also failed to form a capsule and presented no morphologic changes other than degenerative ones, and, in some cases, deposition of calcium salts.

DISCUSSION

Macroscopic observations

Shape. The first macroscopic changes observed on the cultures consisted in a thinning and flattening of the mass, an increase in the initial area and a smoothing of the contours. These changes have been interpreted partly as a manifestation of growth and partly as a re-arrangement of the cells of the colony so as to improve the nutritional conditions in the new environment. The spreading of a tissue culture is a characteristic of explanted material of various origins.¹⁶²

Curving of the longer explants is a macroscopic change the nature of which could not be studied with the methods used. However, it would seem that biomechanical factors are involved, the real nature of which might be disclosed by study of the ultrastructure.

Colour and consistency. The changes in the consistency and the colour of the cultures seem to be closely related to the formation and development of the perichondral membrane. The encapsulation of the cultures by this membrane probably results in the loss of their initial colour and translucency. When no capsule was formed this property remained unchanged throughout the period of cultivation. In the white areas that appeared on the surface of some cultures—usually at their diaphyseal pole—histologic examination demonstrated the presence of calcium deposits.

Migratory activity of the cells

The migratory cell activity was a fairly early characteristic of the cultures. It is known from earlier investigations^{21, 123, 161} that when a fragment of tissue is cultured, cells make their way outwards to the medium. Teleologically, this phenomenon can be considered as resulting in the anchoring of the explants to the supporting medium. However, this migration is an extremely complex process, the real cause of which is still obscure. According to Willmer,¹⁶² when migrating outwards to the medium the cells of a colony are moving to areas of higher oxygen tension, lower carbon dioxide tension and weaker concentration of waste products. A different mechanism of the migratory activity observed in cultures of various origins has been suggested by

Gustafson,* who tentatively ascribes the behaviour of cells in colonies to the frequency of the cell contacts. Cell contacts are, of course, rarer at the periphery than at the centre of the colony, where the cells are closely packed. A diffusion of cells into the medium may occur, therefore, at the periphery of the cell colony. In this and previous investigations^{21, 45} the migratory cell activity was observed later in the case of cartilage than of other tissue. This observation would appear to contradict the findings of Willmer and Gustafson. It is difficult to interpret the wide difference in behaviour of chondrocytes and cells of other tissues.

The wandering cells have been described as embryonic in type and more or less histologically undifferentiated. Such cells are usually called fibroblasts, because they are similar in appearance to the cells in the body that are believed to function in this manner.¹⁶²

Early in this study it was realized that the interpretation of the observed morphologic changes is intimately related to the problem of the origin of the wandering cells. It has been stated earlier that the encapsulation of tissue cultures is a prerequisite of differentiation,⁵⁶ and in the present study the wandering cells were found to be responsible for the formation of the fibrous capsule. Moreover, the development of the changes observed in the cultures and interpreted as the formation of pre-osseous tissue was evidently closely related to the development of the capsule.

Two hypotheses on the origin of the wandering cells may be advanced. (i) They are derived from the perichondral residues in the sectioned specimens, and then the osteogenetic activity of the cultures must be regarded as having the same origin. (ii) The wandering cells originate from certain types of chondrocytes which would thus be directly or indirectly responsible for the osteogenetic activity displayed by the explanted epiphyseal cartilage. In this case the perichondral remnants play no part in the development of the wandering cells.

The former hypothesis is supported by the results obtained by Fell,⁴⁵ who in similar experiments observed that the perichondrium, which remained adherent, gave rise to a broad zone of outgrowth consisting of fibroblasts and numerous highly amoeboid wandering cells. Further support for this hypothesis is found in the fact that perichondral remnants were present in a high percentage of the sectioned controls (94.6 ± 3.72). Accordingly, it must be these perichondral remnants that, by proliferation, enclose the culture, and lead to the formation of a perichondral capsule, which in turn results in the development of pre-osseous tissue. However, if the perichondral remnants are to be considered as responsible for the formation of the perichondral capsule the question arises why these remnants do not form mature bone. Using the hanging-drop technique with homologous plasma and embryo extract as nutrient medium, Fell found that bone readily developed from the rudimentary perichondrium isolated from the cartilaginous limb skeleton of 6-day embryos.^{48, 49}

* Personal communication.

The second hypothesis on the origin of the wandering cells—that they are derived from chondrocytes—implies that the changes observed in the cultures develop according to the following scheme. Immediately the explant is placed in tissue culture there is an intense kinetic activity of the cells, which is probably accompanied by proliferation. Both these activities result in an initial rapid growth in the area of the cultures. Almost simultaneously the perichondral remnants—small pieces of tissue that have been exposed to severe mechanical damage and dried for several seconds during the dissection—die, are detached and fall on the medium. Such cells and cell débris are usually found on the clot-bed beneath the explants during the first subculture. After this phase, which usually lasts for about one week, some groups of chondrocytes in the culture display activity. As has been described above, cultures composed exclusively of hypertrophied and degenerated chondrocytes—the diaphyseal series—failed to display any migratory activity of the cells. On the other hand, it was found that a migratory activity was characteristic of cultures composed of these two groups of cells together with flattened chondrocytes. It may be inferred, then, that it was the flattened chondrocytes that became the wandering cells observed in the cultures.

By way of explanation of the transformation of flattened chondrocytes into wandering cells two mechanisms are suggested:

(i) The peripheral flattened chondrocytes modulate to an indifferent type of fibroblast-like cell, and make their way out into the medium. A similar dedifferentiation has been observed by Fell,⁵⁰ who pointed out that highly differentiated skeletogenous cells—osteoblasts—can dedifferentiate into an indifferent type of cell that is capable of further differentiation into osteoblasts or chondroblasts, according to the environmental conditions. Modulation as a biologic process is also accepted by Chévremont,²⁶ who states that when a tissue fragment is cultivated in a medium rich in growth-promoting substances the cells of the peripheral zone often lack their normal properties. However, when the proliferation decreases or is curtailed the cells again assume their normal function, although some of their morphologic characteristics are modified.

(ii) According to the second mechanism the flattened cells described by Fell⁴⁴ as a type of chondrocyte may be assumed to be mesenchymal cells at a low stage of differentiation. Determination of these cells may occur at later stages in the skeletal development. The morphologic appearance of the cells was found to be quite different from that of mature chondrocytes (Figs. 6 a and 6 b). These cells might provide mature chondrocytes by further differentiation and contribute to the formation or the growth of the perichondrium. A special mechanism of osteogenesis taking place in the region of the epiphysis—termed by Ranvier “la zone de l’encoche d’ossification”—by a direct transformation of chondrocytes into osteoblasts has been reported by several workers.^{132, 44, 27, 126, 86}

The further evolution is characterized by a period during which the wandering

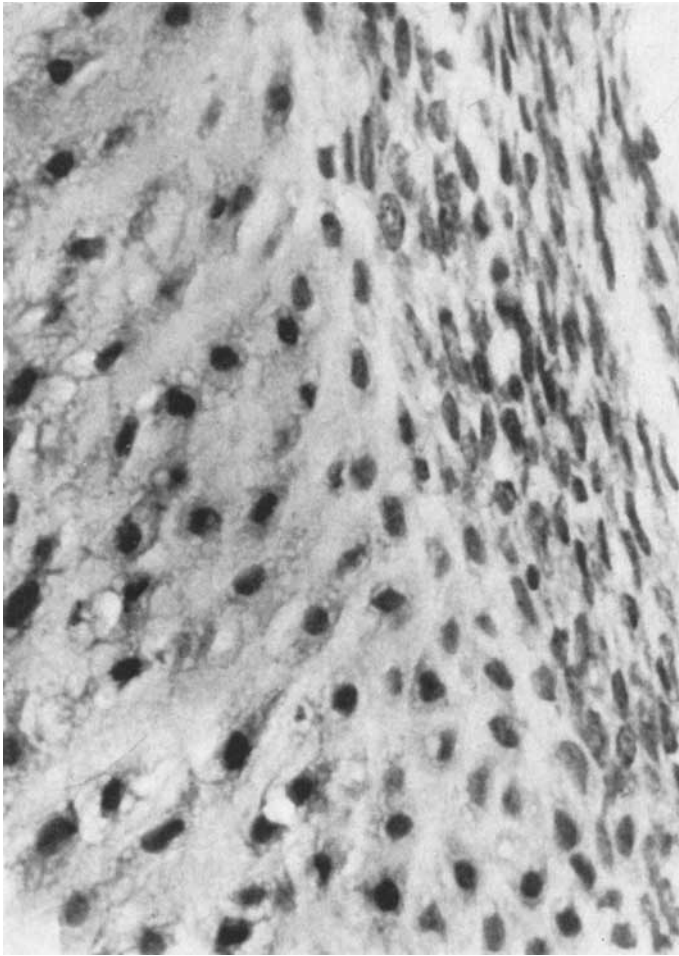


FIG. 21. Section through upper tibial epiphysis of a 10-day chick embryo. Chondrocytes are seen merging with perichondrium cells. Haematoxylin-eosin. $\times 790$.

cells proliferate and form a capsule, which encloses the culture and consequently functions as a perichondrium. The cells of the inner layer of the perichondral capsule so formed differentiate into osteoblasts, which in turn form pre-osseous tissue.

Much evidence was found in support of this hypothesis. The stage at which the wandering cells were first observed is consistent with this course of events. It is difficult to explain why the proliferating cells of the perichondral remnants do not migrate into the medium earlier and in a higher percentage of cultures than was found in this study. It has been proved earlier that cells in cultures of periosteum exhibit an intense migratory tendency as early as at the end of the 1st day of cultiva-

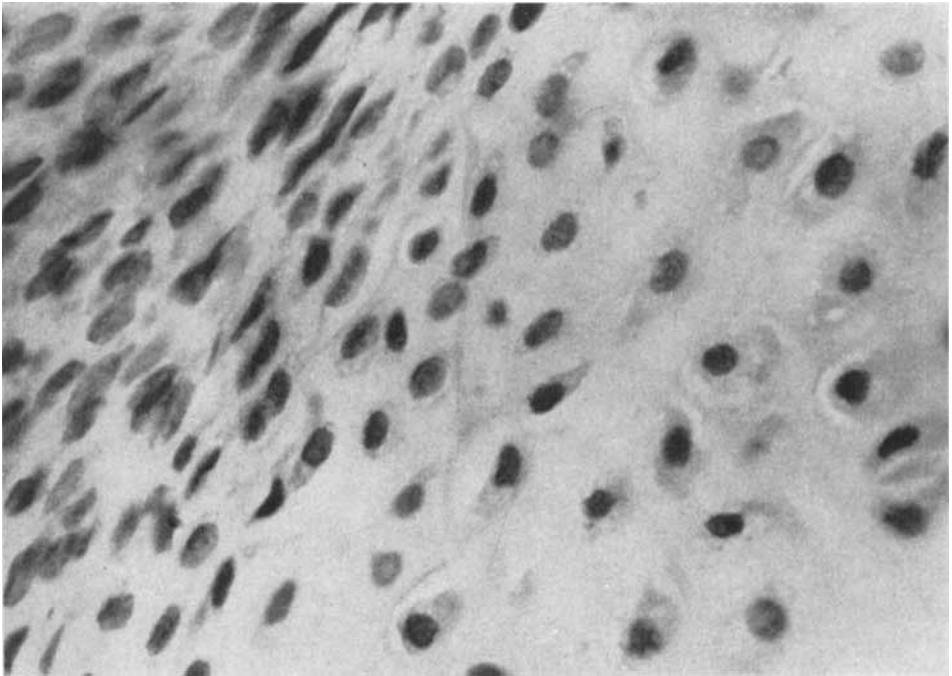


FIG. 22. Section through lower femoral epiphysis of a 2-day-old rabbit. Chondrocytes are seen merging with perichondrium cells. Haematoxylin-eosin. $\times 790$.

tion,⁴⁹ and that perichondral remnants adherent to cartilaginous explants give rise to a broad zone of outgrowth after 48 hours.⁴⁵

Further support for this hypothesis is the fact that in some sectioned controls it was difficult to distinguish flattened chondrocytes from neighbouring perichondral cells (Figs. 21 and 22). Moreover, clear morphologic evidence was found of the transformation of chondrocytes into wandering cells, and a series of intermediate cell types could be observed (Fig. 23).

If the wandering cells derive from chondrocytes, then the flattened cells must be regarded as having the capacity under certain conditions of being transformed into osteoblasts, and hence of forming immature bone. Wilton strongly refutes this possibility. He states categorically that after certain embryonal tissue has been induced (determined)* to an enchondral part, the development of cartilage and bone takes place along previously determined paths, independently of each other.^{163, 164} He thus supports the view that ossification cartilage is a unipotential tissue and maintains

* Determination (cell determination) according to Wilton is the transformation of an "omnipotent" cell (a cell which can give rise to different tissues) to a "unipotent" one (a cell which can give rise to only one kind of tissue).

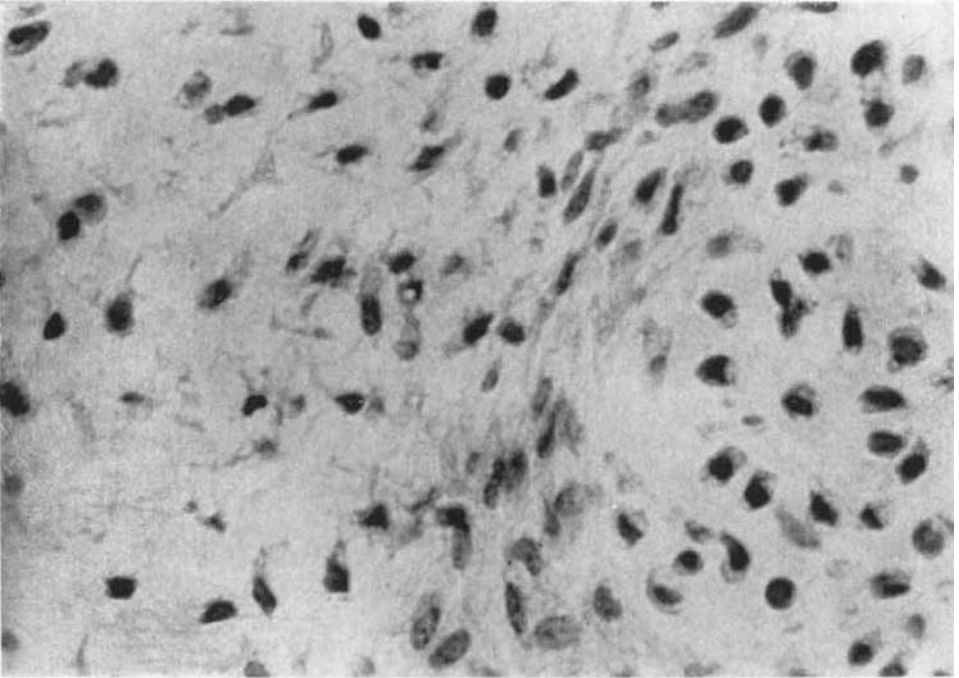


FIG. 23. Flattened chondrocytes merging with wandering cells in culture XVIb, grown for 18 days in homologous medium with fresh embryo extract and normal Tyrode's solution. Haematoxylin-eosin. $\times 790$.

that whereas the development of bone occurs from vessels in the medullary tracts the development of cartilage takes place independently of them.

As has been pointed out earlier, this study does not provide an objective basis for deciding whether the formation of the pre-osseous tissue obtained in the cultures was a result of the activity of perichondral remnants or of a direct or indirect activity of the explanted cartilage tissue—that is to say, a particular group of chondrocytes, the flattened cells.

Attempts to resolve this problem proved unsuccessful. When epiphyseal cartilage from the rabbit and human embryo was dissected and fragments from the centre were explanted in homologous medium the cultures even after 2 weeks still showed no signs of life such as expansion or cell migration. This was attributed to unavoidable impairment of the explants during dissection. In another experiment cartilaginous explants dissected from chick embryos in the usual way were first treated with Tyrode's solution containing no calcium or magnesium salts and then incubated for 15–30 minutes in a 3 per cent solution of crystalline trypsin.¹¹³ This was done to effect an enzymatic digestion of the superficial layers of the explants and consequently of the perichondral remnants; but, here too, the explants failed to show signs of life

after two weeks. Thus, only morphologic evidence was taken into consideration, and as this is invariably more or less subjective the real nature of the osteogenetic activity in the cultures can only be speculated upon.

However, there was morphologic evidence of osteogenesis in the cultures grown in homologous medium. This process was limited to its early stages possibly because under the prevailing experimental conditions the cultures had not the capacity for completing the process by the formation of mature bone tissue. The development of osteogenetic activity by cultures grown in homologous medium is further supported by the biochemical results reported in Part II.

Areal growth

Changes in the initial area of the cultures were followed by the standard method, which provides a measure of their growth. The measure of the growth obtained in this manner is not absolute, however, for only the dimensions of the surface are considered, and growth is, of course, three-dimensional.

The accuracy of the measurements was influenced by various sources of error, the most important of them being the position of the culture—prone, supine or inclined—and the skill of the worker. The standard error of the method was found to be as high as ± 37.1 . However, despite the magnitude of the error the differences between the rate of the areal growth for the experimental groups were statistically significant.

