S T U D I E S

IN

T I S S U E C U L T U R E.

by

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VOLUME I :- See Table of Contents.

VOLUME II :- Illustrations.

Throughout this thesis the word 'avian', unless where otherwise stated, means fowl: as, avian embryo extract = fowl embryo extract.

References are placed at the end of each main section.

ERRATUM.

Reference Drew, H.W., should be

Drew, G.H. J. Path. Bact., 1912-13, 17,581.

Reference. Incomplete

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TABLE OF CONTENTS.

Section I.

		Page
1.	Introduction	1.
2.	Definitions	2.
3.	History	5.
4.	Survival of Cells	9.
5.	Conditions of Growth in vitro	
	(a) Supporting Substance.	11.
	(b) Medium	14.
6.	Other Conditions necessary for Growth in vitro	29.
7.	Tissue Culture Methods	36.
8.	Explantation and Subculture.	51.
9.	Histological Examination of Tissue Cultures	74.
10.	Vital and Supravital Staining of Cells in vitro	87.
11.	References.	

Section II.

1.	Appearances seen during Cultivation of Tissue in vitro	88.
	1. Cell Migration	88.
	2. Cell Proliferation	97.
	3. Spreading out of Explant	99.
2.	General Characteristics of Cells Cultivated in vitro .	105.
3.	The Structure of Cells growing in vitro	121.
4.	Mitosis	126.
5.	Degenerative Changes in Cells in vitro	129.
6.	The Problem of the Differentiation of Cells and Tissues in vitro	133.
7.	Organotypic Growth	140.
8.	References.	

Section III.

Cultivation in vitro of Fragments of Tissues and Organs.

1.	Ectoderm	143.
2.	Amniotic Ectoderm	146.
3.	Skeletal Muscle	147.

TABLE OF CONTENTS.

Section III (cont.)

		Page
4.	Heart Muscle	157.
5.	Smooth Muscle	162.
6.	Cartilage	164.
7.	Kidney	183.
8.	Liver	195.
9.	Stomach	203.
10.	Intestine	204.
11.	Gall Bladder	207.
12.	Allantois	207.
13.	Spleen	211.
14.	Tumours	213.
15.	Pancreas	220.
16.	Lung	221.
17.	Thyroid	223.
18.	References at end of each section.	

Section IV.

Application of Tissue Culture Methods.

1.	The Action of a Cytotoxic Antiserum on Tissue Cultures	230.
2.	The Repair in vitro of Embryonic Skeletal Rudiments after Experimental Injury	279.
3.	The Development in vitro and in vivo of the Avian Patella.	321.
4.	The Formation of Haematoidin in vitro and in vivo	356.
5.	Cytological Changes following Mild Toxic Action in vitro with Special Reference to the Mitochondria .	366.
6.	References at end of each section.	

SECTION I.

INTRODUCTION.

While the importance of the cell as the unit of the tissues had been established by Schwann, Remak and Virchow, Bernard, (1878), appears to have been the first to insist on the necessity for the study of individual cells in the living state. His advocacy of observation and experimentation on isolated parts of the organism did not arise from any lack of appreciation of the co-ordination which exists between the various units of the body, but had its origin in a realisation of the difficulty of investigating the organism as a whole. He emphasised that although each unit has a certain autonomy in virtue of its differentiation, nevertheless the function of each is subservient to the whole, and no part ought to be conceived separately.

The teaching of Bernard has stimulated the search for methods by which the various properties of living protoplasm can be studied. The embryologists, particularly Roux, (1894), adopted early the method of observation and experimentation on living cells isolated from their normal surroundings, but the technique used was only applicable to the study of the early stages of the developing egg. Another method, pursued by Cohnheim, was the study of thin membranes which remained attached to the animal, such as the omentum or mesentery. Leo Loeb, (1902), embedded pieces of embryo guinea-pig skin in agar or

blood clot and implanted the mass into the base of the ear of other guinea-pigs. Until the work of Harrison, (1907), however, no satisfactory method had been obtained for the cultivation of vertebrate tissues outside the animal body so that these remained alive, and the component cells actually underwent multiplication.

DEFINITIONS. Tissue culture is the cultivation in vitro of parts (cells and tissues) of an organism which have been isolated from the whole. This definition of tissue culture, which is usually applied to the cultivation of metazoal tissues, refers also to plant tissues, which, however, proved much less suitable.

A 'tissue culture' is such a piece of tissue which shows migration and proliferation of cells in vitro. The term 'explantation', often used synonomously with tissue culture, should be restricted to the process of isolating the portion of tissue and supplying it with a suitable medium in a convenient vessel or container. The piece of isolated tissue is usually called the 'explant' or 'original fragment'. The term 'zone of outgrowth' is used to denote the cells which migrate out from the explant during cultivation. The terms 'subculture' and 'passage' signify the transfer of the tissue to fresh medium, the first transfer being known as the first

subculture or passage, and so on.

Erdmann's, (1930), contention that a tissue culture in the strictest sense should consist of an actively growing mass of cells in which no trace of the original fragment remains is difficult to realise and can only be approximated to if the tissue has been cultivated *in vitro* for some months. It is quite unnecessary to attempt to fulfil this condition for many of the purposes for which growing tissues can be used.

Growth of tissues *in vitro* can only be obtained provided there is a supply of "growth promoting" substances. These may be provided in the medium or may be present in the explanted tissue. According to Carrel and Fischer, a culture cannot be regarded as suitable for experiment if the medium does not contain substances which are known to have growth-promoting properties. Otherwise, the culture is considered to be merely in a state of 'survival', although migration and proliferation of cells may take place. It is certainly true that for prolonged and maximal activity of growth the presence of added growth promoting substances in the medium is essential. Although such vigorous growth may be required for certain investigations, it is unnecessary for the study of many problems that the tissue should remain alive indefinitely,

a point of view which has been urged especially by Lewis and Lewis. Even when only one of the characteristics of growth *in vitro* is present, viz. migration, provided the cells are healthy as judged by cytological standards, important observations may be made, e.g. on phagocytosis; but, of course, the limitations of the material must be realised.

According to Fischer, (1925), "the only true tissue cultivation is the method which deals with pure cultures", i.e. cultures of one type of cell, fibroblasts or monocytes etc. Carrel, (1927), stated this view even more forcibly and emphasised that no investigations carried out with cultures containing several kinds of cells can have any scientific value. From Carrel's point of view, a culture of connective tissue containing fibroblasts and wandering cells, or a culture of liver consisting of liver epithelium, mesothelium, endothelium, fibroblasts and wandering cells is quite useless. He considers that tissue cultures should be *pure* in the sense that bacterial cultures are *pure*. Such pure strains of cells maintained *in vitro* over a considerable period of time and acclimatised to conditions outside the body are perhaps necessary for the study of cell physiology, of the factors which affect growth-rate, and the influence of X-rays etc. As Maximow pointed out,

however, since bacteria do not combine with one another to form complexes of a higher order they cannot be compared to metazoan cells, the latter being closely associated in the multicellular organism. Therefore cultures of complex tissues can provide information concerning the inter-relations of different types of cells which pure cultures will not yield.

HISTORY. Probably the first reference to tissue culture in vitro is that of Leo Loeb in his book, "Über die Entstehung von Bindegewebe, Leukozyten und Roten Blut Korperchen aus Epithel und uber eine Methoden isolierte Gewebesteile zu zuchten", 1897. He stated that he had succeeded in cultivating in vitro pieces of tissue embedded in agar blocks. (Quoted by Carrel and Burrows, 1911, and others: book not available for reference.) He did not, however, publish a description of the methods used nor any details of the results obtained.

In 1902, Haberlandt published the results of his attempts in 1898 to grow certain specialised cells of the higher plants - palisade cells of Lamium, pith cells from Erchonia, glandular hairs from Pulmonaria. He realised the necessity of supplying the cells with a nutrient medium for which purpose he used Knop's solution with the addition of sucrose and various other substances. He was

able to keep the cells alive for 3 weeks in hanging-drop preparations and reported a considerable increase in size of some of the cells explanted. No cell proliferation, however, was obtained, and in his comment on this, he speculated on the possibility of inducing cell proliferation by adding to the medium extracts of actively growing plant tips or embryo sac sap, a principle adopted later with success in the cultivation of animal tissue.

Harrison, (1907), was the first to describe a method for the cultivation of the tissue of a vertebrate in vitro. His object was "to obtain a method by which the end of a growing nerve might be brought under direct observation while alive in order that a correct conception might be had regarding what takes place as the fibre extends out to the periphery". He isolated from 3 mm. frog embryos pieces of the medullary tube and ectoderm of the branchial region which are known to give rise to nerve fibres in normal development. The fragments of tissue were placed on coverslips in a drop of freshly-drawn lymph from an adult frog and after coagulation of the lymph the coverslips were inverted over hollow-ground slides, the whole preparation being sealed with paraffin wax. The cultures remained alive for from one to four weeks. Harrison found that while differentiation occurred into organs, as such, individual no

cell elements differentiated characteristically. Cells from myotomes developed into muscle fibres, epidermal cells assumed a cuticular character and numerous fibres were formed in the nervous tissue. Emigration and proliferation of mesenchymal cells also occurred. From his observations, Harrison concluded that the nerve fibre is formed as an outgrowth of a single cell, and that the method adopted should prove useful for the study of growing cells.

Early in 1910, Burrows, (1910)[1-2], studied this method of tissue cultivation under Harrison's supervision. He hoped to be able to adapt it to the cultivation of tissues of warm-blooded animals in order to obtain an additional method for investigating the conditions regulating the healing of wounds, a problem which he had been studying in collaboration with Carrel in the Rockefeller Institute, New York. During his work in Harrison's laboratory, he found that frog plasma was a more satisfactory medium than lymph for the cultivation of frog tissues. In addition he succeeded in cultivating tissues of avian embryos in fowl plasma and observed the occurrence of mitosis in some of the cells. After his return to New York he succeeded, with Carrel, (1910)[4], in cultivating many embryonic and adult avian and mammalian tissues as well as tumours of the fowl, rat, dog and man (1910)[2,3,4,5,6,7,] and 1911)[1]. These workers found that according to the nature of the tissue, epithelial or connective tissue outgrowths

were obtained (1911)[1].

The technique was very soon improved by Carrel, (1913)[4], who found that by the addition of extracts of embryonic and other tissues to the plasma medium the rate and extent of growth could be greatly increased and the duration of life *in vitro* prolonged. A strain of fibroblasts isolated by Carrel from fragments of the hearts and blood vessels of 7- to 18-day fowl embryos explanted in January 17th, 1912, and subsequently maintained by Ebeling, (1913), since June 1st, 1912, is still growing actively in a medium composed of plasma and embryonic extract. These cells have thus lived for 10 years longer than the normal span of life of the fowl. In this country, H.W. Drew, (1912-13 was the first to attempt the cultivation of tissue *in vitro*, and succeeded in obtaining growths of certain tissues of the adult frog.

Since Carrel and Burrows, (1911), Carrel (1913) published their technique, methods of tissue cultivation have been employed by many observers for the investigation of biological problems. Various workers have modified Carrel's technique, but the general principles enunciated by him still remain the principles of tissue culture, at least for the cultivation of tissue for a prolonged period.

SURVIVAL OF CELLS.

The cultivation of tissue outside the animal body is rendered possible by the ability of the cells to survive for varying lengths of time after the animal is killed or after they have been removed from the living organism. The length of survival depends upon the type of tissue, the environment in which it is placed and the size of the fragment. Lewis and McCoy (1922, 1 and 2), found that at 37° C., fragments of nerve, skeletal muscle and heart muscle placed in sterile Locke's solution survived for 24 hours, while kidney epithelium, smooth muscle, tracheal cartilage and macrophages survived for 10 days. To establish the survival or non-survival of the tissues concerned, these authors made use of the fact that neutral red stains the nuclei of dead but not of living cells. Most workers have found that small fragments of tissue kept in sterile saline for a few hours at room temperature show no diminution in activity when subsequently explanted in a suitable medium. When the tissue cannot be explanted immediately, it is preferable to keep it at a temperature below that at which metabolism is active, i.e. below 38° C. Fragments of most tissues can be kept in the ice chest at 0° C. for 4 - 5 days and still remain capable of active growth and proliferation when placed in suitable conditions,

connective tissue and skin epithelial cells surviving this treatment better than other types. Bucciante, (1931), has recently investigated the duration of survival of avian embryonic tissues when exposed to temperatures of from 20° C. to -40° C. and used as his criterion of survival, the capacity of the various tissues for subsequent growth in vitro, under suitable conditions. He found that the duration of survival varied in different tissues and that the maximum period of survival, 5 days in the case of liver epithelium and 32 days in the case of the skin and subcutaneous fibroblasts, was obtained at 0° C. At room temperature, survival was from 2 days to 24 days depending on the type of tissue. Below 0° C., the duration of survival rapidly diminishes and at -25° C. the skin, subcutaneous tissue, and cornea, the most resistant to wide variations in temperature of all the tissues investigated, survived for only three hours. These experiments of Bucciante confirm the experience of most workers that the different tissues vary in their capacity for survival and that between 38° C. and 0° C., the lower the temperature, the greater the capacity for survival as evidenced by subsequent growth in vitro.

CONDITIONS OF GROWTH in vitro.

For the successful cultivation of tissue outside the animal body, it is necessary to provide (a) a suitable support for the growing cells, and (b) a nutritive medium.

(a) Supporting Substance. It has been found that metazoan cells become rounded and die rapidly if allowed to float freely in a fluid medium. If provided with some form of support, however, they are able to stretch themselves out and migrate on the supporting surface. Almost any inert or non-toxic substance may be used as a support, some being more suitable than others. They can be divided, however, into two classes, two-dimensional and three-dimensional supports.

When the culture medium is of a fluid nature, the tissue is allowed to rest on the surface of a cover-slip which furnishes a support, the medium being spread out in a thin layer round the tissue. In such cultures, a film of cells may occasionally be seen growing along the upper surface of the liquid, thus showing that the tension at the free surface is capable of supporting the migrating cells.

In fluid medium, however, the support is in two planes only and it has been found that more active migration and growth are obtained when the support provided is in three dimensions. This is available

in the fibrin network of coagulated lymph (Harrison, 1907) or plasma (Burrows, 1910), the latter being the more satisfactory and easily obtainable. The plasma clot is regarded as a fine network of fibrin threads with fluid in the interstices. It is perfectly transparent and is therefore invisible under the microscope by ordinary illumination.

Since, however, different specimens of plasma vary in their characters and the collection of plasma involves considerable preparation and care, several observers have endeavoured to find a more simple and constant substitute for the fibrin network. Lewis and Lewis (1911) obtained emigration of cells in a medium containing agar, and Ingebrigtsen (1912) has used mixtures of agar and serum, agar and embryo extract and agar and gelatine. In the last of these mixtures the gelatine is fluid at 39° C. while the agar is solid. Smyth (1914) has also used agar as the supporting substance in culture media. In general, it has been found that the use of such media merely demonstrates the capacity of cells for taking advantage of any supporting material provided. Growth and emigration occurred, but penetration of the agar coagulum did not take place to any extent and the activity of the cultures was very much less than that

found in a plasma medium. In addition, agar is not so transparent as coagulated plasma and microscopical examination of the cultures during life is thus rendered difficult. Histological preparations are almost impossible to secure as stains are deposited on the agar.

Harrison (1912; 1914) used spiders' webs, Carrel and Burrows (1911,&) used silk veil and cotton-wool, and Fischer (1923,/) used elderberry pith and non-absorbent cotton-wool as supporting substances. According to Fischer, fibroblasts will emigrate and divide quite actively in media containing these substances, but the extent of growth depends on the contact established between the tissue and its support and the amount of fluid present. The fluid present should be limited to that amount which is capable of adhering to the supporting substance by capillary attraction. Such methods are inapplicable to most of the problems which may be investigated by means of tissue culture. The cotton-wool method of Fischer has, however, rendered possible the isolation of individual cells; the significance of this will be referred to later.

Ebeling (1921,/) has devised a method of using fibrinogen, prepared according to Mellanby's method, as a source of fibrin. The fibrinogen forms a clear

transparent clot when embryo extract is added to it and liquefaction of the medium, which tends to take place very rapidly, is diminished by the addition of a trace of sodium linoleate. This method appears to be of value for the investigation of cell physiology, but for morphological and cytological purposes, however, it possesses no advantage over the usual plasma technique.

(b) Medium. In order to obtain growth of tissues in vitro it is necessary to provide the cells with a suitable medium. Theoretically, an ideal nutritive medium would imitate in its physico-chemical constitution the character of the fluids bathing the cells of the tissues, the 'milieu intérieure,' but, since the composition of these fluids is unknown, lymph (Harrison, 1907), plasma (Burrows, 1910,), and serum (Volpino, 1910, 1928.), were employed. In plasma, proliferation of cells was reported. The tissue lived in the plasma medium for, at the most, four days, but Carrel and Burrows, (1911,), and Carrel, (1911,), found that by removing the fragment from the medium, washing it in saline, and transferring it to fresh plasma, the life of the tissue could be prolonged. Other means of increasing the rate of growth and the duration of life were then employed. Ruth, (1911), found that by diluting the plasma with distilled water

the rapidity of emigration could be increased, a finding confirmed by Carrel and Burrows (1911, ?), the hypotonicity apparently being responsible for the improvement. Carrel (1912 , ?) then attempted to prolong the life of the tissue by alternating the phase of activity of the tissue in the incubator with a period of latent life at 0° C. in Ringer's solution, during which time the tissue was washed free of the metabolic products which had accumulated during the period of active life. On the assumption that growth does not occur in serum, he also alternated the period of life in plasma with a period of life in serum at body temperature. None of these methods, however, proved entirely satisfactory, and it came to be realised that, while the blood and intercellular fluids provide the substances essential for the nutrition of cells, they do not promote cell proliferation which, under ordinary conditions, occurs only for the purpose of replacing loss and ceases when that replacement is complete. Many of the problems suitable for investigation by the tissue culture method require cultures of actively growing cells which can be brought under direct observation, and the life of which can be maintained for a considerable period. It was apparent, therefore, that in order to provide an ideal growth-

promoting medium, some adjunct to or modification of normal plasma was essential, an assumption which was encouraged by the fact that, within limits, decrease of the osmotic tension increased the activity of the tissue. In 1907 and 1908, Carrel, (1913,), had studied the action of many substances on the repair of small cutaneous wounds and found that, under certain conditions, the proliferation of epithelium and connective tissue was accelerated by dressings made with pulped tissues and organs. In 1911, in collaboration with Burrows, he found that the growth of chicken tissues in vitro was apparently accelerated by the addition to the medium of extracts of chicken embryos and of Rous chicken sarcoma. In 1912, he investigated more precisely the action of tissue extracts in vitro by comparing the rate of growth of almost identical fragments in normal plasma, and in plasma to which had been added a known quantity of tissue extract. The area of the fragments was measured before explantation and after 24, 48 and 72 hours. It was found that in all cases the presence of tissue extract in the medium increased the rate of growth, as estimated by area, from $2\frac{1}{2}$ to 40 times. This activation was in direct proportion to the concentration of the extract and the most active extracts were obtained from

embryo tissues, adult spleen and Rous sarcoma. The activating power of all the extracts, except that from the spleen, was markedly diminished by heating for half an hour at 56° C., while heating at 70° C. destroyed the activity in every case. Filtration through a Berkefeld filter caused a diminution of activating power, and filtration through a Chamberland filter suppressed it entirely.

Carrel's discovery has been confirmed by many observers, and the growth promoting properties of other tissues have been investigated. Maximow, (1917), recommended the use of a rabbit bone-marrow extract for the growth of rabbit tissue. Drew (1923, 1927) found that autolysed extracts of adult tissues were able to activate growth, and that extracts of mammalian tumours (Jensen's Rat Sarcoma, Carcinoma 63, 206, and Sarcoma 37 of the Imperial Cancer Research Fund) also provided growth-promoting substances. Erdmann (1926) obtained similar results with tumour extracts and recommends a splenic extract when embryos are not available. Heaton, (1926), and, later, Drew (1927) found that substances are present in alcoholic extracts of yeasts which activate the growth of epithelia but not of connective tissue. Carrel, (1924), found that leucocytes

growing in serum or plasma provide substances which are able to activate the growth of fibroblasts explanted into the same preparation.

The growth-promoting substances produced by leucocytes *in vitro* as also those present in tissue extracts have been called 'trephones' and the properties of the latter have been studied extensively by Carrel and others. An attempt has also been made to isolate pure chemical substances which could be substituted for tissue extracts.

The method used by Carrel and his co-workers was to compare the rate of growth of two approximately identical fragments of tissue obtained by division of a single culture which was derived from the permanent strain of fibroblasts isolated by Carrel in 1912. One fragment was explanted in plasma to which had been added the substance to be tested; the other, which was used as a control, was cultivated in plasma and embryo extract. The following is a brief account of the results so far obtained. Carrel and Ebeling, (1923, *l.*), found that although cells growing *in vitro* could not utilise pure protein or bouillon, the growth-promoting substance is contained in the protein fraction of embryo extract, and that this fraction cannot be dialysed;

(Baker and Carrel, 1926[1]); amino acids appear to stimulate cell proliferation, but cannot be utilised as food material (Baker and Carrel, 1926[2]). Payling-Wright, (1926), found, however, that the growth-promoting substances could be dialysed and yet the dialysate contained no protein demonstrable by the biuret reaction, while Burrows and Neymann (1917) had already stated that amino acids were toxic to cells growing in vitro. In further experiments, Carrel and Baker, (1926), found that fibroblasts were able to utilise the lower cleavage products obtained by pepsin digestion of the protein of embryo extract, egg white, rabbit brain and commercial fibrin. Furthermore, they found that in a medium containing Witte's peptone in which a large amount of proteose is present, the activity of the cultures was as great as in embryo extract. In a medium containing the proteoses obtained by hydrolysis and precipitation of Witte's peptone the cells grew twice as actively as in embryo extract. From these experiments, Carrel and his collaborators concluded that cells growing in vitro are able to utilise only the 'split' products of protein such as proteoses and they put forward the hypothesis that enzymes are present in the extract or in the living cells, which are able to perform this cleavage of protein

in vitro. They also state that the plasma clot provides only a support for the growing cells and plays no part in their metabolism. Baker and Carrel, (1928), found that the pepsin hydrolytic products of pure proteins, such as egg albumin, edestin and fibrin, can be utilised by fibroblasts, and that the efficacy of these substances is increased by the addition of glycocol and nucleic acid.

The inadequacy of plasma and serum as culture media for active growth has already been mentioned, and the properties of these substances have also been investigated by Carrel and Ebeling. In most of these experiments, the medium used consisted of fibrinogen coagulated by 2 per cent. embryo extract, according to the method devised by Ebeling, (1921), to which varying amounts of serum were added. The control cultures were cultivated in fibrinogen and embryo extract. Carrel and Ebeling, (1921), found that homogenic serum has an inhibitory effect on the growth of fibroblasts and that this effect is directly proportional to the age of the animal from which the serum is taken. Similarly, the growth rate of tissue in pure plasma varies according to the age of the animal from which the plasma is taken. Heterogenic serum, when the concentration varies within

certain limits, has also an inhibitory effect which varies directly with the age of the animal (Carrel and Ebeling, 1922). Heating homologous serum to 70° C. decreased this inhibiting effect (Carrel and Ebeling, 1922, 2.). The inhibitory action of homologous serum is increased by shaking and that of heterologous serum diminished by the same treatment (Carrel and Ebeling, 1922, 3.). Carrel and Ebeling, (1923, 1.), found that not only growth-inhibiting but also growth-promoting factors are present in serum. Both these factors exist in the globulin fraction and have been isolated by precipitation. The addition of embryo extract to a medium containing 25 per cent. of serum did not increase the rate of growth of the tissue, as it does when added to plasma (Carrel and Ebeling, 1923, 3.).

It might be expected from these results that embryo extract would provide the best possible medium for growth and that, although the support for the cells would have to be supplied by the coverslip on which the tissue rests, growth would be active and vigorous within the limited area provided. This has not been the case in my experience. The growth in embryo extract alone is no greater, and in most cases is less, than that in serum, and is much less than that in plasma only.

Fischer, (1930, Gewebezuchtung, p. 104), has also found apparently that embryo extract is an unsatisfactory medium, since he uses as a fluid medium for flask cultures, a mixture of serum and embryo extract.

As already mentioned, Carrel and Ebeling, (1923, 3.), state that the addition of embryo extract produced no effect on growth rate. In my experience, however, the addition of embryo extract to serum produces a marked effect.

During a study of the growth of various tissues from young mice, I found that mitotic division was very infrequent in the zone of outgrowth when the medium contained serum only. When, however, embryo extract was added, growth was much more vigorous and mitotic figures could always be found, (Figs. 1 and 2). As will be described later, the medium obtained by expressing the fluid from plasm coagulated with embryo extract is more suitable than that consisting of serum and extract. No reason for this has so far been found. Drew, (1923) on the other hand, cultivated tissue successfully in a medium containing saline and tissue extract. He seems, however, to be the only worker who has used such a medium with success.

It is at present impossible to assess accurately

the relative values of plasma or serum and tissue extracts in the medium. In all Carrel's experiemnts, the medium contained protein substances, e.g. plasma, in addition to those present in the embryo extract. A coagulated medium, consisting of plasma and embryo extract, provides optimum conditions, i.e. a large and suitable substratum for migration, and growth-promoting substances; and it is more suitable than plasma alone or embryo extract alone. Similarly, a fluid medium containing serum and embryo extract is more satisfactory than a medium consisting exclusively of one or other of these constituents. Pure plasma is a better medium than either serum or embryo extract, probably on account of the mechanical conditions supplied. It seems likely, therefore, that, whether or not protein in the form supplied in serum or plasma can be utilised by cells growing <u>in vitro</u>, its presence, in the case of plasma, serves some protective function in addition to that of support for the migrating cells. It may, possibly, by altering the viscosity of the medium, render it more suitable for growth. In my opinion, it is an essential constituent for the maintenance of the cells in an active and healthy state, and the protein of embryo extract is not adequate for this purpose. M. R. Lewis,

(1922), stressed the importance of dextrose in the medium. It is significant, however, that in the experiments in which the value of dextrose was studied, the medium contained at least 15 per cent. of muscle bouillon.

While the composition of the medium undoubtedly exercises a very important influence on the growth of tissue in vitro, the effect of cutting the culture out of the medium and transferring it to fresh medium does not appear to have been considered. This is done primarily to remove the waste products of cell metabolism, but it is possible that it exercises a stimulating effect on growth. The growth-rate of a culture is not uniform. Following explantation, there is first a latent period, which is followed by a phase of gradually increasing activity. This reaches a maximum after about 24 to 36 hours and then falls, the diminution being probably due to the accumulation of waste products. Nevertheless, under conditions such as Carrel's flask method, (Carrel, 1923,), in which the tissue remains undisturbed for from 10 to 14 days, during which time the medium is changed every second day and waste products do not accumulate to any extent, the growth rate falls during the latter part of the culture

period, and in order to maintain growth activity, the tissue has to be cut out of the coagulum in the flask and transferred to fresh medium. Likewise, de Haan (1927), who uses an apparatus in which the medium circulates actively round the tissue, cuts the culture out of the clot and transfers it to a fresh coverslip every 20 days although the fluid used for perfusion is still adequate for fresh cultures. Similarly, in hanging-drop cultures in fluid medium which is renewed every two days, emigration ceases after about 8 - 10 days' cultivation; emigration starts again, however, if in addition to being transferred to fresh medium, the edges of the fragment are cut. It would appear, therefore, that the tissue comes into equilibrium with the medium, and that in order to maintain it in a state of activity, it has to be exposed at regular intervals to the mechanical stimulus of '<u>cutting</u>' either out of the clot in the case of cultures on coagulated media, or along the edge of the original fragment in the case of fluid medium cultures.

<u>Heterologous Media</u>.

Before the importance of the inclusion in the medium of tissue extracts such as embryo extract had been thoroughly recognised, efforts were made by various

observers to find more suitable media. These attempts were directed towards the investigation of heterologous plasma and serum and the conditions which determine suitability for growth in vitro.

It was found by Lambert and Hanes, (1911, 1.), that rat sarcoma could be cultivated in heterologous plasma. Of the heterologous media used, only goat plasma proved entirely unsuitable for growth, and, although some were more suitable than others, the degree of suitability did not seem to depend, except perhaps in the case of mouse plasma, on the closeness of relationship of the species. Carrel and Burrows, (1911, 8), found that embryonic fowl tissues grew in plasma from the rabbit, dog and man. All these workers found that growth was more active in homologous than in heterologous plasma, and this has been the case in my experience except in the case of mouse tissues, which grow just as well in rat serum or plasma as in the homologous media. Ingebrigtsen, (1912, 1), also found that the bone marrow of guinea-pigs grew better in homologous serum than in heterologous sera; the latter, however, could be rendered more suitable for growth by heating for half an hour at 56° C. In a later communication, Ingebrigtsen, (1912, 2.), stated that an inverse ratio

existed between the haemolytic power of the heterogenic sera and the extent of growth which takes place in them. I have found that there is a definite correspondence (p.272) between the content of the tissue cultivated in Forssman's antigen and the presence in the serum used for medium of the corresponding antibody.

It has been found that, in avian plasma, all the mammalian tissues investigated grow well. It is a common experience that mammalian homologous and heterologous plasma is liquefied rapidly, particularly by epithelial and tumour tissues whether or not embryo extract is present, and cultivation is thus rendered difficult. Avian plasma, on the other hand, resists liquefaction. Accordingly, by using a medium composed of both homologous and avian plasma, the advantages of the homologous substances and the peculiar liquefaction-resisting properties of the avian coagulum can be obtained. Growth-activity is greater than in either alone. When a large coagulum is required, as for the cultivation of embryonic rudiments, I find, however, that avian plasma alone is more satisfactory than a mixture, as it provides a firmer clot, which seems to be of importance in determining the degree of growth and differentiation.

Carrel and Ebeling (1923,4.), state that heterologous embryo extracts are just as effective as homologous embryo extracts, and this has also been my experience. Kiaer (1925), found that duck fibroblasts grow just as actively in chicken plasma and chicken embryo extract as in duck plasma and duck extract. As stated on p.48 , mammalian embryos are often difficult to obtain, and unless a large number of suitable animals is available, I now use avian embryo extract as a routine in the medium for the cultivation of mammalian tissues. For the cultivation of rat or mouse tissues, I generally use a medium consisting of 1 part rat plasma, 1 part avian plasma and 2 parts avian embryo extract. For coverslip cultures of rabbit tissues, 25 per cent. rabbit plasma is present in the medium. A heterologous fluid medium is also used (p.72).

OTHER CONDITIONS NECESSARY FOR GROWTH in vitro.

Although the supply of a suitable nutritive medium and the provision of a support for the growing cells are essential for the growth of tissues in vitro, other conditions have to be fulfilled. These are:-

1. The use of a saline solution which has the correct constitution and hydrogen-ion concentration,
2. The maintenance of the hydrogen-ion concentration of the medium,
3. Thorough cleanliness and sterilisation of all glassware, etc.,
4. Precautions against bacterial infection during the manipulative procedures,
5. The minimum of injury to the tissue to be cultivated,
6. A supply of oxygen for the growing cells.

1. The saline used for moistening or washing the tissue fragments and for the preparation of embryo extract must be of the correct constitution and hydrogen-ion concentration. Physiological saline (0·9% NaCl) is toxic to cells and should not be employed. Ringer's saline (NaCl, KCl, $CaCl_2$) can be used for washing the cultures and for preparing tissue extracts. Since the optimum hydrogen-ion concentration of the medium for growth lies between P_H 7 and P_H 7·8, it is preferable to use a saline with a P_H value from 7·4 to 7·6 which is

buffered against small variations arising in the reaction of the medium. Two types of buffering system may be employed, a combination of phosphate and carbonate salts, NaH_2PO_4 or $CaH_4(PO_4)_2$ and $NaHCO_3$, and a combination of phosphates, Na_2HPO_4 and NaH_2PO_4. Tyrode's saline and Drew's saline are examples of the former, Pannett and Compton's saline, as modified by Strangeways, of the latter. Tyrode's saline has the disadvantage that it cannot be sterilised by autoclaving on account of the presence of calcium salts and phosphate, and sterilisation by steaming at 100° C. is not reliable since it contains glucose: the solution has to be sterilised by filtration. Drew's saline contains NaCl, KCl, $MgHPO_4$, $NaHCO_3$, $CaCl_2$, $CaH_4(PO_4)_2$. The first three solutions can be steamed together, the others have to be steamed separately. After cooling, the solutions are added to one another in the correct proportions. It is often difficult to obtain solution of the $MgHPO_4$, and the saline rapidly becomes too alkaline, the amount of phosphate present not being adequate to cope with the loss of CO_2. I have found Strangeways' modification of Pannett and Compton's saline to be very satisfactory. Only two stock solutions are required and these are autoclaved separately. When required for use, 1 c.c. of sol. A which contains NaCl,

KCl, $CaCl_2$ and $\overset{Mg}{\wedge}Cl_2$ is added to 22·5 c.c. of sterile glass distilled water, and to this are added 1·5 c.c. of sol. B, which contains NaH_2PO_4 and Na_2HPO_4. If a precipitate forms in solution B, it has to be discarded. The hydrogen-ion concentration of this saline is P_H 7·4. The method of preparation, i.e. the use of concentrated salines to which distilled water is added when required for use, helps to minimise any alteration in concentration caused by the sterilisation.

2. The medium itself must be of the correct hydrogen-ion concentration. As shown by Lewis and Felton, (1921), Fischer, (1925), . optimum growth occurs between a hydrogen-ion concentration corresponding to P_H 7·0— 7·8. The reaction of embryo extract is usually about P_H 7·2 when Pannett and Compton's saline is used, and the plasma-extract mixture is usually about P_H 7·4. This is the ideal reaction of the medium. The reactions of fluid media are usually the same, but that of serum and embryo extract is occasionally P_H 7·6. Precautions which are described on pp. 36, 42 have to be taken to preserve the hydrogen-ion concentration of stored plasma and serum.

3. It is advisable to use glassware made of chemically 'resistant' glass which does not tend to become alkaline.

'Monax' or 'Pyrex' glass are both satisfactory, the former being the better. All the glassware used must be thoroughly clean and should be reserved for tissue culture work only. Petri dishes, test-tubes, watch-glasses, slides, etc., can be cleaned in the usual way, but coverslips, flasks, watch-glasses on which tissues are cultivated, ought to be cleaned in a cleaning solution containing potassium bichromate and sulphuric acid, care being taken to wash away the cleaning solution in running water before drying. Coverslips should be transferred from running water to alcohol, from which they are dried before sterilising. New pipettes should be immersed in cleaning solution and thoroughly cleaned before sterilising. After use, it is advisable to rinse them out immediately, first in boiling distilled water and then in alcohol; periodically, they have to be left in cleaning solution for a few days. Centrifuge tubes for blood, tissue-extract and plasma have to be carefully cleaned and may also require occasional treatment with cleaning solution.

The glassware is sterilised by dry heat - half an hour at 160° C. is adequate. Instruments are sterilised by boiling for 20 minutes. The knives used for cutting the tissue for cultivation are sterilised by dry heat at

120° C. for half an hour. If the temperature is too high or the heating prolonged beyond this time, the steel loses its temper rapidly. The rubber stoppers for centrifuge tubes are sterilised by autoclaving; the saline and other solutions used are also sterilised by autoclaving.

4. The danger of bacterial infection from non-pathogenic and pathogenic organisms can be reduced to a minimum, provided sterilisation of glassware is adequate, if precautions are taken against undue exposure of tissues and fluids to the air and if the plasma and embryo extract are obtained aseptically. The elaborate precautions adopted by some workers for sterilising the culture-room are quite unnecessary. The room should be kept as clean as possible and currents of air avoided while cultures are being prepared as it has been found that most of the infections obtained have been caused by organisms resembling B. subtilis. Some workers prefer to prepare their cultures inside a closed box which can be sterilised. If the bench to be used, however, is covered with a sheet of glass which can be kept clean, the ends of the pipettes kept well within sterile jars (p. 53) and tissues, fluids, etc., kept covered within sterile Petri dishes, it is quite possible to prepare and transfer cultures without

any additional protection. The work can be done much more expeditiously if protective devices are reduced to a minimum, and it has been found that dexterity and rapidity in handling the tissues are most important for avoiding infection and obtaining good growths.