The increase in the initial area of the cultures began almost immediately after explantation, as was demonstrated by measurement of the growth at short intervals during the first 3 days of cultivation (Fig. 3). As mentioned above, the increase in the initial area of the culture during the first stage of cultivation is usually attributed largely to a flattening of the specimen. It is evident, however, from this study that cell proliferation was also responsible for the increase at this stage, evidence for this being found in the rapid increase in the initial area during early stages of cultivation with no simultaneous migration of the cells; an intense activity would be expected to accompany the re-arrangement of the cells in their adaption to the new environment. The characteristic pattern of the areal growth during this period may be considered as additional evidence of the dual mechanism of the increase in the initial area of the explants. The rapid increase in area during the first 24 hours of cultivation may be attributed to the flattening of the cultures. The increase in area from the end of the second day may be attributed to proliferation. It is difficult to accept that proliferation can start during the first hour of cultivation, or to suppose, on the other hand, that the re-arrangement of the cells should be able to continue for days, or even weeks, without these small tissue fragments becoming extremely flat and thin. As has been earlier described in detail, differences in the pattern of areal growth were observed, on the one hand between cultures from the epiphyseal and the diaphyseal part of the cartilaginous cones, and, on the other hand, between cultures

from the epiphyseal part of the cones, but cultivated under different conditions. The differences observed in the former case were attributed to the presence or absence of flattened chondrocytes. Some activities of these cells were assumed to be closely related to the areal changes of the cultures, but the nature of this relationship is obscure. As would be expected, hypertrophied and degenerating chondrocytes displayed no capacity of proliferation. The difference in the pattern of areal growth observed in the latter case was attributed to the varying composition of the medium used in each group. It may be assumed that the differences were due to the mutually antagonistic nature of the processes of differentiation and proliferation.¹⁴⁸

In the cultures of the diaphyseal series, there was no evidence of proliferation or differentiation. No significant changes in the initial area were observed, and no other signs of life were evident during the period of cultivation. However, nor were there degenerative or autolytic changes that would suggest that these cultures had died.

The cultures grown in homologous medium at first showed an intense proliferation that was reflected in a rapid increase in the initial area. Such explants exhibited morphologic and chemical evidence of differentiation, but not until the end of the 2nd week of cultivation. Parallell with the development of the differentiation there was possibly a slight proliferation, at least up to the end of the 5th week.

Cultures in heterologous medium displayed intense but short-lived proliferation; it appeared that this proliferation ceased abruptly, and it was even followed, after an interval, by a gradual decrease in the area. In this experimental group there was morphologic and chemical evidence of an initial differentiation which soon ceased, to be followed by degeneration. The cultures placed first in homologous and then in heterologous medium showed the longest period of proliferation, but here, too, there was morphologic and chemical evidence of only short-lived differentiation.

The cultures of the first group, which were composed only of hypertrophied and degenerating chondrocytes, did not contain the necessary elements for proliferation or differentiation under the prevailing conditions. The cultures of the third group, which were composed of flattened, hypertrophied and degenerating chondrocytes and which were grown in heterologous medium, apparently contained the necessary elements for further development and had displayed intense proliferation, though only for a brief interval; however, there was no differentiation, probably owing to an unsuitable environment. The cultures of the second group were also composed of all three groups of chondrocytes, but they were grown in homologous medium, which provided the necessary conditions for proliferation and differentiation. After two weeks of intense proliferation these cultures displayed incipient differentiation, a process that could be brought to an advanced stage, as shown by morphologic and chemical evidence of the formation of pre-osseous tissue. The last group consisted of cultures that contained all three groups of chondrocytes but they were grown first in homologous and later in heterologous medium. These cultures had the ne-

cessary elements for proliferation and differentiation, and during the first two weeks grew under favourable conditions and developed an intense proliferation and incipient differentiation. However, because the environmental conditions were unfavourable after the 2nd week of cultivation the differentiation soon ceased. With the disappearance of the antagonistic process of differentiation further proliferation was promoted so that the cultures achieved their maximum area of growth. It may be inferred that proliferation was entirely independent of the composition of the culture medium, whereas the process of differentiation was absolutely dependent on this factor.

The tissue cultures displayed an intense proliferation during the early period of cultivation, during which no differentiation took place. Then, when proliferation activity had attained its maximum, differentiation began. When the medium provided favourable conditions differentiation continued with increasing intensity, while proliferation was inhibited. When, however, the conditions for differentiation were unsuitable, proliferation continued intensively and suppressed the differentiation. Hence, the findings of this study support the conception of antagonism between differentiation and proliferation.

Closer study of the areal growth showed a statistically significant decrease at a certain point of cultivation in the majority of the experimental groups. The same observation has been made in an earlier study.¹⁴⁶ At that time no acceptable explanation could be advanced but it was supposed that the decrease in the areal growth might be related to the curving of the explants. However, this change occurred at quite early stages of cultivation. With the progress of the investigation it was found that there was no further evidence of morphologic and chemical differentiation after a certain period of cultivation. This would suggest a limited capacity of these cultures for differentiation under the prevailing conditions, so that the process could not be advanced beyond a certain stage. The fall in the curve for the areal growth of the different experimental groups was then considered as an expression of the limited ability of the cultures to proliferate and differentiate beyond a certain point. It was observed, furthermore, that the fall in the curve of areal growth occurred at the end of the 7th week of cultivation in cultures grown in homologous medium, and at the end of the 5th week in cultures grown in heterologous. It might therefore seem that homologous medium promotes differentiation to a more advanced stage. However, a fall in the curve of areal growth at the end of the 7th week was also observed in the diaphyseal series, which showed no signs at all of proliferation and differentiation, so that the above explanation is not valid here. This is confirmed by the fact that evidence of a chemical differentiation (an increase in the acid phosphatase activity and collagen content) was found in other groups of cultures after the decrease in the areal growth. No satisfactory explanation of this change in the course of the areal growth can at present be advanced.

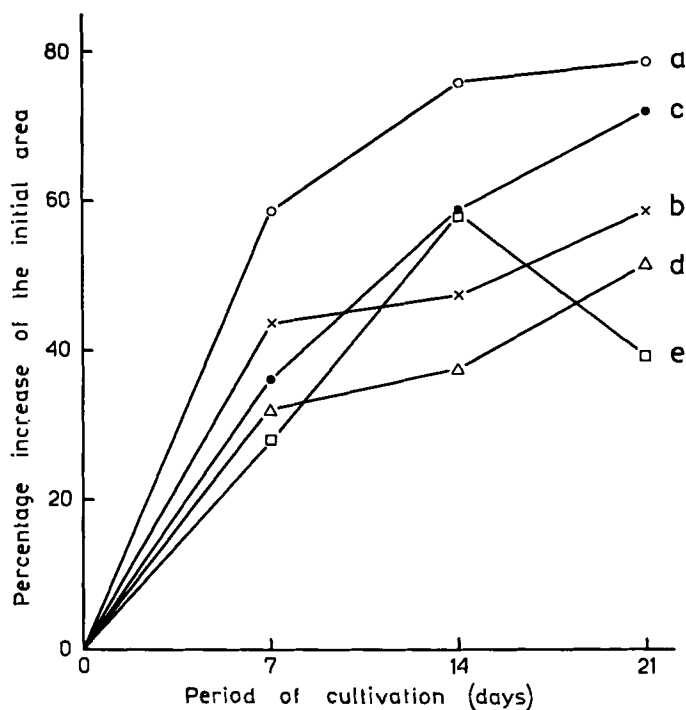


FIG. 24. Areal growth of cultures obtained from chick embryos of different ages grown in homologous medium with fresh embryo extract and normal Tyrode's solution. (a) 10-day embryos, (b) 11-day embryos, (c) 12-day embryos, (d) 13-day embryos, and (e) 14-day embryos.

The rate of the increase of the areal growth differed widely from that reported by other authors. It has been found that cartilage nodules usually increase to three to four times their original size when the cartilaginous extremities of the long bones of 8–8½ day chick embryos are used.⁴⁵ On the other hand it has been reported that explanted embryonic cartilaginous phalangeal and metacarpal bones from human embryos 9–15 weeks old increase to up to 120 to 300 per cent of their original volume.¹⁶⁹ This discrepancy between these results and those of the present study may evidently be attributed to the use by these workers of material at an earlier stage of the embryonic development. This explanation is supported by the findings obtained by explanting in homologous medium the epiphyseal cartilages cones prepared as described above, and derived from embryos incubated for between 10 and 14 days. The areal growth of these groups of cultures was followed up to the 3rd week of cultivation; at the end of the first week the percentage areal growth was found to vary directly with the age of the embryos from which the cultures were obtained. At the end of the 2nd and 3rd week, however, this relation was changed

and the curves for the areal growth of the cultures from 11-, 12-, 13-, and 14-day-old embryos crossed. The cultures derived from 10-day embryos consistently showed a higher pattern of areal growth (Fig. 24).

Observations on sectioned cultures

The morphologic changes displayed by the cultures and characterized by the formation of a perichondral capsule have been discussed in connection with the observed migratory tendency of the cells.

The presence of metachromatic staining has always been considered as related to the turnover of chondroitin sulphuric esters in the intercellular substance of the connective tissues. The study of these substances constituted a special part of this investigation, and the observations on the metachromatic staining of the sectioned cultures will be discussed in the appropriate section (p. 83).

The morphologic changes observed in the cultures and characterized by the formation of a tissue that apparently originated in the inner layer of the perichondral capsule were interpreted as evidence of differentiation, with the formation of bone as a result. The newly-formed tissue closely resembled "osteoid" tissue^{158, 66} and "immature" bone.¹⁶⁰ These terms usually refer to bone tissue in an early stage of development, but at times they have also been used to indicate atypical forms of bone. Pommer¹²⁷ first described a form of uncalcified bone tissue—later called "physiological osteoid"¹⁶¹—a conception that is disputed by MacLean *et al.*^{99, 100} "Osteoid" and "immature" bone are terms that are generally used to denote a tissue that is markedly different in its structure and histogenic details from mature bone, the difference lying in the intercellular substance and the osteocytes.¹⁶⁰ The matrix of such tissue is not calcified,⁵⁸ but has the capacity for calcification.⁵³ The term "pre-osseous tissue", as used in this paper, denotes a non-calcified tissue, morphologically similar to the osteoid or immature bone tissue but with distinct differences from mature bone, and with biochemical characteristics of developing bone tissue.

Pre-osseous tissue was found to develop and fill the narrow structureless zone that stained metachromatically, and which formed between the marginal chondrocytes and the osteoblastic coat of the perichondral capsule. Ham⁶⁶ has described a similar structureless zone, which developed just before the formation of the osteoid tissue. This zone was attributed to formation of bone matrix by the osteoblasts of the osteoblastic layer of the periosteum. No signs of degeneration of the marginal chondrocytes were observed in the present study that might explain the formation of the structureless marginal zone.¹⁴⁶

The formation of pre-osseous tissue that evidently occurred in one of the culture groups is the essential point in the interpretation of the results of this investigation. As it is open to discussion whether true, mature bone can be obtained in tissue culture,² and as the conception of the morphologic character of pre-osseous tissue is

based on subjective criteria it was impossible to decide with any degree of certainty whether the histologic changes observed in the cultures could be interpreted as evidence of the formation of pre-osseous tissue. The biochemical findings, however, bear out the occurrence of differentiation in cultures grown in a certain type of medium.

Calcification was seen in those parts of the culture displaying evidence of extensive degenerative changes of the chondrocytes and was thus of a degenerative nature. In no case were there signs of mineralization in regions where pre-osseous tissue was formed. The presence of calcium salts, although related to degenerative changes of the cultures, was probably not due merely to deposition of amorphous salts. The X-ray diffraction pattern in one case disclosed the presence of hydroxyapatite crystallites. Where calcification was present it had evidently occurred by crystallization.

MEDIUM VARIANTS

The principal differences between the various culture groups were found to be related to the use of homologous or heterologous medium. However, the use of fresh or filtered embryo extract also proved a significant factor. The differences between the results for the homologous and heterologous groups were attributed to inhibition of differentiation by the heterologous medium. Differentiation in heterologous medium has been reported, but this finding was made only in short term cultivation.^{25,76} To judge from the available results, including those of the present study, the choice of medium would appear to have no effect on the differentiation in the early stages of cultivation.

The differences observed between the groups of cultures treated with either fresh or filtered embryo extract were attributed primarily to an oxidation of the vitamin C of the extract during filtration. It has been earlier observed¹³⁰ that osteogenetic cells from chick embryos cultured in plasma obtained from scorbutic guinea pigs failed to form collagen fibres and displayed fatty degeneration, while similar cultures treated with plasma from normal animals exhibited a normal pattern of development. Bourne¹⁷ observed that in the impaired bones of scorbutic animals there was a delay in cell migration to the affected area. He also found a greatly reduced alkaline phosphatase activity in bones of scorbutic guinea pigs, and he maintained that in scurvy calcium in an amorphous form is deposited in various tissues. Similar changes were also observed in the cultures grown in heterologous medium and treated either with filtered or with freshly prepared extract: namely, suppression of the migratory activity of the cells and subsequent formation of the fibrous capsule around the cultures, reduced alkaline phosphatase activity and calcification of a degenerative nature. However, analysis of samples of extract that had been (a) freshly prepared, (b) freshly prepared and stored for 24 hours at 4°, and (c) filtered and stored at -14°

for about 2 months, revealed no significant difference in the vitamin C content, the respective values being 64 γ , 75 γ and 60 γ /ml.*

In this case the difference in the results must therefore be ascribed to other factors than a change in the vitamin C content of the extract. It is conceivable that some component of high molecular weight that promotes differentiation is retained or destroyed on filtration of the extract. The existence in the embryo extract of factors that promote and inhibit growth and differentiation has been suggested earlier.¹⁰⁴

For the above reasons the whole material was considered as falling into two main groups, one consisting of cultures grown in medium containing components promoting both growth and differentiation, and the other consisting of cultures grown in medium containing components that inhibit differentiation. In the former group the promoting components were homologous plasma and freshly prepared extract, while in the latter group the inhibitory component was the heterologous serum, the effect of which was so marked that any difference in the action of the fresh and filtered extracts was masked. Cultures grown first in homologous and later in heterologous medium also belonged to this group.

The cultures in these two groups have been denoted as "differentiating" and "degenerating", respectively.

* Vitamin C determinations were performed according to the method of Roe and Kuether.¹⁴³

PART TWO

BIOCHEMICAL STUDY

A study of certain biochemical reactions characteristic of the various stages of osteogenesis was undertaken with the purpose of ascertaining, on other than morphologic grounds, whether osteogenesis does in fact take place in cultures of the explanted epiphyseal cartilaginous cones. Enzymologic studies of the alkaline and acid phosphatase and phenolsulphatase activities were performed. As the results obtained from these studies were considered to prove that differentiation really occurs in the tissue cultures, the biochemical study was extended to two components of the intercellular substance—collagen and chondroitin sulphate.

No statistical analysis of the results was performed owing to the nature of the material and the difficulty of obtaining sufficient values. In the curves of this part the broken lines are drawn through the averages of the experimental values; the continuous lines represent the assumed development.

ALKALINE PHOSPHATASE ACTIVITY

Since the discovery in the ossifying cartilage of young rats and rabbits of an enzyme, "bone phosphatase", that hydrolysed hexose monophosphoric acid to give free phosphoric acid,^{134, 135} the importance of the role played by a group of similar enzymes (possibly 15 to 20)¹⁵¹ in the biology and biochemistry of the living tissues has become increasingly realized. Such enzymes have been demonstrated in practically all living cells, and especially during the process of cellular differentiation, such as occurs in the embryonic development in mammals,^{80, 42} the chick,^{108, 109} and the sea urchin,^{61, 62} in the repair of wounds and cutaneous burns²⁸ and in the formation of secretions of a protein nature, such as silk.²⁰

"Bone phosphatase" belongs to a group of phosphatases with an optimal pH ranging between 9.2 and 9.4. To the same group belong phosphatases of other organs (kidney, intestine, liver, bladder) with the same⁴³ or slightly different pH optima. There is no incontestable evidence that the alkaline phosphatases from the different sources are not identical.^{110, 18}

Alkaline phosphatase activity has long been considered to be involved in the phase of osteogenesis characterized by mineralization of the intercellular substance. However, this concept has been much debated during recent years, as bone minera-

lization has proved to be a more complex process than Robison believed when he expressed the view that phosphatase liberates phosphate ions until their concentration exceeds the solubility product, at which point they are precipitated with calcium. It has been suggested that, instead of functioning as a hydrolytic agent to furnish inorganic orthophosphate, alkaline phosphatase may participate in the enzymatic transfer of a phosphate group from a metabolic ester to an acceptor in the matrix.⁴⁵ The prevailing opinions on the role played by alkaline phosphatase in cell biology in general, and in osteogenesis in particular, are the following: (i) Alkaline phosphatases are widely occurring enzymes that are essential for the biologic function of several organs. The enzymatic activity is concerned in cell differentiation.^{80, 42, 108, 109, 142} (ii) Alkaline phosphatase is involved in the stage of ossification characterized by mineralization of the intercellular substance*. (iii) Alkaline phosphatase is concerned in the formation of the fibrillar component of the intercellular substance of bone and connective tissue in general.^{51, 14, 15, 110, 7} (iv) Alkaline phosphatase is involved in processes of cell degeneration.^{69, 70}

It would seem to be generally accepted that alkaline phosphatase is in some way involved in osteogenesis but that its precise significance is still obscure.

Experiments

Quantitative determinations of the alkaline phosphatase activity were performed by the method of King and Armstrong⁴³, as modified by Binkley *et al.*⁹ and Gustafson *et al.*⁸¹ Each specimen was weighed and manually homogenized in a glass homogenizer in the presence of 1 ml distilled water saturated with chloroform. The substrate consisted of 5.0 ml of 0.005 *M* di-sodium phenylphosphate in a veronal buffer at pH 9.2, to which 1 ml of a 0.1 *M* solution of magnesium chloride was added as an activator. After incubation at 37°C for 6 hours in a water bath the reaction was arrested by the addition of 2 ml Folin-Ciocalteu's phenol reagent. After centrifugation, 2 ml of a 20 per cent solution of sodium carbonate was added to 5 ml of the centrifugate, and after incubation for 30 minutes at 37°C the colour intensity was read in a Spekker absorptiometer, using an Ilford spectrum red filter no. 608. The optimal conditions for the alkaline phosphatase activity were chosen after preliminary study of the pH, the period of incubation and the concentration (Fig. 25) of the materials and substrates. The optimum pH range for the reaction was 9.2 to 10.2. A value of 9.2 was used throughout as providing the best conditions for the stability of the solutions. The enzymatic activity-time curve was linear up to 12 hours with respect to the maximum extinction values, after which it levelled off. The period used was 6 hours, as a satisfactory hydrolysis of the substrate was then ensured even in samples with a low enzymatic activity. When the activity was expected to exceed the maximum scale reading of the absorptiometer the period of incubation was limited to 3, 2 or even 1 hour and the result was multiplied by 2, 3 or 6, respectively. (The enzymatic activity-time curve was linear in the range in question). If, in spite of the restricted period, a very strong reaction was obtained the final solution was diluted with equal parts, or twice the volume, of distilled water and the

* References: 134, 135, 139, 136, 137, 138, 144, 126, 10, 11, 72, 140, 141, 14, 90.

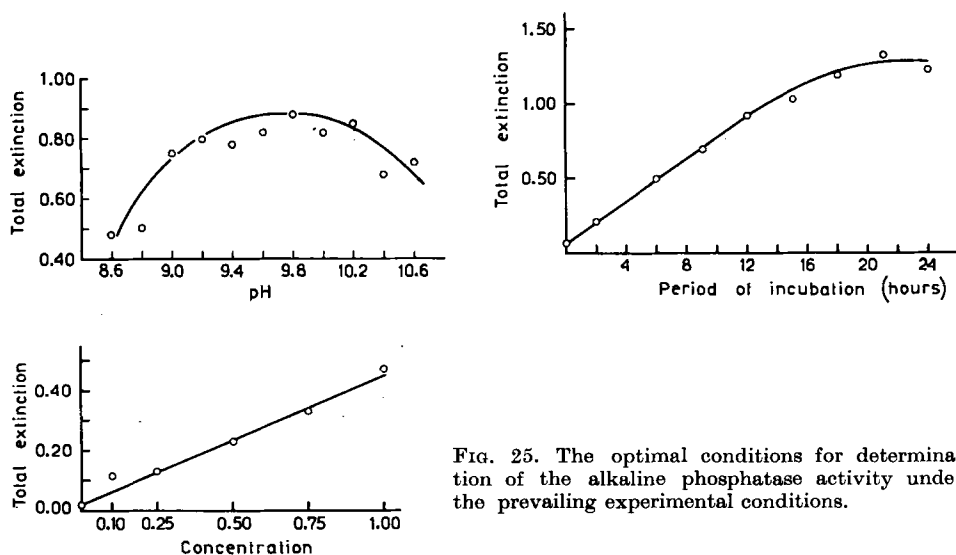


FIG. 25. The optimal conditions for determination of the alkaline phosphatase activity under the prevailing experimental conditions.

result multiplied by 2 or 4, respectively. (The dilution and colour intensity were found to be directly related.)

Histochemical determinations were made by the method of Gomori⁶⁰ as described by Glick.⁵⁹

Quantitative determinations of the alkaline phosphatase activity were performed:

- (i) on cultures grown in homologous medium and treated either with fresh or filtered embryo extract;
- (ii) on cultures grown in heterologous medium, and treated with fresh or filtered embryo extract;
- (iii) on cultures grown first in homologous and then in heterologous medium;
- (iv) on cultures belonging to the diaphyseal series;
- (v) *in vivo*—that is to say, on epiphyseal cartilage cones obtained from chick embryos from the 10th day of incubation until hatching, and from newly hatched up to 5-day-old chicks. (After this age the cartilage cones become honeycombed by perforation of medullary tracts and vessels.) Determinations were also performed on similar specimens of cartilaginous cones, together with the surrounding bony cylinder.

Histochemical determinations were performed on 10 cultures grown for 25 to 48 days in homologous medium and treated with fresh embryo extract (2 cultures for 25 days, 3 for 26 days, 3 for 28 days and 2 for 48 days).

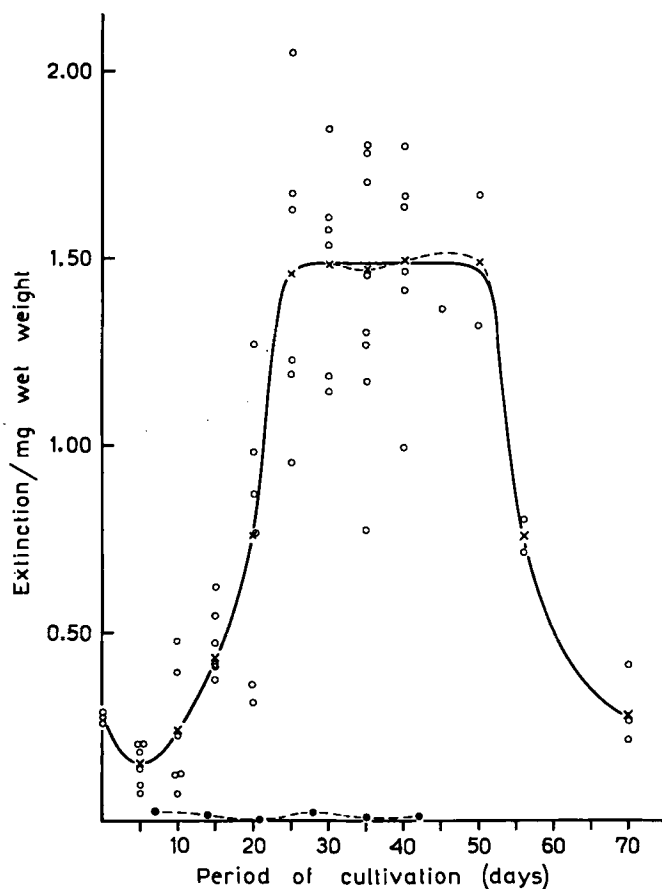


FIG. 26. Alkaline phosphatase activity for cultures grown in homologous medium with fresh embryo extract and normal Tyrode's solution. *Upper curves*: epiphyseal series. *Lower curve*: diaphyseal series.

Results

The course of the alkaline phosphatase activity varied according to the type of medium, and the histologic composition of the cultures.

Homologous media

(a) *Freshly prepared embryo extract.* There was a decrease in the enzymatic activity from the initial values lasting for the first 5–10 days of cultivation, succeeded immediately by a steep rise between the 15th and 25th day, the maximum occurring between the 25th and 50th day. There followed an almost immediate steep fall to approximately the initial value, which was reached on about the 70th day (Fig. 26).

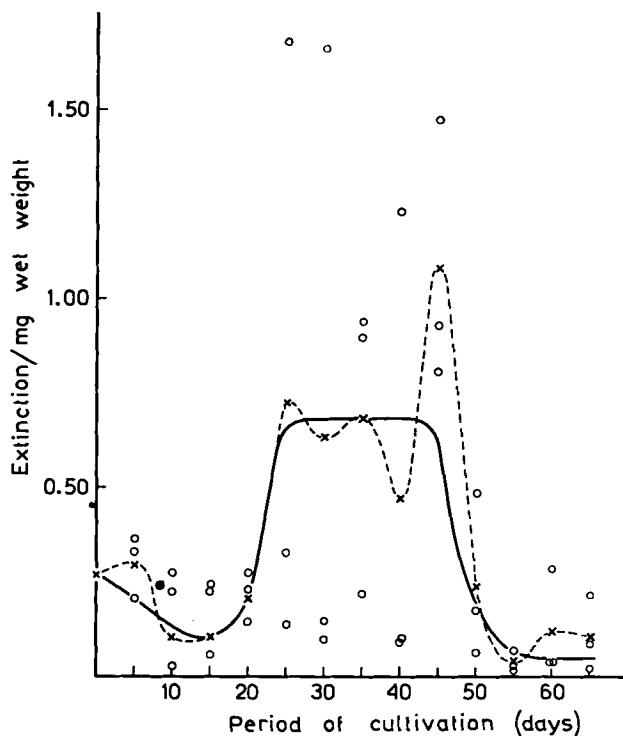


FIG. 27. Alkaline phosphatase activity for cultures grown in homologous medium with filtered embryo extract and normal Tyrode's solution.

(b) *Filtered embryo extract.* The decrease from the initial values was slightly longer than when freshly prepared extract was used. Moreover, the maximum activity was much lower, and the subsequent fall occurred somewhat earlier (Fig. 27).

Heterologous media

(a) *Freshly prepared embryo extract.* There was a more rapid development of the alkaline phosphatase activity, the maximum value being reached as early as the 10th day of cultivation but lasting only 5 days, before a steep fall to slightly below the initial level, reached after the 20th day of cultivation (Fig. 28).

(b) *Filtered embryo extract.* The activity was consistently below the initial value, except for a rise to that level between the 20th and 25th days. After this period no alkaline phosphatase activity could be detected in the cultures (Fig. 29).

FIG. 28. Alkaline phosphatase activity for cultures grown in heterologous medium with fresh embryo extract and calcium-enriched Tyrode's solution.

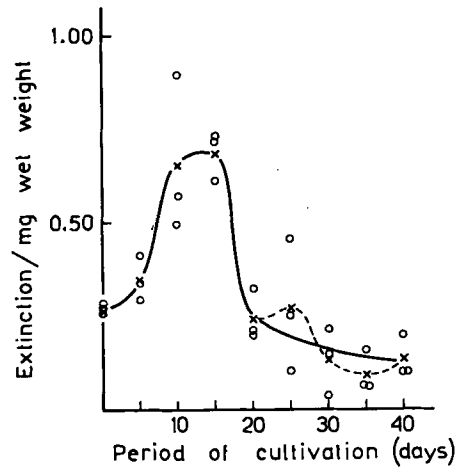


FIG. 29. Alkaline phosphatase activity for cultures grown in heterologous medium with filtered embryo extract and calcium-enriched Tyrode's solution.

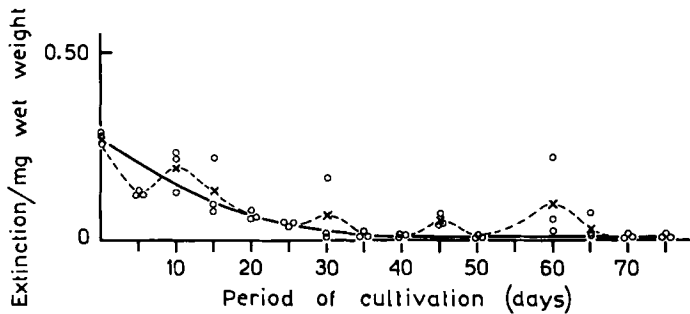
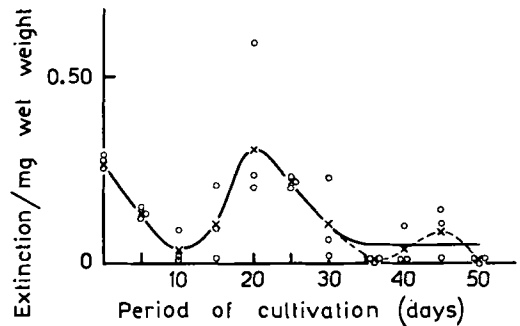


FIG. 30. Alkaline phosphatase activity for cultures grown first in homologous medium for 2 weeks and thereafter in heterologous medium. Filtered embryo extract and calcium-enriched Tyrode's solution were used throughout.

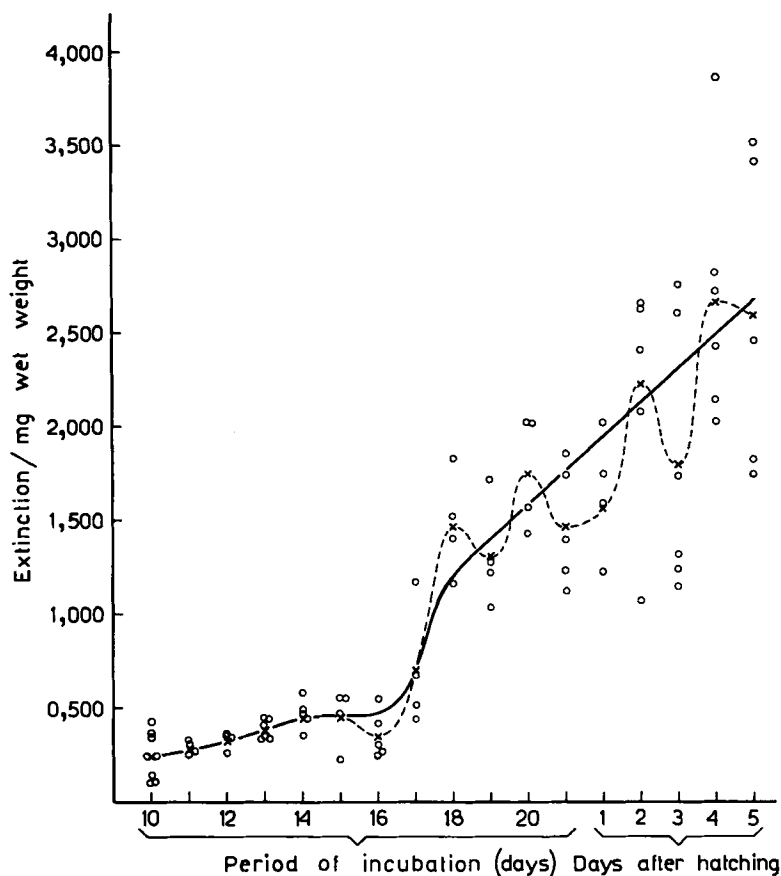


FIG. 31. Alkaline phosphatase activity *in vivo*.

Homologous and heterologous medium

The cultures of this group were treated with filtered embryo extract. The alkaline phosphatase activity gradually decreased until the 35th day of cultivation, by which time it was practically extinguished (Fig. 30).

Diaphyseal series

There were no detectable signs of alkaline phosphatase activity between the 7th and the 42th day of cultivation (Fig. 26, lower curve).

In vivo

A gradual but moderate increase up to the 16th day of incubation was succeeded by a steep and practically constant rise up to the 5th day after hatching (Fig. 31).

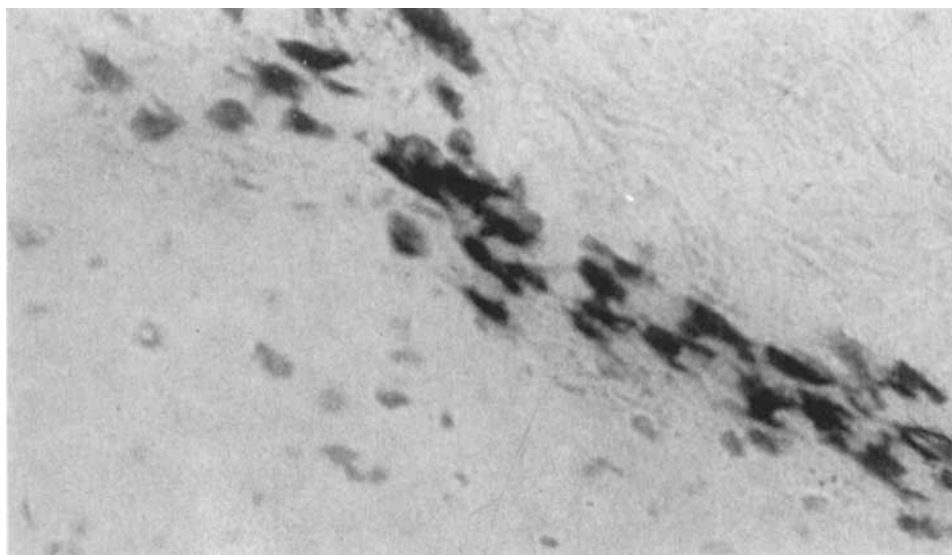


FIG. 32. Alkaline phosphatase activity studied histochemically in culture 248*e* grown for 28 days in homologous medium with fresh embryo extract and normal Tyrode's solution. A positive reaction was obtained in the region of differentiating osteoblasts, whereas the adjacent capsule and cartilage showed no activity. Gomori. $\times 480$.

No appreciable differences were observed between the alkaline phosphatase activity for the cartilage specimens alone and those, of the same age, with the surrounding bony cylinder.

Histochemical study

A positive reaction for alkaline phosphatase was obtained in all the cases. This was characterized by dark brown to black staining at the sites of the enzymatic activity. These, however, were not the same in all cultures. In 3 cases a positive reaction was obtained at places in the perichondral capsule, whereas in 7 cases the capsule was negative. The sites of positive response were located in the inner layer. Sites in pre-osseous tissue gave a positive reaction in 8 cultures, and in the remaining 2 cases there was no morphologic evidence of pre-osseous tissue. In 6 cases weak positive staining was found in limited regions of peripheral chondrocytes—not necessarily adjacent to positive regions of pre-osseous tissue or perichondral capsule—and in the 2 other cases regions of central chondrocytes gave a positive response. In all these cultures there were hypertrophied chondrocytes that displayed a positive reaction. Flattened and degenerated chondrocytes never exhibited a positive reaction.

When a positive reaction was obtained in pre-osseous tissue it was usually very intense in the osteoblasts and less so in the intercellular substance (Fig. 32). When regions of chondrocytes showed positive staining the cell nuclei and protoplasm also stained more intensely than the intercellular substance (Fig. 33).