It is extremely important to observe carefully every manipulation made, so that pipettes, knives, etc., can be discarded if there is any suspicion of their having touched a non-sterile surface. A binocular microscope is often used for the dissection of tissues and for sub-culturing. The stage of the microscope should be protected with a suitable shield which, while allowing free manipulation, protects the tissue from dust or other particles falling directly upon it. The usual bacteriological precautions of flaming the tops of test-tubes, the ends of pipettes, etc., must of course be always observed. Infection of cultures from contaminated plasma or extract has often been described and Erdmann (1930) recommends that all plasma and tissue extracts should be tested bacteriologically as a routine before use. In my experience, I have never had an infection of cultures from these sources. Faulty manipulations and undue delay have been responsible for any infections obtained; it is possible to work with over 3,000 cultures

during a period of nine months without having a single contamination. If the dissection of the tissue takes some time, washing the fragments three or four times with fresh saline will reduce the possibility of infection. Lack of growth is much more liable to be due to damage of the tissue, unsuitable media and filthy glassware than to bacterial infection.

5. One of the commonest causes of lack of growth is injury to the tissue either during dissection or during the cutting into fragments suitable for cultivation or during subculture. This can be minimised if the knives used are sharp; they should be always sharpened and the blades and points examined under the low power of a microscope before sterilising. Damage to the tissue can also be avoided if the fragments of tissue are transferred to the coverslip or culture chamber with a pipette instead of with the point of a knife as recommended by many workers. The vitality of the tissues is much more liable to be impaired by faulty cutting than by keeping the fragments in saline for some time before arranging them on the coverslips, etc. It has been found that vitality remains quite unimpaired for several hours at room temperature; in fact, certain tissues can be kept at 0° C. for several days and still remain capable of growth when

supplied with an appropriate medium (p.9.). Delay is only dangerous when it occurs during the process of arranging the tissue in the medium, etc., since it may then result in drying of the tissue, unless this be kept moist with a sufficient amount of saline during cutting and during the process of making the culture preparation.

6. No special precautions are taken to supply the tissue with oxygen. In all the types of culture-chamber used, however, there is an air-space which if necessary can be kept moist to avoid undue evaporation from the medium, and this seems to be adequate for the requirements of the tissue.

TISSUE CULTURE METHODS.

PREPARATION OF MEDIA.

PLASMA.

Avian Plasma.

The usual method of obtaining plasma is that described by Carrel and Burrows (1911, *l.*) and Strangeways (1924). A bird, not more than two years old, is starved for 24 hours, but is allowed an adequate supply of water. It is anaesthetised with ether and the carotid artery exposed.

An oiled cannula is inserted into the artery and the blood is allowed to flow out of the cannula. The first few cubic centimetres are discarded; thereafter the blood is allowed to flow into ice-cooled paraffined centrifuge tubes. After thorough cooling, the tubes are centrifuged at high speed in ice for five minutes and the supernatant plasma drawn off with a paraffined pipette and transferred to paraffin-lined tubes which are then stoppered and stored in an ice-chest or refrigerator. This is certainly the ideal way of obtaining plasma, and the paraffining of the centrifuge tubes, the cooling and centrifuging in ice prevent coagulation taking place. The method, however, involves a great deal of preparation and the help of at least two assistants. Various workers have attempted to simplify the method by adopting artificial means to prevent coagulation occurring. Foot (quoted by Craciun, 1931) has added citrate to the blood, and others have added oxalate. Awrorow and Timofjewski (quoted by Craciun 1931) have used hirudin. The most useful method of preventing coagulation of the plasma, while preserving its qualities completely unimpaired, has been devised by Craciun (1925).

Craciun (1925), found that heparin, a liver

extract, originally prepared by Howell, prevents the coagulation of the blood and of the plasma in a dilution of 1:20,000 in the blood. Heparin is prepared for use in a dilution of 1 in 2,000 in Ringer's saline. 1 c.c. of the solution is put into ampoules which are sealed and autoclaved at 120° C. for 20 minutes. The solution keeps indefinitely.

The following is the method which I have found to be simplest and most satisfactory. The instruments, etc., are sterilised and allowed to cool. An all-glass syringe of 50 c.c. capacity fitted with a needle is boiled and allowed to cool. The needle should be about 1¼ inches long, of wide bore, and sharp pointed, the bevel of the point being about 45°. After cooling, the syringe is rinsed out with Ringer's saline. 1 c.c. of 1 in 2,000 heparin is taken into the syringe and allowed to flow all over the internal surface of the syringe. Then the heparin is distributed into 4 centrifuge tubes capable of holding 10 c.c. without overfilling, 2 or 3 drops of heparin being left in the syringe. The syringe and centrifuge tubes are then cooled thoroughly on ice. A young bird, not more than 3 or 4 months old, previously starved for 24 hours, is chosen. It is anaesthetised with ether and then tied

down on a board. The feathers are rapidly removed from the thorax and abdomen, and methylated spirit poured over the skin. The abdomen is opened at the lower limit of the diaphragm, the diaphragm cut through, the thorax being then exposed. The rib attachments to the sternum are then rapidly cut through and the sternum drawn back to expose the heart. It is important to avoid cutting the large vessels which pass down on each side of the sternum. The pericardium is then cut open, the heart steadied with forceps and the syringe needle inserted quickly into the right ventricle. The heart, which should be beating actively, pumps the blood into the syringe, the piston being slowly withdrawn. The blood is then put into the centrifuge tubes which are stoppered and allowed to cool in ice for 5 minutes. The tubes are centrifuged at high speed for 5 minutes and the supernatant plasma drawn off with a pipette into small centrifuge tubes or into ampoules. Craciun recommends ampoules but small tubes are more easily handled and are just as satisfactory. It is not necessary to paraffin the tubes in which the plasma is to be kept. The plasma is then stored in the refrigerator and remains fluid for weeks and even months. Often an amorphous deposit forms in the tubes; this sinks to the

bottom, however, and its presence causes no deterioration of the plasma. Various precautions have to be observed during the operation. The anaesthetic has to be skilfully administered so that the heart beats actively during the bleeding. If the blood ceases to flow into the syringe, the needle should be moved gently inside the heart as it often tends to become pressed up against the heart wall. No force should be exercised on the piston: it is better to take a smaller quantity of blood than to exercise suction on the heart. From a bird 3 to 4 months old 40 c.c. of blood are easily obtained and the concentration of heparin in the blood is then about 1 in 80,000. It has been found possible to take blood without any heparin at all and without lining the tubes with paraffin, but if no heparin is used, clotting is liable to take place if the heart is not beating very actively and bleeding is slow. Plasma taken with heparin coagulates rapidly when embryo extract is added to it. The use of an 'activated' extract as recommended by King (1930) is quite unnecessary. M. R. Lewis (1928) recommends heart puncture of the fowl using 0·5 c.c. of a 1 in 500 solution of heparin for every 10 c.c. of blood. Such a high concentration of heparin, however, renders coagulation slower. Erdmann takes

blood from a wing vein, (1930), or from the jugular vein (Erdmann and Ichida, (1927), . If facilities are available for keeping hens, bleeding from a wing vein is practicable; otherwise, bleeding from the heart in the manner described gives the largest yield of blood. So far as tissue-culture is concerned, no difference has been found between the plasmas of venous and arterial blood.

Mammalian Plasma.

Rat plasma is used in the medium for the cultivation both of mouse and of rat tissues. The heparin technique as described by Craciun, (1925), is always employed. The ultimate concentration of heparin in the blood should be about 1 in 20,000. A rat, preferably a male about 9 months old, is chosen and starved for 24 hours. Such an animal normally yields about 10 to 20 c.c. of blood and 1 in 1,000 heparin solution is used so as not to dilute the plasma too much. The instruments are sterilised and the syringe and centrifuge tubes prepared in the same way. The animal is anaesthetised with ether and the heart exposed in the usual way, care being taken to avoid cutting superficial vessels and the vessels which run along the sides of the sternum. The right ventricle is punctured and the blood should flow into the syringe. By pressing on the viscera as the blood flow diminishes, more blood can be

obtained. The blood is cooled and centrifuged and the plasma stored either in ampoules or in small tubes.

Rabbit Plasma.

Rabbit blood can be very easily obtained by heart puncture and 20 c.c. can be withdrawn quite safely. The concentration of heparin should be about 1 in 20,000. The same procedure is adopted for preparing the syringe, etc., and the plasma is obtained after cooling and centrifuging the blood.

Human Plasma.

Blood can be easily obtained from a vein in the antecubital fossa. A 1:20,000 dilution of heparin is sufficient to prevent coagulation. The treatment of the blood is similar to that already described.

THE PREPARATION OF SERUM.

Avian Serum.

If blood is taken quickly from a fowl with the heart beating actively it is extremely difficult to obtain coagulation of the blood even at room temperature. In the incubator at 38° C. coagulation takes place in an hour or so, and after separation of the clot and centrifuging, the serum can be drawn off. Avian serum is

rarely used for culture medium and I prefer to coagulate plasma with extract as described on p. 72 , and use the expressed fluid.

Mouse Serum.

A mouse about a year old is chosen and anaesthetised with ether. The heart is exposed and the right ventricle punctured with a capillary pipette. The blood flows into the pipette and by pressing on the viscera when the flow diminishes, from 1 c.c. to 2·5 c.c. may be obtained. After separation of the clot and centrifuging, the serum is drawn off and put into ampoules which are stored in the refrigerator.

Rat Serum.

The procedure is similar to that already described for the taking of plasma, no heparin, however, being used and ice-cooling of the syringe and centrifuge tubes being unnecessary. The syringe must, of course, be rinsed out with Ringer's saline. Pannett and Compton's saline should not be used for rinsing the syringe as it always causes slight lysis of the corpuscles. I have not been able to find any reason for this, but it is a constant feature. After separation of the clot and centrifuging, the serum is pipetted into ampoules or small tubes and

stored in the refrigerator.

Rabbit Serum.

Rabbits are always bled from an ear vein, from which 10 c.c. can be quickly and easily obtained. The serum is obtained in the usual way and stored in ampoules in the ice chest.

Human Serum.

Blood is taken from a vein in the antecubital fossa and the serum obtained and stored in ampoules in the refrigerator.

PREPARATION OF EMBRYO EXTRACT.

Avian Embryo Extract.

It is convenient to make embryo extract from embryos between the ninth and tenth days of incubation. Slightly older embryos may be employed, but it is not advisable to use embryos in which feather formation has begun, as the feathers cannot be centrifuged. Younger embryos may also be used, but, if taken at too early a stage, several may be required to provide sufficient extract. The 'explosive' type of growth described by Erdmann (1930) in a medium containing extract from very young embryos

has not been observed, and the only disadvantage of using extract made from embryos at an early stage of development is the number of fertile eggs which have to be used.

Before beginning the preparation of the extract, a mug of distilled water must be boiled for at least five minutes and the water kept boiling throughout the operation. Sterile instruments, etc., must also be ready. The egg does not require to be sterilised; any dirt should be removed by washing with water. The blunt end of the egg is tapped with the handle of a scalpel until numerous fine cracks appear. The cracked portion of shell is then carefully flicked off from the underlying shell-membrane with the fingers: it is unnecessary to pick off the pieces of shell with sterile forceps as it can be removed much more quickly and with less chance of injury to the underlying membrane by the method described. When a sufficient area of membrane is exposed, it is carefully removed with forceps round the limit of the broken shell, thus opening into the air space. The lower layer of shell-membrane is then removed and the yolk sac vessels and the embryo lying within the amnion can be seen. By tilting the egg and allowing some of the yolk to flow out, the embryo can be brought forward to a position convenient for removal. The condition of

the embryo should be examined. The heart should be beating actively and the embryo itself translucent. Dead embryos are, of course, useless. In the autumn and winter abnormal embryos are often found; these should be discarded if more eggs are available. The amnion is then slit open with sharp-pointed, angled scissors and the yolk-sac vessels cut on both sides where they enter the body of the embryo. The neck of the embryo is then hooked on to a pair of 'curved on the flat' scissors and carefully removed, avoiding touching the edge of the shell, and placed on a watch-glass contained in a Petri dish. Sometimes the upper layer of shell membrane is torn and a piece of shell falls on to the lower layer. If no pieces fall on to the yolk sac or embryo it need not be discarded. It is advisable to avoid tearing the yolk sac as the embryo then becomes covered with yolk; if this happens, however, the yolk can be removed by repeated washing with saline. The eyes of the embryo are extirpated and the embryo cut into five or six pieces. The blood is removed with a pipette which is then discarded. About 2 c.c. of saline is then pipetted over the embryo, removed and discarded. The pipette is rinsed out several times with boiling distilled water and fresh saline pipetted over

the embryo; this is removed, discarded, and the pipette rinsed out as before. This process is repeated four times, after which most of the blood will have been removed. Longer washing is, of course, necessary if any yolk is present. During the washing, the tissue must be protected from exposure as far as possible with the Petri dish lid. After the last washing of saline has been removed, the embryo is then cut up rapidly into as fine a state as possible with sharp 'curved on the flat' scissors, once more protecting the tissue from exposure to the air with the Petri dish lid. The fragments are then transferred with a wide-bored pipette to a centrifuge tube and ground to a fine mush by rubbing the tissue round the sides of the tube with a glass rod. About 5 c.c. of saline are then added to the tube and mixed with the tissue. A sterile rubber stopper is inserted and the tube centrifuged for from three to five minutes. The supernatant fluid, which is pale and slightly opalescent, is the 'embryo extract.'

It has been found from experience that the extract obtained when 5 c.c. of saline are added to a 9-day embryo, which consists of from 2 c.c. to 2·5 c.c. of tissue, provides a sufficient concentration of growth-promoting substances. For some tissues it may have to

be diluted. Unless, for purposes of experiment, the exact concentration of embryo extract has to be known, it is unnecessary, as Carrel and Erdmann recommend, to centrifuge the embryo tissue and remove the supernatant gelatinous fluid for subsequent dilution with saline. In any case, embryos of the same age are often at different stages of development, and accurate standardisation of the extract is impossible. If older embryos are used, more saline is added; if younger, less is required.

Mammalian Embryo Extract.

The use of mouse and rat embryo extract is rendered difficult by the fact that it is impossible to be sure of the stage of development of the embryos. The best extract is prepared from embryos from the tenth to fourteenth day of development; but, unless very careful mating of the animals can be done, the obtaining of embryos at the right stage of development is a matter of chance. With rabbits, on the other hand, the exact age of the embryos is always known, as these animals can be mated at any stage of the oestrous cycle. Rabbit embryos should be taken at the fourteenth day after mating.

The method of preparation of the extract is similar in all cases. Mice and rabbits are killed by a blow on the head; rats are generally anaesthetised with ether.

In the case of mice and rats, the whole abdominal and thoracic wall is flooded with methylated spirit. As rabbit hair is very fine and floats about in the air, it is advisable, in addition, to shave the abdominal wall. Thereafter, it is again flooded with spirit. In mice and rats, a longitudinal incision is made through the skin from sternum to pubis. The skin is then rapidly reflected on each side and kept reflected with pressure forceps. The abdomen is opened by a transverse incision along the upper limit of the abdomen and by a longitudinal incision along the mid-line which passes down to the pubis. The abdominal contents bulge forwards and the uterine horns are removed by cutting through the ovarian ligaments and the upper end of the vagina. The uterine horns are placed in a Petri dish and they are opened by a longitudinal incision along the antimesometrial side, thus avoiding the placental site. The embryos are removed by cutting through the amnion and the umbilical cord. ~~and the amnion~~. As many as are required are transferred to a watch-glass inside a Petri dish. The tissues are then cut up, washed with saline, etc., and ground into a fine pulp in the manner already described for avian embryo extract. To every 2 c.c. of tissue 5 to 6 c.c. of saline are added, and the extract is obtained by centrifuging.

When removing rabbit embryos, it is advisable to make a transverse incision across the upper limit of the abdomen from the ends of which two longitudinal incisions are made down towards the pubis. The skin is reflected from the abdominal muscles and drawn downwards. The abdomen is opened by a T-shaped incision, the edges being retracted with pressure of forceps. The longitudinal part of the incision should stop about an inch from the pubis; otherwise the abdominal contents bulge forwards too much. The uterine horns are then easily removed and the embryos taken out in the manner already described.

Some workers recommend the use of a Latapie press or some such apparatus for grinding up the tissue, and others reduce the tissue to a pulp with sand in a mortar. For preparing autolysed extracts from adult organs a mincing apparatus is necessary, but all such procedures increase the risk of infection of the tissue and are certainly not necessary for preparing embryo extract. Drew (1923), recommends filtering the embryo extract through a Berkefeld filter to ensure sterility. As already stated, infection of embryo extract has never occurred in my experience and filtration seems unnecessary.

It is usually possible to prepare embryo extract each time it is required, but if this cannot be done the extract should be removed from the tissue and stored in ampoules or small-stoppered tubes in the refrigerator. It will retain its properties unimpaired for several days if kept in this way. If allowed to remain in contact with the tissue, however, it tends to become acid. Mammalian embryo extract has usually to be stored for several days, as pregnant animals are expensive and difficult to obtain. It can still be used after ten days' storage, and Maximow (1928) says that rabbit embryo extract retains its properties unimpaired after it has been kept for six weeks in the refrigerator.

EXPLANTATION AND SUBCULTURE.

The methods of explantation and subculture employed are similar in essentials to those described by Fischer, (1925), Strangeways, (1924), Erdmann, (1930) and Craciun (1931). Each worker, however, varies the technique to suit his individual requirements and the facilities at his disposal; the procedure detailed below is that which I have found most convenient.

Various methods have been devised for the cultivation of tissues *in vitro*. These may be divided into two

classes, (A) those in which the tissue has to be transferred to fresh medium every second or third day during the culture period, and (B) those in which the tissue can remain undisturbed for a relatively long period, up to three weeks. To the first group belong (1) the 'hanging-drop' method, and (2) the watch-glass method devised by Fell. In the second group are included (1) the flask method of Carrel, (2) the coverslip method of Maximow, - a 'hanging-drop' method - and (3) the various 'fluid' medium methods which are also 'hanging-drop' methods. The flask method of Borrel, (1926), and the continuous irrigation method of de Haan, (1926, 1927, 1928) are not described, as I have no experience of the technique employed.

A. <u>The 'Hanging-Drop' Method</u>.

The most convenient arrangement of the necessary apparatus on the bench will be described first. It is convenient to work on a sheet of plate glass 3 feet long by 2 feet wide which can be kept clean and sterilised with 70 per cent. alcohol if desired. Two bunsen burners with tripods should be arranged in convenient positions, one for a mug of distilled water which must be kept boiling throughout the preparation of the cultures, and the other for a mug of paraffin wax which has to be

kept at melting point. Adequate illumination is necessary. A dissecting binocular microscope, fitted with a shield to prevent dust from falling on the stage is often necessary for the dissection of tissue, and should be provided with a suitable lamp.

The following apparatus is necessary:

1. Two sterile 'museum' jars, 8 inches by 6 inches by 2 inches. These rest each on a half tile, with the open ends facing the worker and tilted in such a way that pipettes, etc., do not touch any of the surfaces.
2. Two wooden 'rests' for pipettes and knives, etc. (one for each museum jar), each capable of accommodating four pipettes or knives.
3. A supply of sterile Petri dishes.
4. A supply of sterile Petri dishes containing watch glasses.
5. Sterile pipettes of various diameters; the ends of these should be plugged with cotton-wool.
6. Nipples for the pipettes.
7. Two glass rods each drawn out at one end to a fine point.
8. Sterile coverslips (No. 1 square) contained in a Petri dish. They may be either $\frac{7}{8}$ inch square or $1\frac{1}{4}$ inch square.

9. Sterile hollow-ground slides, extra thick with frosted ends: the sizes most frequently used are 3 inches by 1 inch or 3 inches by 1½ inches. I find the larger coverslips and slides the most convenient; they allow the use of a large quantity of medium and are easier to manipulate than the smaller size.
10. Sterile knives; these are always sterilised in pairs in test-tubes. Fine, thin-bladed knives are the most convenient.
11. A Petri dish containing 25 c.c. of Pannett and Compton's saline.
12. Thick squares of glass, about 2 inches square on which to arrange the coverslips. The bevelled tops of histological staining jars are very suitable for this purpose.
13. Plasma.
14. Embryo extract.
15. Ordinary histological slide-box trays on which to arrange the culture preparations.
16. Brush for applying the paraffin wax.

The tissue to be cultivated is removed from the embryo or animal with the usual aseptic precautions and is placed in a watch-glass in a Petri dish in a little

saline. The heart of a 9-day avian embryo can be used as an example. It is washed several times with fresh saline to remove any blood which may be present. Between each washing, the pipette must be rinsed out in boiling distilled water and saline before fresh saline is transferred to the tissue. Thereafter, the tissue is transferred to a hollow-ground slide with a drop of saline and placed on the microscope stage. The tissue is then rapidly cut into pieces from 1 mm. to 2 mm. in diameter. It must be cleanly cut, not pulled or torn: I have found that the cutting of the tissue into fragments can be done more quickly and with less damage to the cells under the dissecting microscope. When a sufficient number of fragments have been obtained, they are transferred with a pipette to a watch-glass containing about 2 c.c. of embryo extract. Two fine-pointed pipettes are then filled with about 1 c.c. of plasma and embryo extract respectively and placed with their ends well within one of the museum jars. Four coverslips are placed each on a square of glass protected by a sterile Petri dish lid. A fragment of tissue is next placed on each coverslip with a sufficient amount of extract to keep it moist. One or two drops of embryo extract are then discarded from the pipette containing embryo extract and a large drop of extract placed on each

fragment of tissue. A drop is discarded from the pipette containing plasma and a drop of plasma, as nearly as possible of the same size as the drop of extract, placed on each coverslip a little to one side of the extract. The plasma and extract must not be allowed to flow together at this stage, otherwise unequal coagulation will take place. The plasma and extract are then thoroughly mixed with a fine-pointed glass rod and the medium spread out in a thin layer on the coverslip. A hollow-ground slide is taken and two narrow strokes of paraffin wax placed one on each side of the hollow; it is placed on the bench and protected with a Petri dish lid. By this time the plasma has usually coagulated round the first culture prepared. The coverslip is then transferred, culture side downwards, on to the hollow-ground slide. It is fixed in position with a drop of paraffin and then the edges of the coverslip are carefully sealed on to the slide. The same process is repeated with the other three cultures. The remainder of the tissue fragments are then placed on coverslips, supplied with medium, etc., in the same way and mounted on slides. Before adding plasma or extract to the tissue one or two drops must always be discarded from the pipette, so as to wash away any foreign material which may have settled on the end of the pipette. The glass rod with

which the plasma and extract are mixed must always be dipped for a few seconds in boiling distilled water and saline before use to ensure sterility. The pipette with which the tissue fragments are transferred to the coverslips must also be rinsed out in the boiling water and in saline before use. If these precautions are taken, infection of the cultures need not be feared. The cultures are placed on microscopic slide trays, coverslip upwards and incubated at 38° C.

Subculture.

If growth is slow, the cultures do not need to be transferred to fresh medium until the third day after explantation. As a rule, however, if growth is active, as in the case of embryonic avian tissue, or considerable liquefaction of the medium has taken place, it is necessary to transfer the cultures to fresh medium on the second day.

The bench is arranged as before, and a supply of sterile pipettes, Petri dishes, plain microscopic and hollow-ground slides and coverslips prepared. A Petri dish of saline, freshly prepared embryo extract and plasma must also be available. The knives must be sharp and show no inequality of the points under the low power

of a microscope. A sterile microscopic slide - 3 inches by 1 inch - is placed inside a Petri dish and eight large drops of embryo extract well separated from one another are arranged on it.

It is possible to remove cultures from the medium without the aid of a microscope, but I have found that the removal can be done more quickly and with less risk of damage to the culture under a low power of the binocular microscope.

It is convenient to transfer the cultures in batches of eight. The paraffin on the coverslip is removed with a scalpel and four cuts following the outline of the coverslip are made on the paraffin adhering to the slide. The coverslip is then gently elevated with the point of the knife until it can be conveniently grasped with a pair of dental forceps. It is quite unnecessary to sterilise the slide in alcohol before removing the coverslip. It is then placed, culture side uppermost, on a piece of glass, 1½ inches square, lying on the stage of the microscope. The coverslip is kept in position by gentle pressure on the edge and a transverse cut is made along the outer limit of the zone of outgrowth. The coverslip is turned through an angle of 90° and another cut made at right angles to the first one. Four cuts

in all are made in this fashion, so that a small square containing the original explant and the zone of outgrowth is separated from the medium. The cuts should be made lightly: heavy pressure on the plasma compresses but does not cut it. It is important to avoid the inclusion in the mass to be transferred of plasma which is sparsely invaded or uninvaded by cells. A small drop of saline is then placed on the culture and it is transferred by means of a pipette to one of the drops of embryo extract on the glass slide already described. The culture is thus washed free of the waste products which have accumulated during growth. It is not necessary to wash the cultures in several changes of extract unless infection of the culture is suspected.

The remaining seven cultures are then removed from the medium and placed each one in a drop of embryo extract. The blades of the knives should always be dipped for a few seconds in the boiling water and then in saline immediately before use. If the edge of a knife appears to be getting blunt, a fresh one should be taken at once, as the successful removal of the tissue from the plasma depends almost entirely on the sharpness of the knife. The pipette with which the cultures are transferred to the extract should always be rinsed out

in boiling water and saline immediately before use, to ensure its sterility and also to prevent the possible transfer of infection from one culture to another.

The explants are then transferred to coverslips in groups of four, and the medium added in the manner already described. They are sealed with paraffin wax over hollow-ground slides and incubated at 38° C.

Cultures of Epithelium.

Cultures of epithelial organs cause very rapid liquefaction of the medium. It has been found advisable with some tissues to adopt the following procedure, recommended by Fischer, (1922). A drop of plasma and a drop of embryo extract are placed on a coverslip and thoroughly mixed. If a series of four coverslips are prepared one after another, the first is ready for the explant by the time the fourth has been mixed. The piece of tissue to be cultivated is transferred from embryo extract on to the coagulum with just sufficient extract to moisten it. The same procedure is followed in turn on the other three coverslips. The coverslip is then inverted over a hollow-ground slide and sealed with paraffin in the usual way. The slide is inverted on a slide tray so that the coverslip is downwards and the tissue rests on the coagulum. The epithelium is

thus able to grow on the surface of, and to penetrate, the coagulum. Epithelium appears to grow much better under these conditions. Connective tissue cells, on the other hand, grow more actively when the tissue is partially embedded in the clot, and this method of preparation diminishes to some extent the fibroblastic outgrowth which is often so extensive that it prevents the emigration of epithelial cells. Fischer (1922), recommends embedding the explant in the plasma for the first two or three passages. I have found, however, that this is liable to produce a very abundant outgrowth of fibroblasts and endothelium, particularly in cultures of liver, and these cells are liable to form a thick capsule round the epithelial cells, preventing their emigration. I consider it advisable to cultivate epithelial tissues on the surface of the coagulum from the beginning. I have also found that the inversion of the slide so that the coverslip is downwards ensures adhesion of the tissue to the coagulum. If this is not done, adhesion may not take place, particularly if the plasma clot is firm.

Subculture.

Epithelial cultures have to be very carefully subcultured in order to prevent as far as possible the transfer of connective tissue, and to avoid injury to the

delicate epithelial membrane. The use of the dissecting microscope is essential. As far as possible, the connective tissue should be removed and only the epithelial outgrowth transferred. It is extremely important also to avoid transferring plasma in which no cell invasion has occurred, as the smallest amount of uninvaded plasma will prevent the emigration of cells during the next passage.

B. The Watch-Glass Method.

The watch-glass method was devised by Fell. Carrel, (1912, 2.), had attempted to cultivate large pieces of tissue on Gabritschewski plates, but the method of Fell (Fell and Robison, 1929) is the most satisfactory so far devised and is particularly suitable for the cultivation of embryonic skeletal rudiments. A layer of absorbent cotton-wool is placed within a Petri dish, 3 inches in diameter. A circular hole, 1 inch in diameter, is cut in the centre of the cotton-wool so that transmitted light can be used to manipulate and examine the explants. Over the hole in the cotton-wool, a small watch-glass, 1½ inches in diameter, is placed. After sterilisation for half an hour at 140° C. the cotton-wool is saturated with about 20 c.c. of sterile water. In this way an efficient moist chamber is produced. It is not advisable to prolong the sterilisation beyond

the time mentioned as the cotton-wool loses its absorbent properties when overheated. The medium is contained in the watch-glass and consists of 4 drops of fowl plasma and 4 drops of freshly prepared fowl embryo extract, which are thoroughly mixed before clotting. The resulting clot should be firm as its consistency seems to be of importance in determining the degree of growth and differentiation of embryonic rudiments of this type. ~~(p. ———).~~ Fell, in her original communication (Fell and Robison, 1929) used larger Petri dishes, 4 inches in diameter, and larger watch glasses, which were blackened so that direct illumination could be used to examine the explants. A larger amount of medium, however, is required and I prefer transmitted illumination, which allows a more thorough examination of the explanted tissue.

The bench is arranged as before and the tissue to be explanted is transferred from embryo extract on to the surface of the clot and the excess of fluid removed with a fine pipette. It is important that the tissue should be placed as flatly as possible on the surface of the clot, otherwise distortion is liable to occur owing to the unequal growth of the connective tissue of the explant. A considerable amount of water of

condensation often forms on the lower surface of the lid of the Petri dish during incubation. This may be reduced by (a) placing the Petri dishes on pieces of wood laid on the usual metal trays of the incubator, and (b) keeping the Petri dishes in the upper part of the incubator.

Subculture.

Such large pieces of tissues cause a rapid liquefaction of the medium and the tissue has to be transferred to fresh medium every second day. A sufficient number of Petri dishes containing medium are prepared beforehand. The explant is loosened from the clot by cutting very carefully round the zone of outgrowth. This should be done in such a way that an equal amount of connective tissue remains attached on each side of the skeletal rudiment. If there is marked excess in one region, distortion of the rudiment is liable to occur during the subsequent passage. After the explanted tissue has been separated from the medium it is transferred with a wide-bore pipette to a hollow-ground slide, protected by a Petri dish lid, which contains two or three drops of saline. This is removed and a few drops of embryo extract substituted. From the embryo extract the explant is transferred to fresh medium, the excess of fluid removed as before and

the preparation incubated.

Infection of watch-glass cultures is rather more liable to occur for two reasons. The type of tissue cultivated often takes several minutes to dissect from the embryo and is in consequence liable to infection from the air. This risk may be reduced by thorough washing of the explants with saline before placing them in the medium. A much larger area of medium is used and so the risk of infection is greater. The most rigid precautions must accordingly be taken to avoid exposing the medium, and the Petri dish should only be opened either on the microscope stage which is protected with a shield or else in such a way that the watch-glass is protected from foreign material falling on it directly. An infection can generally be seen as a small whitish spot on the medium. A small infection can be got rid of if it is far enough away from the explant by searing it with a red hot platinum loop. The culture should then be transferred to fresh medium as soon as possible. If it is not possible to transfer an infected culture at once, the infection should be seared and the culture kept at room temperature until the tissue can be subcultured. An infected culture should always be transferred to fresh medium at the end of a series and the explant should be washed several times in saline and embryo extract. In

actual practice, very few infections occur if adequate precautions for the protection of the medium are taken.

C. The Flask Method.

This method, which was devised by Carrel, (1923, *l.*), allows the tissue to be maintained in an undisturbed state during cultivation. The tissue is cultivated in small flasks of different types and sizes. The type most commonly used has a diameter of about 3 cms., is 0.4 cms. to 0.5 cms. in height, and has a lateral neck passing obliquely upwards 0.4 cms. in diameter and 2 cms. long, through which the medium and tissue fragments are introduced. The neck is closed with a plug of cotton-wool over which is placed a rubber cap. About 0.5 c.c. of plasma is introduced through the neck and allowed to flow over the bottom of the flask; an equal quantity of diluted embryo extract consisting of Pannet and Compton's saline, 3 parts, embryo extract, 1 part, is added to, and thoroughly mixed with, the plasma. Before coagulation is quite complete the fragments of tissue are introduced with a pipette and placed on the surface of the medium. The excess of fluid is removed, and a thin layer of embryo extract is placed on the surface of the coagulum. The neck of the flask is flamed and the opening closed with a cotton-wool plug covered with a rubber cap. If

too much extract is added or if coagulation is complete before the tissue is placed inside the flask, adhesion of the fragments does not take place and they float in the extract. The embryo extract is removed every second or third day and the surface of the coagulum flooded with fresh embryo extract. This is removed after quarter of an hour and a thin layer of embryo extract placed on the surface as before. If prolonged growth of the culture is desired, the tissue fragments must be removed from the medium every 10 to 14 days with a small platinum spatula, washed in saline and embryo extract, divided into two or more pieces if necessary and placed as before in fresh flasks. The removal of the tissue fragments is often difficult, and considerable practice is necessary before it can be done skilfully. Carrel has devised four other types of flasks. One has an opening on the upper surface which is closed with a coverslip sealed on to the top with paraffin wax. This pattern allows fairly good microscopical examination of the cultures under a low power during growth and the tissue fragments can be extirpated from the medium much more easily if this type is used. It is also possible to place small pieces of coverslip on the bottom of the flask through the opening and these can be fixed in

position with a small amount of plasma and embryo extract. The medium is then introduced in the usual way and the tissue fragments to be cultivated placed over the small pieces of coverslip. The pieces of coverslip with the cultures attached can be removed from the medium through the upper opening when the culture period is complete, and fixed and stained entire for histological examination. Another type has an opening in the base of the flask which is also closed with a coverslip. This method also allows subsequent histological examination of the culture on the coverslip. The fourth type of flask has two necks and the fifth has a neck which passes obliquely downwards instead of upwards.

Maximow's Coverslip Method.

Maximow (1925), attached a small round coverslip by means of a drop of sterile saline on to the under surface of a large square coverslip, the whole being treated as a 'hanging-drop' preparation. The medium used consisted of plasma and embryo extract. Every two or three days the small coverslip was removed from the large one, immersed for 15 to 20 minutes in Ringer's saline and then attached to another large coverslip over another hollow-ground slide. If necessary, more plasma and extract can be added to the culture.

This method was found particularly useful by Maximow (1928), for the study of blood leucocytes. I have found it also useful for cultures of spleen from which many amoeboid cells emigrate which cannot be transferred to fresh medium. The only disadvantage is that the small round coverslip is often difficult to detach from the square one; this difficulty may be minimised by attaching the round coverslip with a very small amount of sterile vaseline which renders it more easily detachable. If this procedure is adopted, the coverslip must not be immersed in the Ringer's saline during the washing of the culture but should float on the surface.

Cultivation in Fluid Media.

Although a coagulated medium such as plasma and embryo extract provides the best conditions for active growth of tissue in vitro, it does not provide the best possible conditions for cytological investigation either during cultivation or after fixation. The plasma is always stained by the histological stains employed and if it coagulates in a thick layer round the zone of outgrowth, it is often impossible to distinguish the finer details of the cells after staining. If fluid media are used they can be removed by immersing the culture for a minute in warm saline before fixation.

Fixation is very rapid and the appearances of the cells can be preserved - provided the proper fixing agent is used - in a condition closely approximating to their state during life. The cytoplasm is spread out in a thin layer on the surface of the coverslip and the cell constituents can be readily studied. While this method of cultivation belongs to the second group - that in which the tissue remains undisturbed for several days on end - the method was adopted not on account of that condition, which is necessitated by the fact that it is impossible to transfer the delicate films of cells which grow out on the coverslip, but as a means of providing optimum conditions for cytological investigation of cells growing in vitro. Lewis and Lewis (1924) have used with great success a medium composed of 80 c.c. Locke's solution containing 0.5 per cent. dextrose and 20 c.c. chicken muscle bouillon. A drop of the medium is placed on a coverslip and the tissue placed in the centre of the drop, which is then spread out in a thin layer on the coverslip. The coverslip is then inverted over a hollow-ground slide to which it is sealed with paraffin. For avian tissues I generally employ the following medium. 1 c.c. of avian plasma and 1 c.c. of embryo extract are thoroughly mixed in a

small centrifuge tube which is then stoppered with a rubber stopper. The tube is put into the incubator for half an hour at the end of which time the clot is broken up with a glass rod. It is then incubated once more for an hour to allow retraction of the fibrin to take place. The fluid is sucked off with a pipette into another tube which is also closed with a rubber stopper. This is used as the culture medium. By incubating the clot for another hour, more fluid may be obtained. The cultures are prepared in the manner described by Lewis and Lewis. I find it advisable, however, in order to ensure contact between the glass and the tissue, to invert the slide so that the coverslip is downwards and the tissue rests on the surface of the coverslip. The drop of medium must be spread out as thinly and as extensively as possible and the explant must be small - 0·5 mm. to 1·0 mm. in size. If large coverslips and slides are used, considerable evaporation of the medium takes place, and I place a drop of sterile distilled water in the hollow of the hollow-ground slide in such a position and of such a size that it does not come into contact with the medium or the coverslip. This precaution is not so necessary when small slides and coverslips are used. Concentration

of the medium may also be prevented by diluting it with one-third of its volume of distilled water.

For mouse tissues, equal parts of mouse serum and mouse embryo extract can be used,and for rat tissues equal parts of rat serum and rat or mouse embryo extract. As mammalian embryos are often difficult to get at the right stage and chicken embryos are usually always available, I generally use a medium composed of one part rat plasma, one part avian plasma and two parts avian extract. This is allowed to coagulate and the fluid is obtained in the manner already described. In this medium, the cells grow more actively than in serum and extract, and just as well as in the fluid expressed from the coagulum formed by equal parts of homologous plasma and homologous embryo extract. Every second day the coverslip is taken off the slide and the medium removed. The cultures are taken in batches of four, and from each in turn the coverslip is removed, the medium sucked off and a drop of embryo extract placed on the explant. Thereafter, the extract is removed and fresh medium added and spread out as before. The coverslips are then placed over hollow-ground slides on to which they are sealed with paraffin in the usual way. Most tissue can be maintained in a healthy state for 10

days by this method, but cell proliferation diminishes after the third or fourth day and emigration gradually ceases.

The methods above described may have to be modified for different purposes. The general principles are the same, however, for each method of cultivation,and any modification necessitated will be mentioned in the appropriate section.

HISTOLOGICAL EXAMINATION OF TISSUE CULTURES.

Tissue cultures can, in many cases, be examined during life both by ordinary and dark-ground illumination. In addition they can also be photographed and filmed under suitable conditions. They can also be fixed, and stained for histological examination either *in toto* or after having been cut into serial sections. It is convenient to describe the general methods used, any modifications required by certain tissues being described in the appropriate sections.

Fixation.

Before fixing, it is advisable to immerse coverslip cultures for about a minute in saline at 38° C. so as to remove any fluid containing coagulable proteins. It is impossible to avoid the coagulation of the medium in plasma cultures round the zone of outgrowth. If liquefaction of the plasma has taken place round the explant, however, the liquefied medium can be removed in warm saline. With fluid medium cultures, the medium can be entirely removed before fixation. Fixatives containing alcohol should be avoided as they bring about a very dense and coarse precipitate of any plasma present. Zenker's fluid without acetic acid, for 5 to 10 minutes,

is quite a satisfactory fixative for morphological purposes. It is not an ideal cytological fixative, but is quite adequate to show the general character of the cells. If 1 per cent. acetic acid is added, a sharper picture is obtained after staining; the nuclei stain more intensely and cytoplasmic outlines are more clearly seen. The acetic acid destroys cytoplasmic constituents, however, and it has the additional disadvantage of causing a rather coarse precipitate of any plasma present. Zenker-formol, (Maximow 1925), does not cause such a coarse precipitate and preserves cell constituents, such as mitochondria, fairly well. Fixation for quarter of an hour is adequate. All these fixatives may be used for fluid medium cultures; five minutes' fixation is adequate.

Bouin's fixative should only be used for fluid medium cultures and plasma cultures in which considerable liquefaction has taken place, as it causes a very dense precipitation of plasma. Fixation should not be prolonged for more than five minutes. Corrosive sublimate - a saturated solution in water - is the best fixative for cultures which have been stained during life with vital dyes. Formalin - 5 per cent. to 10 per cent. formaldehyde neutralised with $MgCO_3$ - can also be used.

It causes considerable shrinkage of the cells, and particularly of the nuclei, and should only be employed if the subsequent staining method requires it. Fischer, (1925), however, recommends fixation in 2 per cent. formalin-Ringer solution. Acetic alcohol, which is used by many workers, is not suitable for subsequent histological examination of the cultures, as it causes marked shrinkage of the cells.

Coverslip cultures fixed in a chrome fixative are placed in a large flat staining jar of water for half an hour. They are then dehydrated by passing them through graded alcohols, 30%, 50%, 70%, 90%, and absolute alcohol, (two changes): five minutes in each alcohol is sufficient. From absolute alcohol, they are placed in absolute alcohol and xylol, equal parts, and cleared in two changes of xylol. From xylol, they are once more brought down to water for staining purposes. I have found that staining with all stains is improved if the cultures are treated in this way. If the cultures are stained directly after washing, the staining is always faint. Cultures which are to be stained for fat, however, unless subjected to prolonged post-chroming, must be stained without this treatment. If it is not possible to stain the cultures immediately

after they have been dehydrated, etc., they should be kept in water containing 0·5 per cent. to 1 per cent. potassium bichromate, as prolonged washing in water or storage in weak alcohol removes the chrome salt entirely and renders satisfactory staining difficult. It is not necessary to treat cultures fixed in solutions containing mercuric chloride with iodine, as the period of fixation is not sufficiently long to cause precipitation of the mercury salt in the fixative. In cultures fixed in saturated corrosive sublimate, however, deposit of mercuric chloride occurs, and they have to be treated with iodine. After fixation in Bouin's solution, it is essential to remove all traces of the picric acid in 50 per cent. alcohol. The cultures are then dehydrated and cleared and brought to water for staining.

Cultures of embryonic organ rudiments such as skeletal rudiments and cultures in flasks which cannot be treated as coverslip preparations are always removed from the culture medium before fixation and are fixed for varying periods of time up to 1 hour, depending on the size of the explant. Zenker's solution with or without acetic acid, Zenker formol or Bouin's fixative may all be used. After fixation, cultures of this type fixed in fixatives containing chrome salts are washed

in several changes of distilled tap-water for not more than one hour. If the washing is prolonged beyond this time, too much of the chrome salt is washed out and subsequent staining is not so satisfactory. Those fixed in Bouin's solution are placed in 50 per cent. alcohol to remove the picric acid.

Staining.

Many of the ordinary histological nuclear stains can be employed for staining cultures in toto, but the methods have to be modified to produce satisfactory results.

The cells of the zone of outgrowth are more difficult to stain than ordinary histological sections, and this is apparently due to the rapid fixation which inhibits the occurence of the changes which take place in the cells in small blocks of tissue during the penetration of the fixative. The production of artefact upon which much of ordinary staining depends is thus prevented to a considerable extent. Even with poor cytological fixatives such as Bouin's solution or Zenker's fluid plus acetic acid, the nuclei do not show the complicated structure seen in histological preparations, although they appear very much more complex than during life. Only in cells which are very degenerate before fixation

are appearances produced which resemble those seen in ordinary fixed and stained preparations. In the centre of the original fragment where fixation is not so rapid, the cells resemble to some extent those seen in histological preparations. The process of embedding in paraffin may in both cases contribute to the production of artefacts, but the cells of the zone of outgrowth of cultures which have been embedded in paraffin and cut into serial sections, although contracted, differ much less from the cells in the zone of outgrowth of an unembedded culture than those of the central fragments.

Counter-stains such as eosin are not taken on by the cytoplasm to any extent and are unnecessary, as the cytoplasm is usually sufficiently tinted by the nuclear stain. With mixed stains such as Giemsa or Eosin Azur II, however, a contrasting cytoplasmic stain can be obtained. Iron haematoxylin is the most useful stain, as it does not tend to fade. The cultures are immersed for half an hour in 2·5 per cent. iron alum at 38° C.; they are then rinsed in water and placed for a similar length of time in Régaud's haematoxylin which is filtered before use. Differentiation is carried out in 0·5 per cent. iron alum and controlled under the microscope. The preparation is then dehydrated, cleared and mounted in

benzol balsam in the usual way. Picric alcohol may be used for differentiation. This method is useful if the mitotic figures are being counted, as it is possible to remove the nuclear staining of resting cells almost entirely while the nuclei in division remain quite sharply stained. Weigert's iron haematoxylin is a useful stain, particularly for cultures which have to be photographed, as it stains the cytoplasm more intensely than Régaud's haematoxylin. The mixture of the two solutions will keep for 2 days. Staining is complete after 2 hours, and the cultures should then be immersed in water for quarter or an hour before dehydrating, etc. If there is much deposit around the explant, ~~no~~ iron haematoxylin can not be used, as the plasma is intensely coloured, and Harris' haematoxylin has to be employed. A 3 per cent. Harris' haematoxylin in water should be used, and the staining should be prolonged overnight. If the stain is undiluted, staining of the nuclei does not take place. After staining, the haematoxylin is 'blued' by immersing the cultures in tap-water for from one half to two hours. If the stain is 'blued' in ammonia water or in hot water, the culture may float off the coverslip. The preparations are then dehydrated, cleared and mounted as before. Toluidin blue, carbol thionin blue, borax

methylene blue can all be used, but the duration of staining varies with different specimens of the stain. As these dyes are easily removed by dilute alcohol, it is necessary to take away the original fragment before staining, so that dehydration may be accomplished as quickly as possible. Maximow's Haematoxylin Eosin Azur II stain can be used after Zenker-formol fixation. The haematoxylin staining should be very faint, the nuclei being only lightly tinted. The eosin and the azur II have, of course, to be mixed immediately before use, and it is advisable to stain in the mixture for at least 12 hours. The preparation should be differentiated in absolute alcohol, not in 95 per cent. alcohol. Giemsa's stain may also be used after corrosive sublimate fixation. A 1 in 10 dilution of the mixture is used, and the cultures should be differentiated in acetone and xylol. Dominici's stain can also be used, and although the results are not so striking as in the best Giemsa staining, a much higher percentage of good preparations can be obtained. For staining fat, Scharlach R. in equal parts of 70 per cent. alcohol and acetone produces the best results. Other methods of staining fat are described later.

As already mentioned, tissue cultures on coverslips are very favourable objects for cytological staining as the cells are spread out in a thin layer on the coverslip, and embedding and section-cutting are unnecessary. A very careful study of the effects of various fixatives has been made by Strangeways and Canti, (1927). These observers illuminated the cells by dark ground illumination and then fixed the same cells with various fixatives. They found that after fixation with a 2 per cent. aqueous solution of osmic acid, the cells present^ed appearances which resembled closely those seen in the healthy living cell. Little or no shrinkage occurred, precipitation of the cell substance was slight, and distortion of the cell constituents minimal. After this fixation, the cells can be stained by mitochondrial stains. The following method of fixation by osmic acid as recommended by Fell (1930) produces excellent results. The culture is fixed for one and a half hours in osmic acid, 2 per cent., freshly prepared. It is then rinsed in water and placed in Zenker's fixative without acetic acid overnight. After washing in water for one hour, the culture is placed in 50 per cent. alcohol, then in 70 per cent.^alcohol coloured brown with iodine to get rid of the mercuric chloride, and dehydrated and

cleared in xylol to remove the fat, brought once more to water and stained by Gatenby's iron-haematoxylin and acid fuchsin method. The treatment with Zenker's solution mordants the cells: if this is not done, staining does not take place. I have found that 3 per cent. potassium bichromate is equally effective. If desired, some of the fat of the cells may be preserved by keeping the culture for 3 days in the bichromate solution. It becomes a bright yellow and resists solution in xylol. Even after staining, the fat retains its colour and is easily distinguished from the other cell constituents. Flemming's solution (strong formula) without acetic acid is an almost equally good mitochondrial fixative, and owing to the smaller amount of osmic acid present is less expensive in use. Two hours' fixation in Flemming's solution is sufficient. The culture is then rinsed in water and may be stained after dehydration and clearing in the usual way. A better result is obtained, however, if the culture is kept overnight in 3 per cent. potassium bichromate before dehydration and clearing. The following staining methods may thereafter be used:

1. Iron haematoxylin after both osmic acid and Flemming's fixation.
2. Iron haematoxylin and acid fuchsin after osmic acid fixation.

3. Bensley-Cowdry's modification of Altmann's method after Flemming's fixation: osmic acid fixation, provided the solution has not been prepared for more than 24 hours may also be employed.

1. Iron Haematoxylin.

Any ripened solution of haematoxylin containing about 10 per cent. alcohol may be used. I find Régaud's haematoxylin very satisfactory. It is advisable when possible to remove the original fragment before staining, as it takes up the stain and makes differentiation more difficult. The culture is mordanted for half an hour in the incubator in 5 per cent. iron alum. It is then rinsed in water and placed in the haematoxylin for a similar time in the incubator. It is differentiated in 0·5 per cent. iron alum until the mitochondria appear distinct, the differentiation being controlled under the microscope. Thereafter, it is dehydrated, cleared and mounted.

2. Iron Haematoxylin and Acid Fuchsin.

The following method is used by Fell (1930) after fixation in osmic acid. The culture is mordanted for one and a half hours in 2·5 per cent. iron alum. It is then rinsed in water and placed in 1 per cent. aqueous haematoxylin for the same time in the incubator. The cells are differentiated with 2·5 per cent. iron alum until the

mitochondria are greyish. Thereafter the culture is placed in Altmann's fuchsin on a hot plate (52° C.) for 5 to 10 minutes, rapidly differentiated in 95 per cent. alcohol, dehydrated, cleared and mounted as before.

3. Bensley-Cowdry's Modification of Altmann's Method.

This method may be used after fixation in Flemming's fixative. 2 c.c. to 3 c.c. of Altmann's fuchsin are placed in a watch-glass in the incubator for 10 minutes. The culture is placed in the stain for quarter of an hour and then rinsed in water. It is dipped first in a jar of methyl green and then moved backwards and forwards in a jar of absolute alcohol until it appears pale green. Thereafter, it is cleared and examined under the microscope. As differentiation is very rapid in the alcohol and is liable to be overdone, the culture should always be cleared before examining under the microscope. When differentiation is complete the culture is mounted in the usual way. This method produces very pretty results but the preparations fade within a few months.

Any type of culture may be cut into serial sections. The celloidin method is apparently the best, as shrinkage of the tissue is reduced to a minimum and it is the only method which gives satisfactory sections of the zone of

outgrowth. It requires, however, a microtome capable of cutting thin celloidin sections. For examination of the explant, paraffin embedding is quite satisfactory, provided certain precautions are taken.

Coverslip Cultures.

The following is the method recommended by Fell. The cultures while still on the coverslip should be tinted with eosin, cleared in cedar-wood oil and then transferred to paraffin (M.P.— 54° C.) in an incubator at 56° C. After quarter of an hour, the paraffin should be removed and fresh paraffin added. This is removed after another quarter of an hour and fresh paraffin added once more. After another half-hour the coverslip is removed from the paraffin and placed on a warm plate. A square is marked out on the surface which encloses the culture and a small amount of the medium. This is removed from the coverslip with a sharp cataract knife and placed in position in a watch-glass of melted paraffin. It can then be cut into serial sections either parallel to or at right angles with the surface of the coverslip. Any histological stain, provided appropriate fixation has been employed, can be used.

Cultures of embryonic rudiments are also cleared in cedar wood oil and the paraffin bath should be changed

twice. The embedding time in paraffin varies according to the size of the rudiment, but impregnation with paraffin is rapid and it is extremely important not to keep the tissue too long in the embedding oven.

VITAL AND SUPRAVITAL STAINING OF CELLS in vitro.

Vital Staining.

Cells growing in vitro may be stained with vital stains such as trypan blue and vital new red. Vital dyes of this type, when present in the medium in sufficient concentration to bring about staining of the cells, tend to inhibit growth, and I find it advisable, when possible, to stain the tissues before explantation by injecting the animal from which they are taken. Newly-born mice and rats tolerate doses of the dye, calculated on a weight basis, quite well. (Cappell, 1929). The injections have to be given subcutaneously or intra-peritoneally.

Supravital Staining.

I have used extensively neutral red and janus green B for supravital staining. As the explants absorb a large quantity of the dye, it is advisable to remove them before staining. This is particularly important when janus green B is used as a high concentration of the dye (1 in 5,000) which is rapidly toxic to the cells, is necessary to produce staining of the mitochondria if the explant is present. A dilution of 1/25,000 will stain the mitochondria of the cells when the explant has been removed, and according to Cowdry, (1924), this dilution produces specific staining. The explant is carefully removed and the coverslip is placed, culture side downwards, in a watch-glass of Pannett and Compton's saline in the incubator for a few minutes. Thereafter, it is placed in a solution of the dye (prepared in Pannett and Compton's saline), in the incubator for quarter of an hour. The dye is washed off with saline and the coverslip mounted and sealed on an ordinary microscopical slide.

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SECTION II.

APPEARANCES SEEN DURING CULTIVATION OF TISSUE in vitro.

When a piece of tissue is explanted in a suitable medium in vitro and incubated, the area occupied by tissue in the medium increases in size. This increase is due to three causes, (1) the emigration of cells which form the 'zone of outgrowth' from the explant into the medium, (2) the multiplication of cells in the explant and in the zone of outgrowth, and (3) the spreading out of the explant on the supporting surface.

1. Cell Migration.

During embryological development the cells of the organism are capable of amoeboid movement and emigration, but after development is complete, the cells of most adult tissues, apart from the leucocytes, do not show active movements under normal circumstances. This capacity, however, manifests itself once more in some cells when the tissue complex is disturbed by a wound or by loss of substance. All cells cultivated in vitro show amoeboid movement and emigrate away from the explant. Before movement can take place, however, the cell must come into contact with a solid or semi-solid substance (see p. //). This induces in the cell a 'stereotropic response' which shows itself in modification of shape and possibly metabolic state. Amoeboid movement and emigration per se depend on other additional

factors such as temperature, composition of the medium, character and condition of the tissue.

Emigration of cells is the first phenomenon observed after explantation, and takes place after a latent period, the length of which depends on the type and age of the tissue, the character of the medium employed, and the injury to which the tissue has been exposed during its removal from the body or during subculture. As a rule, the latent period is shortened when the tissue has been grown in vitro for some time. If the tissue is very embryonic, e.g. avian tissue about the 2nd or 3rd day of incubation, the fragment tends to spread out as a whole, forming a thin sheet, and emigration of cells is not extensive (Fig. 74). In general, however, it may be said that the more embryonic the tissue - from the 6th day of incubation onward in the case of avian tissue - the greater the ease with which emigration takes place. This is probably due to several causes, the most important being the absence of intercellular material and the greater plasticity of the embryonic cell. According to Lewis and Lewis (1924), it is possible that the cytoplasm becomes more rigid with age, and the capacity for emigration is thus diminished. If, however, the tissue contains a large number of migratory cells

which are amoeboid normally in vivo, e.g. spleen and lymph gland, emigration of amoeboid elements takes place within a relatively short time although the organ may be from an adult animal. Cells of avian tissues emigrate more readily than those of mammalian tissues, even when the latter appear to be at a similar stage of embryonic development. It has been noticed during dissection of mammalian and avian tissues that mammalian tissues offer more resistance to the knives and needles, etc. According to Chambers (personal communication) mammalian cytoplasm is more viscous than avian. This probably accounts for the lesser ease of migration of mammalian cells. Connective cells (fibroblasts, mesothelium, endothelium) emigrate after 2 or 3 hours' incubation, while epithelial cells have often a latent period of 10 hours or more before migration begins. Even as between different epithelial tissues, there are marked differences; cells from some organs emigrate after a shorter latent period than others: e.g. liver cells migrate particularly slowly, 18 to 24 hours often elapsing before emigration begins; while epithelial cells from the medulla of the kidney emigrate after a much shorter latent period - 10 hours or less. With regard to the conditions modifying the extent of migration, it has

been found that in a plasma medium, the zone of outgrowth is much denser, cells extend further out into the medium, and emigration continues for a longer time than in a simple fluid medium. This is probably due in part to the larger area provided for migration. Dilution of the plasma increases the extent of migration (Carrel, Burrows, 1911a, Lambert, 1914) i.e. cells wander further away from the original piece of tissue and the zone of outgrowth appears thicker; in addition migration takes place after a shorter period.

Various theories have been put forward as to the causes of migration. Rous,(1913), coloured plasma with litmus and then explanted into it small pieces of chicken tumour and late embryonic chicken heart. He found that the explants were at first blue,but as growth proceeded, the explants and that part of the medium into which cells had migrated became pink, showing that acid formation took place during growth. The medium which was not invaded by cells remained alkaline and diffusion was slow. Burrows, (1913), quoted by Craciun, (1931,p.232), has suggested that these differences in reaction may, by altering the surface tension of the cell,force it into the medium. It might be expected that in cultures in which the medium was kept as far as possible at constant

hydrogen-ion concentration, as in the elaborate 'perfusion' apparatus of de Haan and his co-workers, migration would be diminished. This, however, is not so: actively emigrating and proliferating cultures have been obtained by this method. Burrows, (quoted by Craciun 1931, p.232), also states that when the explant is large, cells migrate further away from the original fragment than when the explant is small, and that this is due to the greater production of acid by the larger mass of tissue. In my own experience, however, (Figs. 147, 154.) cells migrate just as far from small as from large fragments, and they even migrate further in many cases. Lewis and Lewis (1924) have suggested "that the surface film between the fluid medium and the solid may exert a definite pull on the cells, causing them to migrate outwards under tension." Observations on growing cultures of fibroblasts show that there is a marked tendency for cells to emigrate in an approximately radial direction, (Fig. 3) the long axis of the cells being directed at right angles to the surface of the explant. This is particularly noticeable in cultures of fibroblasts when the plasma layer is thin. The radial arrangement when once begun is maintained until an obstacle is encountered; if this can be surrounded, as in fig. 4, the radial arrangement is then resumed.

From appearances of this sort, it has been concluded that substances formed in the medium by the tissue produce at the surface a 'centrifugal pull' which draws out the cells from the explant. To test this hypothesis, Fauré-Fremiet and Wallich (1925) spread out a drop of medium on a coverslip, and covered the surface with talc. When the explant was put in the centre of the drop, the talc gradually retreated from the explant to the periphery of the drop, and a clear zone was formed round the explant into which cells emigrated from the tissue. They observed this phenomenon in cultures of amoebocytes of Arenicola, and cultures of embryonic guinea-pig skin and chicken heart. By an ingenious method of increasing and decreasing tension at the air-fluid interface these authors found that the spreading out of the talc was diminished if the pressure was increased, and increased if the pressure was diminished. They conclude that in growing cultures some catabolic substance diffuses from the tissue which lowers the tension at the surface, producing a centrifugal pull which draws out the cells passively from the explant. Fauré-Fremiet and Wallich note that while amoebocytes arising from a complex tissue show this radial type of emigration, isolated ones do not. Fauré-Fremiet and Ephrussi (1927) conclude later

that there are two factors, (a) amoeboid movement which depends on changes in the cytoplasm, and (b) 'un mouvement d'ensemble' which depends on changes in surface tension. If, however, surface tension played an important part in determining the direction of emigration in cells like fibroblasts, it would be expected that if two pieces of tissue were placed on the same cover-slip in the same drop of medium that a mutual repulsion would occur. In my experience, however, this does not occur; fibroblasts from one culture intermingle with those from another, and approach the explants. (Fig. 75). It might be expected also that the larger the explant, the greater the amount of catabolic substance produced. The resulting increase in centrifugal pull would cause cells from large explants to wander further out than those from small. As already mentioned, this does not occur. Furthermore, an arcade-like arrangement of cells, in which the long axes of the cells are parallel to the explant, is not uncommon, particularly in fluid medium cultures and in cultures of epithelium. Strands of endothelium also show a tendency to migrate parallel to the explants. (Fig. 5.)

It has often been observed in cultures of fibroblasts that if the plasma and embryo extract are insufficiently

mixed and the medium coagulates unevenly, the cells appear to be drawn out along certain lines at right angles to the explant, as if under tension. Lateral processes are absent and the cells are elongated longitudinally. Weiss (1928) devised a method of keeping the medium under tension in small triangular frames of capillary glass. He found that in cultures of fibroblasts the outgrowth was roughly in the form of three radiating bundles, and that in the zones of maximum tension along the normals bisecting the sides of the triangle, the cells were elongated and compact, while opposite the angles, where the tension was least, the cells were loosely arranged. Cultures of macrophages did not react in this way. It seems probable, therefore, that tension in the medium can influence the direction of the outgrowth of some forms of tissue, but it is not in itself an essential factor in determining migration.

According to Tait (1918) amoeboid movement is probably due to local changes of metabolism in the cells, which produce variations in surface tension. When the tension is reduced, pseudopodia are protruded, when it is increased they are retracted. This view was originally put forward by Rhumbler in 1898 and is held by many other observers. Leo Loeb, from studies of amoeboid

movement in the amoebocytes of Limulus (1920, 1921), is of the opinion that local metabolic changes in the cell produce changes in cytoplasmic consistency leading to the formation of pseudopodia. When the cytoplasm becomes more fluid, pseudopodia are pushed out; changes in surface tension occur secondarily. His observations on amoeboid movement in cells under various experimental conditions appear to have a direct bearing on conditions seen in vitro. When amoebocytes are in contact with glass, the cells spread themselves out quickly, and amoeboid movement takes place within a short time. When the cells are in contact with fibrin, movement at first is not so active and the cells never become so spread out. They remain active and healthy, however, for a much longer time than those which were in contact with glass in fluid medium. Similar phenomena have been observed in tissue cultures. Cells in plasma are never so spread out as in fluid medium, (Fig. 32), but they remain healthier for a longer time and proliferation is more active. In fluid medium, after a preliminary period of activity the cells become spread out and activity eventually ceases. They appear as if paralysed by over-stretching. This is particularly well seen in cultures of macrophages. Loeb also noted that at the

point of contact of two cells, pseudopodia are not protruded. He concluded that when a piece of tissue is removed from its surroundings, the cytoplasm at the free end of the cell undergoes changes correlated with the strange environment, and pseudopodia are protruded. The capacity for movement being thus established, it tends to continue once it has begun. The direction of outgrowth he regards as purely fortuitous.

Whatever may be the causes of amoeboid movement and migration of cells, the primary factor is the capacity of the cell to undergo changes of shape when brought into contact with a solid or semi-solid substance, and this characteristic is able to show itself when, under suitable conditions, the normal anatomical restrictions are removed and a substratum for migration supplied.

2. Cell Proliferation.

The multiplication of cells in the explant can only be seen in histological sections, and is more frequent in small thin fragments than in large pieces, and in avian mesenchymal tissues than in avian epithelial or in mammalian tissues of all kinds. In the zone of outgrowth, however, mitotic division can be observed directly, and its occurrence depends on conditions similar to those necessary for active migration, viz. composition of the medium and

type of tissue. It is obvious, of course, that the more suitable the medium for migration, the greater the number of cells capable of mitosis in the zone of outgrowth.

Even when the number of potentially dividing cells in the explant is taken into account, it has been found that cell-proliferation is more active in a coagulated medium than in a fluid medium containing an equal amount of embryo extract. This lessened proliferation is probably correlated with the spread-out condition of the cells, which seems to inhibit mitosis.

Cultures of embryonic mesenchyme, endothelium or mesothelium show more proliferative activity than those of similar tissues from young and adult animals, and avian cells in general have a greater proliferative capacity than cells from mammalian tissues. Epithelial cells of all types do not multiply so rapidly as fibroblasts or cells of other mesenchymal types. During cultivation in vitro, however, the growth rate of adult and mammalian tissues of all kinds increases, and cell-division becomes more active.

Variations in growth-rate of primary cultures from the same organ or tissue are often found: after 2 or 3 passages in vitro, however, the growth-rate becomes

more uniform and appears to depend more on the composition of the medium. It is for this reason that Carrel recommends, for physiological experiemnts, the use of cultures which have become acclimatised to life in vitro.

3. Spreading out of Explant.

Spreading out of the explant is more marked in the case of embryonic than adult tissues, and is more extensive in the case of fragments of some organs than in others. In the case of tissue from very young embryos - avian embryos between the 3rd and 4th days of incubation - increase in area of the culture is brought about more by the spreading out of the explant than by the formation of a zone of outgrowth, and the fragment forms a thin sheet on the supporting surface, only a thin fringe of cells being found at the periphery. (Fig. 74). Even in tissue taken at later stages of development, e.g. intestine from 7-day avian embryos, a similar appearance is often present. In general, however, the older the tissue from which the cultur is derived, the less outspread it becomes.

If the outline of the tissue fragment is projected by means of a camera lucida before incubation and during the period of cultivation, the extent of the spreading out of the explant, and its relation to the gradually increasing zone of outgrowth, can be followed. In the illustrations given, (Figs. 145 - 154), the area is expressed as square

units. It is sometimes difficult to determine the limit of the central fragment. In a culture of mesenchymal tissue after say, 36 hours' incubation, three zones can often be distinguished, (1) a central zone, in which cell outlines cannot be distinguished, (2) a thick zone surrounding this, consisting of closely packed cells with no definite arrangement, and (3) the zone of outgrowth which radiates from the latter. These areas sometimes merge into one another so gradually that it is impossible to demarcate the central area. In such a case, the central area is regarded as that which is enclosed by a line drawn midway between the thick central part and the inner recognisable limit of the zone of outgrowth. In the outline of the zone of outgrowth, isolated cells were not included.

Fig. 145 shows a camera lucida drawing of a piece of choroid and sclerotic, (culture 533) before incubation. In fig. 146, the same culture is shown after 19 hours' incubation, and the area of the central fragment is seen to have increased from 9.5 square units to 12.26 square units. After a further 24 hours' cultivation, the area of the central fragment has increased to 27.0 square units. (Fig.147) Examination of the culture at intervals during the period of cultivation shows that the central fragment gradually becomes less dense, and although this is in part due to the migration of cells into the zone of outgrowth, the fragment as a whole spreads itself out and increases in area,

so that the impression is gained that the cells of the zone of outgrowth are impelled into the medium by the pressure of the spreading explant as well as by their own amoeboid movement. The total area of the culture (Fig.147) was 113.3 square units; that of the original explant was 9.5 square units. These figures convey no idea of the increase in mass of the culture, as the increase in total area is partly accounted for by the spreading out of the central fragment.

When a culture of this type is cut out of the medium, it contracts to a much smaller area. Fig. 148 shows the culture shown in fig. 147 after it has been removed from the coagulum. The zone of outgrowth was preserved as far as possible, so that the area, 11.3 square units, represents almost the whole of the area, 113.3 square units, seen in fig. 143. As will be obvious from an examination of figs. 149, 150 and 151, the central fragment spreads out as before. Although the culture contains more tissue than at explantation, the total area is not much increased - 118 square units as compared with 113 square units. The zone of outgrowth was, however, thicker. An interesting feature which I have often noticed is the increased rapidity of migration after the first transfer to fresh medium. As will be seen from fig. 146 and fig. 149, the total

area more than doubles, after the 1st subculture, that found in the first passage after an almost similar period of incubation. The total areas, however, at the end of the 1st and 2nd passages, did not differ so much, being represented by 113.3 square units and 131.2 square units respectively.

Burrows, (1913), quoted by Craciun, has stated that cells migrate further away from a large than from a small fragment. Fig. 152, (culture 523) shows a small fragment drawn before incubation, the area being represented by 2.2 aquare units: this culture was cultivated at the same time and in the same medium as culture 533. After 19 hours' incubation, the total area is represented by 29.5 square units, and after 43 hours, the area has increased to 146 square units. In fig. 147, the outermost cell was 4 units from the central fragment, in fig. 154, the outermost cell was 7 units from the central fragment. In this culture, the central fragment became very thin and the zone of outgrowth was not so thick as culture 533, but in the migratory zone figured, all the cells were in contact with one another except at the free edge, and isolated elements were not included in the area outlined.

Comparative measurements of area are used to ascertain the effects of different media on growth-rate. According to Ebeling, (1921), provided the

tissue has been grown in vitro for some time and fragments of approximately equal size are used, the rate of growth of two fragments in the same medium only varies within 10 per cent. I have certainly found that after a few passages, the growth-rate, as estimated by area, becomes more uniform. But even when the total areas are approximately the same, the thickness of the zone of outgrowth may differ considerably in cultures of fragments of the same size, and this thickness, which cannot be measured mathematically, is subject to more variations than the area under the same cultural conditions. Accordingly, only gross differences in area measurements ought to be regarded as of any value for indicating the effects of different media.

While fragments of epithelial organs, with the exception of the liver, do not, as a rule, tend to spread out as much as those of mesenchymal tissue, they do so to a certain extent if cultivated during embryonic development, and the process often gives rise to misleading appearances. For example, in the metanephros from a 12-day agian embryo in which the kidney tubules are still surrounded by a large amount of undifferentiated mesenchyme, the spreading out of the explant, particularly when associated with straightening of the coiled tubules, often gives the

impression that typical tubules are actually growing out into the medium. The significance of this is referred to later.

GENERAL CHARACTERISTICS OF CELLS CULTIVATED in vitro.

The appearances seen in cultures depend on the histological structure of the organ or tissue from which the explant has been derived. As most tissues and organs from an early stage of development consist of more than one type of cell, the growths are generally complex at first: after cultivation has been continued for some time, it may be possible to obtain a 'pure' culture, i.e. a culture consisting of one type of cell only. The character of the growth obtained in cultures of tissue from various organs is described on p. 143 et. In order to avoid repetition, the general characteristics of fibroblasts, mesothelium, endothelium, epithelium and wandering cells will be described here, as one or all of these forms may be found in cultures of different organs.

Fibroblasts.

Fibroblasts emigrate from almost every organ or tissue, but are obtained, of course, in greater numbers from some tissues than from others. The choroid and sclerotic from 9-day avian embryos provides a very suitable source of fibroblasts. After 2 to 3 hours' incubation, small tongue-shaped processes of cytoplasm are observed protruding from the edge of the explant. These alter continually in shape, increase in size and

a nucleus passes into each one. Each nucleated mass of protoplasm soon detaches itself from the explant and migrates freely into the medium. This process goes on simultaneously all round the explant, and eventually the original fragment becomes surrounded by a ring of cells which is added to by the progressive emigration of more cells and by the proliferation of the emigrated cells. The cells show considerable polymorphism, from a simple spindle-shaped type to a many-branched, multipolar form (Figs. 27, 24). The cell processes undergo perpetual variation; they may be broad and blunt or may taper to fine points, the latter being more frequently seen than the former. The arrangement of the cells is roughly radial around the explant, (Fig. 3.) but the direction of the long axes of individual cells is very variable. In a fluid medium, the cells appear larger than in plasma medium, this being due to the spreading out of the cells on the surface of the coverslip. The cells are adherent to, and overlap, one another, and where the outgrowth is thick round the explant an apparent fusion of the cells appears to exist. In preparations stained with Harris' haematoxylin, it is impossible in some cases to distinguish cell outlines. With iron haematoxylin, the outlines of individual cells can generally be seen even in the thickest

part of the outgrowth. Apart from observations on fixed and stained material, other evidence points to the absence of fusion of individual cells. In cinematographic films of growing cultures of fibroblasts, the passage outwards of cells from the thickest part of the zone of outgrowth can be followed, and the cells appear to move over one another. The detachment of mononucleated elements from a syncytial mass in the zone of outgrowth does not seem to take place. If a cytotoxic antiserum heated at 56°C. for half an hour (p. 255.) is added to a culture of fibroblasts, the cells become rounded up before death takes place and the cells contract individually, not as a whole. Even in places where clumps of three or more cells are found, each nucleus is surrounded by a narrow zone of contracted cytoplasm which is not continuous with that round the nucleus of a neighbouring cell, thus showing that each nucleus, with its surrounding cytoplasm reacts as an entity. In cultures of fibroblasts from adult tissue, in which cell emigration is often scanty, the independence of each cell is obvious and the cells are as a rule spindle-shaped.