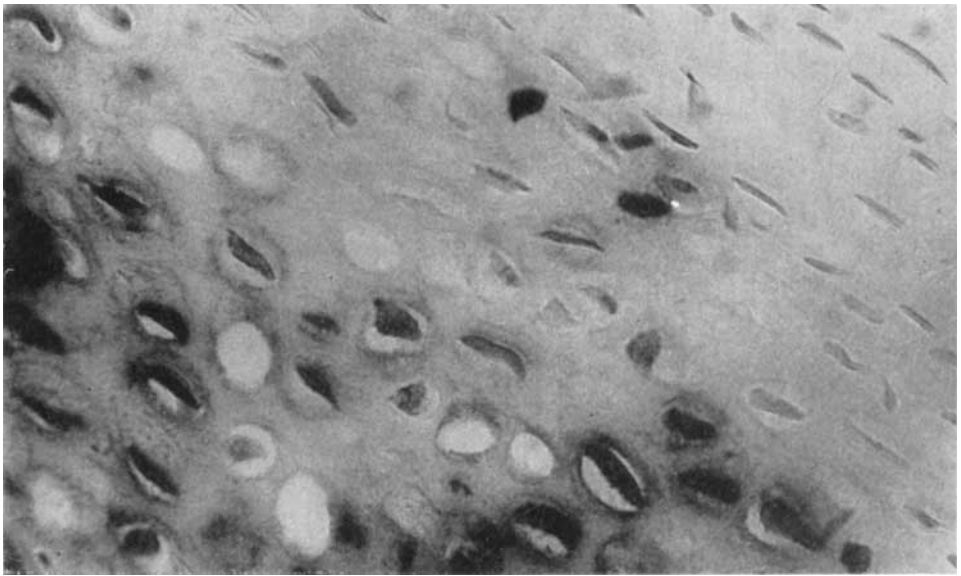


FIG. 33. Alkaline phosphatase activity studied histochemically in culture 263j grown for 28 days in homologous medium with fresh embryo extract and normal Tyrode's solution. A positive reaction was obtained in the region of the hypertrophied chondrocytes whereas the flattened cells showed no activity. Gomori. $\times 480$.

Discussion

The results of the estimations of the alkaline phosphatase activity demonstrate a relationship between this enzymatic activity and the histologic composition of the cultures. Those obtained from the diaphyseal part of the cartilaginous cone displayed no such activity during the period of cultivation. It is logical to infer, therefore, that the presence of phosphatase activity is related to the presence of flattened cells. Furthermore, there was a close relationship between the level of alkaline phosphatase activity and the composition of the culture medium. Cultures grown in a medium that promoted differentiation (p. 53) displayed a high activity. The results obtained in this case confirm earlier findings.^{46, 47}

Variation in the composition of the medium, however, resulted in a change in the enzymatic activity. More specifically, the filtered embryo extract inhibited, but did not eliminate, the activity. On the other hand, cultures grown in heterologous medium showed a low enzymatic activity of short duration when treated with freshly prepared extract, and almost negligible activity when filtered extract was used. For cultures grown first in homologous and later in heterologous medium and treated with filtered extract the activity was also low. As the cultures of this last group displayed intense proliferation it may be inferred that the level of alkaline phosphatase activity does not play any part in the proliferation. This was borne out

by the absence of any enzymatic activity during the early stage of cultivation characterized by intense proliferation. The initial decrease in the alkaline phosphatase activity during this period of cultivation might be ascribed to the marked proliferative activity of the cultures.

The relation between the composition of the medium and the enzymatic activity was similar to that between the composition of the medium and the degree of differentiation. The rise in the alkaline phosphatase activity in the group of "differentiating" cultures may therefore be a biochemical manifestation of the differentiation taking place in these cultures. From the chronologic correlation of the morphologic observations and the alkaline phosphatase for the "differentiating" cultures it is evident that the rise in this enzymatic activity begins during the advanced stage of development of the perichondral capsule. The migratory activity of the cells and the early stage of capsule formation coincide with the extremely low level of enzymatic activity. As the differentiation of the osteoblastic layer of the perichondral capsule occurred almost as soon as this had begun to form and as no traces of phosphatase activity could be detected by histochemical methods in the outer layer of the capsule, the rise in the enzymatic activity may be linked with the appearance of osteoblasts in the inner layer of the perichondral capsule. However, the enzymatic activity remained at a high level throughout the period of formation of the pre-osseous tissue.

No mineralization of the matrix of the pre-osseous tissue and only rare degenerative calcification could be detected in the group of cultures grown in homologous medium in spite of the intense phosphatase activity over a long period. On the other hand, degenerative calcification was observed in 30 per cent of cultures grown in heterologous medium, and as a rule in the absence of any detectable signs of alkaline phosphatase activity. These observations were interpreted as evidence that calcification may occur without simultaneous high alkaline phosphatase activity.

In the "differentiating" cultures the alkaline phosphatase activity was most intense in the osteoblastic layer of the perichondral capsule and at the sites of formation of pre-osseous tissue. Hypertrophied chondrocytes displayed a marked activity, though less than the pre-osseous tissue. These findings would suggest that the alkaline phosphatase activity is related to the various processes of differentiation occurring in cultures. There was evidence that the enzyme played a dual role in that it participated both in the process of differentiation of hypertrophied chondrocytes from flattened cells and in the formation of osteoblasts from the cells of the inner layer of the perichondral capsule. Though they appeared to occur simultaneously there was no apparent connection between these two manifestations of the alkaline phosphatase activity.

The alkaline phosphatase activity *in vivo* showed fairly low, constant values between the 10th and 17th days of incubation, although there is a gradual increase in bone formation at this time. There followed a period of increasing activity. This

may be interpreted as an inverted state of equilibrium, either between the proliferation of the cartilage and the differentiation of osteoblasts or between proliferation of the cartilage and hypertrophy of the chondrocytes. However, as there was no osteogenesis in the parts of the cartilaginous cones used in these experiments the latter assumption must be considered as the more probable. The curve for the alkaline phosphatase activity *in vivo* was similar to those obtained by previous investigators.^{46, 47, 111}

There was a parallel between the alkaline phosphatase activity in "differentiating" cultures and *in vivo*. In both cases an initial period of low activity was followed by a steep rise. However, there was an essential difference between the materials used in the two cases, the *in vivo* determinations being performed exclusively on cartilage in which no osteogenesis takes place, whereas in the cultures there was periosteal osteogenesis. The results are therefore not comparable.

In all cases quantitative determinations showed fairly constant values at the periods of low enzymatic activity, but when a high level was recorded the values displayed wide variation in cultures of the same age. This divergency was ascribed primarily to experimental errors in the chemical analysis, but it may be due in part to the individual pattern of development of the cultures and the chick.

The study of the alkaline phosphatase activity suggests the following conclusions:

(i) The high enzymatic activity detected in "differentiating" cultures may be regarded as biochemical evidence of differentiation.

(ii) The rise in the alkaline phosphatase activity apparently coincided with the differentiation of osteoblasts from the cells of the inner layer of the perichondral capsule developed in these cultures.

(iii) There was a rise in the enzymatic activity during the process of differentiation of hypertrophied chondrocytes from the flattened cells. This finding together with the above (ii) would suggest that the enzyme played a dual role in the development of the cultures.

(iv) Calcification of a degenerative nature, characterized by crystallization of hydroxyapatite, occurred almost invariably in the absence of any detectable signs of alkaline phosphatase activity.

(v) There was a certain parallel in the course of the alkaline phosphatase activity in differentiating cultures and *in vivo* but no conclusions may be drawn since it is not known whether the materials are comparable.

ACID PHOSPHATASE ACTIVITY

Acid phosphatase is, after alkaline phosphatase, the most widely distributed enzyme in nature.¹⁴² It has been found, however, that most tissues displaying an intense activity of the latter enzyme are practically devoid of the former.^{82, 29, 24, 120} Both phosphatases are present in the mesoderm tissue from the 4th day of incubation.¹⁴³

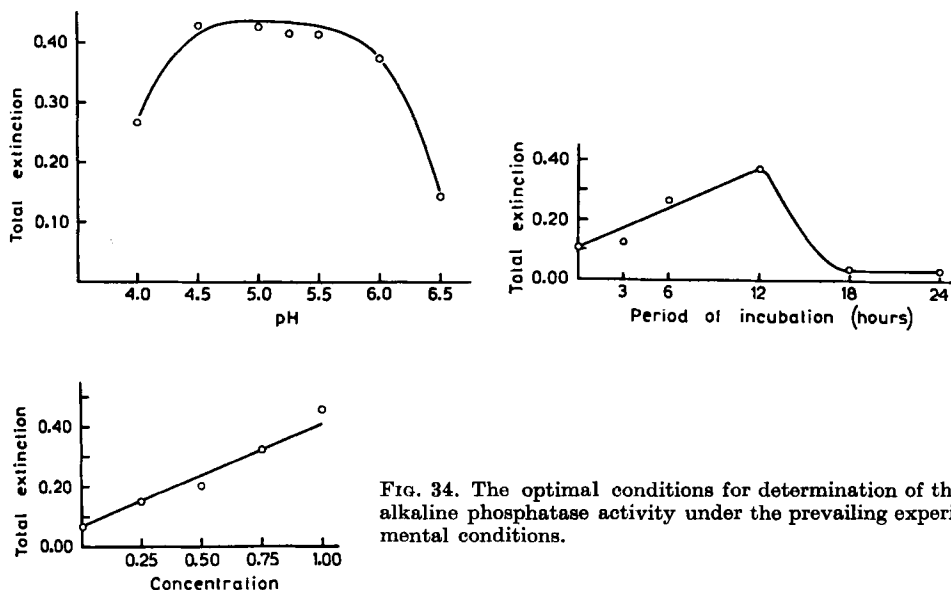


FIG. 34. The optimal conditions for determination of the alkaline phosphatase activity under the prevailing experimental conditions.

A rise in the acid phosphatase activity in connection with the development of the chick skeleton has been observed earlier²³ and a dual role played by acid and alkaline phosphatases in bone metabolism has been suggested,¹⁵⁵ but on the whole the relationship between the acid phosphatase activity and the process of osteogenesis has received little attention.

Experiments

As in the estimation of alkaline phosphatase activity, the method described by Binkley *et al.*⁹ with slight modifications was used for the quantitative determinations of the acid phosphatase activity. Five millilitres of a 0.005 *M* solution of di-sodium phenyl-phosphate in an acetate buffer of pH 5.0 was used as substrate. The reagents and the analytical procedure were identical with those used in the study of alkaline phosphatase.

A preliminary study of the incubation time, the concentration and the optimal pH (Fig. 34) was made in order to ascertain the most suitable conditions for the experiments.

The optimal pH of the reaction was found to lie between 4.5 and 5.5, and 5.0 was chosen for these estimations. It was found that the enzymatic activity-time curve was linear with respect to the maximum extinction values up to the 12th hour of incubation but that it decreased sharply thereafter until, after 18 hours, only traces of activity could be detected. In order to ensure satisfactory hydrolysis of the substrate even in tests with low enzymatic activity an incubation time of 12 hours was used throughout. However, when the enzymatic activity was expected to exceed the maximum scale reading the time of incubation was, as in alkaline phosphatase, restricted to 6, or even 3, hours and the results were multiplied by 2 or 4, respectively.

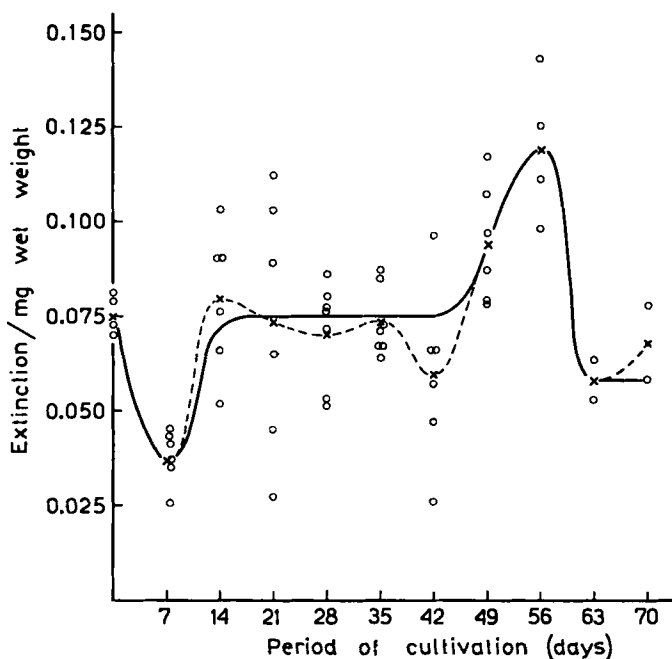


FIG. 35. Acid phosphatase activity for cultures grown in homologous medium with fresh embryo extract and normal Tyrode's solution.

Determinations of the acid phosphatase activity were performed:

- (i) on "differentiating" cultures,
- (ii) on cultures grown in two variants of heterologous medium, and
- (iii) on *in vivo* material, obtained as for the alkaline phosphatase experiments.

Results

In the case of this enzyme, too, different types of activity were obtained, depending on the composition of the culture medium.

(i) In cultures grown in homologous medium and treated with freshly prepared embryo extract a decrease in the enzymatic activity was observed during the 1st week of cultivation, followed by a slight increase during the 2nd week, after which the values remained fairly constant until the end of the 6th week. There was then a surge of activity and maximum values were attained at the end of the 8th week. A steep fall in the activity occurred during the 9th week and the values remained constant until the end of the 10th (Fig. 35).

(ii) Estimations of the acid phosphatase activity in "degenerating" cultures showed that where heterologous medium and freshly prepared embryo extract were used there was an initial decrease lasting to the end of the 2nd week, after which

FIG. 36. Acid phosphatase activity for cultures grown in heterologous medium with fresh embryo extract and calcium-enriched Tyrode's solution.

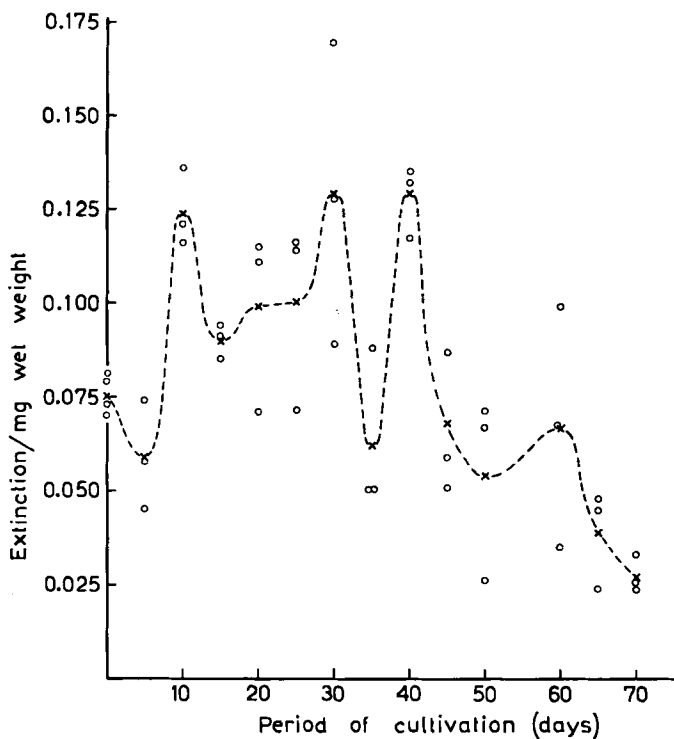
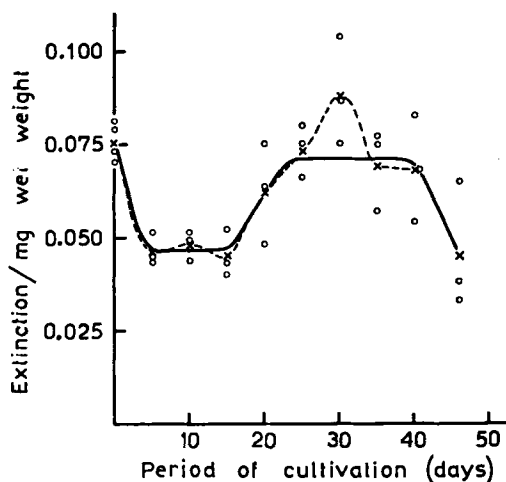


FIG. 37. Acid phosphatase activity for cultures grown first in homologous medium for 2 weeks and thereafter in heterologous medium. Filtered embryo extract and calcium-enriched Tyrode's solution were used throughout.

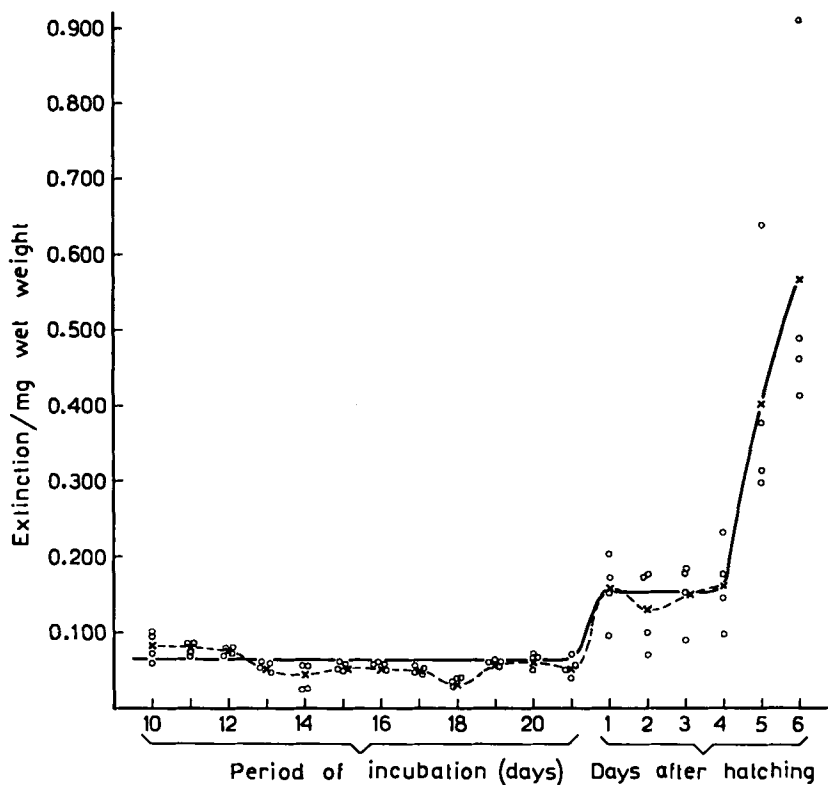


FIG. 38. Acid phosphatase activity *in vivo*.

there was a slight rise, the values then remaining constant between the 25th and 40th days of cultivation, when they fell again (Fig. 36).