Considerable difference of opinion however still exists as to whether embryonic mesenchyme growing <u>in vitro</u> forms a syncytium or an adherent reticulum. Levi (1925), considers that fibroblasts may form either a syncytium or a reticulum, and that during growth <u>in vitro</u>, a syncytium

may form by fusion of cells which were previously adherent to one another. He claims to have observed the passage of mitochondria from one nuclear area to another. Laser, H. (1925), is of the opinion that actual anastomoses between cells exist, and he states that vitally stained granules pass from one cell to another. Fischer (1930) also considers that connections exist between cells, but that these may be broken and reformed. He states (1923, /.), that isolated fibroblasts obtained from cultures on cotton wool threads never divide; and, before proliferation can occur, the cells must be in intimate relation with one another. I have often found that fibroblasts at the periphery of cultures which are quite isolated from other cells divide, and cells which are about to divide move away from other cells rather than remain in contact with them.

Lewis, W.H., (1922, /) from a careful study of mesenchyme cells during life <u>in vitro</u>, does not consider that they form a syncytium but is of the opinion that they give rise to an adherent reticulum when present in sufficient numbers. The results of micro-dissection operations on cells support this view. Chambers (1924) found that if the nucleus of one cell, which was in such close relation to another that cell boundaries could not be

distinguished, was punctured, changes took place in the nucleus, cytoplasm and mitochondria of the injured cell resulting in cell death. The other cell remained healthy, and its outline became apparent as the injured cell retracted from it. If one daughter nucleus of a pair of cells in the late telophase stage, which were connected by a thin strand of cytoplasm, was punctured, death of the punctured cell was followed in a short time by the death of the other cell , showing that where protoplasmic continuity exists, the injury to one nucleus is transmissible to another; these results have been confirmed by Chambers and Fell, (1931). In this study, these workers found what at first appears to be an apparent contradiction to the above statement. Puncture of one nucleus of an isolated binucleated cell was not always followed by degenerative changes in the cytoplasm and the other nucleus resulting in cell death. Changes occurred in the cytoplasm immediately surrounding the punctured nucleus but these disappeared and the cell regained its former appearance, the only sign of injury being the presence of the punctured, shrunken nucleus. Puncture of the other nucleus results in cell death which is preceded by typical nuclear, cytoplasmic and mitochondrial changes. These workers conclude that in the case of a binucleated cell, the

presence of another nucleus causes a reversal of degenerative changes in the cytoplasm. It does not invalidate the conclusion that when a protoplasmic connection exists between two cells, the injury inflicted on one is transmitted to the other.

Mesothelium.

The term 'mesothelium', as defined by Maximow (1927), is used to describe the continuous layer of flattened cells which lines all the serous cavities and invests the free surfaces of the organs which lie therein. Outgrowth of mesothelium may, therefore, be obtained from a fragment of any thoracic or abdominal organ or tissue on which the mesothelial covering is present at explantation, and is readily obtained from the heart and from the mesentery or omentum. The outgrowth consists of thin sheets or membranes of flattened cells which are in close contact with one another, and between which during life, while the cells remain healthy, no intercellular spaces or cell boundaries can be seen. The outlines of the individual cells, however, are readily made out after fixation and staining. (Fig. 141. 10,A). The cells are polygonal inshape and small intercellular spaces may be seen between them. Long thin striae which pass from one cell to another are also seen

in stained preparations and these are regarded by some workers as evidence of the existence of cytoplasmic continuity between the cells. In some places, the cells appear to overlap one another.

At the periphery of the outgrowth, the membranous arrangement of the cells may persist, but, as a rule, during cultivation the cells lose their polygonal shape, acquire typical processes and become undistinguishable from fibroblasts. (Fig. 10, 14f.). These separated cells are quite healthy; they emigrate into the medium and undergo mitotic division in the usual way. This phenomenon has been observed by Lewis (1923), who also describes the reverse process, namely, the formation of mesothelial membranes by the transformation of bi-polar and multi-polar mesenchymal fibroblasts in cultures from embryo chicken hearts. Maximow (1927) also describes the transformation in vitro of mesothelial cells growing in the form of a membrane into fibroblasts, but does not appear to have observed the reverse process described by Lewis. Maximow also observed the formation in vitro of colonies of typical fibroblasts from the desquamated mesothelial cells obtained from peritoneal exudates.

Endothelium.

The outgrowth of vascular endothelium occurs frequently in cultures of various organs and tissue, and in primary cultures, at least, it grows in a typical fashion. From the cut end of a blood vessel, either a vein or an artery, the endothelium grows out in the form of bundles or sprouts of long spindle-shaped cells which are closely adherent to one another. At the peripheral end of the sprout, the cells are often spread out in a roughly fan-shaped fashion and resemble fibroblasts. When the endothelium penetrates a thick coagulum, the character of the outgrowth resembles closely the capillary sprouts seen in repairing wounds in vivo. After one or two subcultures, this type of outgrowth no longer persists and the cells apparently become undistinguishable from fibroblasts. The formation of endothelial sprouts from the vessels of the pia mater has been described by Maximow (1925). He found that the primary form of outgrowth was not maintained and that the cells became undistinguishable from fibroblasts. Silberberg (1929-30), arrived at similar conclusions.

The endothelium from the larger vessels, aorta and great veins, and from the embryonic avian liver, shows a different type of outgrowth. The endothelium grows

out in the form of broad strands or sheets of elongated and closely adherent cells which do not show the fine branched processes found in fibroblasts. (Figs. 5, 12, 25, 78). Many of the cells break off and resemble fibroblasts and, after one or two subcultures, the outgrowth assumes a fibroblastic character. W. H. Lewis (1922) described similar appearances in cultures of endothelium from the avian liver, but does not record the transformation of the endothelial cells into a fibroblastic type.

Epithelium.

Certain types of epithelium emigrate in vitro much more readily than others, but the character of the outgrowth of epithelial cells, whether from organs of ectodermal, endodermal or mesodermal origin, is essentially similar, although individual variations, depending on the type of epithelium explanted, are found.

Epithelial cells differ from fibroblasts in that they form a continuous sheet or membrane in which the individual cells, with the exception of those at the free edge, are in contact with one another on all sides. Such a membrane may surround the explant entirely (Fig. 48.). Frequently, however, particularly when the explant contains a considerable amount of connective tissue, several small membranes may be found in the same

culture. ~~(Fig.)~~ Liver and tumour epithelia usually grow out in the form of broad spade-shaped or finger-shaped processes and rarely form continuous sheets round the explant. Epithelial outgrowth can be distinguished as a rule from mesothelium by the adhesion of the cells at the outer margin and, unless the culture becomes degenerate, the detachment of individual cells at the outer part of the zone of outgrowth does not take place. In an organ such as the kidney, in which the cortex consists of several functionally different kinds of epithelium, more than one type may participate in the formation of the membrane (p. /83) and it is impossible to determine the origin of any individual part of the sheet unless the organ has been vitally stained before explantation. Two epithelial sheets (Fig. 43.) from different kidney cultures, one from the cortex, the other from the medulla, may coalesce and continue to grow out on each side.

Observations during life on the growth of epithelial organs show how the membrane is formed. After several hours' incubation, in the case of an organ such as the kidney, the cells at the cut ends of a tubule abutting on the medium push out cytoplasmic processes, spread themselves out on the surface of the coverslip

or coagulum in a single layer which consists at first of three or four cells. This small group of cells is added to by the emigration of cells further back in the tubule and by mitotic division of the emigrating cells. Similar outgrowths take place all round the explant, and the cells at the lateral margins come into contact with one another, thus giving rise to a continuous membrane. At the free edge, the cells possess amoeboid processes which are continually altering in shape. These cells, in virtue of their movement, exercise traction on the rest of the sheet and, aided by the addition of cells from the explant, bring about the movement of the membrane as a continuous carpet. Craciun (1927) has described similarly the formation of epithelial membranes in cultures of the kidney of guinea-pigs. Occasionally, tubules at a considerable distance from the cut surface of the fragment can be seen growing out. In such a case, the tubular arrangement persists until the zone of outgrowth is approached. At that point, the cells spread themselves out in the manner described, and contribute to the formation of the membrane in the zone of outgrowth.

Sometimes tubules or glandular acini may be seen projecting from the edge of the explant. This appearance has led many workers to the conclusion that epithelial

cells can grow out in a manner resembling their morphological arrangement in vivo. Such complexes, however, usually remain unchanged during the passage: if growth does occur, it assumes a membranous character.

While the cells remain healthy no cell boundaries or intercellular spaces are to be seen during life, and the membrane thus appears to be a syncytium. Chambers and Fell (1931), however, found that, if the nucleus of an epithelial cell is punctured, the cytoplasm, except at two opposite ends, shrank away from the surrounding cells, and death of the cell supervened after the usual cytoplasmic and nuclear changes had occurred. The injury was not transmitted to the neighbouring cells, thus indicating that protoplasmic connection, in spite of the apparent continuity, does not exist between the cells of the membrane. When degeneration of the membrane takes place, however, the cells tend to retract from one another and spaces can be seen between them, thus giving the appearance of cell boundaries. During fixation, cell boundaries become obvious. When osmic acid is used, little or no retraction of the cells occurs, and in the cytoplasm remote from the nuclei fine striae appear which define cell areas. After staining with iron haematoxylin, the cell boundaries stain intensely (Fig. 15.)

and each cell can be seen to consist of a central nucleus surrounded by the cytoplasm in which lie mitochondria and fat globules. With ordinary fixatives, a certain amount of retraction may occur, resulting in the formation of intercellular spaces in cultures in which previous to fixation none had been present. Isolated epithelial cells are occasionally seen. These are always degenerate and are found only in cultures in which degeneration is taking place or in cultures in which, for some reason, emigration of the epithelium has not occurred.

The shape of epithelial cells varies in different parts of the same culture whether they are cultivated in plasma or fluid media, and the variation does not depend on differences in the type of epithelium in the explant. They may be polygonal or they may be elongated either parallel to, or at right angles to, the surface of the explant. At the periphery of the outgrowth they are often parallel to the surface of the explant and an arcade-like arrangement of cells is thus produced. According to Uhlenhuth (1916), the appearance of epithelial cells depends entirely on the consistence of the medium. From his descriptions, however, it is apparent that the differences observed were due to the occurrence of degeneration in the cultures. The consistence of

the medium only affects the extent to which the cells are stretched out on the supporting surface. In fluid medium, the further they migrate from the explant, the more outspread they become, forming a very thin sheet which is only one cell thick. The nuclei are ovoid and flattened. On the surface of a plasma medium, the cells are not nearly so stretched out, and the nuclei tend to be spherical, and the membrane may consist of two or three layers superimposed on one another. When the epithelium actually penetrates the coagulum, the cells are even less stretched out. Mitotic figures are numerous in plasma medium. In a fluid medium, probably owing to the stretched-out condition of the cells, they are much less abundant.

Cultures of epithelium liquefy plasma with great rapidity. When this occurs, the epithelial sheet may break away from the explant, and the cells retract and form a thick ring round the liquefied area. (Fig. 32.). These cells may then migrate once more into the unliquefied plasma and proliferate by mitotic division. Another layer of cells may also grow out on the surface of the coverslip from the explant. Sometimes the detachment is not complete and bands of contracted cells may connect the separated part to the explant. (Fig. 33.).

The presence of isolated degenerate cells has been mentioned. When the culture is left too long without being transferred to fresh medium, the cells at the periphery withdraw their amoeboid processes and become somewhat spherical. If the adjacent cells also become rounded, the edge of the epithelial carpet presents a rolled over appearance. These cells may then become detached and migrate away from the rest of the zone of outgrowth. They never undergo mitotic division and, when stained supravitally with neutral red, show numerous degeneration granules and vacuoles.

Wandering Cells.

The wandering cells of the blood and tissues migrate as individual units and do not unite except under exceptional conditions, such as the presence of foreign bodies (Lambert, 1912) or tubercle bacilli (Maximow, 1924). Various types of wandering cells are to be found. Polymorphonuclear leucocytes usually die within 48 hours and are phagocytosed by other cells. Lymphocytes migrate out rapidly from cultures of spleen or lymph nodes. (Fig.70.). These cells are usually elongated and possess small globular pseudopodia, the nucleus lying in the anterior end. When movement ceases they become rounded up and are similar in appearance to the lymphocytes

seen in blood films. Mitotic division occurs in lymphocytes, but many seem to die without undergoing any change. Others, however, increase in size and come to resemble monocytes. Monocytesare found in cultures of spleen and may be obtained from the buffy coat of the blood. They show the typical rosette-like arrangement of granules round the centriole when stained supra-vitally with neutral red. During cultivation, monocytes hypertrophy, assume the character of highly phagocytic macrophages, and, after supravital staining with neutral red, show numerous granules and vacuoles in the cyto-plasm. Macrophages or clasmatocytes (Figs.67,68) are found in cultures of any tissue but are more numerous in some, such as the spleen, than in others. They move with extreme rapidity, and are very phagocytic, engulf-ing red blood corpuscles, dead leucocytes and other cells. They appear to be actually attracted by dead cytoplasm. Chambers and Fell (1931) found that if a localised injury was produced on a skeletal myoblast, the macrophages in the vicinity surrounded the injured area with great rapidity. If a localised infection appears in a plasma culture, they emigrate towards it rapidly. The form of the macrophages is continually changing and they possess broad, wavy, membranous

pseudopodia of which only part may be in focus. Macrophages often appear to have fine filiform processes; these, according to Carrel (1926) are really part of a 'membrane ondulante' or large membranous pseudopodium. Macrophages contain numerous granules and vacuoles which have a strong affinity for neutral red: binucleate macrophages are quite common. According to Maximow (1928), Carrel and Ebeling (1922), Fischer (1925), de Haan (1926), and others, transformation of monocytes and macrophages into fibroblasts occurs during cultivation in vitro. Lewis and Lewis (1924) and Strangeways (1929) consider that this transformation has not been proved.

The Structure of Cells growing in vitro.

Considerable complexity of structure of cells growing in vitro has been described by many workers. After a prolonged study of tissue culture cells under varying conditions, it has been found that much of the detail recorded has been due to the use of unhealthy cultures for examination. During the course of cultivation, degenerative changes of varying degree of intensity and importance occur in the cells: these are due to the accumulation of waste products and to alteration of the hydrogen-ion concentration of the medium. These changes are

not necessarily fatal, and when fresh medium is supplied, the cells recover their previous appearance. While the study of the degenerative changes which take place during cultivation has provided information on the transformations which may occur in the cell constituents, the state of the healthy cell must be known before these can be interpreted. Fibroblasts and epithelial cells growing in fluid media are very suitable objects for examination. The cultures should be taken within 24 hours after explantation and should be mounted on flat slides to give optimum optical conditions. If possible, the explant should be removed without injuring the cells of the zone of outgrowth. Ordinary and dark-ground illumination can be used. The cultures should be examined on a hot stage so that intracellular movement can be watched, but they should also be examined at room temperature, as this allows a better appreciation of the morphology of the constituents.

Cytoplasm.

The cytoplasm itself is optically structureless and appears homogeneous. No visible cell membrane delimits the cells, but the outlines of isolated fibroblasts can be made out owing to the difference in refractivity of the cytoplasm and the surrounding medium. The boundaries

of epithelial cells cannot be seen in the healthy state. An ectoplasm and an endoplasm cannot be distinguished during life, and the cytoplasmic network seen in fixed and stained preparations does not exist as such in the living cell. In the cytoplasm are embedded the nucleus, the mitochondria and fat globules.

Nucleus.

The nucleus is ovoid or spherical in shape and, being slightly less refractive than the cytoplasm, can be distinguished by its slightly greater opacity. With ordinary illumination, no nuclear membrane can be detected, but with dark-ground illumination a thin white line can be seen delimiting the nucleus from the cytoplasm. The substance of the nucleus appears structureless and is optically homogeneous. One or more nucleoli are found in each nucleus. These alter continually in shape and position. Under ordinary illumination they are homogeneous, but with dark-ground illumination they appear faintly granular. The granules and complicated network seen in the nucleus in fixed and stained preparations cannot be seen in the healthy living cell and are due to the coagulation or precipitation of the nuclear substance brought about by fixation, a process which can be directly observed under the microscope. The work of

Chambers and Fell (1931) has shown that the nucleus is a fluid body which collapses when punctured and in which the nucleoli can be freely moved about.

Mitochondria.

The mitochondria can be seen with ordinary illumination but are much more readily studied with dark-ground illumination. After supravital staining with Janus green, they become a deep blue colour. In the healthy cell, they are in the form of filaments, short threads and rods, the first two types predominating. The branching forms described by Lewis and Lewis, (1924) are also to be seen. The number present varies considerably in different cells. While the cell remains healthy, they are not orientated in any particular fashion either in epithelial cells or fibroblasts. On the warm stage, they show very active writhing and darting movements, and transverse division of a mitochondrium has been observed.

Fat Globules.

Most tissue culture cells contain highly refractile globules which move slowly in the cytoplasm and which stain with fat stains. Their number, size and constitution vary according to the age of the culture and the

length of time during which the cell has existed in the zone of outgrowth. Some types of cell, e.g. liver epithelial cells, contain much more fat than others after the same period of time in the zone of outgrowth. Recently emigrated cells contain very few fat globules. I have never seen an emigrated fibroblast or epithelial cell which did not contain at least one fat globule. In an explant of mesenchyme at explantation, no fat globules can be seen in the cells. The appearance of one or two fat globules in a cell at the periphery of the explant indicates that such a cell is about to migrate and, although the conclusion is not implied that fat globules must be present before migration occurs or are associated directly with migration, since fat globules appear later in cells in the explant which do not participate in the zone of outgrowth, the presence of fat is a constant accompaniment of the metabolism of cells which show activity. In explants which for some reason die without showing cell activity, no fat appears in the cells. The fat globules do not prevent the occurrence of mitosis even when present in quite large numbers. The nature of the fat has not been accurately ascertained, but in the early stages, it consists of neutral fats.

Centrosphere.

In the healthy living cell I have only occasionally been able to identify the centrosphere, which lies in close proximity to the nucleus. According to Chambers and Fell, (1931), it is clearly visible in fibroblasts.

Cell Granules and Vacuoles.

In the clasmatocyte, cell granules and vacuoles are numerous, but in the healthy fibroblast or epithelial cell they are not to be seen. During cultivation, they may develop in the cytoplasm, and have a marked affinity for neutral red (Lewis, W.H., 1919). Their origin is discussed later. The presence of a few granules does not prevent the occurrence of mitotic division, but if vacuolation is considerable, mitosis does not take place.

Golgi Apparatus.

I have never been able in the living cell to identify a structure which resembles the Golgi apparatus.

MITOSIS.

The process of mitotic division has been described by many workers and full accounts have been given by Levi (1916), Strangeways (1922), and Lewis and Lewis (1924). The descriptions given by these workers differ

only in the time allotted to each phase of mitosis, and they all agree that the spindle area is homogeneous, the spindle fibres seen in fixed and stained preparations not being visible as such during life.

Amitosis.

Amitotic division has been reported by many workers, but as Lewis and Lewis (1924) point out, the statement has been based on fixed and stained preparations. I have never seen direct cleavage of the nucleus take place, although appearances suggestive of amitosis have often been seen in stained cells. Lewis and Webster (1921) have observed only one case of amitotic division of a nucleus.

Abnormal Mitosis.

Mitotic division of the nucleus without division of the cytoplasm has been observed by many workers. I have always noticed that the connecting strand of cytoplasm in the late telophase stage becomes very thin and appears as if it were going to divide. The daughter cells approach one another once more, however, the connecting strand thickens, and after a period of from 1 to 3 hours, no trace of constriction can be seen. Simultaneous mitotic division of both nuclei of a

(Fig. 143)
binucleate cell, resulting in the formation of a cell containing 4 nuclei of equal size has also been observed.

The Appearances of Cells after Fixation and Staining.

Methods of fixation and staining are discussed on p. 74 The normal appearances are described on p. 105, and cytological characteristics are dealt with on p. 366 The differences found in cells of cultures of the various organs is described in section III. . One point, however, must be emphasised. During life, the nuclei of all types of tissue culture cells appear similar in structure, although variations in size, shape and superficial nucleo-plasmic ratio occur. Even after fixation and staining by methods which are productive of artefact, it is impossible, with a few exceptions, to determine the type of cell to which a given nucleus belongs. The nuclei of clasmatocytes, however, are denser than those of fibroblasts, and those of polymorpho-neuclear leucocytes can of course always be recognised; and tumour cell nuclei, such as those of carcinoma M.63 and particularly carcinoma 2046 of the Imperial Cancer Research Fund, can as a rule be distinguished by the greater complexity of their structure in stained preparations.

DEGENERATIVE CHANGES IN CELLS *in vitro*.

The factors which bring about degenerative changes in cells growing *in vitro* are difficult to analyse, but they occur if the medium is not of the correct constitution and hydrogen-ion concentration, and if accumulation of waste products is allowed to take place. Such changes occur more rapidly in a fluid than in a coagulated medium and, on the whole, take place with greater rapidity in epithelial and tumour cells than in mesoblastic elements. Changes which occur during cultivation such as liquefaction of the medium and lowering of the hydrogen-ion concentration (a constant accompaniment of active growth) also contribute to the onset of degeneration. Accordingly, in a rapidly growing culture, degenerative changes may appear more rapidly than in one in which activity is less pronounced. It is possible that the rapid onset of degenerative changes in epithelial cells in coagulated medium is due in part to the extensive liquefaction which accompanies epithelial growth. Liquefaction of the medium, however, is not always accompanied by gross degenerative changes of the cells themselves, and the liquefied medium is not entirely unsuitable for the growth of

epithelial cells which often migrate on the surface of the coverslip, (Fig. 32). By renewing the medium or transferring the culture to a fresh coagulum, the cells may recover their healthy appearance. If the degenerative changes are marked, however, recovery does not occur.

In a degenerate culture, cell-division ceases and changes take place in the cells. As a rule, these changes consist in the appearance of granules and vacuoles, the granules being the first to appear. As degeneration proceeds, the granules and vacuoles increase in number so that the cytoplasm becomes reduced to a thin framework between the cell constituents. W.H. Lewis, (1919) gave a very complete account of the appearance and arrangement of the granules and vacuoles of degenerating cells. He has found that both have marked affinity for neutral red and accumulate round the centrosphere area, the vacuoles tending to form round the granules. He considers that the granules and vacuoles have no relation to the mitochondria but are formed, in part at least, by the breaking down of cell cytoplasm. The relation of mitochondria to the granules will be discussed later. In mesenchymal cells, enlargement of the centrosphere area also takes place during degeneration, and this also has been fully

described by W.H. Lewis (1920). Changes described on p.346 *et seq.* occur in the mitochondria and shrinkage of the nuclei takes place in the later stages of cell degeneration. The number of fatty globules in degenerating cells seems to depend on the rapidity with which the changes have occurred. Where the degenerative process is slow, fat tends to accumulate. When it is rapid, as in the experimental conditions described on p.366, the accumulation of granules is more pronounced. Alterations in shape are also seen. In the case of fibroblasts, cell processes are slowly withdrawn and the cell becomes rounded. In epithelial membranes, the cells retract from one another so that spaces appear between them. Later, the epithelial cells separate from one another, stretch themselves out once more, and migrate slowly into the medium. Even where degenerative changes are pronounced, cell death, as evidenced by the penetration of the nucleus with neutral red, takes place slowly under ordinary conditions. It is often impossible to distinguish between degenerate fibroblasts, epithelial cells and macrophages: but, although they may survive for several days, they are very sensitive, however, to injury of any kind.

The capacity for amoeboid movement seems to be the last property to disappear in cells growing *in vitro*. Cells which by all cytological standards are quite degenerate still retain their capacity for movement, and migrate slowly in the culture medium. This phenomenon is particularly characteristic of cells in which vacuolation is slight and the degenerative changes are indicated by the accumulation of fat and granules.

The problem of the differentiation of cells and tissues in vitro.

During normal development, the cells of the organism by a process of differentiation come to possess distinctive characteristics and form specific tissues and organs, each of which subserves a specialised function correlated with its anatomical structure. During such development cell proliferation takes place in an orderly manner and 'mutual restraining influences' prevent the overgrowth of any one organ or tissue. In the adult animal, the differentiated character of the various cell groups, when once established, persists throughout life unless there occurs disturbance by certain external agencies or by the factors which lead to tumour formation. Accordingly, under normal adult conditions, cell proliferation only occurs to replace loss.

From the description already given (p. /65) of the general appearances seen in cultures of tissues growing in vitro and of the growth characteristics of the various organs and tissues (p. /43.), it is apparent that in the zone of outgrowth of a culture in vitro the 'restraining influences' which produce and maintain the normal anatomical arrangement of the elements in vivo is lacking.

When a piece of suitable tissue is explanted in vitro, proliferation of cells takes place, and this proliferation is preceded by, and associated with, another phenomenon, cell migration. For example, in a culture of intestine, in which all the elements present in adult life are already differentiated, there may be found outgrowth of epithelium, mesothelium, smooth muscle, fibroblasts, clasmatocytes and sympathetic nerve fibres. This outgrowth is quite disorderly: the epithelial cells are in the form of sheets in which no gland-like structures can be made out, and the smooth muscle cells and fibroblasts show no constant relation to one another or to the epithelium. The growth is thus different in character from that which would have occurred during the process of development from the late embryonic to the adult form. To growth of the type observed in vitro, Thomson (1914) has applied the term 'uncontrolled'; Maximow (1925) calls it 'histioid' or 'histiotypic'; others designate it 'unorganised'. But, while the individual cells in the zone of outgrowth are not related to one another anatomically, each type of cell maintains certain peculiarities of growth, and sometimes even of function. The epithelial cells retain contact with one another, just as they do in the organism, and in cultures of

intestine they secrete mucin. Again, smooth muscle cells continue to show spontaneous contraction. Similarly, skeletal muscle may contract on stimulation (Chambers and Fell, 1931), and glycogen can be demonstrated in liver cells even after long periods of cultivation. Although mesothelium and clasmatocytes may become transformed into fibroblasts, this change cannot be regarded as peculiar to tissues growing in vitro, since it has also been recorded in vivo.

Accordingly, cells which are growing actively in vitro lose their normal arrangement; but some workers go so far as to assert that they undergo dedifferentiation, that is to say, that they revert to a primitive type. Champy (1914, 1915, 1913.) considers that all cells grown in vitro, e.g. thyroid epithelium, kidney epithelium and smooth muscle etc. become transformed into an indifferent type of cell, which is comparable to that found in the early stages of the blastoderm; and he has regarded this as an adaptation of the cells to conditions prevailing in vitro. As a result, mesenchymal and epithelial elements become indistinguishable from one another. The rapidity of dedifferentiation is held to be related to the rapidity of proliferation. As has been stated

on p. , however, degenerate isolated epithelial cells and degenerate fibroblasts resemble one another closely, and it seems possible that Champy has misinterpreted a purely degenerative phenomenon. While most workers do not agree with the view that cells revert in vitro to so primitive a condition, the character of the out-growth of epithelial cells is usually described as "undifferentiated" or "de-differentiated", but it seems that what is really meant may, for lack of another term, be described as 'partial dedifferentiation'.

In contrast to Champy, other workers (Carrel, Ebeling, Chlopin etc.) have described the active outgrowth of tubular structures, and consider that from a fragment of an organ, growth which has an organised form, may occur in the migratory zone. As stated on p.13/ this conclusion may rest upon misleading appearances brought by several factors — thus, spreading out of the explant at one place, or a local contraction of epithelial cells where an area of coagulated plasma has undergone liquefaction may cause appearances simulating tubule formation. (pp.13/163) In Ebeling's experiments on the cultivation of the thyroid gland, it is possible (p.223) that he has interpreted such changes which have occurred in the explants as actual growth in the migratory zone.

The process of partial dedifferentiation has been stated to be reversible under certain conditions, and

Drew, Fischer and others consider that the addition of connective tissue to a so-called undifferentiated sheet of epithelium (kidney) may result in a redifferentiation of the epithelial elements with the production of tubules. Skin undergoes keratinisation under similar circumstances. The fact, however, that epithelial outgrowths of a membranous character occur in the presence of luxuriant outgrowths of connective tissue gives the impression that other factors, beside the mere presence of fibroblasts, must contribute to the redifferentiation of the epithelium. In Drew's and Fischer's figures, the redifferentiated epithelium appears always in the centre of the culture, being surrounded by fibroblasts. The phenomenon of differentiation, therefore, seems to be in part a mechanical one. When the fibroblasts, in virtue of their higher proliferative power and rapidity of emigration, surround the epithelium and prevent its migration, the latter reassumes its capacity for tubule formation and the normal histological appearances are again produced. In support of this is the observation that differentiation <u>in vitro</u> always occurs in the centre of a mass. For example, I have observed (p.347) that if tissue of an embryo limb rudiment which is about to form cartilage is

divided into 2 parts, the subsequent differentiation of cartilage cells does not appear at the periphery, although potential cartilage cells are present in that situation, but always at some distance from it. Similarly in Fell's experiments, (1928, 1932), differentiation of cartilage and the production of osteoblasts and bony matrix always occurred in the centre of the culture; in addition, the production of the last appeared to occur when the tissue was in a contracted state.

Recently, Fischer and Parker have re-emphasised the antagonism between differentiation and proliferation, and have ascribed the differentiation of cartilage which they observed to the absence of growth-promoting substances from the media used. It must be noted, however, that in addition to the diminution of cell proliferation in differentiating cultures, there was a corresponding diminution of cell migration and, as already noted, the differentiating cells were always surrounded by other cells. In the centre of a culture, mitotic division is always deficient as compared with that seen in the zone of outgrowth, even when the culture contains growth-promoting substance. Accordingly it appears essential that for differentiation to occur <u>in vitro</u>, migration

of the potentially differentiating elements should be prevented by the presence of a surrounding zone of other cells. Diminution in mitosis of the differentiating cells also favours the process of differentiation. It is to be noted that in the case of a culture containing ectoderm, when the cells of the latter have completely surrounded the explant, they proceed to keratinise. The question, however, remains as to whether keratin formation is an evidence of differentiation or merely of senescence.

Organotypic Growth.

Mention must be made here of 'organotypic' or 'organoid' growth (Maximow) which takes place if an embryo or fragment of an embryo is explanted in vitro. Thomson calls this type of growth 'controlled' and the term 'organised' is also used.

Maximow, (1925), found that cultures of parts of rabbit embryos rounded themselves off and became surrounded with epithelium of various sorts. In the interior, differentiation took place, histiotypic growth being absent although it occasionally occurred in the later stages of cultivation. The explants behaved as 'independent organisms'.

Fell, (1928), observed progressive differentiation of the explanted otocyst from 3-day embryos. The differentiation was purely histological, and the otocyst retained its primitive vesicular form. Within the vesicular otocyst, all the epithelial constituents of the fully-formed auditory labyrinth appeared.

The growth of skeletal rudiments in vitro described by Fell and Robison, (1929), represents another type of organotypic growth. A femur taken from an embryo on the 6th day of incubation increased in size, retained a comparatively normal form and showed

progressive histological differentiation ~~(p.)~~. I have obtained similar results during a study of the repair of cartilage and bone in vitro (p.~~2~~). From an examination of the figures in Fell and Robison's paper, it might be imagined that the cartilaginous epiphyses abutted directly on, and grew into, the medium. The appearance figured, however, is due to removal of the rudiments and subsequent fixation. During cultivation the explants were surrounded entirely by a thick zone of fibroblastic cells, and within this covering, the progressive development of the rudiment in an orderly manner took place.

An interesting point was observed with regard to the development of the epiphyseal surface. At explantation, the extremities of the rudiments merged imperceptibly into the surrounding mesenchyme. During development in vivo this condition is maintained until about the 10th day of incubation. In vitro, however, the peripheral cells within a few hours flatten themselves out parallel to the surface of the epiphyses and form a limiting perichondrium from which, later, fibroblasts radiate into the medium.

The 'organotypic' growth described above

demonstrates the remarkable capacity of embryonic tissue to undergo self-differentiation when removed from its normal surroundings. The 'intestinal' organisms of Fischer, (1922), on the other hand, which consisted of fully developed intestinal tissue obtained from embryos almost 21 days old and which are usually regarded as typical examples of 'organotypic' growth, demonstrate the capacity of epithelium to grow round a cut surface and show that cells tend to maintain their normal anatomical relations with one another when migration is prevented. No new formation of intestinal tissue occurred.

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S E C T I O N III.

CULTIVATION in vitro OF FRAGMENTS OF TISSUES AND ORGANS.

Ectoderm.

The rapidity and ease with which emigration and growth of ectoderm takes place depends on the age of the embryo from which it has been taken. If the skin from a 5-day avian embryo is cultivated on coverslips in the usual plasma and embryo extract medium, the ectoderm grows out rapidly in the form of spade-shaped epithelial sheets in which mitotic figures are fairly numerous. Emigration of fibroblasts and of wandering cells also occurs, but during the period of active outwandering of epithelium, no keratinisation takes place, although the fibroblastic outgrowth is quite abundant. After the culture is transferred to fresh medium, the ectoderm tends to grow round the explant instead of emigrating outwards. Only by cutting the fragment in two parts is it possible to obtain a fresh outgrowth of epithelium. If the explant is not divided into two pieces, keratinisation of the epithelium takes place after 4 days' cultivation. Keratinising explants produce a very rapid liquefaction of the plasma. If a piece of skin is included in the explant of a skeletal rudiment from a 5-day avian embryo, keratinisation takes place rapidly. From older embryos the outgrowth of skin epithelium takes place more slowly

and in the case of an 8-day embryo, does not begin until the second or third day of activation.

The epithelial membrane is as a rule only one layer thick in plasma medium, and intercellular spaces cannot be seen as long as the cultures remain healthy. At the free margin, the cells show numerous amoeboid processes and are more spread out than those near the explant. After fixation and staining, however, intercellular spaces are often found and the membrane appears almost reticular. (Fig. 71.) The cells are elongated and are often spindle-shaped, the long axes being directed parallel to the surface of the explant. In fluid media, growth is often difficult to obtain; when it occurs, however, it is always membraneus in type.

If the skin of an almost full-time mouse is cultivated in vitro, outgrowth of the skin epithelium does not take place until the end of the third or fourth day of cultivation, and often it does not grow out at all. The epithelium has a marked tendency to grow round the explant and keratinisation of the investing layer takes place.

The character of skin outgrowths was described by Lambert, (1912). He found that the skin showed a typical epithelial formation resembling the membranes

obtained from epithelial organs.

Uhlenhuth, (1914, 1915, 1916) cultivated the skin of the adult frog and found that the epithelial outgrowths were formed from the basal layer only. By using media of varying consistence, he concluded that the character of the outgrowth depended on the type of media employed: in a stiff coagulum, the cells formed a membrane; in a fluid medium, the outgrowth consisted of isolated elements, and all intermediate stages were found according as the medium varied between these two extremes. From Uhlenhuth's description, it seems much more likely that the variations described were due to degeneration of the cultures, as I have found that if skin epithelium is cultivated in a stiff coagulum, the cells remain healthy for a longer time than when cultivated in a soft clot. In the latter, degenerative changes occur in the cells fairly rapidly, and the membrane becomes broken up into groups of cells or into isolated elements. In a fluid medium, degeneration often occurs rapidly, resulting in the production of isolated elements.

Drew, (1923), obtained growth of embryonic skin in the form of sheets. When connective tissue cells were added to pure cultures or when a sufficient amount

of connective tissue was present in the zone of outgrowth keratinisation of the skin epithelium occurred. As stated above, I have never observed keratinisation of the epithelium in the zone of outgrowth.

Fischer, (1924), cultivated skin epithelium and obtained a 'pure' culture unmixed with fibroblasts. In cultures which had become thick and in which the growth was irregular and scanty, he found that keratinisation occurred in the central part of the culture. In contrast to Drew, Fischer does not consider that the presence of connective tissue cells is necessary for the keratinisation of ectoderm cells, but is of the opinion that it is always the result of inadequate growth conditions.

Amniotic Ectoderm.

Amniotic ectoderm grows out in the form of a typical epithelial membrane, the cells being in close contact with one another. The epithelium, however, differs from embryonic skin ectoderm in that the cells are much less spread out in both coagulated and fluid media. In fixed and stained preparations, the cytoplasm of the cells takes up the stain with great intensity: the reticular arrangement of cells often seen at the periphery of sheets of skin ectoderm is never observed. (Fig. 18.)

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Cultivation of Skeletal Muscle.

I have cultivated skeletal muscle from 7, 8, 10, 12, 14 and 17-day fowl embryos and from almost full-term mouse embryos. In all cases, the muscle of the thigh was used. The tissue was cultivated by the usual 'hanging-drop' method, both coagulated and fluid media being employed. The former consisted of equal parts of avian plasma and avian extract, the latter was similar to that described on p.72. In some cases, the plasma was diluted with one-third of its volume of distilled water. The cultures in plasma were transferred to fresh medium every second day, the zone of outgrowth being preserved intact as far as possible; those in fluid medium were washed with embryo extract and the medium renewed every second day. It was found that the most interesting growths were obtained when the explants were small (0.5 mm. in diameter or less). When the cultures in plasma, however, became of sufficient size, they were divided into two pieces. The maximum period of cultivation was 20 days for plasma cultures and 10 days for fluid medium cultures. Most of the cultures were fixed and stained entire. In some cases, the cultures were embedded in paraffin and cut into serial sections. In addition to the usual stains, Mallory's connective tissue stain and van Gieson's stain were employed. Many of the cultures in fluid

medium were fixed in osmic acid, and stained for mitochondria in the usual way.

The muscle from 7-day embryos consists of long multinucleated strands which show no trace of cross-striation and in which only fine, longitudinal striae can be demonstrated in stained preparations. In 8-day embryos cross-striation is present in the muscle in most cases, and from the ninth day onwards, the muscle fibres show well marked longitudinal and cross-striation. The amount of connective tissue between the muscle fibres is greater in the younger than in the older embryos. The muscle from the mouse embryos was highly differentiated and showed well marked longitudinal and cross striation.

The appearances found in cultures of muscle from 10-day embryos will be described first. Within 2 hours after incubation a fringe of mesenchyme cells can, as a rule, be seen round the edge of the explant. Two to three hours later, numerous protoplasmic buds or sprouts can be seen extending outwards from the edge of the explant. In small fragments continuity between these buds and the muscle fibres present in the explant can be traced. These buds increase rapidly in length and form long protoplasmic strands which extend in all directions outwards into the medium and which are easily distinguishable from the

mesenchyme of the outgrowth by their refractile nature and cylindrical appearance (Fig.21.). Their cytoplasm appears denser and less transparent than that of the mesenchyme cells. These muscle fibres often send off branches which unite with other branches or muscle fibres; a very complex network is thus produced in some cases, (Fig.23). While the general characters of the outgrowths are similar, there are considerable variations in detail in different cultures. The extent of the anastomosis between the strands is variable, and in some cultures the distal ends of the muscle fibres are rounded, in others the ends are stretched out and terminate in numerous fine processes. The muscle strands contain numerous nuclei usually arranged in rows (Fig.76) at irregular intervals: clumps of two or three nuclei, however, are often seen, and in the expanded ends, several nuclei are often found. No trace of cell outlines could be seen in the living state and the muscle fibre appears to be a syncytium. During cultivation, the cytoplasm of the muscle fibres often becomes thinned out in places and the distal part, which may contain one or more nuclei, (Figs.19,20) becomes separated off from the proximal part and emigrates freely into the medium, (Fig.19). Branches also become split off from the parent strand. The

cytoplasm of these separated parts retains its characteristic dense character. Those which contain only one nucleus and which may be called 'myoblasts' have as a rule a spindle-shaped appearance which is in marked contrast to the polymorphic outline of the mesenchyme cells. (Fig. 19.) Cells similar to those which have been seen to break off directly from muscle fibres in the zone of outgrowth also emigrate from the original fragment and have probably become isolated from muscle fibres in the explant. While they can as a rule be distinguished from the mesenchyme cells, it is not always possible to be certain of their nature if the mesenchymal outgrowth is very dense.

The cytoplasm of the muscle fibres contains a few fat globules, generally less numerous but larger than those found in mesenchyme cells. Their mitochondria owing to the dense character of the cytoplasm are not so readily made out with ordinary illumination as are the mitochondria of the mesenchyme cells. No trace of longitudinal or cross-striation was seen in the living state. The nuclei are ovoid in shape and contain two or more nucleoli. Mitotic division has been observed in nuclei at any part of the fibre in the living state. When

this occurs an oval-shaped area of less transparent cytoplasm which is quite sharply demarcated from the rest of the fibre can be seen. The nucleus undergoes the ordinary changes characteristic of cell division and at the telophase stage, the cytoplasm may or may not divide. The reunion of almost separated cytoplasm has been observed. The spindle-shaped myoblasts also undergo mitotic division. In a series of over 300 cultures, I have never seen spontaneous contraction of the muscle fibres.

In cultures in which the plasma was diluted with distilled water, (Fig. 24.) mononucleated myoblasts were very numerous, and fragmentation of the strands was more marked.

The muscle strands diminished in number after successive subcultures while the mesenchymal outgrowth became more abundant. Even after 20 days' cultivation, however, a few muscle strands were still present in the zone of outgrowth. In fluid medium the appearances were similar, the zone of outgrowth being, however, less dense. The muscle strands, however, are flattened out on the coverslip and do not appear so cylindrical as in a plasma medium.

In histological preparations the muscle strands stain more intensely than the mesenchyme cells and show,

particularly after fixation in Zenker's fluid plus acetic acid, fine longitudinal striae (Fig. 76) resembling those seen in the muscle from 7-day embryos, which extended a considerable distance along the fibre. Similar striae are seen in the myoblasts and in the separated multinucleated strands. These striae are much finer than the myofibrils seen in fully differentiated muscle. No trace of cross-striation was seen in any of the muscle fibres in the zone of outgrowth, although the muscle fibres of the explant showed cross-striation and, in favourable cases in very thin explants, direct continuity between a striated fibre in the explant and its non-striated continuation in the zone of outgrowth could be seen. The cytoplasm surrounding nuclei in mitotic division stains more intensely than the rest of the fibre. The muscle fibres in the zone of outgrowth could not be distinguished either by Mallory's connective tissue stain or by van Gieson's stain, although the muscle in the explant reacted specifically.

The mitochondria are long, slender and rod-shaped, and are arranged in longitudinal rows parallel to the long axis of the fibril in the peripheral layer of the cytoplasm. Granular forms were seen in some fibrils in slightly unhealthy cultures. Where mitotic division was

occurring, the mitochondria in the associated cytoplasm were shorter and plumper and similar in appearance to the mitochondria in dividing cells of other tissues. The longitudinal striae are much longer and finer than the mitochondria, from which they can easily be distinguished. Since these structures are only seen in histological preparations they apparently do not exist as such in the living cell, but assume this form after fixation. No trace of cell outlines could be seen in any of the muscle strands.

The type of outgrowth obtained from muscle of 8, 9, 12 and 14-day embryos was similar in character to that described above. No spontaneous contraction of the muscle fibres was seen. In the cultures of muscle from 8-day embryos the mesenchymal outgrowth was more abundant. In the cultures of muscle from 14-day embryos, muscle buds did not begin to make their appearance until 12 to 24 hours after incubation. In muscle from 7-day embryos, the muscle strands were thinner and mononuclear myoblasts were more numerous. (Fig. 22.).

In the muscle from 17-day embryos, only a few fibroblasts and wandering cells appeared during the first 48 hours' incubation. After the first subculture, changes were seen in the muscle of the explant. The cross-striation

appeared less conspicuous and highly refractile fatty globules appeared in the muscle substance. Thin, multinucleated strands which anastomosed with one another, then began to grow out into the medium. These strands occupied various planes of the medium so that it was impossible to tell by direct observation whether or not they were continuous with pre-existing muscle fibres in the explant. Cultures were therefore fixed in osmic acid, post-chromed, cut into serial sections and stained for mitochondria. Many of the muscle fibres showed, in places, the absence of cross-striation. Spaces where fat had apparently been dissolved were seen, and short, rod-shaped mitochondria were present. In many places the continuity between these altered muscle fibres and strands in the medium could be traced. The changes described in the muscle fibre of the explant did not extend throughout its entire length, but were confined to the part in continuity with a fibre which was growing out into the medium. Many of the growing ends of muscle fibres remained confined to the explant.

In cultures of mouse muscle, only fibroblasts and wandering cells were obtained in the zone of outgrowth.

The appearances described in these cultures of skeletal muscle correspond to the changes which occur

in the early stages of muscle regeneration *in vivo*, (Muir,1929). Multinucleated buds grow out from the cut ends of divided muscle fibres, and the cytoplasm of these buds appears homogeneous and unstriated. Lateral buds are also said to occur, and detached mononuclear elements (Maximow,1931) have been described. *In vivo*, however, differentiation into striped muscle-substance occurs. In the cultures described, no cross-striation appeared in any of the muscle outgrowths either during life or after fixation. Lewis and Lewis (1917) described in one or two instances the appearance of cross-striation in muscle buds in the zone of outgrowth in fixed specimens. It is uncertain from M.R. Lewis (1920) whether such have also been seen in the living state. Lewis and Lewis (1924) state, however, that they are uncertain as to whether or not cross-striation developed *in vitro* during life. Cross-striated fibres are frequently observed in histological preparations in the tissue surrounding cultures of limb-bone rudiments isolated from 5½ day avian embryos, (Fig.142) and these have been noted by Fell and Robison (1929). Harrison (1907) also observed differentiation of striated muscle cells in explants of the myotomes of 3 mm. frog embryos. In muscle tissue from older embryos differentiation does not appear to continue, and in explants of 9-day muscle

the cross-striation apparently disappears. (See p.). It seems, therefore, from the observations described, that a position unfavourable for emigration favours the development of cross-striation, at least in stained preparations, and this only occurs in tissue which at explantation is relatively undifferentiated. In muscular tissue in which differentiation is already established, the specialised characters apparently disappear although multinucleated strands persist for a considerable time. Levi (1923), on the other hand, has described the appearance of typical striation in mononucleated myoblasts in the zone of outgrowth from cultures of skeletal tissue of 7-day embryos, in which no striation was apparent before explantation. In cultures of older skeletal tissue he obtained an outgrowth somewhat similar to that described, and consisting of multinucleated strands. From his observations on such cultures he confirms the view of Duesberg (1910) that myofibrils are derived by transformation of the mitochondria, and he considers that this change is reversible. From the preparations examined (p.154) no evidence of the latter transformation has been obtained.

The appearances of the cultures which have been studied correspond closely to the description given by Lewis and Lewis (1917). These authors, however, failed

to observe division of the nuclei during life in the multinucleated muscle strands, nor did they obtain convincing evidence of it in stained preparations. They concluded that the nuclei came from the parent muscle fibre. The absence of mitosis in their cultures may be due to their use of fluid medium.

Spontaneous contraction has been recorded by M.R. Lewis (1915, 1920), Lewis and Lewis (1917), and Friedheim (1931). I observed no trace of it in the cultures examined; and de Reny and Hogue (1931), Chambers and Fell (1931) also failed to observe spontaneous contraction, although all these authors found that the muscle fibres contracted on stimulation. The capacity for spontaneous contraction apparently depends on conditions so far undetermined, as it does not, according to Lewis and Lewis, invariably occur in all cultures.

Heart.

Fragments of the heart from embryonic and young animals have been used extensively by tissue culture workers, as the heart is an organ which is easily isolated without much risk of infection, and one from which migration of cells is readily obtained. Burrows (1910) cultivated the heart of avian embryos and found in a small percentage of his cultures that the muscle grew

out in the form of short chains of striated cells which contracted rhythmically.

Considerable difference of opinion still exists concerning the appearance and identification of heart muscle growing in vitro. As Lewis (1926) has pointed out, the heart from an early stage of development contains, in addition to heart muscle, fibroblasts, mesothelium, endothelium, clasmatocytes, and sympathetic nerve cells and fibres. Any or all of these types of cell may be found in the zone of outgrowth. In this communication, W.H. Lewis (1926) reported the results of his investigations conducted over a period of more than 10 years on the cultivation of avian embryonic heart tissue. Only at the end of this prolonged study did he obtain emigration of heart muscle. He found that in 8% of cultures from the hearts of 6 to 7-day embryos, heart muscle appeared; in embryos taken at the 4th day, heart muscle was obtained in 11% of cultures, and from 11-day embryos, only 2% of the growths contained muscle. Mesenchyme and endothelium appeared in almost all the cultures, and mesothelium and clasmatocytes were common, these mesenchymal elements showing migration within 24 hours after incubation. The heart muscle did not begin

to grow out into the medium until the second day of incubation and often appeared much later. The heart muscle migrated outwards in the form of a reticulum in which cell outlines could be demonstrated by appropriate methods. Both the reticulum in the zone of outgrowth and the muscle of the explant showed rhythmic contraction, but no true fibrillae or cross-striations were ever seen during life. In fixed and stained preparations, fibrillae were often found and cross-striations were observed in a few cases. Glycogen was also demonstrable in the cytoplasm. Occasionally isolated muscle cells which contracted rhythmically were found. Lewis regards the presence of contraction as the only reliable criterion by which a heart muscle cell can be distinguished during life, although he has found that after some experience it is possible to identify by their appearance non-contracting heart cells, the identification being confirmed later by the onset of contractions.

In this communication, Lewis reviews the results found by various workers and is of the opinion that Levi (1916, 1923), who has also studied extensively heart muscle growing in vitro, has confused muscle and mesenchyme. Drew (1923) also cultivated heart muscle from mouse embryos. From an examination of Drew's figures, Lewis is

of the opinion that the cells figured are probably endothelial.

Cultivation of Heart Muscle.

I have cultivated heart muscle from avian embryos from the 7th to the 10th day of incubation and from late embryonic and newly-born mice and rats in coagulated and fluid media. In the case of the avian material, outgrowth of mesenchymal elements of the type mentioned by Lewis was always abundant; but, although contraction of the explant was frequent, only in a few cases was contractile tissue observed in the zone of outgrowth. Fig. 17. shows part of a reticular outgrowth in which contraction was observed during life, the culture being derived from the heart of an 8-day embryo. The outgrowth began at the end of the 2nd day of incubation and the culture was fixed at the end of the 3rd day. The appearance seen corresponds fairly well with Lewis' fig. 1 and fig. 8. On the other hand, in fixed and stained preparations, fibrillated cells, similar to those described by Levi and regarded by him as myoblasts, were frequently seen. Such, however, never showed contraction during life and were undoubtedly of mesothelial or endothelial origin.

Mammalian Heart Tissue.

Fragments of the hearts of late embryonic and newly-born mice and rats were cultivated in coagulated and fluid media, and as a rule, a vigorous outgrowth of mesenchymal elements was obtained. As before, the explants often showed rhythmic contraction and the emigrated cells were often pulled by the contracting explant. In no case, however, were contracting elements found in the zone of outgrowth, and I have never obtained outgrowth of mammalian heart muscle. In fixed and stained preparations, I have frequently seen cells similar to those figured by Drew, (1923). My observations, though much less extensive than those of Lewis, tend to confirm his views at least with regard to the infrequency with which migration of heart muscle occurs in tissue from avian embryos and also with regard to the reticular character of the outgrowth. Drew undoubtedly obtained outgrowth of heart muscle in his cultures, but later expressed the view (personal communication) that the cells figured by him were probably of endothelial or mesothelial origin as stated by Lewis. Levi, too, has probably obtained outgrowth of heart muscle cells, but in his case also the cells described did not represent the actual contractile heart muscle elements.

Smooth Muscle.

The amnion from 7-day avian embryos was used as the source for smooth muscle, since, as M.R. Lewis (1920) has pointed out, it consists of a layer of smooth muscle cells which cover a layer of epithelial cells, the amniotic ectoderm, both of which can be readily distinguished *in vitro*.

The tissue fragments were cultivated in equal parts of avian plasma and embryo extract. Spontaneous contraction of the explant occurred in many cultures. Outgrowth of smooth muscle cells took place from any part of the explant after about 24 hours' incubation. They could readily be distinguished from the ectoderm cells by their elongated appearance and the peculiar refractility of the cytoplasm which is also seen in skeletal muscle growing *in vitro*. Many of the cells were elongated and band-like, and were in contact with one another both laterally and by their proximal and distal processes, which overlapped those of neighbouring cells, (Fig.58). These cells often showed spontaneous contractions either in groups or individually. Isolated cells were also found and many of these were spread out and polygonal in shape. Mitotic figures were quite numerous amongst the spread-out cells and isolated elongated cells. In

the reticular strands of elongated cells I have never observed mitotic division. Fibrils were not observed in the living state, but after fixation and staining were seen in both the elongated and spread-out type of smooth muscle cell.

Champy (1914) observed in fixed and stained preparations that smooth muscle cells in mitosis did not show fibrillae. On this evidence, he concluded that smooth muscle cultivated in vitro dedifferentiated to form a common indifferent type of cell, and this dedifferentiation was due to the rapidity of mitosis. I have not maintained cultures of amnion in vitro for more than 10 days; but, during that time, the smooth muscle cells retained the appearances seen in primary cultures. M.R. Lewis (1920) described the appearance of smooth muscle in cultures of amnion. She confirmed Champy's observation regarding the absence of fibrils in cells fixed during mitosis, but found that fibrils were demonstrable by fixation in daughter cells after mitosis had occurred.

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Cartilage.

Cultivation of Cartilage.

~~The~~ Differentiation of cartilage in vitro is dealt with on p. 321. The cartilage of 7- to 8-day avian skeletal rudiments - femur and tibia - has been cultivated in vitro. At this stage of development, the centre of the shaft consists of a broad zone of hypertrophic cells surrounded by a thin layer of bone and osteoblastic tissue. On both sides of the hypertrophic area are two zones of flattened cells which merge on each side into the small-celled cartilage of the epiphyses. The latter are not sharply demarcated by an epiphyseal perichondrium from the surrounding mesenchyme, but the epiphyses themselves can be isolated fairly easily. It is possible to remove the perichondrium and bone from the rest of the rudiment almost entirely. Fragments of cartilage from all three zones were cultivated in the usual plasma and embryo extract medium.

Epiphyseal Cartilage.

Fragments of epiphyseal cartilage were cultivated on coverslips. Small pieces of mesenchyme and developing muscle were occasionally found adherent to the cartilage, but by cutting the tissue into small fragments it was possible to obtain explants of cartilage in which

no mesenchyme was present, the cartilage being in direct contact with the medium. Emigration of cartilage cells began within 12 hours of explantation. The cells were small, resembled monocytes and could be easily distinguished from fibroblasts (Fig. 29). As cultivation continued, they increased in size and became more spindle-shaped (Fig. 30): the most recently emigrated cells, however, were small in size. Mitotic figures were numerous and the zone of outgrowth was preserved as far as possible. After the first passage, the cells which emigrated were larger than those which appeared in the primary culture. After the second passage, the cartilage mass, which appeared denser, was surrounded by a capsule of cells resembling fibroblasts and the zone of outgrowth consisted of cells which could not be distinguished from fibroblasts. After 12 days' cultivation, the cultures consisted of a nodule of cartilage considerably larger than the original fragment, and surrounded by a capsule of cells from which radiated into the medium cells indistinguishable from fibroblasts. In sections of such cultures, a typical area of small-celled cartilage surrounded by an actively chondroblastic perichondrium was found. The chondroblasts were separated by more

cartilage matrix than was present at explantation. The perichondrium merged on the outer side into a narrow zone of cells, indistinguishable from fibroblasts, which passed out into the zone of outgrowth. It is apparent, therefore, that the cartilage cells in the centre of the original fragment preserved their histological characters and continued to form cartilage matrix. The cells at the periphery emigrated into the medium, and acquired a fibroblastic form. During subculture, when the culture was cut out of the old medium, they became aggregated round the central mass of cartilage. Emigration into the medium and proliferation, however, again took place after each transfer, but, as the zone of outgrowth was preserved at each subcultivation, more and more cells became included in the explant, where they formed an actively chondroblastic perichondrium, through the activity of which cells were constantly being added to the central mass. From a fragment of embryonic epiphyseal cartilage it is thus possible to obtain a culture consisting of cartilage, a perichondrium and cells indistinguishable from fibroblasts. In some of the cultures, the cartilage cells did not always migrate from the cut surface, and this was found to occur in fragments which were derived from the zone next to the

flattened layer of the skeletal rudiment. In this area, a considerable amount of cartilage matrix is present. As stated on p.319 , the greater the amount of intercellular matrix, the more susceptible are the cells to trauma, and the injury caused by cutting probably accounts for the lack of growth *in vitro*. Fischer, (1922, 1) obtained a 'pure' culture of cartilage cells from the *pars cartilagino sclerae* of the eye-ball of the embryo chick; the age of the embryo was not stated. He found that little or no growth took place if the explant was embedded in the coagulum. When placed on the surface of the coagulum, active growth occurred after 3 to 5 subcultures. The cells were at first small and resembled lymphocytes; later they increased in size, and the outgrowth assumed a membranous character and resembled epithelium. The condition of the explant was apparently not examined. In my experiemnts, I found no difficulty in obtaining active migration and proliferation of the cartilage cells from the first day of cultivation, and it was not necessary to place the explant on the surface of the coagulum. In the later stage of cultivation, the cells in the zone of outgrowth were indistinguishable from fibroblasts, and, although strands of cells were often seen, a membranous

type of growth resembling epithelium was not found. Fischer and Parker, (1929), state that a 'pure' culture of chondroblasts was obtained from the os frontale of 10-day fowl embryos. The original fragment became necrotic and was removed, and the zone of outgrowth, which consisted of cells indistinguishable from fibroblasts was cultivated in a medium consisting of plasma or plasma and serum only. In the centre of the cultures, after several passages, differentiation of cartilage and/or a bone-like substance was obtained. These workers made no attempt to determine the character of the 'ground-subatance' by staining methods, but from the figures shown, it appears to be of a cartilaginous nature. They concluded that it is necessary to diminish proliferation by reducing the amount of growth-promoting substances in the medium before differentiation can be obtained. Fell, (1928,), obtained differentiation of cartilage in cultures of limb-bud rudiments from 3-day avian embryos in a medium containing abundant embryo extract. In the centre of the explant of actively growing cultures of epiphyseal perichondrium from 10-day embryos, I have frequently observed the differentiation of cartilage in a medium containing equal parts of plasma and embryo extract. Fischer and Parker found in their cultures

that the original fragment, which consisted of cartilage, underwent necrosis within a short time. Since they apparently did not use very small fragments, the necrosis was probably due to the large size which is attained in quite a short time by cartilage, and which does not allow of diffusion of nutritive material, or removal of waste products. By reducing the quantity of embryo extract in the medium, the activity of the culture was diminished, but the cells which still retained their capacity for forming cartilage proceeded to do so when they came to occupy a position in the centre of the explant which was unfavourable for migration and proliferation. If this differentiation had occurred in the zone of outgrowth, the constitution of the medium could then be considered as playing an all-important part in determining differentiation. Since, however, it occurred in a region in which cell proliferation is always diminished, in which active migration of cells is inhibited, and in which differentiation of cartilage occurs even when the medium contains embryo extract, diminution in proliferative activity brought about by the restriction of growth-promoting substances cannot be regarded as the primary cause of differentiation. The interest of Fischer and Parker's experiments, in my opinion, lies in the capacity of the

fibroblastic cells, obtained from the zone of outgrowth of cultures of cartilage, to form cartilage or some such substance when they become incorporated in the centre of the explant.

Cartilage from the Flattened Zone.

The cartilage of the flattened zone was also cultivated on coverslips in the usual way. Only a few cells derived from tiny scraps of adherent perichondrium emigrated from the explant even after several subcultures. Sections of the cultures after four passages showed that the intercellular matrix had increased in amount in some cultures, but a considerable number of necrotic cells was found.

Hypertrophic Cartilage.

The hypertrophic cartilage cells are very sensitive to injury and I therefore cultivated the whole central mass on watch-glasses. No emigration of cells occurred for the first 48 hours, with the exception, in some cultures, of a few fibroblasts which had not been removed from the cartilage. After 60 hours' incubation, a scanty emigration of degenerate-looking, fat-laden cells took place from the cartilage. They tended to adhere to one another, but were so small in number that they could not

be transferred 'en bloc' to fresh medium. As cultivation continued, the cartilage became softer in consistency, and after each subculture a few more cells emigrated from the medium, but after 12 days' cultivation no further emigration occurred. The explants were then fixed, cut into serial sections and stained with Mallory's connective tissue stain. Round the periphery of the sections, many empty cartilage capsules were seen and from these the degenerate cells which emigrated into the medium were derived. In the centre, the cartilage cells were almost completely necrotic. The cartilage matrix stained very poorly with the aniline blue of Mallory's stain, and the picture presented was very different from that seen in cultures of whole rudiments from 7- to 8-day embryos cultivated for a similar period. In these, the hypertrophic cartilage which is invested by a layer of bone and periosteal tissue remains quite healthy for 12 days, the cartilage continuing its normal histological developmmet. It thus appears that diaphyseal cartilage which has become differentiated requires the presence of surrounding tissue in order to maintain a healthy condition <u>in vitro</u>; it is incapable of active emigration and proliferation. Unlike epithelial cells, it does not dedifferentiate in the sense stated on p. 134.

Fischer, (1931), also found that the shaft and the differentiated joint cartilage from avian embryos when isolated from perichondrium and bone becomes necrotic within a short time when explanted *in vitro*. When explanted along with cultures of fibroblasts, the latter rapidly grow around the cartilage, which remains healthy. In addition, the fibroblasts assume chondroblastic activity and form new cartilage on the surface of the original cartilaginous explant, and he concludes that cartilage is able to induce the formation of cartilage by non-cartilage-forming cells.

Kidney.

The cultivation of kidney tissue has been described by many observers, and conflicting results have been reported by different workers. Carrel and Burrows, (1910) cultivated the kidney of young cats, and described the appearance of structures resembling urinary tubules in the zone of outgrowth. Dadda, (1912), also cultivated kidney tissue, but could not distinguish between fibroblastic and epithelial outgrowths. Lewis and Lewis, (1912,1), cultivated small fragments of kidney tubules from chick embryos, and found that, from the cut ends, epithelial cells grew out in the form of small membranes. H. W. Drew, (1913), described the growth of epithelial cells in the form of tubules from the kidney of the adult frog. Champy, (1914,1,2) described in detail the results of his attempts to cultivate kidney tissue from embryonic and young animals. He observed the emigration and proliferation of epithelial cells from the cut ends of tubules, and the formation of epithelial membranes. He also found that outgrowth of connective tissue cells occurred, and described the 'de-differentiation', during cultivation, of the epithelial cells which reverted to an indifferent type indistinguishable from fibroblasts. Champy also found that in some cases the epithelium grew round the explant, completely investing

it, while in others, cells indistinguishable from fibroblasts, which he regarded, however, as of epithelial origin, formed an investing capsule. From this 'epithelium de cicatrisation', cells of indeterminate type often invaded the medium. Champy concluded from his observations that kidney epithelial cells dedifferentiate to an indifferent type which is comparable to that seen in the earliest stages of embryonic development, and he relates the rapidity of dedifferentiation to the rapidity of cell division.

A study of the development in vivo and in vitro of the metanephros of the chick embryo was made by Rienhoff (1922). This worker found that differentiation of undifferentiated nephrogenic tissue occurred in the explant. In the zone of outgrowth, endothelium, fibroblasts and epithelial outgrowths were found. The latter were usually in the form of thin membranes, but when the mesenchymal outgrowth was thick, tubules carrying with them nephrogenic tissue grew into the mesenchyme, and there continued their differentiation. From an examination of Rienhoff's figures, the possibility suggests itself that the presence of kidney tubules in the zone of outgrowth could have been produced by the spreading out of the explant on the coverslip, and by the

straightening out of pre-existing kidney tubules; both these processes are very common in kidney explants of this type. A. H. Drew (1923) cultivated the kidney of young rats and found that the epithelium grew out in the form of sheets. When a culture of fibroblasts was added to the pure culture of epithelium, the epithelial cells arranged themselves in a tubular fashion. Policard (1925) cultivated the kidney of young cats and found that the epithelium grew out in the form of sheets in which no tubular structures could be found; the glomeruli did not participate in the zone of outgrowth.

Graciun (1927) found that kidney epithelium grows out in the form of membranes, and did not report the presence of tubules in the zone of outgrowth. De Ligneris (1928) considered that he obtained growth of endothelial cells only. From his figures, however, the outgrowths obtained resemble typical epithelial membranes. Nishibe (1928,1929) stated that he obtained only outgrowth of endothelial and mesothelial cells from cultures of kidney of the adult toad. The figures shown in his communication resemble degenerating cultures of epithelium. Nordmann (1929-30) cultivated fragments of kidney of adult rabbits and guinea pigs and found that

outgrowth of epithelial membranes took place. These he considered were derived from the collecting tubules, as in sections of the explants he found that the convoluted tubules became necrotic within a few hours. During cultivation, the character of the epithelial cells altered and the cells came to resemble fibroblasts. From his account of the changes which took place in the epithelial cells, it is apparent that the alteration in type was accompanied by marked degenerative processes such as occur in unhealthy cultures.

Cultivation of Kidney.

Since there is such considerable difference of opinion concerning the character of the outgrowth, and the type of cell which emigrates, I have made a careful study of the growth of kidney tissue in vitro.

The kidney of newly born and young rats up to 6 weeks old has been used almost exclusively for the investigation. Mouse kidney has also been grown, but it was found that good growths were much more difficult to obtain and, although many variations of the medium were tried, no reason for the difference in growth capacity in vitro has so far been found.

The kidney is removed from the animal with the usual precautions to prevent infection, and washed in several changes of saline. It is then cut transversely

into two or more pieces, which are transferred to a hollow-ground slide, the cutting up into fragments being done with the aid of the dissecting microscope. By this means, it is possible to obtain fragments from either the medulla or cortex. The cutting should be done carefully, and crushing or tearing of the tissue should be avoided. The fragments should be about 1 mm. in size, and may be cultivated in either coagulated or fluid media. The character of the outgrowth obtained from medulla and cortex will be described separately.

Medulla of the Kidney.

The fragments were taken from the central part of the medulla, and so consisted mainly of collecting tubules and the distal part of Henle's loop, a small amount of intertubular connective tissue being, of course, always present. The coagulated medium used consisted as a rule of 1 drop avian plasma, 1 drop rat plasma and 2 drops avian embryo extract. The explant was sometimes embedded in the clot and sometimes placed on the surface. The fluid medium used was similar to that described on p.72. It is essential that the fluid medium should be spread out on the coverslip in as thin a layer as possible, a tiny drop of sterile distilled water being placed on the hollow of the hollow-ground slide to prevent undue

evaporation from the medium. The preparations should always be inverted so that the tissue lies on the coverslip.. In plasma medium, emigration of epithelial cells begins as a rule within 12 hours after explantation, and sheets of epithelial cells soon form round the explant. The manner in which the epithelium spreads out has been described on p.114. Several epithelial sheets may grow out at different levels in the coagulum, and sometimes a very complicated outgrowth, such as is seen in Fig. 37 may be found. The epithelial sheet may be more than one cell thick, and mitotic figures are numerous. Rapid liquefaction of the plasma takes place, particularly if growth is active, and the phenomenon of separation of the epithelial carpet from the explant with subsequent emigration into the uncoagulated medium is often seen. Another sheet of epithelium may grow along the surface of the coverslip in the liquefied area. Like all epithelial outgrowths, kidney epithelium is rather difficult to transfer to fresh medium, and if it is necessary to prolong the cultures for some time it is advisable to cultivate the tissue on the surface of the coagulum, as liquefaction in that situation does not take place so rapidly; the epithelium can then be transferred with greater ease. While the epithelial outgrowth

remains on the surface of, or in the interior of, the coagulum, mitotic division is active. In the epithelial carpet which may grow on the coverslip, mitotic division is usually scanty and the cells are spread out in a very thin layer on the surface of the coverslip. Fibroblasts are only occasionally found in the zone of outgrowth in primary cultures, but in the second or third subculture they are more common. The cytological structure of the epithelial cells is described on p. 366. In healthy cultures, cell boundaries are not visible in the living state. When degeneration takes place, the carpet may break up into individual cells which migrate away from one another. These cells are very degenerate and never undergo mitotic division.

In fluid medium, growth begins equally rapidly and a wide membrane of epithelial cells soon forms round the original fragment, the cells being spread out in a thin layer. Mitotic division is not very active. In order to maintain the cells in a healthy condition, it is necessary to wash the culture in embryo extract about every 36 hours and add fresh medium. Emigration usually ceases after the third or fourth day, but the carpet may remain healthy for 8 or 9 days. After that time, degeneration usually occurs, but fresh emigration may

always be brought about by cutting away the tissue at the periphery of the fragment and transferring it to a fresh medium. I have never been able to transfer, uninjured, a sheet of kidney epithelium growing in fluid medium to a fresh coverslip. If too much medium is added to the fragment or if it is not very carefully spread out, emigration of epithelial cells may not occur. The epithelium grows round the explant itself, forming a complete investment corresponding to the 'epithelium de cicatrisation' of Champy. Such a fragment may become locally distended with fluid, and a spherical excrescence may form in which stretched out kidney tubules are seen. The formation of an 'epithelium de cicatrisation' is rarely seen in plasma cultures. If such an explant is divided into two parts and explanted in fresh medium, outgrowth of epithelium generally occurs.

Cortex of the Kidney.

Outgrowth of cells from the cortex of the kidney does not begin in plasma medium until about 24 hours after explantation, and the growth-rate of the epithelium is not as a rule so rapid. The formation of a membrane surrounding the explant usually takes about 48 hours to form; and, quite often, sheets derived from different parts, which do not join up with one another, are found. The

general character of the epithelial outgrowth is, apart from the slower growth-rate, similar to that found in cultures of the medulla, and it was impossible to distinguish different types of cells, or to determine the elements of the kidney tubules from which they had been derived. Mitotic figures are numerous, and the difference in growth-rate between the epithelium derived from the cortex and that derived from the medulla appears to be due more to a slower initial rate of migration than to a diminished capacity for cell proliferation in the zone of outgrowth. Emigration of fibroblasts and vascular endothelium also occurs, and macrophages are often seen. In subculture, it is essential, if the growth of the epithelium is to be maintained, to avoid transferring the fibroblasts, as these often form a capsule round the explant and prevent migration of the epithelium. Under the dissecting microscope, it is sometimes possible to sear a fairly large area of connective tissue outgrowth with a fine-pointed platinum wire without injuring the epithelium. Liquefaction of the plasma does not as a rule take place so rapidly as in cultures of the medulla. If degeneration occurs, separation of the epithelial cells takes place, as described on p.131 . Tubules sometimes project slightly from the original fragment into the zone of outgrowth.