In the group of cultures grown first in homologous and then in heterologous medium there was also a decrease, which lasted one week and which was succeeded by a period, between the 10th and 40th days, when large variations were recorded. There followed a continuous fall from the 40th to the 70th day (Fig. 37).

(iii) The *in vivo* study revealed constant values of the acid phosphatase activity at a low level from the 10th day of incubation to hatching. On the 1st day thereafter a rise was noted, followed by a rather stable period until the 4th post-embryonic day, at the end of which there was a steep rise in the activity to the 6th day (Fig. 38).

No appreciable differences were observed between the acid phosphatase activity for the cartilage specimens alone and those, of the same age, with the surrounding bony cylinder.

Discussion

The intense activity of acid phosphatase displayed by the "differentiating" cultures may be regarded as further biochemical evidence of differentiation. As the differentiation of these cultures led to the formation of pre-osseous tissue, the rise in the acid phosphatase activity is probably associated with some stage of this process.

As in the case of the alkaline phosphatase activity, the results of the study of the acid phosphatase were compared with the morphologic observations, viz. (i) intense proliferation during the initial period of cultivation, (ii) formation of the perichondral capsule, and differentiation of osteoblasts from its inner layer, and (iii) formation of pre-osseous tissue. The rise in the acid phosphatase activity observed between the 6th and the 9th weeks of cultivation corresponded approximately in time to the last of these processes, in which it would thus appear that the enzyme is involved.

There was a distinct relationship between the course of the acid phosphatase activity and the composition of the medium. In cultures grown in heterologous medium only a negligible rise in the enzymatic activity was recorded. Owing to the highly variable activity for the cultures grown in the two types of media the validity of the determinations in this case is open to question.

As in the case of the alkaline phosphatase, the initial decrease in activity was attributed to the intense proliferation of the explants during that period of cultivation. However, this drop was followed by a moderate rise and, in contrast to the case of the alkaline phosphatase activity, the subsequent values were constant. The significance of the slight rise following the initial decrease is difficult to interpret.

There was a similarity between the acid phosphatase activity *in vivo* and that observed in the "differentiating" cultures. There was in both cases a period during which the activity was at a fairly constant low level; there followed a two-step rise.

The course of the acid phosphatase activity *in vivo*, as in the case of alkaline phosphatase, may be interpreted as indicating a marked variation in the intensity of the processes of osteogenesis during the successive stages of the embryonic and early post-embryonic development, and taking the form of a disturbance of an existing state of equilibrium.

The suggested dual role of alkaline and acid phosphatase in bone formation¹⁵⁵ seems to be further supported by these results. It is difficult, however, to subscribe to the view that both enzymes are involved in the same process of osteogenesis, since the rise in the activity of each enzyme began, and attained a maximum, at a different period of cultivation.

The study of the acid phosphatase activity suggests the following conclusions.

(i) The rise in the acid phosphatase activity occurring in "differentiating" cultures during the period of cultivation may be considered as further biochemical evidence of differentiation.

(ii) The acid phosphatase activity was probably related to the actual formation of pre-osseous tissue.

(iii) In spite of the similarity observed between the course of the acid phosphatase activity in "differentiating" cultures and *in vivo* it is doubtful whether the results obtained in these two cases can be compared.

PHENOLSULPHATASE ACTIVITY

Hydrolysis of potassium indoxyl sulphate to free indoxyl and potassium hydrogen sulphate has been shown to involve the action of an enzyme,³¹ which is also capable of hydrolysing potassium phenylsulphate.^{116, 117, 118} This enzyme, phenolsulphatase, has been found to be present in lower plants,¹¹⁷ lower animals¹⁴⁷ and in higher animals, and to be most abundant in the liver, adrenals and kidneys.^{71, 145, 1} The enzymologic study was extended to an examination of this enzyme, as it has been suggested that phenolsulphatase is involved in the detoxification of aromatic amino-acids produced during the synthesis of collagen from the protein reserve in the course of embryonic development of echinoderms,⁸¹ and as little is known of the role of this enzyme during osteogenesis.

The method used for the quantitative determination of this enzyme was the same as that described for acid phosphatase, with appropriate modifications proposed by Gustafson.* A 0.005 *M* solution of potassium phenylsulphate (1.06 Gm in 500 ml distilled water) was used as the substrate with an acetate buffer. Follin-Ciocalteu's phenol reagent, a 20 per cent solution of sodium carbonate and water saturated with chloroform, were used.

The examination of the phenolsulphatase activity was restricted to the preliminary study of the optimal conditions for the enzymatic reaction. pH values from 4 to 6.5 were used, as this range has in other experiments been found to favour this enzymatic activity.⁷³ Incubation was performed up to 24 hours at 37°C,³⁴ although the rate of reaction has been reported to be greater at 50°.¹

Under these experimental conditions no phenolsulphatase activity could be detected in the epiphyseal cartilage from the 12-day chick embryos, or in a number of samples taken at random from the "differentiating" cultures. Further study of this enzyme at regular stages of cultivation therefore seemed unnecessary. It may be concluded that there is probably no phenolsulphatase in the epiphyseal cartilage of the 12-day embryonic chick and that this enzyme is not involved in osteogenesis.

* Personal communication.

COLLAGEN

The connective tissues—connective tissue proper, bone, cartilage and haemopoietic tissue—are of mesodermal origin and generally composed of cells with the intercellular spaces filled with fibres and matrix. Fibres consist of a special group of proteins which includes elastin, reticulin and collagen.⁷⁷ Collagen is the most abundant fibrous protein in connective tissue occurring in vertebrates and lower animals.⁵ The collagen of invertebrates differs so distinctly from that of the vertebrates that it cannot be considered as collagen in the accepted sense of the term.⁴⁰ Collagens from various species of mammals and birds on the other hand, differ little in their fibrillar structure¹³¹ and their chemical composition.¹¹⁹

Collagen is arranged in fibres, the elementary unit of which is the collagen "fibrilla", visible only under the electron microscope. Closely packed aggregates of many such fibrillae constitute the primitive fibres, which are just resolvable under the light microscope. These fibres in turn form the fibre bundles, which constitute the easily recognizable fibrous unit of bone structure.¹⁵⁹ In newly formed embryonic bone the fibre bundles are disposed with no apparent order; in mature lamellar bone, however, the fibre pattern is remarkably uniform and regular.¹²⁹ Widely divergent opinions are held on the origin and mode of formation of the collagen fibres, and it is much debated whether they are formed by the extracellular fluids or by cellular elements under the influence of these fluids.

The principal opinions can be summarized as follows. (i) Collagen fibres develop extracellularly and involve the conversion of fibrin into collagen.^{3, 115} (ii) They develop extracellularly but are not derived from fibrin, although the fibrin threads may form a basis for the collagen fibres during their formation.^{95, 98, 107, 112, 121} (iii) Collagen fibres originate within the cell.^{93, 87, 166, 128} (iv) It has been demonstrated by tissue culture experiments that the elementary "fibrillae" of the connective tissue are formed outside the cytoplasm—that is to say, in the intracellular substance—by the influence of cellular secretions.³⁵ (v) It has recently been suggested that fibroblasts produce a soluble protein procollagenous substance, which precipitates collagen fibre in the presence of a carbohydrate fraction in the ground substance.³⁷ (vi) Collagen fibres are extracellular elements but their precursors can be traced back into the cytoplasm.⁷⁷

Collagen has a very high molecular weight and is composed of a variety of amino-acids.^{156, 119, 40} A characteristic of collagen is its high content of proline (15–16 per cent), hydroxyproline (13.5 per cent), and glycine (27–29 per cent)^{119a, 40} and a very low percentage of aromatic and sulphur-containing amino-acids.^{63, 64, 119} Collagen is the only protein besides elastin that contains hydroxyproline.^{119a, 40}

Since no reliable microbiologic method was available for estimating hydroxyproline, and since quantitative chemical^{79, 119a} or paper chromatographic⁹⁷ techniques had not then been adapted for micro-estimation of this amino-acid the total proline was taken as a measure of the collagen level in the cultures.

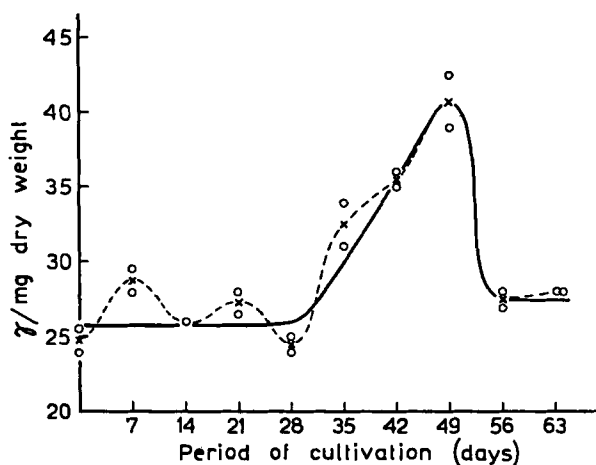


FIG. 39. Collagen content of cultures grown in homologous medium with fresh embryo extract and normal Tyrode's solution.

Experiments

Estimation of proline was performed by the method evolved by Dunn *et al.*,³⁶ with slight modifications and using *Lactobacillus brevis*, ATCC 8287. The growth of the micro-organism was measured with samples and with standards of 10–40 γ -proline per tube by the conventional acidimetric procedure. The modification of Dunn's method consisted in the use of the technique of Banhidi *et al.*⁴ in the preparation of the stock cultures of *L. brevis*.

Samples of 5 to 12 specimens of different stages were lyophilized with 2.5 *N* hydrochloric acid for 10 hrs at 120°C in fused pyrex tubes, and the pH adjusted to 6.8 with 1 *N* sodium acetate and 0.5–1.0 mg of dry substance per ml before testing for proline.

Estimations of the proline were made (i) on cultures grown in homologous medium, and (ii) *in vivo*, as in the case of alkaline and acid phosphatases.

Results

(i) The curve compiled from the values obtained from the estimation of proline in "differentiating" cultures showed that epiphyseal cartilage cones from the long bones of 12-day chick embryo—0-day samples—contained a fairly large amount of proline. Until the end of the 4th week the proline remained more or less constant; it then rose to a maximum at about the end of the 7th week. An immediate decrease ensued from the end of the 8th week to the end of the 9th, after which the values were nearly the same as during the initial period (Fig. 39). Estimation of the proline in cultures grown in homologous medium, but with homogenization of the samples before hydrolysis, showed the increase to occur about one week earlier, the period of high values being prolonged by one week.

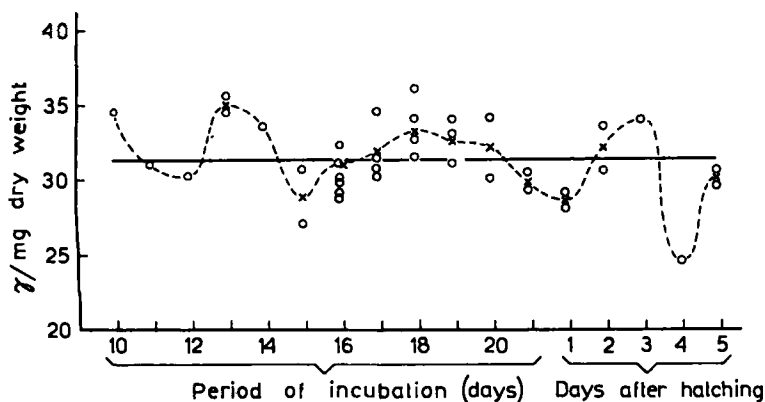


FIG. 40. Collagen content *in vivo*.

(ii) From the curve of the *in vivo* values it is seen that in the epiphyseal cartilage cones from embryonic and early post-embryonic bones the proline was fairly constant between the 10th day of incubation and the 5th day after hatching. The small waves in the curve are probably of no significance (Fig. 40).

No appreciable differences were observed between the proline content of the cartilage specimens alone and those, of the same age, with the surrounding bony cylinder.

Discussion

The increase in the collagen content that is reflected in the observed increase in the proline level provides further biochemical evidence of differentiation in cultures grown in homologous medium, since collagen synthesis during osteogenesis has been considered as a specific activity of the osteoblasts involved in the formation of the organic components of the intercellular substance of bone. However, as collagen is a component of the intercellular substance of cartilage and other forms of connective tissue, the rise in the collagen in this case might be related to the formation of intercellular substance of cartilage or of pre-osseous tissue. Nevertheless, since the rise occurred at a late stage of cultivation and did not coincide with the initial period of proliferation of the cartilage, or to the formation of the perichondral capsule, it is reasonable to suppose that the rise in the collagen content was related to the formation of the intercellular substance of pre-osseous tissue.

The initial decrease in the alkaline and acid phosphatase activity observed in almost all the cases was attributed to the coincident intense proliferative activity of the cultures. The absence of any initial decrease in proline may be ascribed to the production of collagen simultaneously with the proliferation of the cultures. On the other hand, it has been held that once collagen has been laid down its turnover is

very slow, a fact that may also explain the equilibrium of the collagen level during the early stages of cultivation.

The fairly stable collagen content of the epiphyseal cartilage cones *in vivo* throughout the embryonic and early post-embryonic development may have been due to the absence of any formation of intercellular substance of bone or cartilage.

The alkaline phosphatase activity rose and attained maximum intensity much earlier than the collagen content. The rise in the collagen level, however, occurred during the period of intense alkaline phosphatase activity. It would be hazardous on the basis of these findings to attempt to draw definite conclusions on the nature of the relationship between the activities of alkaline and acid phosphatase and the collagen content of the cultures, as no special study of this problem has been performed. However, the coincidence between certain morphologic and biochemical observations warrants discussion. The proliferation phase of the "differentiating" cultures was characterized by a decrease in the alkaline and acid phosphatase activities, while the collagen level remained stable. During the period of formation of the perichondral capsule and the differentiation of osteoblasts from its inner layer there was a steep rise in the alkaline phosphatase, while no significant change in the acid phosphatase and collagen was detectable. The formation of pre-osseous tissue coincided almost exactly with the rise in the collagen content and the acid phosphatase. During the same period the alkaline phosphatase remained at a very high level, but there was no evidence that differentiation of osteoblasts had ceased by this time. All the processes showed an almost simultaneous decline, which may be attributed to the limited osteogenetic capacity under the prevailing experimental conditions. The parallel between the alkaline phosphatase, acid phosphatase and collagen levels in "differentiating" cultures was not evident *in vivo*.

The study of the collagen content suggests the following conclusions.

(i) The rise in the collagen content observed in "differentiating" cultures during the period of cultivation may be regarded as further biochemical evidence that differentiation occurred in this group of cultures.

(ii) It is possible that small amounts of collagen were formed during the period of proliferation.

(iii) The rise in the collagen content occurred almost simultaneously with the rise in the acid phosphatase level and displayed a parallel development.

(iv) The rise in the collagen occurred during high alkaline phosphatase activity. However, the alkaline phosphatase rose long before the collagen.

(v) The variation in the collagen content in *in vivo* material bore no resemblance to that observed in "differentiating" cultures.

SULPHOMUCOPOLYSACCHARIDES

The sulphomucopolysaccharides, the sulphuric acid esters of the mucopolysaccharides, constitute an important group with a wide distribution in various organs and tissues. Compared with other tissues, connective tissue is rich in sulphomucopolysaccharides, which are believed to occur as protein-bound components of the ground substance. Sulphomucopolysaccharides play a dominant role in cartilage biology. Cartilage in fact represents the richest store of chondroitin sulphate in the body. On hydrolysis, chondroitin sulphuric acid yields galactosamine (chondrosamine) uronic acid, sulphate and acetic acid. Chondroitin sulphate is one of the main constituents of hyaline cartilage. Meyer^{102, 103} states that three different chondroitin sulphates, A, B, and C, could be identified, which could be distinguished by virtue of differences in their solubilities, optical rotation, digestability with hyaluronidases and their colour reactions. Chondroitin sulphates A and C are of interest in the study of cartilage or bone. The mucopolysaccharide pattern of growing bone is different from that of cartilage.¹⁰²

The importance of chondroitin sulphate in the biologic function of cartilage has been widely studied and divergent opinions are held on the role played by the chondroitin sulphate, and the sulphomucopolysaccharides in general, in osteogenesis. It has been held that the maintenance of a high level of the sulphate is a device by which cartilage is protected against calcification,⁶⁸ but it is doubtful whether this itself can be regarded as a protective mechanism; it is more probable that its removal is a pre-requisite for the action of the bone-forming osteoblasts. The concept of an antagonism between alkaline phosphatase and chondroitin sulphate finds support in the fact that depletion of chondroitin sulphate in cartilage precedes calcification, while a high alkaline phosphatase activity appears during ossification and mineralization of the ossifying cartilage.^{152, 153} A connection between, on the one hand, depletion of sulphate and, on the other, the formation of bone^{89, 152, 153} or calcification,^{38, 39, 30, 6, 2} has been suggested. On the other hand, chondroitin sulphate has been considered as largely responsible for the binding of calcium ions.¹⁸ Histochemical studies of developing teeth have revealed that mucopolysaccharides are widely distributed in several components of this organ. There was a gradual increase in the amount of mucopolysaccharide in functional ameloblasts and odontoblasts, and with the onset of calcification the dentine showed a corresponding increase in mucopolysaccharide.⁸

Experiments

The study of the sulphomucopolysaccharides consisted principally in the estimation of the mucopolysaccharide in various materials. However, as it is known that most inorganic ³⁵S sulphate introduced in experimental animals is incorporated in sulphomucopolysaccharides,^{38, 39, 12, 13} this isotope was used for examination of the synthesis of mucopolysaccharides in the material of the study.