This appearance, which at first sight is suggestive of the outgrowth of tubules as such, is due partly to the thinning out of the periphery of the explant, brought about by the migration of cells from the margin, and to the straightening out of the tubules themselves. As a rule these projecting tubules remain unchanged, but if growth does take place, it is always membranous in character. The outgrowth of glomeruli was not observed.

Since the cortex of the kidney consists of several different types of epithelium, convoluted tubules, loops of Henle and collecting tubules, an attempt was made to ascertain if the more highly specialised types of epithelium took part in the formation of the zone of outgrowth. Young rats were therefore injected subcutaneously with 0·2 c.c. to 0·3 c.c. of vital new red daily for 4 days. The animals tolerated the dye in all cases, and it was found, as stated by Cappell (1929) that the dye was deposited in the epithelial cells of the proximal convoluted tubules only. Small fragments of kidney cortex were then cultivated in plasma and the migration of the epithelial cells watched. The migration of unstained epithelial cells took place first. After 36 hours' incubation, dye-stained epithelial cells in which mitotic division occurred were seen to be

emigrating in several places at the margin of the explant and these came to form part of the epithelial membrane (Fig.42). The amount of dye in the cells in the zone of outgrowth appeared less than that in the tubules in the explant or in histological sections of the kidney from the other side of the same animal. This may be due either to the spreading out of the epithelial cells, resulting in the distribution of the dye over a larger area so making it appear less in amount, or to the diminution in dye-content brought about by mitotic division of dye-containing cells. In fluid medium, outgrowth of epithelial cells occurs after a latent period of at least 24 hours, and vitally stained epithelium is also capable of growing out. The epithelial cells seem to remain healthy for a longer time than those from the medulla.

During the above experiments, the slides containing the cultures were in some cases inverted so that the coverslip was downwards, and, in others, they were placed in the usual 'hanging-drop' position. In the latter, the retraction of the epithelial carpet, which results from liquefaction of the plasma, took place more quickly than when the slide was inverted, and the phenomenon was carefully studied. I found that in many cultures in which, before the onset of liquefaction,

the explant was surrounded by a typical epithelial membrane, detachment was not always complete, and a separated ring of cells often remained attached to the explant by a thick strand of contracted epithelial cells which simulated a tubular structure growing out of the explant. I am of the opinion that the tubular structures described by Carrel and Burrows, and H.W. Drew were of this nature.

The appearances of the epithelial sheets seen in my cultures of kidney resemble closely the descriptions given by Policard, A.H. Drew, and Craciun, and from very tiny fragments of kidney the outgrowth of epithelial cells in the manner described by Lewis and Lewis has also been observed. Only in degenerate preparations have I ever seen appearances resembling those recorded by Champy. Nordmann is the only worker who has attempted to determine the type of kidney epithelial cells which migrates in vitro, and states that the cells seen in the zone of outgrowth are derived from the collecting tubules, the convoluted tubules becoming degenerate within a short time. In my cultures, I found that the cells of the convoluted tubules in the original explant remained healthy for at least 12 days, and in the experiment with vitally stained kidney I found that the cells of the proximal convoluted tubules are capable of migration and proliferation.

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Liver.

Cultivation of Liver.

The capacity of liver epithelial cells to show emigration and proliferation is very variable. I have obtained growth from cultures of avian liver up to the 17th day of incubation. The liver from younger embryos, on the other hand, quite often fails to show growth *in vitro*. Similarly, with explants of mammalian liver, growth has been obtained from mice and rats up to the 5th day after birth, while absence of growth has often been found in cultures of embryonic liver. Although excessive proliferation of endothelium often prevents the emigration of the liver parenchyma, the condition of the liver itself seems to play an important part. For example, I have noticed that the liver from avian embryos of the same age often differs macroscopically. In some embryos it is reddish in colour, in others it is brownish-red and appears to contain fat. From the latter type, emigration of epithelial cells rarely takes place. In mice and rats, the same variations have been noted with similar absence of growth in explants of liver containing fatty substances. After some experience, it is possible to tell by the macroscopic appearance alone whether or not the liver epithelial cells will emigrate *in vitro*.

Avian Liver.

The most satisfactory results have been obtained in cultures of liver from embryos at the 8th to the 10th day of incubation. At that stage of development, the liver consists of columns of epithelial cells, and separated by blood sinusoids in which haematopoiesis is still active. The explants should be small, about 1 mm. in diameter and should be explanted on the surface of a plasma clot consisting of equal parts of plasma and embryo extract, the latter diluted with saline in the proportions of 1:3. After a few hours' incubation, outgrowth of cells takes place, and this consists mainly of endothelium, derived from the sinusoids. It grows out in the form of broad strands of elongated cells in close contact with one another. The appearances have been described on p. 112 . Endothelial sprouts are also seen penetrating the coagulum. In fig. 55. the outgrowth of the sinusoidal epithelium from between the columns of liver cells is well seen. Emigration of liver cells begins after 18-24 hours' incubation and proceeds slowly. If it does not begin within 24 hours after explantation it rarely occurs, as the endothelium grows actively and has a marked tendency to grow round the explant, thus surrounding the epithelium and preventing the occurrence

of epithelial migration. This is shown in fig. The culture was fixed after 48 hours' incubation and shows the formation of a capsule of endothelial cells, from which rapidly proliferating endothelial cells migrate into the medium.

As already stated, emigration of liver cells is slow and occurs in the form of finger-shaped or spade-shaped processes, (Fig. 52); a complete membrane of epithelial cells is rarely seen. The cells at the margin of the zone of outgrowth do not show the amoeboid processes found in cultures of other epithelial cells, but the arrangement of the cells is typically epithelial in that, except at the free margin, the cells are in contact with one another on all sides, (Fig. 5/). Numerous fatty droplets, which increase in number during cultivation, (Fig. 52) are present, and these consist of neutral fats and lipoids. Liver epithelial cells even in fluid media are never so spread out as the epithelial cells of other organs. Mitotic division is quite active. From an examination of living cultures, the impression is given that the cells are less stereotropic than those from other organs, and that increase in number in the zone of outgrowth is brought about more by proliferation than by migration of cells from the original fragment.

Isolated epithelial cells are sometimes observed. They occur in degenerating cultures or in cultures in which typical epithelial outgrowth has not taken place (Fig. 52). They are degenerate and show the usual accumulation of degeneration granules and vacuoles.

Haematopoiesis can be seen in thin explants in the sinusoids at the periphery. From cultures derived from 10-day embryos and onwards, (Fig. 54.) considerable emigration of wandering cells, many of them of the monocyte and clasmatocyte type, is often found. Outgrowth of mesothelium also occurs if the explant has been derived from the surface of the liver.

The growth of the epithelial cells is more easily maintained by subculture, in cultures derived from 10-day embryos than in cultures taken from younger embryos, as the endothelial outgrowth is less prolific in the former case. Extreme care must be taken to prevent as far as possible the inclusion of endothelium in the explant during subculture.

Mammalian Liver.

The medium used for the cultivation of mouse and rat liver consisted of 1 part rat plasma, 1 part avian plasma and 2 parts avian embryo extract diluted with saline in the proportion of 1:3. As before, the explants

were placed on the surface of the coagulum, and the cultures were transferred to fresh medium every second day. The fluid medium described on p. 74 was also used, the cultures being washed and supplied with fresh medium every second day; the embryo extract was diluted in the proportion stated above.

Migration of wandering cells, mainly of the clasmatocyte type, began a few hours after cultivation, (Fig. 144.). These multiplied by mitotic division and were actively phagocytic. Lymphocytes, polymorphs and nucleated red cells were also found in the zone of outgrowth. During cultivation, haematoidin sometimes appeared in the macrophages. This phenomenon is described on p. 356 *et seq.*

Emigration of liver cells began after 24 hours' incubation and was always preceded by a marked spreading out of the explant on the surface of the coagulum or coverslip. The migration of the liver cells was similar in character to that found in cultures of avian liver. Mitotic division was quite active. In fixed and stained preparations, the liver cells were polygonal in shape and did not show the polymorphism seen in cultures of other epithelial organs. Intra-cellular fatty globules were numerous.

Outgrowths of endothelium in the form of capillary

sprouts, of mesothelium and of fibroblasts were also found. The migration of these mesoblastic elements was not nearly so extensive as in the cultures of avian liver, and the maintenance of the epithelial outgrowth in sub-culture was easier than in the avian cultures.

Smirnow, (1916) cultivated the liver of newly born mice, rabbits and guinea-pigs. He obtained outgrowth of epithelial cells similar to those described, and noted the occurrence of large numbers of fatty globules in the cells.

Lynch, (1920,1921) cultivated avian liver from 5 - 14-day embryos in Locke-Lewis' medium and obtained out-growth of liver epithelial cells. He noted the slow rate of migration, but states that mitotic division of the liver cells does not occur <u>in vitro</u>. As already mentioned, mitoses were quite numerous in my cultures.

Kapel, (1926) cultivated avian liver from embryos between the 9th and 15th days of incubation. He obtained an abundant emigration of wandering cells which invaded fragments of heart muscle which were placed in the medium. He considered that these cells were of epithelial origin. From his description, however, the cells in question appear to have been of the macrophage class. He did not obtain outgrowth of epithelial cells in the form of sheets.

Doljansky, (1929, 1, 2; 1930, 1, 2.) has studied extensively the growth of avian liver *in vitro*. He also found that it was advisable to dilute the embryo extract in order to obtain growth of the liver parenchyma. The outgrowth of the epithelial cells was similar to that found in my cultures, and the cells multiplied by mitotic division. He found that by inhibiting the growth of the mesenchymal elements either by using embryo extract inactivated by heating, or by adding small amounts of arsenious acid to the medium, pure cultures of liver epithelium could be obtained, and these maintained the typical epithelial character. Glycogen could be demonstrated in the epithelial cells: it was absent, however, during the phase of active growth.

Ephrussi (1930) also cultivated liver tissue, but failed to obtain outgrowth of liver epithelial cells.

Recently, Bisceglie (1931) has given a very complete account of the appearances found *in vitro* of cultures of the avian liver from embryos at the 8th to the 18th day of incubation. He also found that a diluted embryo extract favoured proliferation of the liver cells, and his description of his cultures corresponds exactly to that found in my cultures of avian liver. He did not, as a rule, however, observe emigration of epithelial

cells until after 40 hours' incubation, as compared with 24 hours in my experiments. The membranous character of the epithelial outgrowth was maintained for 85 days *in vitro* without showing any morphological change. In his experience, as in mine, isolated liver epithelial cells are degenerate and soon die off.

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Stomach.

Fragments of the stomach of 9-day avian embryos were cultivated on coverslips on the surface of a coagulum consisting of equal parts of plasma and embryo extract. The epithelium lining the glands began to grow out within a few hours after explantation and soon formed a complete membrane round the explant similar to that described by Lewis and Lewis, (1912). (Figs. 48, 14.). Two or three membranes were often found growing at different levels in the coagulum. The cells showed numerous mitoses, were quite indistinguishable from the epithelial cells obtained in cultures of gall-bladder and intestine, and were all of the same type. Near the explant, each cell contained one or two large fat globules. As cultivation proceeded, small fat globules appeared in the cells, so that the cells at the periphery of the zone of outgrowth contained in addition to the one or two large fat globules, numerous small fat globules, and all transitions between these cells and those round the explant were seen. After 36 hours' cultivation, rapid liquefaction of the plasma took place, and in many cultures the epithelial sheets became detached from the explant. In primary cultures the outgrowth of fibroblasts and smooth muscle cells was not very abundant, and in many cultures

it was absent. After one or two passages, however, the mesenchymal and muscle outgrowth became profuse, and overgrew in many cases the epithelial outgrowth. Spontaneous contraction of the explant took place for 10 days, and the smooth muscle in the zone of outgrowth also showed spontaneous contraction. In fixed preparations, the presence of mucin was detected in the cells surrounding the explant. As the limit of the zone of outgrowth was approached the mucin diminished in amount, and none was found in the peripheral cells. Even after subculture, mucin could always be detected in the central part of the zone of outgrowth.

Intestine.

Fragments of intestine from 14-day avian embryos were cultivated in a medium consisting of equal parts of plasma and embryo extract. In order to obtain active out-wandering of the cells, the intestine has to be carefully cut up along its length under the dissecting microscope and the intestinal epithelium exposed. The explants should be small, about 1 mm. in size, and should be placed on the surface of the coagulum. The fragment soon shows rhythmic contraction. Outgrowth of intestinal epithelium takes place within a few hours after incubation, and large sheets of

epithelial cells, amongst which numerous mitotic figures are to be found, soon form around the original fragment (Fig.43.). These are similar in appearance to the epithelial cells obtained from cultures of stomach, and show the same arrangement of fat globules (Figs.44,47). Rapid liquefaction of the plasma takes place, and the membranes may become detached from the explant and grow out into the uncoagulated medium, while a new sheet of epithelial cells grows out on the surface of the coverslip in the liquefied area. Migration of fibroblasts and smooth muscle cells begins somewhat later than the epithelial outgrowth, and often becomes abundant when the plasma has liquefied. Macrophages are also seen in considerable numbers after 36 hours' cultivation, and mesothelium was found in some cultures. Sympathetic nerve fibres also grow out occasionally in the primary culture but, as a rule, after the first subculture. The free ends of the nerve fibres show numerous radiating processes which change shape continually during life. No fibrillae were seen in the living state, but after fixation and staining they were easily seen.

In fixed cultures, stained with thionin blue, mucin granules were detected in the epithelial cells

(Figs. 45, 44.)

surrounding the explant, and the distribution was similar to that seen in cultures of stomach. It was present in the same situation after each subculture.

After 3 or 4 subcultures, the epithelium was overgrown by the rapid proliferation of fibroblasts and smooth muscle, and emigration of epithelium ceased.

The epithelial membranes obtained in cultures of stomach and intestine were similar to those described by Lewis and Lewis (1912), from explants of these organs. These workers, (1912), also described the outgrowth of nerve fibres in cultures of the intestine. Fischer, (1922), cultivated segments of intestine from embryos approaching hatching; in some cases the cylinders of intestine were turned inside out so that the epithelium was on the outside. He found that little or no outgrowth was obtained, and the epithelium grew all over the surface of the explant. These ' intestinal organisms' remained healthy for a month in vitro. I have also found that no epithelial outgrowth occurred if segments of intestine were placed in the medium. Mesothelium sometimes grew out in small sheets and fibroblasts and smooth muscle cells usually emigrated from the cut ends. After the second subculture, the mesothelium grew round the cut ends and cell migration

ceased. A cyst-like structure was formed from which fluid escaped when it was punctured. Only if the segment of intestine was turned inside out, did the epithelium grow over the cut surface in the manner described by Fischer.

Gall Bladder.

Cultures of the gall-bladder from 12-day avian embryos were prepared. At that stage of development the gall-bladder contains bile and it is necessary to wash the gall-bladder in saline before cutting the tissue into fragments for explantation. The epithelium can be quite easily seen under the dissecting microscope, and only tissue fragments with an epithelial layer should be taken. The tissue was explanted on the surface of the coagulum, which consisted of equal parts of plasma and embryo extract, the whole preparation being inverted so that the coverslip was underneath.

The epithelial cells grow out very quickly after 6 - 12 hours' incubation, in the form of a typical epithelial membrane in which mitotic figures are numerous (Fig. 50.). The appearance of the cells is so similar to that of cultures of intestinal epithelium that it is impossible to distinguish the one from the other. The plasma is liquefied very rapidly and the

epithelium retracts from the edge of the explant and grows out into the unliquefied medium in the manner already described in intestinal and stomach cultures. Another layer of epithelium often migrates outwards on the surface of the coverslip in the liquefied area. Fibroblasts, mesothelial cells and smooth muscle cells also grow out from the explant. The epithelium has been maintained in subcultures for 6 passages, the usual precautions being necessary to avoid as far as possible transferring the fibroblasts and smooth muscle cells. Small pieces of epithelium often become detached from the main outgrowth, and these increase in size by mitotic division. Owing to the delicate character of the cells, however, it has not been possible to maintain them by subculture.

In stained preparations, the epithelial cells show the usual characters. The fatty globules stain bright red with Scharlach R. The smooth muscle cells show the fine fibrillae which are always present in fixed and stained preparations. When preparations are stained with 0.5% aqueous solution of thionin blue, numerous fine granules of mucin are seen in the epithelial cells surrounding the explant, as in cultures of

intestinal epithelium. In the peripheral cells of the zone of outgrowth, however, no mucin is present.

Cultures of avian gall-bladder have been described by Erdmann, (1931). Erdmann did not obtain outgrowth of epithelial cells until the second or third day of explantation, and in several of her cultures obtained smooth muscle only. This was probably due to absence of epithelium in the explant. She found, in the cultures containing epithelium, that two types of cells were obtained: in the one type the nuclei were large and stained intensely, while in the other type, the nuclei were small and less intensely-staining. The former type disappeared from the cultures, but the latter type of epithelial outgrowth was maintained by passage for about two months in vitro. In my cultures, I could not distinguish two types of epithelial growth after 2 weeks' cultivation.

Allantois.

The allantois of 7-day avian embryos was cultivated on coverslips in a medium consisting of equal parts of plasma and embryo extract. The thin fragments of allantois were placed as flatly as possible on the surface of the coagulum in order to prevent rounding up of the explants. The explant spreads out as a thin

sheet on the surface of the coagulum, and from the periphery an abundant outgrowth of mesenchyme cells and vascular endothelium in the form of strands similar to that seen in liver cultures (p.), takes place within 24 hours. Outgrowths of epithelium also occur in the form of typical membranes, resembling ectodermal epithelium in stained preparations, (Fig.). In the spread-out explant, capillary blood vessels could be seen in which haematopoiesis was taking place. The plasma liquefied rapidly. Owing to the rapid growth of the mesenchyme, the epithelium disappeared from the cultures after the second passage.

Lewis and Lewis, (1912, 1) obtained outgrowth of epithelium from the allantois.

Katzenstein, (1925), cultivated the allantois from 5-day avian embryos. Plasma alone proved the most satisfactory medium, the addition of embryo extract apparently preventing growth. This was not the case in my experience. Katzenstein found that the allantoic fragments tended to round themselves up in the manner sometimes seen in cultures of intestine: as stated above this can be avoided by spreading out the tissue at explantation. He obtained, however, outgrowth of mesenchyme and epithelium, and, in some cases, found mesothelial cells in the zone of outgrowth.

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Cultivation of Spleen.

I have cultivated the spleen of newly-born and young rats and mice up to three months old, both in coagulated and in fluid media. The former consists either of a mixture of rat plasma and mouse embryo extract or equal parts of a mixture of rat and avian plasma and avian embryo extract. A mixture of rat serum and mouse embryo extract was used for fluid medium. The plasma cultures were not removed from the clot but were washed with embryo extract every second day, more plasma being added if necessary. Maximow's method (p. 68.) was also employed.

Emigration of lymphocytes and polymorphonuclear leucocytes begins within a few hours after incubation, and soon a fairly thick ring of cells is seen round the explant. Monocytes also emigrate into the medium. After 48 hours' incubation, most of the lymphocytes in the outer part of the zone of outgrowth have become rounded up and many die. The polymorphonuclear leucocytes also die within a short time. After 36 hours' incubation, migration of cells of the macrophage class begins, and these show the typical segregation apparatus when supravitally stained with neutral red. These cells are very phagocytic and ingest dead lymphocytes, (Fig. 70) (Fig. 68.)

polymorphs and red blood corpuscles: the fate of the last is discussed on p. . The macrophages undergo mitotic division and their number appears to be increased in addition by the transformation of monocytes into macrophages. After about 2 days' cultivation, sprouts of cells resembling sprouts of endothelium are often seen. (Fig. 66) These extend outwards into the zone of outgrowth, and many of the cells become detached, emigrate as isolated individuals in the medium, and show phagocytosis. If the spleen has been stained intra vitam with vital new red, these sprouts of cells show small vitally stained granules similar to those described by Cappell (1929) in the cells lining the venous sinusoids of the spleen. It is concluded, therefore, that some at least of these sprouts are derived from the endothelial lining of the sinusoids. In such vitally stained fragments of spleen, the phagocytic cells which emigrate during the second day usually contain numerous dye granules. Binucleate phagocytes are quite common after several days of cultivation. Mesothelium and fibroblasts are often found in cultures of spleen, particularly from mouse tissue.

In fluid media, migration of lymphocytes and monocytes takes place rapidly. The lymphocytes die very quickly, however, and mitotic division occurs only in the

monocytes. The macrophages which appear tend to become spread out on the surface of the coverslip and do not show such active proliferation as in a coagulated medium. After 3 to 4 days, mitotic division and amoeboid movement cease, and the cells become widely extended on the surface of the coverslip. They still retain their capacity for ingesting freely divided particulate matter and when a suspension of India ink is added to the culture, it is soon taken up by these cells.

Fazzari (1926) cultivated the spleen of avian embryos and young mice. Erdmann, Eisner and Laser (1926), cultivated the spleen from foetal, young and adult rats. All these workers describe in detail the character and origin of the phagocytic wandering cells, and derive them from the reticulum cells of the spleen pulp and from the endothelial cells lining the venous sinuses. They do not appear to have observed the outgrowth of the sinus endothelium in the form of endothelial sprouts.

Tumour Cells.

Carcinomata and sarcomata of animals and man have been cultivated by many workers. As early as 1910, Carrel and Burrows reported the cultivation of Rous' sarcoma, Jensen's rat sarcoma, a spontaneous breast carcinoma of the dog, a sarcoma of the fibula and a

mammary carcinoma of man. Volpino (1928-29) states that he cultivated before 1910 an adeno-carcinoma of the mouse in horse serum, and claims priority in the field of tumour growth in vitro. Lambert and Hanes (1911) described the appearances of sarcomata of rats and carcinomata of mice growing in vitro. They found that the sarcomata grew in a manner characteristic of tissues of mesenchymal origin, while the carcinomata grew in a typical epithelial fashion.

Most workers have found that tumour cells are much more difficult to cultivate in vitro than cells of normal tissues, and various methods have been adopted to overcome the technical difficulties. It was found that tumour cells liquefy homologous plasma with great rapidity. Fischer (1927) has studied extensively the growth conditions of tumours in vitro and found that the addition of avian plasma to the medium prevented the rapid onset of liquefaction; in a medium containing equal parts of a mixture of 75 per cent. rat plasma and 25 per cent. avian plasma and avian embryo extract, good growths of Ehrlich's transplantable mouse carcinoma could be obtained. In addition to his contributions to the technique of tumour cultivation, Fischer (1930) has also established the important fact that 'pure'

cultures of carcinoma maintained in vitro for over a year retain their virulence and are capable of producing tumours when injected into mice. He has also found that tumour cells growing in vitro infiltrate normal tissues just as they do in vivo.

Cultivation of Tumour Tissues.

The following tumours have been cultivated in vitro:

1. Carcinoma M.63 of the Imperial Cancer Research Fund.
2. Tar carcinoma 2146 of the Imperial Cancer Research Fund.
3. A transplantable melanoma of mice (obtained from Professor Passey, Leeds).
4. Five spontaneous adeno-carcinomata of mice.
5. A spontaneous sarcoma of a mouse.
6. A sarcoma of man.

1. Carcinoma M.63.

The type of medium used and the general characteristics of carcinoma M.63 are described on p.251. It is to be noted that the nuclei of the cells in fixed and stained preparations more closely resemble those seen in ordinary histological sections than nuclei of normal tissues growing in vitro.

2. Carcinoma 2146.

This tumour was more difficult to grow than M.63 and I found it advisable either to use a 1 in 8 solution of embryo extract or to substitute rat serum for embryo extract. Growth occurred in only about 10 per cent. of the cultures and was epithelial in type, numerous small membranes occurring round the explant. The cells were often somewhat spindle-shaped,and cytoplasmic staining was never so intense as in the cells of M.63. The nuclei were very hyperchromatic and showed a much more complicated structure than those of normal tissue cells fixed by the same fixative. Degeneration of the cultures took place very rapidly.

3. Transplantable Melanoma.

Attempts were made to cultivate this tumour in vitro in order to ascertain, if possible, from the character of the outgrowth, whether the tumour cells were of epithelial or mesoblastic type. Two strains of the tumour were obtained. In one, there was a large amount of pigment; in the other, very little pigment was present. The tumour did not grow readily and the medium employed for the cultivation of carcinoma M.63 was found to be the most suitable. From the pigmented strain, emigration of isolated mononuclear cells containing

pigment granules took place after about 48 hours' incubation. In their appearances, these cells resembled pigment-containing macrophages, and when stained with neutral red, they showed a segregation apparatus similar to that seen in macrophages. In sections of this tumour, cells resembling mononuclear phagocytes containing melanin pigment were found and it was concluded that the cells which appeared in vitro belonged to the macrophage class, which had phagocytosed pigment released from the tumour cells. In the less pigmented type of tumour, in addition to a few pigmented cells similar to those described above, a fairly abundant outgrowth of non-pigmented spindle-shaped cells resembling fibroblasts was sometimes obtained. Whether these were tumour cells or were derived from the stroma of the tumour was not determined, and the nature of the neoplasm was not ascertained.

4. Spontaneous Tumours.

In contrast with the transplantable tumours, spontaneous tumours were relatively easy to grow in vitro. The usual undiluted embryo extract was employed in combination with avian and rat plasma. Quite good growths were sometimes obtained in fluid media. After the cultures had been prepared, a series of mice was

always inoculated subcutaneously with fragments of the tumour.

In four out of the five tumours cultivated, outgrowth of typical epithelial membranes and fibroblasts was obtained. (Figs. 72,73.). The membranes resembled those seen in cultures of normal epithelial organs; and, after fixation and staining, the nuclei were similar in appearance to those of normal cells in vitro. In all cases, the grafts injected into mice regressed after a few days. Histologically, these tumours were typical adenocarcinomata. In the fifth case, only one culture out of a series of 20 showed emigration of cells in vitro, and the type of outgrowth resembled that seen in cultures of carcinoma M.63. Histologically this tumour was more encephaloid than adenomatous in type and resembled carcinoma M.63. The grafts were carried through two passages in mice, after which they regressed. These results suggest the possibility that the ease with which growth of a spontaneous tumour is obtained in vitro varies inversely as its capacity for transplantation.

5. Mouse Sarcoma.

This tumour was not grafted into other mice. It was found in the neck of an adult mouse and had invaded the lower jaw. Histologically, it was a spindle-celled

sarcoma. The tumour was cultivated in mouse serum and outgrowth of cells which were quite indistinguishable from fibroblasts was obtained. A few wandering cells were also found in the zone of outgrowth.

6. Human Sarcoma.

This tumour was a round-celled sarcoma of the femur and was cultivated in human serum and mouse embryo extract. Outgrowth of mononucleated cells, some resembling fibroblasts and others resembling macrophages, began after 24 hours' incubation, many degenerate cells being present. After 4 days' incubation, migration ceased and the cultures died.

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Pancreas.

Kapel, (1926) cultivated avian pancreas and found an abundant emigration of isolated cells which invaded a piece of muscle explanted in the same medium. He considered that these cells were epithelial in origin. Pinkus, (1929) cultivated the pancreas of newly hatched chickens and obtained outgrowth of epithelial cells in the form of a membrane. He found that many of the cells contained secretion granules, and identified gland cells, duct cells, and cells corresponding to those of the islets of Langerhans in his cultures.

Chlopin (1929-30) cultivated the pancreas of young rabbits. He described the outgrowths of epithelial membranes and isolated epithelial cells inhis cultures. He stated that growth occurred later in the form of cysts and gland-like structures and that the epithelium became stratified. I have never observed such appearances in cultures of pancreas.

Cultivation of Pancreas.

I have cultivated the pancreas of newly-born mice and rats in a coagulated medium. Emigration of macrophages and fibroblasts was abundant and the latter tended to grow round the explant. (Fig. 64.) Occasionally outgrowths of epithelium in a typical membranous form were obtained. Stratified epithelium, as described by Chlopin was never seen in my cultures: isolated epithelial cells were always degenerate.

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Cultivation of Lung.

Fragments of lung remote from the pleural surface of 14-day avian embryos were cultivated in equal parts of avian plasma and avian extract on coverslips. After 24 hours' incubation, many fibroblasts and endothelial sprouts were seen in the zone of outgrowth. Within the next 24 hours the explants had spread out on the coverslip and the bronchi could be seen. Emigration of wandering cells began during this period. Most of these were typical macrophages, and when the cultures were stained supravitally with neutral red, showed the presence of typical granules and vacuoles. Lymphocytes and monocytes were also seen. Some of the wandering cells showed numerous fine neutral red granules and could not be placed in any definite class. During the second 24 hours' incubation emigration of epithelium began, and this appeared to be derived from the cut ends of bronchi which were in contact with the medium. The epithelial cells (Figs.59,60) resembled skin epithelium, the cells being elongated and spindle-shaped. In spite of the abundant outgrowth of fibroblasts, the epithelium was easy to maintain in subculture and remained remarkably healthy. The larger the amount of epithelium in the zone of outgrowth, the more rapid was the plasma liquefaction. The lymphocytes and

wandering cells which contained the fine neutral red granules became less numerous as cultivation continued, but the macrophages remained abundant in the zone of outgrowth.

The lung of embryo cats was also cultivated in a mixture of equal parts rat plasma and mouse embryo extract. A similar type of outgrowth was obtained, but the bronchial epithelium grew out more rapidly and formed large sheets. The same capacity for growth in subculture was also noted in these cultures. Wandering cells were abundant, but these consisted mainly of macrophages. A few wandering cells, containing fine neutral red granules similar to those seen in the avian cultures, were found; but they disappeared after the second subculture; the origin of these cells was not determined.

Carleton, (1925) was the first to describe the cultivation of lung tissue, his object being to investigate the phenomena of phagocytosis. He cultivated the lung from foetal and adult cats and rabbits and obtained outgrowth of fibroblasts, epithelial cells and wandering cells. He found that the wandering cells were very phagocytic towards coal and carmine particles. Considerable liquefaction of the medium took place, and he described in detail the detachment of the epithelial

sheets and the growth of epithelium round the explant. He considers that the epithelium in the zone of outgrowth was derived partly from the bronchi and partly from the epithelial lining of the alveoli. In my experiments, the epithelium always appeared to be derived from the bronchi. Binet and Champy, (1926), cultivated lung tissue and described the outgrowth of epithelium and fibroblasts.

Lang, (1926) has also investigated the growth of lung tissue in vitro and obtained outgrowth of fibroblasts, epithelium, endothelium and phagocytes. Like Carleton, he was particularly concerned with the problem of phagocytosis, but arrived at a different conclusion regarding the origin of the alveolar phagocytes.

Thyroid Gland.

The cultivation of the thyroid gland of mammals was first described by Carrel and Burrows (1911). The thyroid cells grew out as continuous epithelial sheets or in the form of tubules. Champy (1915) also cultivated the thyroid gland of mammals. He states that the epithelium showed proliferative activity within 24 hours, and that the outgrowth of fibroblasts was inhibited by the proliferation of the epithelium. The epithelial cells showed at first a membranous arrangement, but after 72 hours' cultivation it was impossible to distinguish them from

fibroblasts, outgrowth of which began after 48 hours' incubation. He concluded that the growth of the epithelium inhibits to some extent the outgrowth of fibroblasts, and that the cells of the thyroid in vitro dedifferentiate to form an indifferent type which is indistinguishable from fibroblasts.

Ebeling (1924,1.), described the morphological appearances of cultures of the avian thyroid from 18- to 19-day embryos. He found (1924,2,), that if a culture of fibroblasts was placed close to a culture of thyroid, the fibroblasts grew round the thyroid culture and encapsulated it. The growth-rate of the fibroblasts was greater than that of control cultures in which no thyroid tissue was present. In sections of the cultures of thyroid and fibroblasts he found that the epithelium possessed an acinar arrangement and in the lumen of the gland-like structure, colloid material could be demonstrated . In a later paper, Ebeling (1925), stated that he obtained pure cultures of thyroid cells from the thyroid gland of 18-to19-day avian embryos. He cultivated the tissue on coverslips,and after a few subcultures, he transferred them to flasks. In the primary cultures, he found that the epithelial cells grew out in the form of a membrane on the surface of the coagulum. Tubular

structures, which he considered to be epithelial in nature, penetrated the clot. At the distal end, i.e. the end farthest from the explant, the cells of these 'tubules were spread out and it was impossible to distinguish them from fibroblasts. The cultures in flasks were fixed after varying periods of cultivation, up to 129 days, and were sectioned and examined histologically. In the sections, he found in many cases that the epithelial cells had assumed an acinar arrangement and surrounded spaces containing colloid material.

Cultivation of the thyroid gland.

I cultivated fragments of avian thyroid, from 14-day embryos, on coverslips on the surface of a coagulum consisting of equal parts of plasma and embryo extract. The cultures were maintained for 12 days by subculturing every second day, care being taken to avoid transferring the mesenchymal elements of the outgrowth. After 18 hours' cultivation, sheets of epithelial cells resembling those seen in cultures of lung were seen both on the surface of the coagulum and in the interior of the clot. (Figs. 61, 62). The epithelial cells on the surface of the clot were more spread out than those which had penetrated it; otherwise no difference between the outgrowths could be detected. The general character of the cells remained the same

throughout the culture period, and no tubular formation was observed. Fibroblasts also emigrated from the explant and, in some cultures, many wandering cells were present. In quite a number of cultures, typical endothelium appeared in the zone of outgrowth, and those endothelial sprouts which had penetrated the coagulum had a somewhat tubular appearance; the cells at the distal end were spread out, however, and resembled fibroblasts. In stained preparations, the various types of cells could be easily distinguished from one another and the 'tubular' structures in the coagulum consisted of flattened cells identical with endothelial cells from other organs.

From the observations recorded above, I am of the opinion that the tubular structures described by Carrel and Burrows, and by Ebeling were endothelial and not epithelial in nature. Carrel and Burrows' figures bear a strong resemblance to endothelium. Ebeling, from his observations, is of the opinion that he obtained a pure culture of thyroid epithelium, and he concludes that thyroid epithelium 'does not necessarily dedifferentiate' in vitro but is able to maintain its normal anatomical form and part at least of its physiological activity, the cells growing out in the form of vesicles, the

epithelium of which secretes colloid material.

In order to justify the statement that a pure culture of epithelium was obtained, it is essential that the original fragment should have been removed and the culture built up entirely by the proliferation of sheets of epithelial cells. Only in this way is it possible to be sure that no fibroblasts are present in the explant. Fibroblasts apparently did not occur in the zone of outgrowth; but, as Ebeling did not remove the original fragment and does not say whether or not they were present in that situation, it cannot be inferred that they were absent from the culture. Accordingly, it is impossible to conclude that the cultures were 'pure' cultures.

As evidence for the statement that thyroid epithelium 'does not necessarily dedifferentiate' in vitro but grows out in the form of colloid-secreting vesicles, Ebeling adduces the presence of the tubular structures in primary cultures, which I consider are endothelial and not epithelial in nature, and the appearance, in sections of cultures of typical thyroid vesicles after varying periods of cultivation. He does not state specifically whether or not these were found in the central mass or in the zone of outgrowth, although from his figures they appear to have been found in the explant itself. Accordingly, two

interpretations of this result are possible: (1) the persistence of the original thyroid tissue on the explant, and (2) the re-differentiation of thyroid epithelium which at some period formed part of the zone of outgrowth and later became incorporated in the central mass. Such re-differentiation would then be determined by the position of the cells, which is unfavourable for migration, and possibly also by the presence of fibroblasts, the absence of which has not been proved. These conditions were present in the cultures of thyroid gland which were surrounded by fibroblasts. The most convincing evidence, viz. the direct observation of the formation of vesicles, and the gradual accumulation of the secretion within them, has not been given. Accordingly, I do not consider that Ebeling has proved either (1) that he has obtained a pure culture of thyroid epithelium, or (2) that thyroid epithelium differs from other types of epithelium in that it is able to grow out into the medium in vitro in a manner resembling the form seen in vivo. Ebeling has only shown that typical colloid-secreting vesicles are present in cultures of thyroid gland after 129 days' cultivation in vitro.

The phenomena of dedifferentiation to an indifferent type of cell described by Champy was not observed by me and I

did not find that epithelial cells inhibit the proliferation of fibroblasts; in fact, the epithelial outgrowth was prevented in some cases by the presence of fibroblasts.

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S E C T I O N IV.

The cultivation of tissue outside the animal body, since the establishment of the fact that cells are capable of active proliferation *in vitro*, should be regarded only as a method of research, or technique, whereby the properties of cells under varying conditions can be investigated. In the succeeding section, several applications of tissue culture methods are reported.

THE ACTION OF A CYTOTOXIC ANTISERUM ON TISSUE CULTURES.

C O N T E N T S.

	Page		Page
I. Methods	230.	IV. The action of the cytotoxic serum on cultures of tissues from other species	264
II. Action of cytotoxic serum on cultures of mouse tissues	239.	V. The complement-fixing haemolytic and precipitating properties of the cytotoxic serum during the course of immunisation	268
III. The appearances of tissue cultures after treatment with cytotoxic serum, in fixed and stained preparations	259.	VI. Summary	276

The early investigators of cytotoxic antisera action aimed at the production of an antibody specific for a given organ by injecting emulsions of the tissue into animals of another species. They tested the effect of such cytotoxic sera (I) by exposing the antigen (cell emulsions) to the antiserum, and (II) by injecting the antiserum into the animal body parenterally. Landsteiner (1899) von Dungern (1898) Metchnikoff (1899) Besredka (1900) Delezenne (1900) and Lindemann (1900) all concluded that the injection of a particular organ or tissue into an animal of different species resulted in the production of antibodies in the serum to that particular organ or tissue although it appeared that the antiserum acted on other tissues in addition. The first method of testing

is of little value, as the cells in emulsions are probably considerably damaged by the process involved in their preparation; the latter method, as Pearce (1904) has pointed out, is worthless since the haemolytic and haemagglutinating properties possessed by practically all cytotoxic sera tend to cause thrombosis, embolism and haemorrages with secondary necrosis of organs. Later investigators, Ranzi (1904) Schutze (1908) Miller (1908) and others using the complement fixation reaction failed to demonstrate any specificity of cytotoxic sera for the particular tissue or organ used as the antigen.

The use of tissue cultures offers a very suitable method for the study of cytotoxic substances. Lambert and Hanes (1911) were the first to employ such cultures for this purpose. They injected rats and guinea-pigs subcutaneously with mouse sarcoma and rat sarcoma respectively, and found that after two injections the plasma of these animals proved unsuitable for the growth of the tumours. The plasma of a guinea-pig which had received an intraperitoneal injection of 5 c.c. of defibrinated rat blood was also inhibitory to the growth of rat sarcoma. The authors considered that the inhibition of growth was due to the presence of a

cytotoxin which is, however, not specific for sarcoma cells. Lambert (1914) extended these observations. He injected guinea-pigs with embryonic rat skin, rat sarcoma and defibrinated rat blood, and obtained almost complete inhibition of growth in vitro of both rat skin and sarcoma in the plasma from the injected animals. The plasma of the animal injected with defibrinated rat blood allowed only a feeble growth of those tissues, while control cultures made with the plasma of normal animals grew well. Similarly the plasma of guinea-pigs injected with either chicken heart or intestine inhibited the growth of both these tissues. Lambert concluded that the cytotoxins formed after the injection of various animal tissues into a foreign species are not specific for the tissue injected. In none of Lambert and Hanes' experiments were more than two injections given, and these, except in the case of defibrinated blood, were always subcutaneous. It is to be noted that these workers did not investigate the effect of the "immune" plasma on tissue cultures which were in the stage of active growth, but merely tested for inhibitory effects. Foot (1912) injected a rabbit three times intraperitoneally with chicken bone-marrow and found that the plasma of the injected animal allowed no emigration of marrow cells

explanted in it, and that a dense precipitate formed in the medium. A parallel series of cultures prepared in normal rabbit plasma showed some emigration of cells, but many were degenerate, and amœboid movements were not so active as in cultures prepared in chicken plasma. No precipitate, however, was found in normal rabbit plasma. It was concluded that the injection of chicken bone-marrow into a rabbit resulted in the appearance of antibodies in the plasma of the injected animal. Like Lambert and Hanes, Foot also did not describe the effect of his immune serum on growing cultures.

Recently Lumsden (1925, etc.) has made use of the technique of tissue culture to test antisera to various tumours, particularly Jensen's rat sarcoma, carcinoma M.63 of the Imperial Cancer Research Fund and human mammary carcinomata. Kimura (1927,1928) obtained antisera from rabbits to chicken tissues which he tested on cultures of these and heterologous tissues.

The importance of such work in connection with the problems of tumour growth suggested that a more complete study should be made than has so far been undertaken of the action of immune cytotoxic sera on tissues growing in vitro. For this purpose an antiserum was obtained by injecting emulsions of mouse embryo tissues into a

rabbit. The action of this antiserum was tested at various periods in the course of a prolonged series of immunising injections. At the same time the action of the antiserum on cultures derived from the tissues of other animals was examined and an attempt was made to analyse the mechanism of the cytotoxic action. In addition to investigating the microscopic appearances of the fresh cultures when subjected to the action of antiserum, the tissues were also fixed and stained in various ways and the histological appearances were studied in detail. Further, methods of vital staining were used in order to follow the harmful effects of the antiserum on the cells of cultures. In addition, the properties of the antiserum were examined in order to detect complement-fixing, haemolytic and precipitating effects.

I. Methods.

Tissue culture. The tissue culture methods were, in general, modifications of those described by Drew (1923) and Strangeways (1924). Cultures were prepared from the tissues of newly-born mice or embryos at an advanced stage, heart being principally used on account of the relative ease with which good growths are obtained, especially of the fibroblasts which it contains. The

cultures were prepared to cover glasses inverted over hollow ground slides, sealed with a mixture of paraffin and vaseline and incubated at 37·5°C., the so-called hanging drop method.

Two types of medium were used, a fluid and a coagulated one. The former consisted of embryo juice, mouse serum and buffered saline (Drew's) in approximately equal parts; this is the more useful, since it can be removed almost entirely and replaced by antiserum, a procedure not possible with a solid medium. For the preparation of embryo juice (according to a modification of Drew's method) the attempt was always made to obtain embryos at about the end of the second week of pregnancy. After anaesthetising the mouse with ether the uterine tissues were removed aseptically, the embryos dissected, cut up into six to eight pieces, quickly washed with three changes of buffered saline and finally converted into pulp by snipping with fine scissors. Undue prolongation of the washing process or cutting up into too small pieces should be avoided, since the tissue juices are lost thereby. After adding an equal volume of buffered saline, the mixture was pipetted into a centrifuge tube and frozen in a mixture of ice and salt and then allowed

to thaw at 37·5°C., this procedure being carried out three times. When it was desired to use the extract on the following day the mixture was frozen overnight at - 10° to - 14°C. After a final period of incubation for thirty minutes at 37·5°C., the mixture was centrifuged until a clear fluid of a faint yellowish colour was obtained. This was removed, diluted with an equal quantity of buffered saline and pipetted into ampoules which were sealed and stored in the ice chest. This constituted "embryo juice"; it retained its growth-promoting qualities practically unimpaired for about a week. It was found desirable not to freeze the extract. In the coagulated medium, rat plasma or a mixture of rat and chicken plasma was substituted for the serum. The method of obtaining the plasma has already been described.

Tissues of embryonic and newly born mice grow readily in both these media. After twenty-four to forty-eight hours' incubation, growth is generally well established, and out of a series of, say, twenty cultures, fifteen to nineteen are in approximately the same condition and can be used for experimental purposes. Provided that the explant is derived directly from the embryo or from a vigorous culture, the course of events

is as follows. After a period of lag of four to eight hours, emigration of cells becomes active and increases progressively up to thirty hours,after which it diminishes, ceasing after about sixty hours. Mitoses make their appearance after about twenty hours, reach their maximum after thirty hours and disappear some hours before emigration ceases. So long as the cultures are viable, i.e. up to six days, removal of the medium and its substitution by a further quantity of fresh medium leads to renewed emigration and mitoses, but not to an extent equalling that originally seen. After ten days in spite of renewal of culture medium every forty-eight hours the culture becomes quiescent. In order to obtain prolonged vigorous growth, it is necessary to transfer a portion of the original culture to fresh medium, i.e. to make a subculture, every forty-eight hours; but even under these conditions after repeated subcultures mammalian tissues lose their activity and, in general, the most vigorous growths are obtained in the first three or four cultures within a period of six days after obtaining the tissue.

Mention must again be made here of the appearance in cells growing in vitro of vacuoles known as "degeneration vacuoles"(W.H. Lewis, 1919). These vacuoles develop in numbers after several days (three or more) in all types of cells, especially in primary cultures; in fluid medium

they appear more quickly than in plasma. The vacuoles often contain one or more granules which show an active dancing movement and these seem to be the starting points round which the vacuoles develop. Both the granules and other contents of the vacuoles have a marked affinity for neutral red.

Antiserum. Before commencing immunisation with mouse embryo it was necessary to determine whether normal rabbit serum showed any cytotoxic effect. Accordingly two young female rabbits were selected, "I" (weight = 1·6 kilos) and "C" (weight = 1·45 kilos). From each about 5 c.c. of blood were taken rapidly from an ear vein under aseptic conditions. After separation of the clot and centrifuging, the serum was stored in capillary tubes and ampoules of hard glass and kept frozen. The P_H of both sera was 7·5.

Cultures of embryo heart in the unheated serum of both these animals showed such emigration of cells as one might expect in a medium which is protective rather than growth-promoting. The sera were devoid of cytotoxic action. One of these animals "I" was therefore used for immunisation, while the other "C" was kept in order that its serum might serve as a control and indicate the action to be expected from that of a normal animal of similar age.

Immunisation. The injections were given intraperitoneally

and each consisted of approximately 5 gms. of the tissue of mouse embryos approximately seventeen days old. The embryos were minced into small fragments, washed free of blood and suspended in Ringer's solution. The injections were made at intervals of seven to ten days. Occasionally, however, owing to lack of material, a fortnight elapsed between them, and on one occasion between 5/8/27 and 23/9/27, there was an interval of seven weeks. About five days after each of the earlier injections, and at longer intervals after the later ones, the animal was bled to the extent of about 5 c.c. from the ear vein. The serum was collected and stored as above described. The P_H varied from 7·5 to 7·7. The serum was tested from time to time on cultures of cells growing in vitro.

II. Action of cytotoxic serum on cultures of mouse tissues.

In order to detect cytotoxic action of the serum at the early stages of immunisation when only weak effects were to be expected, the method adopted was that already described for normal serum, i.e. the inhibition test. Later on, when the antiserum was more powerful, it was tested by two methods on cultures which were at the stage of active growth. (I) the culture medium was removed as thoroughly as possible by means of a sterile glass pipette attached to a weakly-acting water pump and was replaced by anti-

serum which was allowed to remain in contact for varying periods of time, from a quarter of an hour onward. The effects were watched with the cultures on the warm stage. (2) After allowing the antiserum to act on the cultures in the incubator for varying periods of time, the serum was removed and the cultures then washed with saline and fresh medium added. Therafter they were incubated for times varying from half an hour to several days. The object of this procedure was to determine whether any recovery of damaged cells or fresh outgrowth took place. Stained preparations were made under both conditions and after various intervals of treatment.

Alterations in the action on tissue cultures undergone by rabbit's serum in the course of immunisation. A considerable time elapsed before the serum of the injected rabbit showed any harmful effect on tissues already growing in vitro. Thus cultures of embryo mouse heart, made in the serum of the rabbit after the first injection of embryo emulsion, showed considerable emigration of cells which was practically equal to that of the cultures in normal rabbit's serum. With the serum obtained after the second injection, emigration of cells was less marked than in the control. After the antiserum had been allowed to act for seventy-two hours, the cultures were washed and mouse serum or normal

rabbit serum substituted; thereupon increased emigration took place. Thus it was clear that the cells were still active and capable of responding rapidly to a fresh supply of suitable protective medium. After the third injection, anti-properties, though slight, were more marked, and ten fibroblasts was the largest number seen in an individual culture out of a series of more than twenty made with the serum at this stage, whereas in the control cultures the number of cells was apparently uncountable. With the serum obtained after the fourth injection, no emigration of fibroblasts or other cells took place, even after removal of the serum, washing and substitution of normal rabbit or mouse serum and reincubation. Inhibition of emigration is thus clearly evident at this stage.

Thereafter, the antiserum was tested at frequent intervals for cytotoxic action on cultures in a medium containing embryo juice as described above. In regard to such tests, it must be noted that normal rabbit's serum has also a slight degree of cytotoxic action which must not be mistaken for evidence of the presence of immune antibody (see Lumsden, 1927; Pybus and Whitehead 1929). This effect of normal serum is detected when serum is applied to actively growing cultures in a suitable nutritive medium, i.e., one containing embryo-juice. It is then observed that outlying cells, which are often degenerate,

withdraw their processes and become rounded up, and the fatty droplets coalesce and form irregular masses. The nuclei become opaque, diminish in size and show an obvious nuclear membrane. These changes take place slowly and cell death, as evidenced by the penetration of the nuclei by neutral red, does not occur as a rule until six to eight hours after the application of the serum. Stained preparations also show pyknosis of the nuclei and loss of cytoplasmic staining. Autolysis of these killed cells occurs and after twelve hours only granular debris remains. This cytotoxic action of normal rabbit serum has been examined in eight animals at various ages and in one animal "C" over a period of two years commencing at the age of four months. The results indicate that individual sera may vary considerably in their cytotoxic power, but that age is an important factor. In rabbit "C" the action was practically absent to begin with and increased up to the age of one year, but has remained practically stationary for the subsequent sixteen months. In one animal aged six weeks the serum was not toxic to cultures of mouse and chicken tissue, but was definitely toxic six weeks later. The toxic effect, however, can in all cases be removed by heating at 56°C. for half an hour.

Up to the twelfth injection the serum of the injected rabbit produced effects very similar to those described for normal serum, but the action was more rapid and

healthy cells tended to withdraw their processes and assume a rounded form. Recovery of many cells could be brought about, however, by washing the cultures after two hours, adding fresh medium and reincubating. The effect of the antiserum at this stage might be described as an intensification of normal properties. After the twelfth injection, toxic effects of the antiserum such as have been described were still more rapid in onset, and involved all the cells which had grown out of the explant. With serum after the sixteehth injection all the cells became rounded within half an hour. At this point an interruption of seven weeks occurred before the next injection, and the serum obtained after this interval was found to have diminished markedly in toxicity; ten more injections were necessary to restore it to the previous activity. After about the thirtieth injection the action upon cells *in vitro* appeared to have altered in a qualitative sense and this change was pronounced after the thirty-seventh injection; the serum might now be described as "rapidly acting". Since the fortieth injection no further change has occurred in the course of twenty additional injections beyond a slight increase in toxicity. The qualitative change in the action of the serum just referred to consists in the disappearance of

the stage of preliminary rounding; the nuclei and cytoplasm now shrink rapidly and the cytoplasmic constituents disappear within half an hour at 37·5°C. Washing with saline and application of fresh medium at this point does not bring about recovery or fresh outgrowth from the explant.

The effects of rapidly acting cytotoxic serum on tissue cultures. The following is an account of the changes observed over a period of two hours after the addition of rapidly acting antiserum (obtained after forty-two injections of embryo emulsion)to a culture of embryonic mouse heart; the cells which show activity are mainly fibroblasts, with a small admixture of macrophages, etc. Mesothelial cells also occasionally grow out in the form of sheets.

(a) Fibroblasts. Within five minutes after the application of the antiserum, cell nuclei become more distinct, opaque and delimited by a well marked but thin nuclear membrane. Shrinkage of the nuclei begins and continues slowly and the nuclear substance becomes granular. After a quarter of an hour the nucleoli begin to swell, become less conspicuous, lose their regular shape, and in a proportion of cells move towards the periphery of the nucleus. After fifty-five minutes the

nuclei are very irregular in shape, indented slightly in places and the nuclear membrane appears much thicker; the nucleoli are now apparent in only a few cells and by this stage they have become shrunken and distorted. The process of mitosis stops abruptly. The spindle area, which in normal dividing cells is of different refractivity from the cytoplasm and quite homogeneous in character, assumes a granular appearance. The chromosomes themselves become clumped together in one or two masses, depending on the phase. Simultaneously with the commencement of nuclear changes, i.e. within 5 minutes, the cell outlines become indistinct, ragged and irregular. This stage is followed by one which is well marked after twenty-five minutes, at which period cell outlines can be easily distinguished and the cells are bounded by a very thin, refractile pellicle which is apparently of different consistence from the cytoplasm, an appearance very unlike that seen in normal cells. Some cells show the extrusion of bleb-like processes, either empty or containing one or two dancing granules and bounded by an equally thin pellicle. Five minutes after the commencement of the observations, the cytoplasm becomes very granular in appearance, and a quarter of an hour later the mitochondria cannot be distinguished. The granularity is much finer after thirty-five minutes. Shrinkage of the cytoplasm is very marked, so that the total area of

the cell after two hours' application of the antiserum is only about one third of its original area (Figs. 81, 82), (Figs 83, 84). The processes of fibroblasts are not withdrawn under the influence of the antiserum obtained at the advanced stage of immunisation (compare the results at earlier stage, p. 242).

(b) Mesothelium. The destructive effects on sheets of mesothelial cells were similar, but, in addition, the cytoplasm becomes heaped round the nuclei, while the cells remain connected with each other by irregular granular bridges, (Figs. 95, 96.)

(c) Macrophages. which are scanty in such cultures, show similar changes in both nucleus and cytoplasm to those exhibited by fibroblasts, except that the finest cell processes disappear, whereas the coarser ones persist as in the fibroblasts. Macrophages, which are abundant in cultures of other tissues, e.g. spleen, liver and subcutaneous tissue, behave in the same way, but shrinkage of the cytoplasm is specially prominent.

Reaction of cells to neutral red. The process of death of the cells has also been followed by examining their reaction to neutral red. It is generally accepted that dilute solutions of this dye, e.g. 1:5000-1:50,000, when brought into contact with healthy cells stain only the so-called segregation apparatus, the healthy nucleus remaining unstained. When death of the cell is occurring

one of the earliest signs of injury is staining of the nucleoli; tinting of the nucleus as a whole is indicative of cell death. The method followed was to prepare a series of parallel cultures, to each of which antiserum was added. After fifteen minutes and at five-minute intervals thereafter up to two hours, a culture was treated with fresh neutral red solution (1-5000) in buffered saline. After twenty-five minutes, penetration of the nucleoli occurs and ten minutes later, i.e., thirty-five minutes after the application of the antiserum, the entire nucleus takes the stain. A segregation apparatus is not a prominent feature of mouse fibroblasts growing in vitro, and after the death of the cells it does not become stained. In the macrophages, prior to cell death the segregation apparatus is well stained, but coincidently with the staining of the nucleoli, it usually ceases to hold the stain, and the cytoplasm assumes a transient pink colour.

Estimation of the potency of the antiserum. The powerful cytotoxic action of the antiserum having been established, it was of interest to determine the degree of dilution of the serum at which the effect was still produced. A series of cultures was prepared in which the medium contained doubling dilutions of the rapidly acting antiserum (in place of a similar quantity of normal serum),

varying from 1:2 to 1:32. In one out of the four cultures in which the dilution of antiserum was 1:32, four cells were found to have emigrated after thirty-six hours. A dilution of 1:32, therefore, just inhibits growth. The effect of the rapidly acting antiserum on actively growing cultures was also tested in doubling dilutions with buffered saline from 1:2 up to 1:32. These dilutions were applied to a series of cultures. After incubation at 37·5°C. for two hours, the serum was removed, the cultures washed and fresh nutritive medium added. Twenty-four hours later it was found that in dilutions up to 1:8 all the cells had been killed, and that no further outgrowth had taken place. With higher dilutions all the cells of the original outgrowth had not been killed, further outgrowth had taken place and mitotic figures were numerous. Lying amongst the living cells could be seen dead cells with shrunken nuclei and cytoplasm, unaffected by autolytic change (see below).

Action of the antiserum on cultures of epithelium. The action of the antiserum was also tested on cultures of epithelium from various organs. It must be pointed out that normal vigorous growths of such cells consist of membranes which are one cell thick, the cells being so closely applied as to present the appearance of syncytium without

(Figs. 93 & 94.)

recognisable cell boundaries. (In old cultures, however, owing to the development of intercellular spaces, cell outlines may be seen with high magnifications.) Membranes of epithelium, of renal, intestinal and hepatic type, are killed as quickly as fibroblasts, but owing to the morphological arrangement, the gross effects of shrinkage appear more dramatic. After half an hour, the area of the membrane is reduced to one half or one third of its original size. The epithelial sheets in many cases break away from the explant, so that gaps are left, but the cells remain in contact with one another by means of granular bridges. Nuclear and cytoplasmic changes are similar to those seen in fibroblasts. By way of control, the action of normal rabbit's serum was tested on cultures of epithelium from kidney, liver and intestine. In each of these, the cells at the free margin showed signs of injury and some were killed, but the rest of the epithelial sheet remained unchanged.

Late effect of the antiserum. With the antiserum obtained at the earlier stages of immunisation, before the thirtieth injection, it was found that if the serum was removed from a heart culture after two hours, and the culture was washed with saline and fresh medium added, then on reincubation for twelve hours, practically complete lysis of the cells occurred, only a few shrunken nuclei and

cytoplasmic debris remaining. Membranes of renal epithelium disappeared completely. If the antiserum was left in contact with the cultures no recognisable remains of cells were found after twelve hours, although the explant persisted intact. Autolytic change must therefore have taken place. With the antiserum obtained after the thirty-seventh injection very different appearances resulted. The action of the antiserum remained unaltered after death had taken place even when the cultures were kept for three weeks at 37·5°C. Evidently, the cells were not only killed but also in a sense fixed by the antiserum, so that autolysis was inhibited by some means which need not be discussed here. No recovery of cells or fresh outgrowth took place if the antiserum was removed and the cultures were washed and fresh medium added (see also Lumsden, 1926).

The effect of rapidly acting antiserum on tissue en masse. Small fragments of mouse heart about 1 mm. in diameter were immersed in antiserum in hollow-ground slides for two hours at 37·5°C. Similar fragments were treated in the same manner with normal rabbit's serum. All the fragments were then washed in buffered saline, and two out of each set were cut into two parts. All the pieces, both cut and uncut, were explanted separately in a medium consisting of equal parts of rat plasma and mouse embryo

juice, which was chosen because of its great capacity for promoting growth. Those fragments which had been treated with normal rabbit serum showed vigorous outgrowth after twenty-four hours. The uncut explants treated with antiserum showed, after twenty-four hours, only extensive liquefaction of the plasma, while the cut pieces showed a slight emigration of cells on one side. No mitotic figures were present. The antiserum had therefore not only acted on the tissue at the surface but had penetrated it to a considerable extent.

Action of the antiserum on cultures of tumour cells. Considerable difficulty was experienced in obtaining satisfactory cultures of carcinoma M.63 (Imperial Cancer Research Fund). In the fluid medium used for normal tissues, growths were only occasionally obtained. Emigration of cells into the medium surrounding the explant was marked, but careful examination showed that they were mainly isolated cancer cells which showed degeneration vacuoles and granules in their cytoplasm. Scattered amongst them were a few fibroblasts and cells of the macrophage class. The degenerate cells, which never undergo mitotic division, often remained in an almost moribund state for several days, exhibiting slight movement; but they were easily killed by adverse conditions which would

not harm normal tissue cells. Thus these cultures of tumour cells were apt to be killed by mere renewal of the medium, especially if the reaction was slightly alkaline (P_H-7·8), and they were rapidly killed by normal rabbit's serum. Accordingly, it was decided that such cultures were unsuited for investigating specific cytotoxic action. It has been found that, in contrast to normal tissues, a more suitable medium for tumour cells results when the concentration of embryo juice is diminished, and this has also been observed by Drew (unpublished). The following proved a useful medium - a mixture of two parts chicken plasma with one part rat plasma is placed on a cover glass and coagulated by the addition of a small drop of dilute embryo juice prepared immediately beforehand by adding one part of stock embryo juice to three parts of buffered saline. (See also Fischer, 1927). The explant is then placed on the surface of the coagulum, and is surrounded by a thin layer of the dilute embryo juice. Under these conditions it is possible to obtain active growth of tumour cells into the medium in the form of finger-like and spade-shaped outgrowths (Fig. 86). Mitotic figures are fairly numerous. But even in these conditions, which are found to be optimal for carcinoma M.63, the extent of growth is not so great as that of normal mouse tissues under the best conditions;

considerable variation likewise occurs in a series of cultures prepared from the same material, and it is necessary to select with care those intended for comparison. The arrangement of the cells is epithelial but the mosaic-like appearance of normal epithelial sheets is not prominent and the cells are quite different in appearance, as has also been observed by Fischer. Thus, in fixed and stained preparations, the cells closely resemble those seen in sections of the tumour growing in vivo, the cytoplasm staining intensely with haematoxylin and the nuclei being hyperchromatic as compared with those of cultures of normal tissues. Pluripolar mitoses occur occasionally. The cellular arrangement in some cultures resembles that seen in human mammary cancers of encephaloid type. The tumour cells were killed by the antiserum in the same fashion as normal cells, and similar nuclear and cytoplasmic changes occurred (Fig. 90, Fig. 87). Distortion of cell outlines and clumping of lipoid droplets were found. The tumour cells did not show any greater susceptibility to the antiserum than those of normal mouse tissues. With normal rabbit serum, no cytotoxic effect was observed on the healthy tumour cells, but those cells which to begin with showed evidence of degeneration were very rapidly killed. In addition, a precipitate appeared in the antiserum, due probably to the presence of embryo juice; it is not possible

to remove this completely in plasma cultures. (see also p.)

Cultures were obtained of a spontaneous adeno-carcinoma from a mouse, probably mammary in origin, which failed to yield growths when transplanted into mice. The growths in vitro were very similar to normal epithelium, and the antiserum had the same action upon them as upon those of transplantable tumour cells or normal tissues. Normal rabbit's serum had no effect on the cultures.

Influence of temperature on cytotoxic action. In general, the occurrence of cytotoxic action on cultures has been investigated at 37.5°C. In order to determine whether or not effects were produced at room temperature and 0°C., a series of cultures was prepared, and after they were growing actively, rapidly-acting antiserum was applied to them in the usual way. Some were incubated at 37·5°C., others were kept at room temperature and the remainder were packed in ice. In the first set, cell death took place as usual within half an hour. In the second series, no effect was observed for seventy minutes, then nuclear and cytoplasmic changes became apparent, and all the cells of the cultures were killed after two and a quarter hours. The third series showed no change after two hours; several of these cultures were then incubated at 37·5° C. and cell death took place with great rapidity, all the cells being killed within ten to fifteen minutes. The rest of the cultures

showed no change even after two hours further at 0°C.

The effect of antiserum heated at 56°C. The rapidly acting antiserum and normal rabbit's serum were heated at 56°C. for periods of time varying from half an hour to thirty hours. The heated sera were applied to a series of actively growing cultures of mouse fibroblasts obtained from embryo heart tissue, previously washed in saline to remove the original medium. The effect of the heated antiserum was the same in all cases, despite the different duration of heating. The first obvious change in the cells was vacuolation of the cytoplasm, which became obvious within half an hour of the application. Cell processes were slowly withdrawn, the cells becoming rounded up, and nuclear and cytoplasmic shrinkage with coalescence of fatty droplets took place. Two hours after the application of the antiserum, a solution of neutral red 1:5000 in buffered saline showed the presence of a large number of vacuoles of degeneration, but the nuclei were unstained. The striking feature of this change was the rapidity with which it occurred, since the vacuolation, otherwise closely similar, which arises spontaneously in old cultures, is always of slower onset and development. (see p.237.). For some hours the cells remained in this condition, clumped together in groups and showing no change. At intervals, cultures were stained supravitally with neutral red; after fifteen hours,

staining of the small shrunken nuclei was observed in a few of the cells, indicating complete cell death; but although the majority of the cells appeared inactive their nuclei exhibited no obvious change beyond shrinkage. General death of the cells, as indicated by nuclear staining, did not take place for at least thirty hours after the application of the antiserum (Fig. 85). Thereafter autolysis of the dead cells took place. No emigration of cells occurred from the explant on removal and renewal of the medium after forty-eight hours. The heated normal rabbit's serum had no effect on similar cultures, and cells continued to migrate, though less actively than in a good nutritive medium. The contrast between the effects of the unheated and the heated antiserum was marked, and that between the latter and unheated normal rabbit's serum equally so.

Sheets of growing epithelium from the kidney under the influence of the heated antiserum showed shrinkage of the cells from each other and the diminution in size was marked. The protoplasm became greatly vacuolated, and the nuclei gradually became granular, this change being well seen after twenty hours. Finally, after between twenty and thirty hours, almost complete autolysis had occurred.

Reactivation of heated antiserum by normal rabbit's serum. It was found that the heated antiserum could be completely reactivated by the addition of unheated normal rabbit's serum.

To two equal amounts of rapidly acting antiserum which had been heated for half an hour and ten hours respectively at 56°C., fresh normal rabbit's serum was added so as to constitute one third and one quarter of the total volume in each case. These mixtures killed cultures of actively growing mouse fibroblasts from embryo heart in the same manner as unheated antiserum. When normal rabbit's sera "C," previously heated at 56°C. for one half and for ten hours, were substituted for unheated serum in the mixture, the result on the cultures was the same as that produced by heated antiserum alone.

Keeping properties of the antiserum. Antiserum after eight months' storage at - 10°C to - 14°C. was just as actively cytotoxic as fresh serum when tested both on fibroblasts and on kidney epithelium from mouse embryos. After ten months, however, the antiserum was not quite so active, and a few cells in each culture remained unaffected. Antiserum kept at room temperature under sterile conditions for six weeks had completely lost its effect on cultures of fibroblasts or epithelium.

The action of antiserum after treatment with the homologous tissue. The rapidly acting antiserum was absorbed at 37·5°C. with an equal quantity of suspension of embryo mouse tissue for one and a half hours. After centrifuging, the fluid was removed and applied to cultures of mouse fibroblasts. The cells were killed in the usual way, as by untreated antiserum. The experiment was repeated, but the tissue was allowed

to remain in contact with the antiserum for six hours and was changed twice during that period. After such prolonged and intense treatment, no cytotoxic action on cultures of fibroblasts or liver epithelium was produced by the treated antiserum. In the latter experiment dilution by the saline in which the tissue was suspended was reduced to a minimum by using tissue "packed" by centrifuging.

Injection of the antiserum into normal and pregnant mice. On several occasions normal mice were injected intraperitoneally with 0·5 c.c. of rapidly acting antiserum within twenty-four hours of the withdrawal and clotting of the blood. Half an hour after the injection the animals were distinctly less responsive to external stimuli than those which had received the same amount of a similar specimen of normal rabbit's serum, but two hours after the injection, they had completely recovered from the effects. Pregnant mice in which the pregnancy had progressed about four-fifths to three-quarters of the normal course were also injected intraperitoneally with the same amount of antiserum without ill effect, and in all cases the offspring were normal at birth. The uterine horns of two such animals were examined within twenty-four hours after parturition and no dead fœtuses were present. Accordingly no evidence was afforded by these experiments that the

antiserum exerted toxic effects in vivo.

111. The appearances of tissue cultures in fixed and stained preparations.

The experimental and control cultures in all cases were fixed and stained in various ways. The cultures were washed at 37·5°C. for five minutes with saline to remove the serum and were then fixed.

Normal appearances. As already described after fixation with a 2 per cent. solution of osmic acid in distilled water, the cells of tissue cultures present appearances which more closely resemble those seen in the healthy living cell than after any other fixative (Strangeways and Canti,1927). On staining osmic-fixed cultures with iron haematoxylin after chroming with Zenker's fluid minus acetic acid, the nuclear substance of normal cells at high magnifications (x750) is almost homogeneous and of a greyish colour; the nucleoli are intensely black and no nuclear membrane is present in perfectly healthy cells, although, owing to the difference in character between nucleus and cytoplasm, the nuclear limit is distinct. No linin network or chromatin granules can be seen. The cytoplasm is very finely granular and almost unstained, but embedded in it can be seen cell granules and mitochondria of various shapes. In some cells the centrosphere can also be made out. The spaces previously occupied by fatty droplets are easily recognised; their outline is smooth and they are

arranged usually in clusters. No cell membrane is present, but cytoplasmic limits are easily discernible in fibroblasts, and in epithelial and mesothelial sheets a narrow line of demarcation between the individual cells is seen. Iron haematoxylin is the only stain which gives satisfactory results after osmic acid fixation; since unfortunately it also stains with intensity fibrin and any debris in the culture, it is unsuitable for plasma cultures when extensive liquefaction of the medium has occurred. and has been used only for special purposes. Bouin's fluid,Zenker's solution without acetic acid and Zenker-formol (10 c.c. formalin and 90 c.c. Zenker's fluid without acetic acid) were the fixatives commonly used; the last is a very good fixative which does not cause any serious shrinkage of cells. Subsequent staining with Harris' haematoxylin diluted 1:10 with distilled water gave good micro-anatomical differentiation, after two hours in the case of cultures in fluid medium, and three hours for those in plasma. By this method of fixation and staining the nuclei of fibroblasts show a fine but well-marked nuclear membrane and well-stained nucleoli. The nucleoplasm is granular and small chromatin dots are often present. The cytoplasm is always tinted with haematoxylin and is of "ground glass" appearance. The vacuoles previously occupied by fatty droplets are distinct. No cell membrane is apparent but cell

limits are fairly definite. Bouin's fluid destroys the mitochondria which are of course not stained by ordinary haematoxylin. Iron haematoxylin also fails to stain them satisfactorily after Zenker or Zenker-formal fixation. The fibrillae, so common in fixed mesothelial cells (Lewis, 1926), are well stained by iron haematoxylin and Harris' haematoxylin after these methods of fixation. Sheets of mesothelium and epithelium also show fine but definite cell outlines. The nuclei and cytoplasm of epithelium stain in the same way as after osmic acid fixation but the details appear coarser. The appearance of tumour cells in fixed and stained preparations have already been mentioned (see p.252). Sudan III stains the fatty droplets of tissue culture cells with great intensity; they vary in size and have smooth outlines from round to oval in shape. They tend to occur in groups, but isolated globules are often found, e.g. in cell processes.

The appearance of cells in mitosis depends on the phase at which fixation has taken place. In preparations fixed with osmic acid and stained with iron haematoxylin the cytoplasm stains with greater intensity than that of resting cells and the mitochondria are shorter and more rod-like. The spindle area, when present, stains homogeneously and so can be distinguished from the rest of the cytoplasm; fibrils are not present. The chromosomes are black in

colour and stand out conspicuously. With Harris' and iron haematoxylin after the other fixatives the cytoplasm is also well stained, and the spindle area may show a suggestion of fibrillation.

Histological effects of antiserum. In order to obtain a criterion for judging the state of fixed and stained cells, a careful study was made of cultures of fibroblasts and epithelium to which rapidly acting antiserum had been applied, and in which penetration of the nuclei by neutral red clearly indicated the occurrence of cell death. The most remarkable feature was the uniform intensity of nuclear staining after all fixatives (Figures 82, 84). The shrinkage was very apparent, and many nuclei showed crenation of outline (Figure 94). In some of these pyknotic nuclei, more darkly staining dots represented the remains of nucleoli. The shrunken cytoplasm, on the other hand, did not stain at all, and showed an amorphous structure, more coarsely granular than that of normal cells, but cell boundaries could be traced easily. "Tigroid-like" granules, described by Kimura (1927) in the cytoplasm of chicken fibroblasts after treatment with antiserum and subsequent staining with haematoxylin were not seen. After staining with sudan III, the fatty droplets showed an irregular shape, due apparently to partial coalescence of individual droplets (Figure 96). The

mitochondria could not be demonstrated by any staining method (iron haematoxylin after fixation with osmic acid or Flemming's solution minus acetic acid, methyl-green fuchsin by the Bensley Cowdry method). The fibrillae of mesothelial cells were never found, nor were the centrospheres (figure 96.). The chromosomes of cells in mitosis were clumped together into one or two groups depending on the phase, and were smaller than normal. The spindle area disappeared completely. The cytoplasm of tumour cells (carcinoma M.63) did not stain with haematoxylin, the nuclei were shrunken and pyknotic and cytoplasmic inclusions could not be demonstrated.