QUANTITATIVE ESTIMATION

Estimation of mucopolysaccharide in the cartilage cultures was performed by the chromatographic method described by Gardell.⁵⁷ This is based on chromatographic determinations of the two amino-sugars, glucosamine and galactosamine.

The tissue samples, about 10 cultures for each determination, were freeze-dried, pulverized and further dried in vacuo over phosphorus pentoxide at 100°C. Samples of the dry powder were hydrolysed with 6 *N* hydrochloric acid for 8 hours on a boiling water bath. After hydrolysis, the mixture was placed in a small beaker and evaporated to dryness in a desiccator over sodium hydroxide and concentrated sulphuric acid. The residue was taken up in a suitable volume of 0.3 *N* hydrochloric acid and applied to the top of the ion exchange column. Thereafter the analysis was conducted as described by Gardell.

Quantitative determinations of the mucopolysaccharides were performed (i) on "differentiating" cultures (ii) on "degenerating" cultures and (iii) *in vivo*, the material in this case being obtained in the same way as for the corresponding determinations of the alkaline phosphatase.

UPTAKE OF ³⁵S

The method used for determining the incorporation of the radioactive inorganic sulphate (³⁵S) was that used by Boström,¹² with a modification of the ashing procedure.³³

A number of cultures, usually 4 to 10, of the same age were sliced finely by hand and the material was divided into two approximately equal parts. Each sample was placed in 4 ml sulphate-free Krebs-Ringer's solution containing 1–2 ml of a solution of inorganic ³⁵S-labelled sodium sulphate (2 μ C per 150–500 ml Krebs-Ringer). After incubation in a water bath for 3 to 6 hours at 37°C the tissue fragments were washed with distilled water and incubated overnight in saturated sodium sulphate solution at the same temperature. The samples were then dried, weighed and ignited. The ash was dissolved in 2.0 ml distilled water and plated. The count rate of the samples was then determined in a standard position under a thin-windowed Geiger-Müller tube. In each experiment 0-time-inhibited samples were used. Inhibition was performed by iodo-acetic acid.

Estimations of the uptake of ³⁵S-labelled sodium sulphate were performed

(i) on "differentiating" cultures, the determinations being performed on two series of cultures at weekly intervals and on cultures at daily intervals during the 1st week of cultivation;

(ii) on "degenerating" cultures; and

(iii) *in vivo*, the same material being used as for similar determinations of the alkaline phosphatase activity.

As the radioactive solutions and incubation times in the experiments were not identical, the results for the various series are not quantitatively comparable.

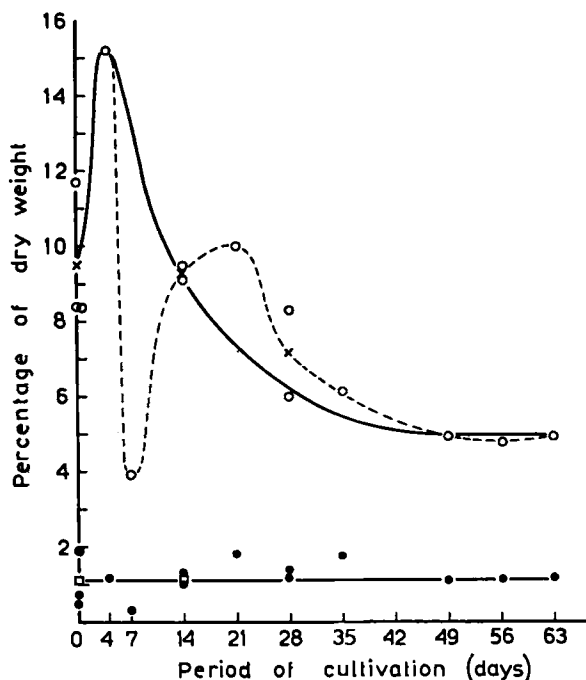


FIG. 41. Galactosamine (upper curves) and glucosamine (lower curve) content of cultures grown in homologous medium with fresh embryo extract and normal Tyrode's solution.*

Results

MUCOPOLYSACCHARIDES

(i) The galactosamine (chondroitin sulphate) content of cultures grown in homologous medium was studied at weekly intervals until the 9th week of cultivation, with one determination on the 4th day. The curve compiled from the values obtained at various stages of cultivation showed that the epiphyseal cartilage cones in 12-day embryos, 0-day samples, were rich in galactosamine, which constituted about 10 per cent of their dry weight. There was a pronounced rise in the galactosamine on the 4th day, followed by a steep fall to the end of the 1st week, which then gradually leveled out. This fall was ascribed to erroneous determination, as such a low value of the galactosamine was never observed.

The glucosamine level was fairly constant up to the 63rd day of cultivation, and much lower than that of the galactosamine, even initially (Fig. 41).

(ii) Estimation of the mucopolysaccharides in cultures grown in heterologous medium was performed at 10-day intervals until the 70th day of cultivation. The variation in the galactosamine and the glucosamine levels in this group of cultures

* The galactosamine and glucosamine were determined as the hydrochloride, dry substance.

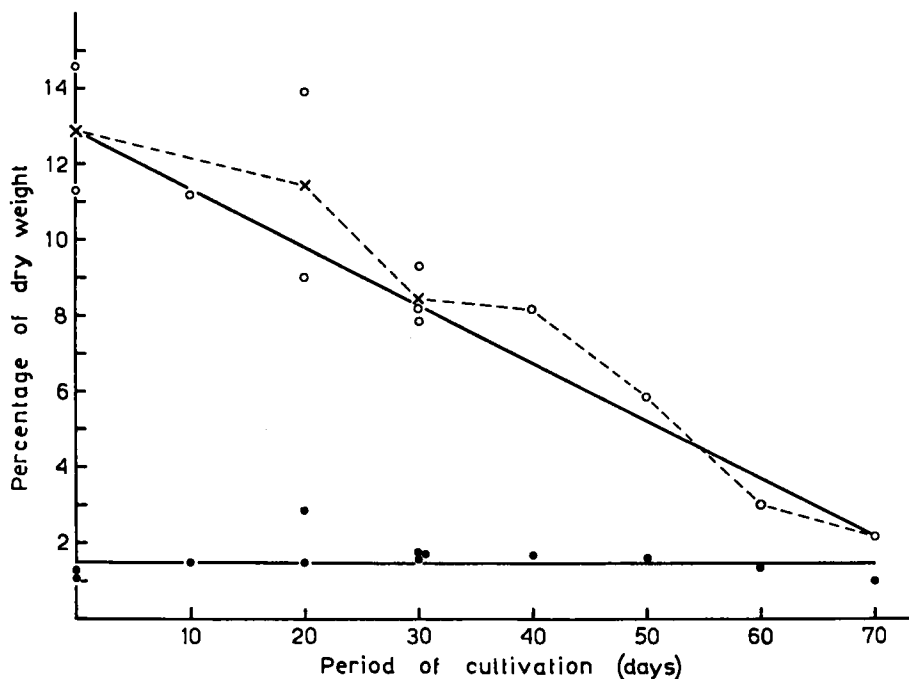


FIG. 42. Galactosamine (*upper curves*) and glucosamine (*lower curve*) content of cultures grown in heterologous medium with filtered embryo extract and calcium-enriched Tyrode's solution. The twenty-day values are based on infected material.

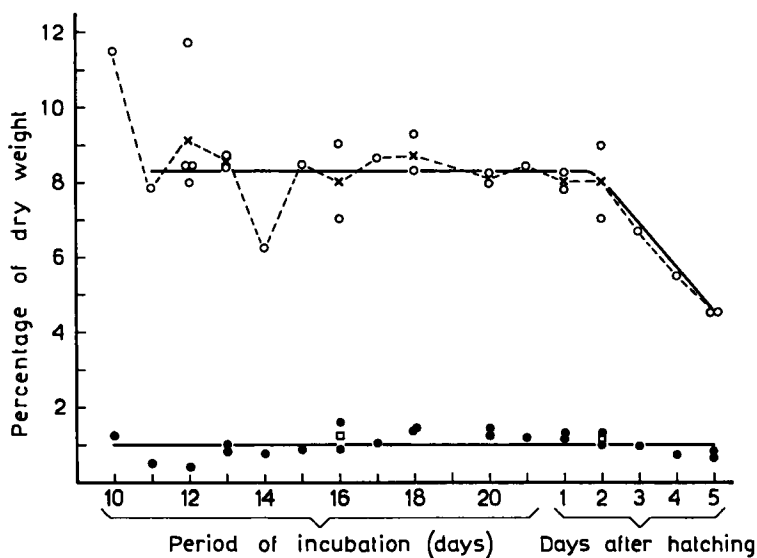


FIG. 43. Galactosamine (*upper curves*) and glucosamine (*lower curve*) content *in vivo*.

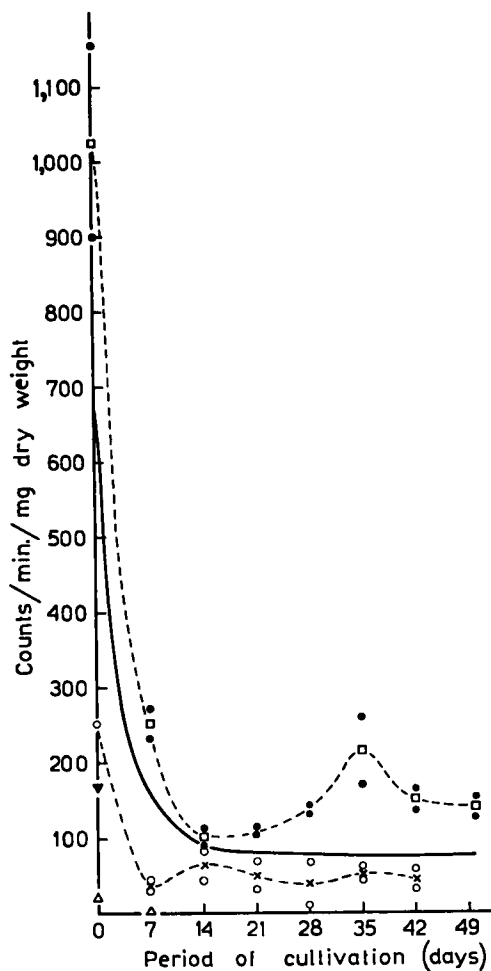


FIG. 44. Uptake of ^{35}S by cultures grown in homologous medium with fresh embryo extract and normal Tyrode's solution; two series of experiments. $\triangle$ and $\blacktriangledown$ denote the values of 0-time-inhibited samples.

was almost identical with that for the "differentiating" cultures, galactosamine showing a gradual decrease, whereas the glucosamine was constant throughout the period of cultivation (Fig. 42).

(iii) The mucopolysaccharide content *in vivo* showed that there was a stabilization of the galactosamine values from the 11th day of incubation to the 2nd day after hatching, when a progressive decrease occurred. The glucosamine content of the samples remained almost constant throughout the period of observation (Fig. 43).

UPTAKE OF ^{35}S

(i) In the "differentiating" cultures a high initial uptake of ^{35}S was recorded for 0-day samples. This was followed by a steep fall until the end of the 1st week, and

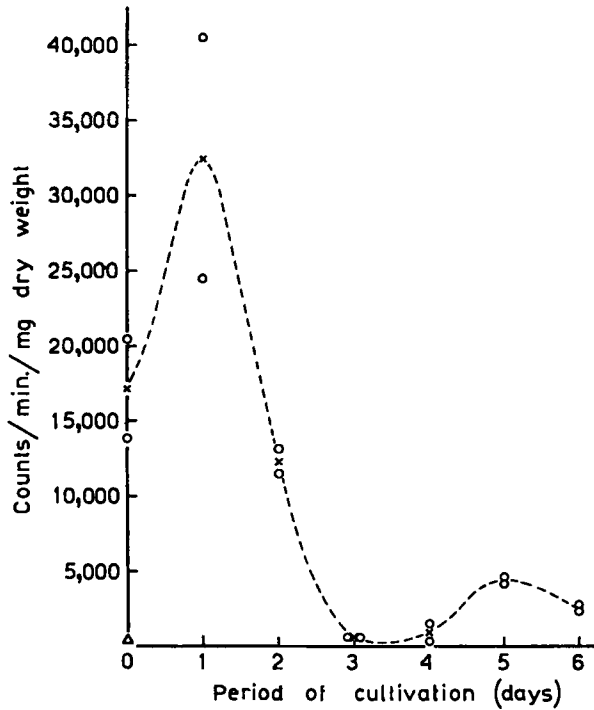


FIG. 45. Uptake of ^{35}S during the first week of cultivation by cultures grown in homologous medium with fresh embryo extract and normal Tyrode's solution. $\triangle$ denotes a value of the 0-time-inhibited samples.

from then onwards there was only a very low uptake, the values remaining at about the same level as those of the 0-time inhibited samples (Fig. 44). A third series of determinations was performed in order to study the steep decrease in the uptake of ^{35}S during the 1st week. Thus, at the end of the 24th hour of cultivation there was a significant rise in uptake, followed by a steep decrease, a very low value being recorded from the 3rd to the 6th day of cultivation. In this experiment, too, the values were approximating to those for the 0-time inhibited samples (Fig. 45).

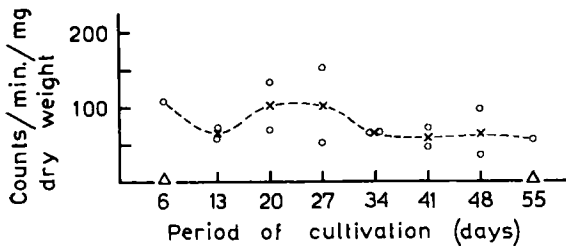


FIG. 46. Uptake of ^{35}S by cultures grown in heterologous medium with filtered embryo extract and calcium-enriched Tyrode's solution. $\triangle$ denotes the values of the 0-time-inhibited samples.

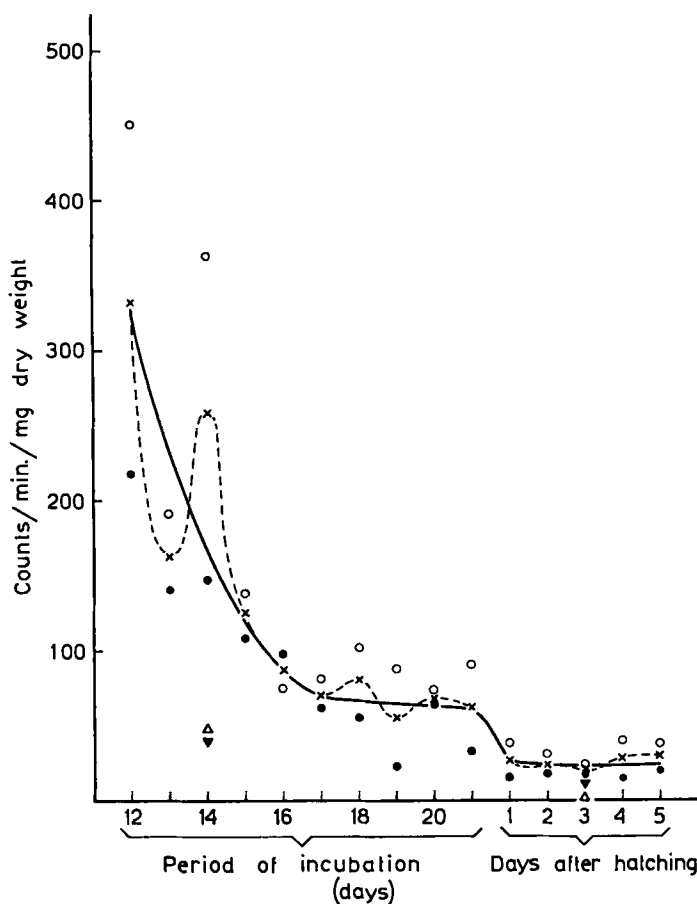


FIG. 47. Uptake of ^{35}S *in vivo*. Two readings were performed on each sample with a 3-hour interval. Δ and ∇ denote the values of the 0-time-inhibited samples.

(ii) Weekly determination of the uptake of ^{35}S by “degenerating” cultures of various periods of cultivation from the 6th up to the 55th day showed constant values but here the level was appreciably higher than that for the 0-time inhibited samples. In contrast to the case of the “differentiating” material, no 0-day samples were used here (Fig. 46).

(iii) *In vivo* determinations of the uptake of ^{35}S showed that the rate of the uptake of ^{35}S by the epiphyseal cartilage fell steeply between the 12th and the 17th day of incubation, after which fairly constant values were recorded until hatching. On the 1st day there was a new decrease, followed by a period of low constant values to the 5th day (Fig. 47).

Discussion

As the main polysaccharide in cartilage is chondroitin sulphuric acid, this is represented in the samples by galactosamine. The presence of glucosamine in cartilage indicates the existence of hyaluronic acid, keratosulphate, heparine, neutral glucoproteins or a mixture of these components.

The estimation of mucopolysaccharides revealed a similar variation in the level in "differentiating" and "degenerating" cultures. There was a gradual decrease in the amount of galactosamine, and hence of chondroitin sulphate, throughout cultivation, whereas the glucosamine level remained stable. In 0-day samples and during the early stage of cultivation, galactosamine was by far the more abundant of the two components, but there followed a gradual change in the ratio until they were present in approximately equal quantities. However the decrease in the galactosamine may have been only apparent. As this was expressed as a percentage of the dry weight, the presence of calcium salts or organic substances would influence these values. Little being known of the role of these amino-sugars in the biology of the cartilage, it is difficult to conceive the significance of the variations in the level. However, there was evidence that there was no synthesis of chondroitin sulphate throughout the period of cultivation except for an initial increase in the galactosamine on the 4th day of cultivation in cultures grown in homologous medium.

Even during the 1st week the rate of uptake of ^{35}S by cultures grown in homologous medium showed a steep decrease, the very low subsequent values still being slightly higher than in the 0-time inhibited samples. For cultures grown in heterologous medium no ^{35}S estimation was performed on 0-day samples, but as the values were found to be constant at a low level between the 6th and the 55th day of cultivation, a higher rate of uptake of ^{35}S could be expected in 0-day samples than was the case in differentiating cultures.

Parallel with the increase in the chondroitin sulphate there was a marked rise in the uptake of ^{35}S during the early stage of cultivation. However, as chondroitin sulphate was not determined more than once during the first week of cultivation (on the 4th day) it is difficult to compare the rise in the two cases. However, the increase in the rate of uptake seemed to occur slightly earlier than the increase in the chondroitin sulphate, since by the time the chondroitin sulphate level had reached its maximum the ^{35}S had already fallen to its minimum. The initial rise in the uptake of ^{35}S by cartilage *in vitro* has been observed earlier under different conditions. Boström* noted that when articular cartilage of young calves was stored over-night in a refrigerator it showed a higher uptake of ^{35}S than before. However, as both this and the chondroitin sulphate content were higher during the early stage of cultivation, and as there was apparently an intense proliferation of the cartilage explants at the same time, the increase in chondroitin sulphate and uptake of ^{35}S might be

* Personal communication.

considered as related to the production of new cartilage tissue in the cultures. On the other hand the high rate of incorporation of ^{35}S on the first day of cultivation is not compatible with the view that, at least during the first 24 hours of cultivation, only a flattening of the cultures occurs, proliferation beginning somewhat later. Moreover, the short-lived rise in chondroitin sulphate might be considered to conflict with the view that the sulphate content is related to proliferation; hence this process has been assumed to continue to about the end of the 2nd week of cultivation. In the morphologic study the frequency of metachromatic staining differed for the various groups of sectioned cultures. However, in no case were there reliable signs of metachromatic staining in sectioned control specimens. Furthermore, in the sectioned cultures of all the groups metachromatic staining did not appear until the 3rd week of cultivation. On the other hand, as pointed out above, the highest level in sulphomucopolysaccharides was always found in 0-day samples. It is widely accepted that metachromasia is related to the presence of sulphomucopolysaccharides in the metachromatically stained tissues. In the light of the results obtained in the present investigation it is doubtful whether this is, in fact, the case. However, as the sectioned specimens were fixed in formaldehyde before sectioning and staining and as this substance dissolves carbohydrates, it is unjustified to draw conclusions on this point.

The study of the sulphomucopolysaccharides suggests the following conclusions.

- (i) The sulphomucopolysaccharide content of "differentiating" and "degenerating" cultures and *in vivo* material decreased gradually with age.
- (ii) Synthesis of such substances occurred in the "differentiating" cultures during the earliest stage of cultivation, when there was proliferation. Thereafter, there was little if any synthesis.
- (iii) Mucopolysaccharides were not involved to any appreciable degree in the process of differentiation in the cultures.

GENERAL DISCUSSION

The osteogenetic capacity of the isolated embryonic epiphyseal cartilaginous cones of the 12-day chick was studied in tissue cultures. In one group of cultures grown in homologous medium with fresh embryo extract there was morphologic evidence of differentiation and of formation of pre-osseous tissue with the appearance of uncalcified, immature bone. This differentiation was influenced by the histologic composition of the cultures, those having no flattened chondrocytes failing to display differentiation. The differentiation was also influenced by the composition of the culture medium. Cultures grown in either heterologous medium or in homologous medium but treated with filtered embryo extract, displayed no formation of pre-osseous tissue. It was therefore inferred that differentiation depended on the presence of flattened chondrocytes in the explants and on some properties of the homologous medium and the freshly prepared embryo extract that promote differentiation.

Differentiation was preceded by an initial period during which the cultures displayed proliferation, this being manifested as an increase in their initial area. It appeared that the proliferation was inhibited at the time differentiation began; this confirms the conception of an antagonism between these processes.

In a further stage of cultivation a perichondral capsule was formed, which gradually enclosed the culture. From the cells of the inner layer of the capsule osteoblasts differentiated, these in turn forming the pre-osseous tissue, which must therefore be regarded as periosteal in type.

The formation of the perichondral capsule was consistently found to coincide with the migratory activity of the cells, from which it may be inferred that these cells were responsible for the formation of the capsule.

As adherent perichondral remnants were found on a high percentage of sectioned control specimens and as attempts to obtain cultures free from such remnants were unsuccessful, no definite conclusions can be drawn as to the origin of the wandering cells and hence of the formation of the perichondral capsule. This might occur either from the perichondral remnants or from the cells of the original explant. The former possibility is unlikely. Moreover, as the formation of the capsule was characteristic of cultures containing flattened chondrocytes, these might be considered responsible for the formation of the capsule. The transformation of the flattened cells into cells of the perichondral capsule might occur in one of two ways—viz., either by modulation of these chondrocytes under the prevailing experimental conditions or in the course of the normal function of the flattened cells, which contribute to the growth of the perichondrium.

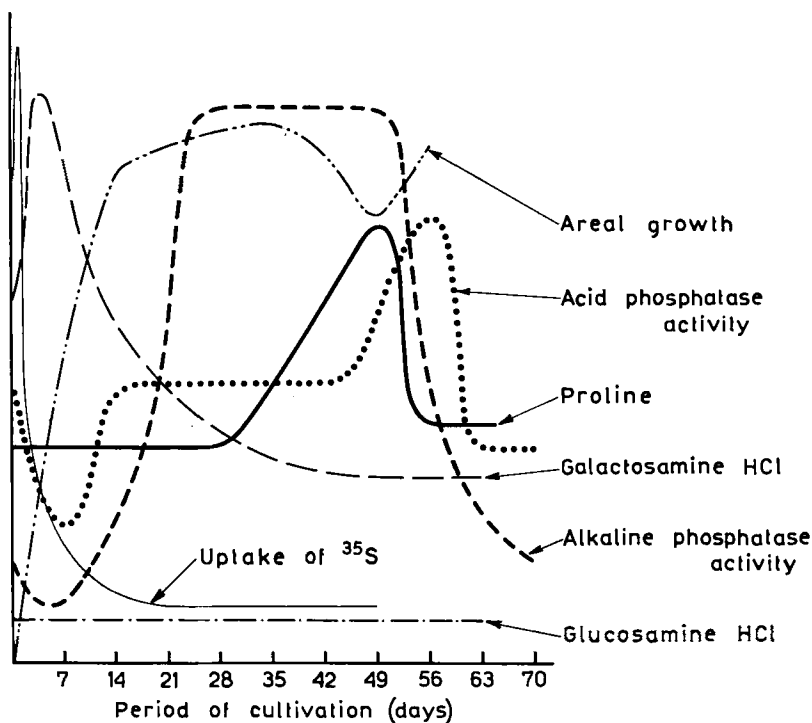


FIG. 48. Differentiating cultures. Each curve has a different ordinate scale, for which see the respective individual figures.

The morphologic study showed that no mature bone was formed in the cultures. This, however, proved an advantage in that the early stages of osteogenesis could be studied independently of mineralization by the applied method.

As the criteria for the interpretation of the various morphologic changes were highly subjective, objective support was sought for the occurrence of differentiation by studying various enzymes and chemical substances known to be involved in osteogenesis. Estimation of the alkaline phosphatase revealed a marked increase in the activity of this enzyme during the period of cultivation. The acid phosphatase activity also increased, the rise occurring here later than in the case of the alkaline phosphatase. Moreover, an appreciable increase in the amount of collagen was observed at later stages. It was inferred from the biochemical study that the increase in the alkaline and acid phosphatase activities and in the collagen content provided objective evidence of differentiation in cultures grown in homologous medium with fresh embryo extract.

The course of events in "differentiating" cultures (surveyed in Fig. 48) may be summarized as follows. During the earliest stage of cultivation there is a re-arrange-

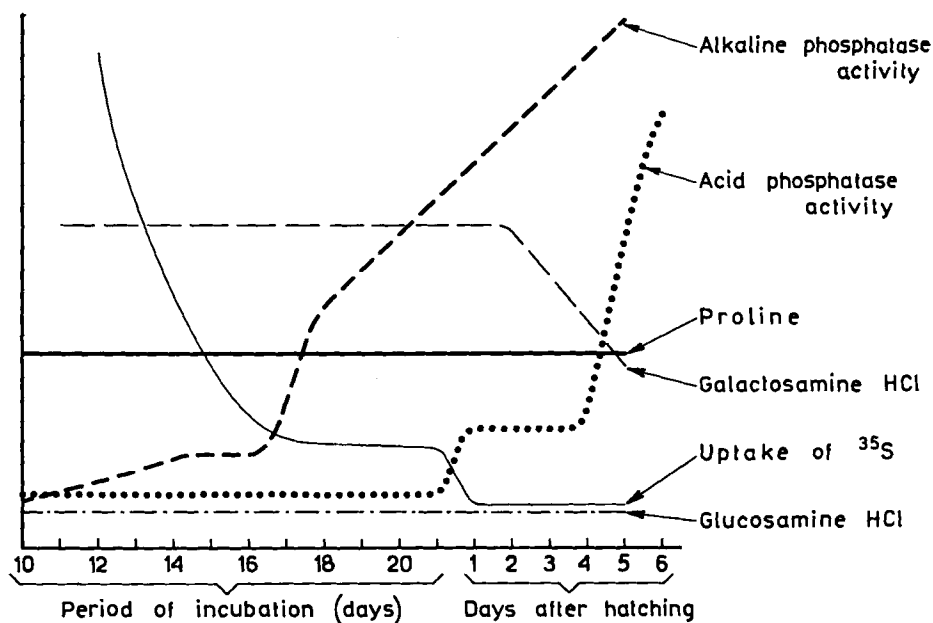


FIG. 49. Material *in vivo*. Each curve has a different ordinate scale, for which see the respective individual figures.

ment of the cells, followed immediately by intense proliferation; these phenomena are manifested as an increase in the initial area of the culture. During this period there is only a brief increase in the chondroitin sulphate content. At the end of the phase of proliferation flattened chondrocytes display a migratory tendency and form a perichondral layer, which encloses the culture. At the same time differentiation begins, characterized by the appearance of osteoblasts from the inner layer of the perichondral capsule. There is simultaneously a steep rise in the alkaline phosphatase activity, the maximum probably coinciding with the increase in the differentiating activity of the osteoblasts. At the end of the period of high alkaline phosphatase activity and before any decline is evident there is an increase in the collagen content, with an almost simultaneous rise in the acid phosphatase activity. During this phase, there appears to be an elaboration of the ground substance as the formation of the pre-osseous tissue is completed. However, owing, probably to the limited osteogenetic capacity of the cultures under the prevailing experimental conditions, all activities then showed a steep decline.

The results obtained from *in vivo* biochemical studies (surveyed in Fig. 49) show that the alkaline and acid phosphatase activities are constant during a certain period of embryonic development. A rise follows at later stages of incubation and during the early part of the post-embryonic period. The course of the activities of

these enzymes may be a manifestation of the advance of a certain process of osteogenesis at various stages of embryonic and early post-embryonic development at the expense of another process, this resulting to the disturbance of a state of equilibrium. The collagen content remains fairly constant throughout the period of observation, while the chondroitin sulphate content is stable until the 2nd day after hatching, when there is a decline. Although in the course of the various biochemical studies there was a similarity between "differentiating" cultures and material *in vivo*, no conclusions can be drawn as it is doubtful whether the materials are comparable.

SUMMARY

1. A morphologic and biochemical investigation has been carried out in order to ascertain whether epiphyseal cartilaginous cones from chick embryos differentiate and form bone when grown as tissue cultures. The material was obtained from the long bones of the leg of 12-day embryos. The original "watch-glass" tissue culture method described by Fell and Robison⁴⁶ was followed in the majority of the cases, a static, homologous medium being used. In some other cases, however, static heterologous medium was used, as described by Chen.²⁵ Altogether 4523 explants were cultivated for periods of up to 10 weeks.

2. Two main groups of cultures could be recognized; the first consisted of cultures grown in homologous medium with freshly prepared embryo extract, and the second group, of cultures generally grown in heterologous medium. In both groups an increase in the areal growth was demonstrated within the first 3 weeks of cultivation, this being followed in the former group of cultures by a moderate rise and in the latter group by a decrease in area. Moreover, the cultures displayed a migratory activity of the cells. The rate of the areal growth and the migratory activity of the cells was higher in the cultures of the first group. This group exhibited morphologic evidence of differentiation. In the second group, on the other hand, there were morphologic signs of degeneration.

Differentiation depended on the histologic composition of the explants and the composition of the culture medium. Cultures containing no flattened cells displayed no differentiation. Those grown in heterologous medium showed only initial differentiation.

3. The first morphologic change seen in sectioned cultures comprised the formation of a perichondral capsule, which enclosed the culture. The cells forming this capsule were apparently derived from flattened chondrocytes that had moved out into the medium as wandering cells. This capsule later played the role of a true perichondrium, and osteoblasts were differentiated from the cells of its inner layer. These subsequently produced a pre-osseous tissue, which in spite of prolonged cultivation did not mineralize and form mature bone. Calcification of a degenerative nature was found in 30 per cent of the "degenerating" and in a few "differentiating" cultures. The X-ray diffraction pattern of one of the cultures of the former group revealed the presence of non-oriented hydroxyapatite crystallites.

4. The alkaline phosphatase activity was studied by quantitative and histochemical methods in cultures, and quantitatively in embryonic material *in vivo*. In "differentiating" cultures an initial period of low enzymatic activity was observed,

which corresponded to a period of intense proliferation. This was followed by an increase in activity to a maximum, after which a period of constant values led to a steep decline.

During the whole period of high activity no mineralization of the pre-osseous tissue occurred. There was a parallel between the curves of the enzymatic activity in "differentiating" cultures and *in vivo* material.

"Degenerating" cultures, on the other hand, displayed a low alkaline phosphatase activity in the early stages of cultivation. In a high percentage of this group of cultures calcification of a degenerative nature was observed, usually at a late stage of cultivation and in the absence of any detectable signs of enzymatic activity.

Determinations of the alkaline phosphatase by histochemical methods showed that the highest enzymatic activity was located in the region of the osteoblasts and at places where formation of pre-osseous tissue was observed. An intense activity was also present in hypertrophied chondrocytes. No apparent connection between these two manifestations of the enzymatic activity could be assumed, though they occurred simultaneously.

5. The acid phosphatase activity was also studied by quantitative methods in cultures and *in vivo*. This enzyme showed a significant rise in activity in the "differentiating" cultures at a later stage of cultivation. "Degenerating" cultures displayed no significant changes in the acid phosphatase activity. There was a similarity between the curves of the enzymatic activity for "differentiating" cultures and *in vivo* material.

6. Since no traces of phenolsulphatase activity could be detected in epiphyseal cones from 12-day chick embryos and "differentiating" cultures taken at random, it was inferred that this enzyme did not participate in the process of osteogenesis.

7. An increase in the collagen content of "differentiating" cultures was established during cultivation. This variation was strikingly parallel to that of the acid phosphatase activity, a finding that suggests a relationship between the two properties. The collagen content *in vivo* was constant throughout the period of observation.

8. Estimations of the chondroitin sulphate content and studies of the synthesis of this substance by means of ^{35}S -labelled sodium sulphate showed a decrease in the chondroitin sulphate content and a very low ^{35}S uptake in "differentiating" and "degenerating" cultures and *in vivo* material throughout the period of observation. A short-lived increase in the chondroitin sulphate was present only during the initial stage of cultivation. These findings suggest that chondroitin sulphate is of little, if any, significance in osteogenesis.

REFERENCES

1. ABOTT, L. D., and EAST, M. K.: *Fed. Proc.*, 8: 178, 1949.
2. AMPRINO, R.: In *Ciba Foundation Symposium on Bone Structure and Metabolism*, J. & A. Churchill Ltd., London, 1956.
3. BAITSSELL, R.: *Jour. Exptl. Med.* 21: 455, 1915.
4. BÁNHIDI, Z. G., SJÖSTRÖM, A. G. M., GRÖMMER, S., and LUNDIN, H.: *Arkiv f. Kemi*, 5: 393, 1953.
5. BEAR, R. S.: *Advances in Protein Chem.* 7: 69, 1952.
6. BELANGER, L. F.: *Canad. Jour. Biochem. Phys.*, 32: 161, 1954.
7. BEVELANDER, G., and JOHNSON, P. L.: *Anat. Rec.*, 108: 1, 1950.
8. ——— *Jour. Dent. Res.*, 34: 123, 1955.
9. BINKLEY, F., SHANK, R. E., and HOAGLAND, C. L.: *Jour. Biol. Chem.*, 156: 253, 1944.
10. BODANSKY, A., BAKWIN, R. M., and BAKWIN, H.: *Jour. Biol. Chem.*, 94: 551, 1931.
11. ——— *Jour. Biol. Chem.*, 118: 341, 1937.
12. BOSTRÖM, H.: *Jour. Biol. Chem.*, 196: 477, 1952.
13. ——— *Arkiv f. Kemi*, 6: 43, 1953.
14. BOURNE, G. H.: *Quart. Jour. Exptl. Physiol.*, 32: 1, 1943.
15. ——— *Jour. Anat.*, 82: 81, 1948.
16. ——— *The Biochemistry and Physiology of Bone*, Academic Press Inc., N.Y., chap. IX, p. 251, 1956.
17. ——— *Ibid.*, chapter XVIII, p. 539, 1956.
18. BOYD, E. S., and NEUMAN, W. F.: *Jour. Biol. Chem.*, 193: 243, 1951.
19. BRACHET, A.: *Intern. Monatschr. Anat. Physiol.*, Leipzig, 10: 391, 1893.
20. BRADFIELD, R. G.: *Nature*, 157: 876, 1946.
21. CARELL, A., and BURROWS, M. T.: *J. A.M.A.*, 55: 1379, 1910.
22. CAREY, E. J., ZEIT, W., and MCGRATH, B. F.: *Amer. Jour. Anat.*, 40: 127, 1927.
23. CASTELLÁ, B. B.: *Annal. Facult. Veter. Madrid*, 4: 402, 1952.
24. CENDRANGULO, F., and RUFFO, A.: *Arc. Sci. Biol.*, Italy, 24: 59, 1938.
25. CHEN, J. M.: *Exptl. Cell Res.*, 7: 521, 1954.
26. CHÉVREMONT, M.: *Cytologie et Histologie*, édit. Desoer, Liège, 1956.
27. DAHL, B.: *Skrift. Norske Vidensk. Akad. Oslo, Mat. Naturv. Kl.*, 1936.
28. DANIELLI, J. F., FELL, H. B., and CODICEK, E.: *Brit. Jour. Exptl. Pathol.*, 24: 196, 1943.
29. DAVIES, D. R.: *Biochem. Jour.*, 28: 529, 1934.
30. DAVIES, D. V., and YOUNG, L.: *Brit. Jour. Anat.*, 88: 174, 1954.
31. DERRIEN, M.: *Bull. Soc. Chim. France*, 9: 110, 1911.
32. DIXON, W. J., and MASSEY, F. J.: *Introduction to Statistical Analysis*, McGraw-Hill Book Co., Inc., N.Y., 1954.
33. DOHLMAN, C. H.: *Arkiv f. Kemi*, 11: 255, 1957.
34. DOLJANSKI, K.: *Zeitschr. Zellforsch. mikr. Anat.*, 8: 789, 1928/29.
35. DOLJANSKI, L., and ROULET, F.: *Virchows Arch. Pathol. Anat. Physiol. klin. Med.*, 291: 260, 1933.

36. DUNN, M. S., MCCLURE, L. E., and MERRIFIELD, R. B.: *Jour. Biol. Chem.* 179: 11, 1949.
37. DUNPHY, J. E., and UDUPA, K. N.: *New England Jour. Med.*, 253: 847, 1955.
38. DZIEVIATKOWSKI, D. D.: *Jour. Exptl. Med.*, 93: 451, 1951.
39. ——— *Ibid.*, 99: 283, 1954.
40. EASTOE, J. E.: In Bourne's *Biochemistry and Physiology of Bone*, Chap. IV, p. 81, 1956.
41. EBELING, A. H.: *Jour. Exptl. Med.*, 34: 231, 1921.
42. ENGEL, M. B., and FURUTA, W.: *Proc. Soc. Exptl. Biol. Med.*, 50: 5, 1942.
- 42a. ENGSTRÖM, A.: In *Ciba Foundation's Symposium on Bone Structure and Metabolism*, p. 1, 1956.
43. FEIGIN, I., WOLF, A., and KABAT, E. A.: *Fed. Proc.*, 8: 232, 1949.
44. FELL, H. B.: *Jour. Morphol. and Physiol.*, 40: 417, 1925.
45. ——— *Arch. exptl. Zellforsch.*, 7: 390, 1928/29.
46. FELL, H. B., and ROBISON, R.: *Biochem. Jour.*, 23: 767, 1929.
47. ——— *Ibid.*, 24: 1905, 1930.
48. FELL, H. B.: *Arch. exptl. Zellforsch.*, 11: 245, 1931.
49. ——— *Jour. Anat.*, 66: 157, 1931/32.
50. ——— *Proc. Roy. Soc., London, Ser. B.*, 112: 417, 1932/33.
51. FELL, H. B., and DANIELLI, J. F.: *Brit. Jour. Exptl. Pathol.*, 24: 196, 1943.
52. FELL, H. B., and MELLANBY, E.: *Br. Med. Jour.*, 1950/II: 535, 1950.
53. FOLLIS, R. G. Jr.: In *Ciba Foundation Symposium on Bone Structure and Metabolism*, p. 63, J. & A. Churchill Ltd., London, 1956.
54. FROMAGEOT, G.: In J. Summer and K. Myrbäck's *The Enzymes*, vol. I, part I, Academic Press Inc., N.Y., 1950.
55. GAILLARD, P. J.: *Protoplasma*, 23: 145, 1935.
56. ——— In G. Bourne and J. Danielli's *International Review of Cytology*, 2: 331, 1953.
57. GARDELL, S.: *Acta Chem. Scand.*, 7: 207, 1953.
58. GARDNER, E.: In G. Bourne's *The Biochemistry and Physiology of Bone*, chap. XIII, p. 359, 1956.
59. GLICK, D.: *Technique of Histo- and Cytochemistry*, Interscience Publ. Inc., N.Y., 1949.
60. GOMORI, G.: *Proc. Soc. Exptl. Biol. and Med.*, 42: 23, 1939.
61. GUSTAFSON, T., and HASSELBERG, I.: *Exptl. Cell Res.*, 1: 371, 1950.
62. ——— *Ibid.*, 2: 643, 1951.
63. GUSTAVSON, K. H.: *Advances in Protein Chem.*, 5: 353, 1949.
64. ——— *The Chemistry and Reactivity of Collagen*, Academic Press Inc., N.Y., 1956.
65. GUTMAN, A. B.: *Trans. 2nd Conf. Metab. Interrel.*, Josiah Macy Jr., Found., p. 167, 1950.
66. HAM, A. W.: *Histology*, 2nd edit., J. B. Lippincott Co., Philadelphia 1953.
67. HARRIS, H. A.: *Bone Growth in Health and Disease*, Oxford Med. Publ., London, 1933.
68. HASS, G. M.: *Arch. Pathol.*, 35: 275, 1943.
69. HENRIKSEN, E.: *Exptl. Cell Res.*, 11: 115, 1956.
70. ——— *Ibid.*, 11: 403, 1956.
71. HOMMERBERG, C.: *Zeit. physiol. Chem.*, 200: 69, 1931.
72. HUGGINS, C. B.: *Biochem. Jour.*, 25: 728, 1931.
73. HUGGINS, C. B., and SMITH, D. R.: *Jour. Biol. Chem.*, 170: 391, 1947.

74. HÄGGQVIST, G.: *Acta Chir. Scandin.*, 65: 180, 1929.
75. ——— *Nordisk Medicin*, 13: 72, 1937.
76. ITO, Y., and ENDO, H.: *Endocrinologia Japonica*, 3: 106, 1956.
77. JACOBSON, W.: In *Randall's Nature and Structure of Collagen*, Butterworths Scient. Publ., London, 1953.
78. JOHNSON, A.: *Quart. Jour. Microsc. Science* 23: 399, 1883.
79. JEPSON, J. B., and SMITH, I.: *Nature*, 142: 1100, 1953.
80. KABAT, E. A., and FURTH, J.: *Am. Jour. Path.*, 17: 303, 1941.
81. KATE, T. C.: *Arch. Néerland. Zool.*, 10 (suppl. 1): 108, 1953.
82. KAY, H. D.: *Physiol. Rev.*, 12: 384, 1932.
83. KING, E. J., and ARMSTRONG, A. R.: *Canad. Med. Assoc. Jour.*, 70: 376, 1934.
84. KRASSOWSKAYA, O. W.: *Cytologia*, 6: 217, 1934.
85. LACROIX, P.: *L'organisation des os*, Edit. Desoer, Liège, 1949.
86. LANGENSKIÖLD, F.: *Acta Chir. Scandin.*, 95: 367, 1947.
87. LEWIS, M. R.: *Contr. Embryol. Carneg. Inst.*, 6: 45, 1917.
88. LILLIE, F. R., and HAMILTON, H. L.: *Development of the Chick*, H. Holt and Co., N.Y., 3rd edit., 1952.
89. LOGAN, M. A.: *Jour. Biol. Chem.*, 110: 375, 1935.
90. LORCH, I. J.: *Quart. Jour. Microsc. Sci.*, 90: 381, 1949.
91. LUBOSCH, W.: *Gegenbaurs Morphol. Jahrb.*, p. 49, 1924.
92. MACEWEN, W.: *The Growth and Shedding of the Antler of the Deer*, Glasgow, 1920.
93. MALLORY, F. B.: *Jour. Med. Res.*, 10: 334, 1903.
94. MAINLAND, D.: *Canad. Jour. Res.*, 22E: 1, 1948.
95. MAXIMOW, A.: *Zeit. mikr. Anat. Forsch.*, 17: 625, 1929.
96. MAXIMOW, A., and BLOOM, W.: *A Textbook of Histology*, Saunders Co., Philadelphia and London, 1946.
97. MCFARLANE, W. D., and GUEST, G. H.: *Canad. Jour. Res.*, 17B: 139, 1939.
98. MCKINNEY, R.: *Arch. exptl. Zellforsch.*, 9: 14, 1929.
99. McLEAN, F. C., and BLOOM, W.: *Anat. Rec.*, 78: 333, 1940.
100. McLEAN, COMPERE, E. L., CAREY, E. J., and BENNETT, G. A.: In *Lectures on Regional Orthopaedic Surgery and Fundamental Orthopaedic Problems*, J. W. Edwards, Ann Arbor, Mich., p. 110, 1946.
101. McLEAN, F. C., and URIST, M. R.: *Bone, an Introduction to the Pathology of Skeletal Tissue*, Univers. of Chicago Press, 1955.
102. MEYER, K.: In *Ciba Foundation's Symposium on Bone Structure and Metabolism*, J. & A. Churchill Ltd., London, p. 65, 1956.
103. ——— In the *Harvey Lectures*, series 51, p. 88, Academic Press Inc., N.Y., 1957.
104. MISZURSKI, B.: *Arch. exptl. Zellforsch.*, 23: 80, 1939.
105. MIYAZAKI, Y., KATSUTA, H., AOYAMA, Y., ENDO, H., TAKAOKA, T., and OISHI, Y.: *Trans. Soc. Path. Japan.*, 43: 1, 1954.
106. ——— *Japan. Jour. Exptl. Med.*, 27: 331, 1957.
107. MOELLENDORF, W.: *Zeit. Zellforsch.*, 15: 131, 1932.
108. MOOG, F.: *Proc. Nation. Acad. Sci. U.S.*, 29: 176, 1943.
109. ——— *Biol. Bull.*, 86: 51, 1944.
110. ——— *Biol. Rev. Cambridge Phil. Soc.*, 21: 41, 1946.
111. ——— *Jour. Exptl. Zool.*, 115: 109, 1950.
112. MOMIGLIANA-LEVI, G.: *Zeit. Zellforsch.*, 16: 389, 1932.
113. MOSCONA, A.: *Exptl. Cell Res.*, 3: 535, 1952.
114. MURRAY, P. D. F., and HUXLEY, J. S.: *Jour. Anat.*, 59: 379, 1925.

115. NAGEOTTE, J., and GUYON, L.: *Comp. Rend. Assoc. Anat.*, 29: 408, 1934.
116. NEUBERG, C., and KURONO, K.: *Biochem. Zeit.*, 140: 295, 1923.
117. NEUBERG, C., and WAGNER, J.: *Ibid.*, 161: 492, 1925.
118. ——— *Zeit. ges. exptl. Med.*, 56: 334, 1927.
119. NEUMAN, R. E.: *Arch. Biochem.*, 24: 289, 1949.
- 119a. NEUMAN, R. E., and LOGAN, M. A.: *Jour. Biol. Chem.*, 186: 559, 1950.
120. NGUYEN VAN THOAI, G. I.: *Bull. Soc. Chim. Biol.*, 23: 1277, 1941.
121. OLIVO, O. M.: *Boll. Soc. Ital. Biol. Sper.*, 8: 589, 1933.
122. PAFF, G. H.: *Proc. Soc. Exptl. Biol. Med.*, 68: 288, 1948.
123. PARKER, R. C.: *Methods of Tissue Culture*, Cassel and Co Ltd., London 1950.
124. POLICARD, A., and BOUCHARLAT, M.: *Comp. Rend. Soc. Biol.*, 93: 26, 1925.
125. POLICARD, A., PÉHU, H., ROCHE, J., and BOUCAUMONT, J.: *Bull. Hist. Appl. Phys. Path. Techn. Microsc.*, 13: 645, 1931.
126. ——— *L'appareil de croissance des os longs*, Masson et Cie., Paris, 1941.
127. POMMER, G.: *Untersuchungen über Osteomalaciae und Rachitis*, Leipzig, 1885.
128. PORTER, K. R., and VANAMEE, P.: *Proc. Soc. Exptl. Biol.*, N.Y., 71: 513, 1949.
129. PRITCHARD, J. J.: In *Bourne's Biochemistry and Physiology of Bone*, chap. VII, p. 179, 1956.
130. QUERIDO, A., and GAILLARD, P. J.: *Acta Brev. Needer. Physiol. Pharmac. Microb.*, 9: 70, 1939.
131. RANDAL, J. T., FRAZER, R. D. B., JACKSON-FITTON, S., MARTIN, A. V. W., and NORTH, A. C. T.: *Nature*, 169: 1029, 1952.
132. RANVIER, L.: *Traité Technique d'Histologie*, 1^o édit., Paris, 1875.
133. RETTERER, E.: *Jour. d'Anat. Physiol.*, 42: 218, 1906.
134. ROBISON, R.: *Biochem. Jour.*, 16: 809, 1922.
135. ——— *Ibid.*, 17: 286, 1923.
136. ROBISON, R., and SOAMES, K. M.: *Ibid.*, 18: 740, 1924.
137. ——— *Ibid.*, 17: 847, 1926.
138. ——— *Biochem. Jour.*, 24: 1922, 1930.
139. ROBISON, R.: *Ergeb. Enzymforsch.*, 1: 280, 1932.
140. ROCHE, J., and MOURGUE, M.: *Compt. Rend. Acad. Scient.*, 214: 809, 1942.
141. ROCHE, J.: *Experientia*, 2: 325, 1946.
142. ——— In *Sumner and Myrbäck's The Enzymes*, vol. I, chap. 11, p. 473, Academic Press Inc., N.Y., 1950.
143. ROE, J. H., and KUETHER, C. A.: *Jour. Biol. Chem.*, 147: 399, 1943.
144. ROSENHEIM, A. H.: *Biochem. Jour.*, 28: 699 and 708, 1934.
145. ROSENFELD, L.: *Biochem. Zeit.*, 157: 434, 1925.
146. SEVASTIKOGLU, J.: *Exptl. Cell Res.*, 12: 80, 1957.
147. SODA, T.: *Jour. Fac. Sciences, Tokyo*, 3: 149, 1936.
148. STRANGEWAYS, T. S. P.: *Tissue Culture in Relation to Growth and Differentiation*, Heffer & Sons Ltd., Cambridge, 1924.
149. STRANGEWAYS, T. S. P., and FELL, H. B.: *Proc. Roy. Soc., London*, 99: 340, 1926.
150. STUDITSKY, A. N.: *Arch. f. exptl. Zellforsch.*, 13: 390, 1933.
151. SUMNER, J. B., and SOMERS, F. G.: *Chemistry and Methods of Enzymes*, 2nd edit., Academic Press Inc., N.Y., 1947.
152. SYLVÉN, B.: *Jour. Bone Joint Surg.*, 45: 745, 1947.
153. ——— *Ibid.*, 45: 973, 1947.
154. TODD, W.: *Jour. Anat. Physiol.*, 47: 177, 1913.

155. TONNA, E. A.: Amer. Acad. Orthop. Surg., Annual Meet., Prelim. Program, p. 28, 1957 (Abstract).
 156. TRISTRAM, C. R.: In H. Neurath and K. Bailey's *The Proteins*, vol. 1, Academic Press Inc., N.Y., 1953.
 157. VAN DER STRICHT, O.: Arch. de Biol., 10: 1, 1890.
 158. VIRCHOW, R.: Verhan. physikal. med. Gesellsch. Würzburg, 2: 150, 1851.
 159. WEINDENREICH, F.: In von Möllendorf's *Handbuch der mikroskopischen Anatomie des Menschen*, Springer, Berlin, 1928.
 160. WEINMANN, J. P., and SICHER, H.: *Bone and Bones*, 2nd edit., C. V. Mosby and Co., St. Louis, 1955.
 161. WIELAND, F.: Jahrb. Kinderheil., 70: 539, 1909.
 162. WILMER, E. N.: *Tissue Culture*, Methuen and Co., London, 1954.
 163. WILTON, Å.: *Tissue Reactions in Bone and Dentine*, H. Kimpton, London, 1937.
 164. ——— In *Nordisk Lærobok i Ortopedi*, in press.
 165. WJERESCHINSKY, A. O.: *Virchows Arch.*, 253: 592, 1924.
 166. WOLBACH, S. B.: *Am. Jour. Pathol.*, 9 (suppl.): 689, 1933.
 167. YOSHITOMI, M.: *Jour. Biochem.*, Tokyo, 21: 37, 1935.
 168. ZAAIJER, J. J. P.: *Embryonale pijpbeentjes van de mens in vitro Thesis*, E. J. Brill Publ., Leyden, 1953.
 169. ZAAIJER, J. J. P., and SAUER, E. K.: *Excerpta Med.*, 8: 403, 1954 (Abstract).
- MURRAY, M. R., and KOPECH, G.: *A Bibliography of the Research in Tissue Culture*, Acad. Press Inc., N.Y., 1953.
- SPENCER, M. C., and UHLER, K.: *The Structure Composition and Growth of Bone, 1930-1953, A Bibliography*, Washington, 1955.