Cultures of tissues treated with normal rabbit's serum, when fixed within twelve hours, showed, on the whole, the usual histological picture of normal cells in fixed and stained preparations. Those scanty cells which had been killed by the toxic action of the normal serum were usually rounded in outline. The nuclei showed varying degrees of pyknosis and the cytoplasm was unstained. In cultures which had been kept at 37·5°C. for a longer period e.g. twenty-four hours, the appearances due to autolysis predominated.

IV The action of the rapidly acting antiserum on cultures of tissues from other species.

Rat. Cultures were made of the heart, kidney and liver of embryo and newly-born rats in fluid medium and in plasma, just as in the case of the mouse. On all these tissues the antiserum when undiluted had the same effect and killed the cells as rapidly as those of mouse tissue cultures. It was noted, however, that the normal rabbit's serum, although toxic for mouse tissues, did not have any effect on rat cultures: this will be discussed later. In order to kill cultures of rat tissues, a higher concentration of the antiserum was necessary than sufficed to kill mouse tissues (see p.247). Many cells were unharmed by a dilution of 1:2, and dilutions from 1:4 up to 1:32 had no effect. In the inhibition test dilutions of 1:4 to 1:32 did not inhibit growth, although a dilution of 1:2 was inhibitory.

Guinea-pig. Cultures of fibroblasts were obtained from the heart of embryo guinea-pigs. The antiserum, when applied to them, killed the outgrowth. Normal rabbit's serum, however, had a similar effect. In both cases the killed cells became autolysed and fresh emigration took place later from the explant.

Rabbit. Cultures of the heart of the newly-born offspring of the injected rabbit "I" (born after the 55th injection) were prepared in a medium consisting of chicken plasma and mouse embryo juice. After growth was established, the antiserum and normal rabbit's serum respectively were applied to the cultures. Neither of these had any effect, and cell emigration continued as before.

Chicken. The antiserum was tested on cultures of chicken fibroblasts obtained from the embryo heart, i.e. on tissue from a species far removed from that yielding the antigen. In order to control the effects of the antiserum, the sera of six normal rabbits were also tested; three of these had a very rapid cytotoxic action and all the cells of the outgrowth were killed in half an hour. (Fig. 89) On reincubation without removal of the antiserum, however, autolysis of the dead cells took place and a fresh outgrowth arose. Within eighteen hours the film of cells surrounding the explant was as great as the original outgrowth and sometimes exceeded it; numerous mitotic figures were present. Thus it appears that while the immediate effect of the antiserum or of normal rabbit serum was as intense as that of the former on homologous tissue, the action became rapidly exhausted. The immediate nuclear and cytoplasmic changes were similar to those described for mouse cells after the application of antiserum, but

the cytoplasm showed considerable vacuolation. After fixation and staining, however, it was observed that while the nuclear changes of the killed cells were similar, the cytoplasm still showed slight staining with haematoxylin and was more vacuolated than that of mouse cells killed with antiserum. The normal cytoplasmic constituents could not be demonstrated. The serum from two other normal rabbits was not quite so toxic, and that from a young rabbit six weeks old had no effect. Six weeks later, however, the latter was also markedly toxic. The cytotoxic action for chicken tissue was destroyed in both the normal serum and the antiserum by heating for half an hour at 56°C. This cytotoxic effect of normal rabbit serum on cultures of chicken tissue was apparently not observed by Kimura in his investigations on the action of an antiserum (rabbit v. chicken embryo tissue). It is thus seen that the toxic action of the antiserum is exerted only on the tissues of the mouse and of a closely related animal, the rat, amongst the animals tested, and that accordingly the antiserum possesses specific properties.

Effect of absorption of antiserum and of normal rabbit serum by heterologous tissues. The rapidly acting antiserum and normal rabbit's serum from rabbit "C" were absorbed at 0°C. for one hour with an equal volume of

suspension of adult guinea-pig organs (heart and kidney), which had been packed by centrifuging to prevent dilution of the sera. The treated sera when applied to cultures of chicken (Fig. 88) and guinea-pig fibroblasts produced no cytotoxic effect, but when the treated antiserum was applied to cultures of mouse fibroblasts the cells were killed as before. On rat cultures the cytotoxic effect was slower than that produced by untreated antiserum, and the cells were not killed for about an hour after its application. The treated normal rabbit serum, of course, had no effect on cultures of mouse or rat tissues. The antiserum and normal rabbit's serum from rabbit "C" were treated in the same way with rabbit organs (heart and kidney). This treatment did not influence the effect produced by these sera on mouse, rat, chicken, and guinea-pig cultures. The results of these absorption experiments are summarised in the table on p. 268.

It is of importance in this connection that the tissues of the mouse, guinea-pig and fowl all contain Forssman's antigen and that rabbits' serum tends to contain naturally the corresponding antibody, or to develop it as a result of immunisation with any of the tissues mentioned. As will be seen later, both the normal and the immunised

rabbit developed this antibody in equal amount (see p.) and there is a definite correspondence between the susceptibility of tissues to the cytotoxic action of normal rabbits' serum and their content in Forssman's antigen. The results of the absorption experiments bear this out. Thus treatment of the antiserum, as well as of the normal rabbit's serum "C", with guinea-pig tissues removed cytotoxic properties for cultures of chicken as well as for guinea-pig tissues;

Species of tissue used for absorption.	Antiserum: effect on cultures of				Normal rabbit's serum ("C"): effect on cultures of			
	Mouse.	Rat.	Guinea-pig.	Chicken.	Mouse.	Rat.	Guinea-pig.	Chicken.
None. .	+ + + +	+ + +	+ + +	+ + +	+	–	+ + +	+ + +
Mouse .	–	...	...	...	–	...	...	...
Guinea-pig	+ + + +	+ +	–	–	–	–	–	–
Rabbit .	+ + + +	+ +	+ + +	+ + +	+	–	+ + +	+ + +

– indicates the absence of cytotoxic action.
+ + + + indicates the occurrence of cytotoxic action which is intense and prolonged.
+ + +, + +, + indicate progressively diminishing degrees of such action.

and the slight toxic property possessed by normal rabbit serum for mouse tissue is removed by the same treatment. On the other hand, the powerful cytotoxic properties of the immune serum are removed only by treatment with mouse tissues. Thus it appears that the immune antibody possesses a high degree of species specificity.

V. The complement-fixing, haemolytic and precipitating properties of the antiserum (rabbit v. mouse embryo tissue) during the course of immunisation.

As the injections were in progress the serum of rabbit

"I" was examined at intervals to determine the presence of complement-fixing and haemolytic antibodies; its precipitating action on embryo juice was also tested. The development of these properties, as will be seen from the tables, was very slow. The serum from normal rabbit "C" was used as a control throughout.

Complement-fixation. Table I shows that after twelve injections, the antiserum in conjunction with the homologous antigen fixed completely 4 M.H.D. of guinea-pig's complement. The normal rabbit's serum gave no fixation. With the antiserum obtained after fifty-four injections (table II) 14 M.H.D. of complement were completely fixed and with 20 M.H.D., less than I M.H.D. of complement remained free. With normal rabbit's serum at this stage, however, between 3 and 5 M.H.D. of complement were required to give complete lysis of the test corpuscles. Table III shows that an emulsion of tumour tissue was just as effective for fixing complement in conjunction with the antiserum as mouse embryo tissue. The experiment detailed in table IV was undertaken (1) to see if treatment of the antiserum with guinea-pig organ at 0°C. for one hour reduced the complement-fixing power in the presence of the homologous antigen, and (2) to determine if the antiserum

TABLE I.

The amount of guinea-pig's complement fixed by antiserum (rabbit v. *mouse embryo tissue) + corresponding antigen.*

Antigen 0·5 c.c. suspension of embryo mouse tissue.

Antiserum obtained after immunisation had been in progress for three months (12 injections) heated at 56° C. for half an hour, 0·05 c.c.

Normal rabbit's serum heated at 56° C. for half an hour, 0·05 c.c.

Complement { M.H.D. / c.c.	2 / 0·012	3 / 0·018	4 / 0·024	5 / 0·03	7 / 0·042
Antigen + antiserum	0	0	0	tr	c
Antigen + normal rabbit's serum	c	c	c	...	...
Antigen alone	c	...	c	...	...
Antiserum alone	c	...	c	...	...

The results in this and the following tables indicate the lysis of 0·5 c.c. of 3 per cent. suspension of ox or sheep's red blood corpuscles sensitised with at least 5 M.H.D. of the corresponding immune body from the rabbit. The complement used throughout is that contained in guinea-pig's serum.

In all these experiments a preliminary test was made in which 0·5 c.c. of varying dilutions of the antigen were incubated along with two and four doses of complement for one and a half hours at 37·5° C. Then the test corpuscles were added and the mixture kept at 37·5° C. for a further period of an hour and a quarter. The highest concentration of antigen which permitted of complete lysis of the corpuscles with both amounts of complement was that used for the experiment.

As controls, the action on complement of the antigen by itself and of the antiserum added to 0·5 c.c. of saline were always tested.

0 = no lysis. ftr = faint trace of lysis. d = distinct lysis.
ac = almost complete lysis. jc = just complete lysis. c = complete lysis.
... = not done

TABLE II.

Similar to table I. except that the antiserum was obtained after immunisation had been in progress for 20 *months* (54 *injections*).

Complement { M.H.D. / c.c.	2 / 0·015	3 / 0·0225	4 / 0·03	5 / 0·0375	7 / 0·0525	14 / 0·105	20 / 0·15
Antigen + antiserum	0	0	...	0	0	0	ftr
Antigen + normal rabbit's serum	m	ac	...	c	c	c	c
Antigen alone	c	...	c	...	...	...	...
Antiserum alone	ac	...	c	...	...	...	...

before and after treatment fixed complement along with a heterologous tissue antigen (emulsion of chicken tissue). As will be seen, 14 M.H.D. of complement were almost completely fixed by the treated antiserum with mouse embryo

tissue antigen, while the untreated antiserum gave complete fixation. It may be deduced, therefore, that the complement-fixing power of the antiserum with

TABLE III.

Similar to table II. except that both embryo mouse suspension and tumour tissue suspension (carcinoma M. 63, Imp. Cancer Res. Fund) were used as antigens. 0·01 c.c. of antiserum (rabbit v. mouse embryo tissue) was used in this experiment.

Complement { M.H.D. / c.c.	2 / 0·01	3 / 0·015	4 / 0·02	5 / 0·025	7 / 0·035	10 / 0·05
Embryo mouse antigen + antiserum	0	0	...	0	0	ftr
Tumour antigen + antiserum	0	0	...	0	0	ftr
Embryo mouse antigen alone	m	...	c	...	...	...
Tumour antigen alone	m	...	c	...	...	...
Antiserum alone	m	...	c	...	...	...
Embryo mouse antigen + normal rabbit's serum	m	c	...	c	c	c
Tumour antigen + normal rabbit's serum	m	c	...	c	c	c

TABLE IV.

Similar experiment to table II. except that (1) *0·25 c.c. of mouse embryo tissue suspension and 0·25 c.c. of embryo chicken tissue suspension were used as antigens, and* (2) *the antiserum and normal rabbit's serum were treated at 0° C. for 1 hour with an equal volume of "packed" suspension of adult guinea-pig organ (heart and kidney). The amount of untreated or treated antiserum and normal rabbit's serum used was 0·03 c.c., and of sensitised sheep's red blood corpuscles, 0·25 c.c.*

Complement { M.H.D. / c.c.	3 / 0·015	5 / 0·025	7 / 0·035	10 / 0·05	14 / 0·07
Mouse embryo antigen + treated antiserum	0	0	0	0	ftr
Mouse embryo antigen + untreated antiserum	0	0	0	0	0
Chicken embryo antigen + treated antiserum	ftr	jc	c	c	c
Chicken embryo antigen + untreated antiserum	0	m	c	c	c
Mouse embryo antigen + treated normal rabbit's serum	m	c	c	c	...
Mouse embryo antigen + untreated normal rabbit's serum	ftr	jc	c	c	...
Chicken embryo antigen + treated normal rabbit's serum	m	c	c	c	...
Chicken embryo antigen + untreated normal rabbit's serum	ftr	ac	c	c	...

The antigen, normal serum and antiserum controls all gave almost to just complete lysis with two doses of complement and complete lysis with four doses of complement.

the homologous antigen was not much reduced by treatment, a result which confirms the findings of the tissue culture experiments (p.244). With chicken tissue as antigen, the untreated serum fixed completely 3 M.H.D. of complement, and lysis was complete with 7 M.H.D.; the treated antiserum, in combination with the same tissue, gave complete lysis with 5 M.H.D. of complement. The amount of complement fixed by the untreated antiserum in combination with chicken embryo emulsion is not so great as one might have expected from the tissue culture experiments (p.266). The results with normal rabbit's serum in the case of both mouse and chicken tissue antigen show a diminution in the amount of complement fixed by the treated normal rabbit's serum. With the latter tissue, the results are almost the same both with antiserum and with normal rabbit's serum and are probably to be explained by the content of the sera in heterophile antibody (see p.273.).

Hœmolytic properties of the antiserum. (a) Heterophile antibody. It is well known that tissues of the mouse as well as those of the guinea-pig and fowl contain Forssman's antigen, i.e. when injected into rabbits they cause the development of a haemolytic immune body for sheep corpuscles, heterophile or Forssman's antibody.

Accordingly the sera of the immunised rabbit "I" and the normal animal "C", were examined from this point of view. After the twelfth injection of mouse embryo 0·1 c.c. of the antiserum from rabbit "I" caused complete lysis of 0·5 c.c. of a 3 per cent. suspension of sheep's corpuscles, while the same amount of normal rabbit's serum "C" did not do so (see table V). In the serum of the latter, however, heterophile antibody gradually developed, and at

TABLE V.

The amount of hæmolysin for sheep's red blood corpuscles in the antiserum (rabbit v. *mouse embryo tissue).*

Antiserum obtained after immunisation had been in progress for three months (12 injections), previously heated at 56° C. for half an hour.

Normal rabbit's serum previously heated at 56° C. for half an hour.

Serum in c.c.	0·001	0·01	0·1
Antiserum	0	tr	c
Normal rabbit's serum . .	0	0	0

The results in this table indicate the amount of lysis of 0·5 c.c. of a 3 per cent. suspension of sheep's corpuscles by the antiserum (rabbit *v.* embryo mouse tissue) in varying amounts. The method adopted was to add to 0·5 c.c. of 3 per cent. suspension of sheep's red blood corpuscles varying amounts of serum (antiserum and normal rabbit's serum respectively), and 5 M.H.D. of guinea-pig's complement which had previously been treated with washed sediment of sheep's corpuscles at 0° C. in order to remove any natural immune body. This amount of the treated complement by itself produced no lysis of the test corpuscles.

M.H.D. of treated complement = 0·005 c.c.

the time when rabbit "I" had received forty-two injections, both its serum and that of the normal rabbit in a dose of 0·05 c.c. caused lysis of 0·5 c.c. of suspension of sheep's corpuscles. Accordingly it is clear that, even after a

prolonged course of injections of mouse embryo, the content of the animal's serum in heterophile antibody did not exceed the limit met with in normal animals (see also Mackie,1925). This contrasts with the appearance in the serum of specific antibodies for mouse tissue.

It has already been seen that normal rabbits' serum and the antiserum exert a considerable degree of cytotoxic action on cultures of guinea-pig and chicken tissues, and that normal rabbit's serum also has a slight effect on mouse tissues. In view of the fact that all these species contain Forssman's antigen in their organs, and that the rabbits' serum contains normally corresponding antibody, it was of importance to determine whether any relationship existed between these two properties of the serum.

On treatment both of the immune serum and the normal serum with an equal volume of packed suspension of guinea-pig's organs (heart and kidney) for one hour at 0°C. the haemolytic immune body of the serum was practically removed. Thus while the M.H.D. of both sera in the untreated state (after heating for half an hour at 56°C.) was 0·05 c.c for 0·5 c.c. suspension of sheep's corpuscles, 1·0 c.c. of treated sera (similarly heated) caused only a faint trace of lysis. Since the presence of this large amount of treated serum might itself inhibit the action of the com-

plement, the experiment was also varied in the following manner. Th treated normal serum and the treated antiserum (both heated at 56°C.) were added to the red corpuscle suspension, and, after contact for one and a half hours at room temperature, the fluid was removed by centrifuging and replaced by normal saline. Then 5 M.H.D. of complement were added and the tubes were incubated at 37·5°C. The result was the same as that above described.

Hœmolytic antibody for mouse red blood corpuscles. As tested in the usual way with 0·5°c.c. of 3 per cent. mouse red blood corpuscle suspension and guinea-pig complement (5 M.H.D. as reckoned for sheep's corpuscles with immune body from the rabbit), the M.H.D. of the heated antiserum was 0·06 c.c., whereas, with 0·1 c.c. of the normal serum "C" only a faint trace of lysis resulted.

Precipitation reaction with mouse embryo juice. The antiserum in amounts of 0 .1 and 0·01 c.c. when added to 0·3 c.c. of 1:10 dilution of embryo juice, as used for cultivation purposes, gave rise to a distinct precipitate which developed within half an hour. With 1:100 and 1:1000 dilution of embryo juice, no precipitate appeared even after eight hours. With 0·1 c.c. of normal rabbit's serum "C" no precipitation occurred with any of the concentrations of embryo juice. It is not possible to compare

these results quantitatively with those of Kimura.

VI. Summary.

I. A study has been made of the cytotoxic property, as manifested on tissue cultures, which developed in rabbit's serum as the result of injecting suspensions of tissues of mouse embryos.

2. The first evidence of cytotoxic action in the serum was an inhibition of emigration of fibroblasts when tissue, e.g. embryo heart, was cultured in the serum. Later on, death of the cells was produced when actively growing culture was exposed to the serum for a short time (half an hour). On substituting a suitable nutritive medium, no further activity was shown by the cells.

3. To produce a powerful cytotoxic serum it was found necessary to continue immunisation over a long period. It was noted that the mode of action of the anti-serum underwent also a qualitative change. At first, under the influence of the serum, death of cells took place only after several hours, and autolysis then followed; the persisting cells of the cultures resumed activity, and emigration and mitoses occurred when a suitable medium

was supplied. At a later stage of immunisation, the rapidly acting cytotoxin which had been developed, whilst killing the cells, appeared to inhibit the subsequent autolysis.

4. The antiserum acted on cultures of normal epithelial cells (kidney, liver, intestine), carcinoma M.63 (Imperial Cancer Research Fund) and a spontaneous adenocarcinoma of the mouse, as well as on various mesoblastic elements, fibroblasts, mesothelial cells, macrophages, etc. The changes produced were studied in fresh preparations, alterations in morphology and in the behaviour of cells to supra-vital staining by neutral red being examined. In addition, the changes in the cells were investigated after fixation and staining by various methods.

5. The cytotoxic action took place most rapidly at 37·5°C., more slowly at room temperature and not at 0°C. At 0°C. fixation of cytotoxin by the cells occurred, since on transferring to 37·5°C. the cytotoxic action took place with great rapidity.

6. When the rapidly acting antiserum was heated at 56°C., its cytotoxic action was not entirely destroyed, but underwent a modification characterised especially by delayed action and the production of vacuoles in the cytoplasm. The action of the heated antiserum was completely

restored by the addition of normal rabbit's serum which by itself was devoid of rapidly acting cytotoxic properties. Accordingly, complement appears essential for the occurrence of rapid and intense cytotoxic action, although a thermostable antibody may by itself produce a certain amount of damage to the cells, shown by histological changes.

7. The antiserum was tested on cultures of the tissues of other animal species. On those of the embryo and newly-born rat, the effect was similar to, but slightly weaker than, that on mouse tissues. On embryo chicken and guinea-pig tissues, the antiserum did not act more intensely than did normal rabbits' serum, and this effect appears to be related to the normal content of rabbits' sera in heterophile antibody. This is borne out by absorption experiments with the various tissues. The conclusion is reached that a specific antibody has been developed for mouse tissues which acts also on those of a related species, the rat.

8. The antiserum shows in addition to its cytotoxic properties a slight degree of hæmolytic, precipitating and complement-fixing action.

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THE REPAIR *in vitro* OF EMBRYONIC SKELETAL RUDIMENTS AFTER EXPERIMENTAL INJURY.

THE REPAIR *in vitro* OF EMBRYONIC SKELETAL RUDIMENTS AFTER EXPERIMENTAL INJURY.

The following observations on reparative processes in embryonic skeletal rudiments have been rendered possible by the methods which Fell and Robison (1929) applied to normal development. These observers found that the femoral rudiments isolated from 5½-day and 6-day fowl embryos are capable of undergoing a considerable degree of histological and morphological growth and differentiation when cultivated *in vitro*. The course of development which these authors established was as follows. The femur, which at this stage consists of early cartilage, develops a diaphysis and epiphysis of typical avian character, and periosteal bone and a periosteal membrane appear. The cartilage becomes differentiated into three zones, with rounded, flattened and hypertrophic cells respectively, characteristic of normal development. The hypertrophic zone occupies the centre of the shaft and is bounded on each side by a zone of flattened cells. The extremities are composed of small rounded elements which are fairly well demarcated from the flattened zone;

the hypertrophic and flattened areas, however, merge into one another. The hypertrophic cells are larger than the other constituents of the cartilage and contain a large clear spherical nucleus in which two or more nucleoli can be seen: the cytoplasm is very vacuolated and stains faintly. These cells do not multiply by mitosis; their number increases by the addition of cells from the flattened zone, which undergo similar changes as development proceeds. Increase in size of the rudiment is brought about to a considerable extent by the formation of intercellular material. The flattened zone consists of chondroblasts which are compressed in the horizontal plane and which merge on the one hand into the small round chondroblasts of the extremities and on the other into the hypertrophic area. Mitoses are often seen amongst the flattened chondroblasts. The extremities are composed of small round chondroblasts which are irregularly arranged except at the articular surfaces, where they lie parallel to the surface. Mitoses are numerous amongst them, particularly in the superficial parts. The tissue surrounding the central zone of cartilage becomes differentiated to form a periosteum in which two layers can be distinguished, an outer

layer of elongated fibroblasts separated by fine collagen fibrils, which forms the fibrous layer of the periosteum, and an inner layer of polygonal cells, the osteoblasts, which form bone on the surface of the hypertrophic cartilage. The appearances described correspond to those which are found in early development *in vivo* (Fell (1925)). Simultaneously with this histological development, the femur increases in length and acquires a form which resembles the normal anatomical structure. Fell and Robison (1930) have also shown that the distal part of Meckel's cartilage from a 6-day fowl embryo, which forms part of the embryonic skeletal rudiment of the lower jaw but which does not undergo periosteal ossification, increases in length during cultivation *in vitro*. The chondroblasts of Meckel's cartilage, however, do not, either *in vitro* or *in vivo*, undergo the characteristic histological development found in the cartilage of long bone rudiments in which periosteal bone appears.

Such isolated femora and Meckel's cartilages from early embryos, if injured either before or during cultivation *in vitro*, provide useful material for the study of repair in cartilage and bone uncomplicated by the presence of a blood- or nerve-supply. Again, by

injuring rudiments isolated from older embryos the repair of cartilage and bone which have already attained some degree of histological development in vivo can be compared with that of cartilage and bone which have differentiated in vitro.

The observations to be recorded also supply a means of studying the proliferative capacity of cartilage cells, regarding which there has been diversity of opinion. Many authors (e.g. Maximow (1930), Schaffer (1930)) hold that in the adult independent regeneration of cartilage does not occur.

Methods.

The culture technique employed was similar to that used by Fell and Robison (1929) for the cultivation of isolated femoral rudiments. It has already been described on page 62.

The material used consisted of the femoral and tibial rudiments isolated from fowl embryos, after from 5 to 10 days' incubation, the distal part of Meckel's cartilage taken from 6- to 9-day embryos, and femora and tibiae from embryo mice. When fowl tibiae were used, the tarsal element which fuses with it during development was sometimes removed along with the long bone rudiment. The rudiments were injured,

either before explantation or after different periods of growth in vitro, by cutting with a sharp knife on the surface of a glass slide. The region of the cut depended on the area of the tissue in which it was desired to study repair. In some cases, the rudiments were cut through at the centre of the shaft at right angles to the long axis, in others at the junction of the upper and middle thirds. In order to study epiphyseal repair, cuts were usually made into the epiphyseal surface parallel to the long axis. The Meckel's cartilages were often cut in several places. The rudiments were then transferred to the surface of the clot and the cut ends placed in apposition; sometimes, however, the rudiments were not completely severed. Two explants were placed on each clot. Every 48 hours, the explants were loosened from the clot by cutting round the zone of outgrowth and after washing in saline and embryo extract, were transferred to fresh medium.

Fixation and staining. — Explants were fixed in Bouin's solution or Zenker's fluid plus acetic acid; Flemming's solution (strong formula without acetic acid) was used for small explants. After dehydration, clearing and embedding, serial sections were cut and stained

with Mallory's triple stain, safranin or magenta and picro-indigo-carmine, or by Masson's trichromic staining methods. Rudiments isolated from embryos of the same age and of corresponding development were fixed as controls so that the degree of differentiation of the tissue before explantation could be determined. In some cases, the femur and tibia of one limb were used for experiment while those from the other limb were fixed as controls. Since the processes of repair are similar in both femoral and tibial rudiments isolated from embryos at the same stage of development, it is unnecessary to describe them separately. It has been found that during the winter months, the embryos are less advanced in development than those taken from eggs during the spring and early summer after an equal period of incubation. Any modification in technique necessitated by this variation will be mentioned.

REPAIR OF LONG BONE RUDIMENTS FROM FOWL EMBRYOS.

Repair of femora and tibiae, isolated from 5-day fowl embryos.

The femur and tibia at this stage of development consist of an early type of cartilage, which shows no trace of differentiation into epiphyses and diaphysis (fig.97.). The cells in the centre are arranged with

their long axes at right angles to the long axis of the shaft and are separated by more intercellular material than those of the future epiphyses. Towards the two ends the cells are smaller and more irregularly disposed and become indistinguishable from the surrounding tissue. The shaft is surrounded by a zone of somewhat compact cells, which merge into the surrounding mesenchyme, but there is no trace of bone or of a definite perichondrial membrane. It is convenient to cut such rudiments on the surface of the plasma clot on which they are subsequently cultivated, as otherwise it is difficult to determine the cut surface of cartilage, and correct apposition is not easy to obtain.

Appearances after repair in vitro. — Union of the two fragments takes place with great rapidity, and after 24 hours' cultivation complete fusion has occurred. Fibroblasts and wandering cells emigrate on to the surface of the medium from the periphery of the explant. The cartilage is much more distinct than it was before explantation and has increased in length. No trace of the cut can now be seen, and this is confirmed by histological examination (fig. 98.). The cells of the centre of the shaft are larger than in the control specimen and the cartilage already shows differentiation

into three zones, the cells of the future epiphyses being separated from those of the diaphysis by two narrow zones of flattened cells. The region of the enlarged cells is covered externally by a layer of flattened cells which form a rudimentary perichondrium. The appearances correspond in all respects with those of a 5-day rudiment which has been cultivated for 24 hours in vitro without previous injury (fig. 99).

After 6 days' cultivation in vitro of femora and tibiae from 5-day embryos which have been cut across, considerable increase in length has taken place, the epiphyses are enlarged and the explants exhibit a form which resembles closely that of the normal structure. Histological examination shows that the cartilage has advanced in development and is arranged in the three zones (hypertrophic, flattened and round-celled), characteristic of normal histogenesis, both in vitro and in vivo (fig. 100). Around the hypertrophic area, the periosteum, which has developed by differentiation of the layer of compact cells surrounding the shaft, consists of an inner layer of polygonal cells, the osteoblasts, and an outer layer of elongated fibroblasts between which fine collagen fibrils can be seen. Between the osteoblastic layer and the hypertrophic

cartilage is a thin sheet of early bone. Such appearances correspond to those of uncut 5-day rudiments which have been cultivated for 6 days *in vitro*. It appears from these results that the cartilage of a long bone rudiment isolated from a 5-day fowl embryo is capable after injury of undergoing complete repair by cartilage, and the rudiment as a whole continues its histological and morphological development, just as if it had not been cut.

If such 5-day rudiments are cut in two and the fragments cultivated separately, increase in size takes place, but the cut ends become rounded and do not enlarge to form epiphyses. Similar absence of regeneration was observed by Levi (1930), who cultivated fragments of rudiments from 5½- to 6½-day fowl embryos. The epiphyseal end acquires its appropriate shape; for example, the proximal half of the divided femur develops a head and a trochanter while the distal half develops condyles. The cartilage also undergoes histological differentiation during the culture period in those situations in which this would normally occur. After 9 days' cultivation the epiphysis consists of small-celled cartilage and is separated by a zone of flattened cells from an area of normal hypertrophy which extends almost to the

cut end of the rudiment. Osteogenesis has proceeded as usual in the region of hypertrophic chondroblasts, and the osteogenic tissue has grown round the cut end and deposited a thin layer of bone on the surface, continuous with that of the shaft. Levi also observed histological differentiation of fragments of rudiments *in vitro*.

Repair of femora and tibiae, isolated from 5½-day fowl embryos.

Femora and tibiae, isolated from 5½-day fowl embryos are slightly more advanced in development than those from 5-day embryos (fig./0/). The chondroblasts in the centre of the shaft are larger and are separated by more matrix than those at the two ends, but there is no differentiation into three zones. The shaft is surrounded by a broad zone of compact cells in which there is no trace of bone or of a perichondrial membrane. Such rudiments can be cut very conveniently on a glass slide and transferred by means of a pipette to the culture medium. It is important that the fragments should be placed in as accurate apposition as possible, for at this stage of development the rudiments grow very rapidly and are liable to become very distorted.

Appearances after repair in vitro. - Immediately after the rudiment has been cut, the cartilage in direct relation to the injury loses its semi-translucent character and appears black by transmitted light. Union of the fragments takes place quickly, but an opaque area persists at the site of fracture. Considerable increase in length and breadth of the rudiment takes place during the culture period; the epiphyses develop and in their form resemble closely the normal development. After 6 days' cultivation in vitro the chondroblasts above and below the site of fracture have become hypertrophic and vacuolated and the intercellular material is much greater in amount than in the control specimen. This hypertrophic cartilage is separated from the small-celled cartilage of the epiphyses by two broad zones of flattened cells. The outer layer of cells around the central area of the shaft becomes differentiated to form a periosteum in which two layers can be distinguished, an inner stratum of polygonal cells which have formed a thin sheet of early bone on the surface of the hypertrophic cartilage, and an outer layer of elongated fibroblasts arranged parallel to the long axis of the shaft with fine collagen fibrils between them. At the site of

section (fig. 102, 103.) the cut ends of the fragments become approximated more closely by the growth and development of the cartilage, but no union of healthy cartilage occurs and the site of fracture remains visible, outlined on each side by a zone of necrotic chondroblasts. Union of the fragments is brought about by the proliferation of the periosteal cells and of the surrounding mesenchyme, which bridge across the gap. Cells from the osteoblastic layer of the periosteum may also grow between the cut ends to a varying extent depending on the degree of separation and the rapidity of development of the chondroblasts; that is to say, where the gap is wide, either by faulty apposition or owing to relative failure of the cartilage cells to undergo their normal hypertrophy, the cells from the osteoblastic layer tend to spread over the surface of the necrotic chondroblasts and fill the gap across the line of fracture. No trace of proliferation of chondroblasts in the hypertrophic area has been seen, but mitotic figures have been observed in the osteoblastic cells in the gap across the site of fracture. Osteoid tissue is often formed by these cells, which retain their character in this situation and never become transformed into chondroblasts (fig. 106) This osteoid tissue, however, becomes compressed by the

continued development of the cartilage, and where cartilage hypertrophy is abundant, the resulting close apposition and compression of the cut ends may even prevent osteogenesis in this situation.

In favourable cases, the explants retain their shape after cutting. Distortion, however, often occurs and this usually takes the form of angulation at the site of fracture. (Fig. 107.) Twisting and curving of the shaft occur also in unfractured rudiments and according to Fell and Robison (1929) are due to two main causes, prolific outgrowth of the epiphyseal perichondrium, which may anchor the ends so that increase in length can only take place by bending, and the formation of tough bands of fibrous tissue which, extending between and attached to the epiphyses, prevent elongation taking place along the normal axis. This latter factor, these authors consider, can be eliminated by removing as much as possible of the zone of outgrowth during subculture. These observations have been confirmed and in fractured rudiments it has also been found that the normal elongation of the shaft is often inhibited by the presence of the osteoblastic tissue and the zone of dead chondroblasts near the site of fracture, so that distortion may occur in spite of the greatest care

during subculture. The following histological features are similar both in fractured and unfractured rudiments. More bone is found in the concavity of the curvature than over the convexity (Fig. 107). In the latter situation, the cells appear to be stretched, the various layers are thinned out and osteogenesis is diminished; previously formed bone may even disappear in this situation. In the concavity, on the other hand, the osteoblastic layer is thicker, and there is an accumulation of osteoblasts within the fibrous coat which is more fibrotic than usual. Much more bone is formed than would have occurred if the rudiment had remained straight. The effect of curvature is particularly well seen in cultures of rabbit embryonic femora at a corresponding stage of development. Differentiation of the osteogenic tissue is much more difficult to obtain in these, however, than in similar avian material. This is probably due to the degeneration resulting from the diminished vitality of all mammalian tissue when cultivated in vitro. When rabbit femora become curved during cultivation, the formation of the periosteal layers and of osteoid tissue is markedly greater in the concavity than on the convexity, where differentiation is minimal and little or no osteoid tissue is formed.

The thickness of the zone of osteoid tissue is never so great in the most healthy rudiments which remain straight as it is in the concavity of those which become curved and which may be as a whole more degenerate. Similar appearances have also been described by Murray and Selby (1930) in chorio-allantoic grafts of isolated avian femora. These workers consider that the approximation of the epiphyses on the concave side resulting from the curvature causes a loosening of the perichondrium, while on the convex side the perichondrium is pulled taut. They are of the opinion that the conditions thus established favour ossification. In addition, however, the present observations on living explants in vitro suggest strongly that the pressure in the concavity favours fibrosis of the fibrous periosteum, which separates from the shaft and subsequently shortens; in the potential space thus produced the osteoblasts multiply and form osteoid tissue. The results described above show that although fracture of a $5\frac{1}{2}$-day rudiment causes a certain degree of irreparable damage to the cartilage, further histological and morphological development takes place and a continuous sheath of periosteal bone is laid down, completely covering the site of fracture.

Repair of rudiments cultivated in vitro for 6 to 9 days before injury.

In 5½-day rudiments which have been cultivated for 6 to 9 days in vitro, the cartilage shows the usual differentiation into hypertrophic flattened and small-celled zones. The periosteal layers are well-developed and between the hypertrophic cartilage and the layer of osteoblasts a thin sheet of early bone, which is often calcified, has been formed. When such rudiments are injured repair occurs within 48 hours. The site of injury in the cartilage is always indicated by an opaque area in the cartilage, the extent of which varies according to the amount of trauma to which the bone has been subjected. The continuity of the fibrous layer of the periosteum is restored within 48 hours and the layer of osteoblasts also becomes complete. At the site of injury in the cartilage numerous necrotic cartilage cells are present. The osteoblasts fill up the gap between the cut ends to a varying extent and tend to accumulate round the margins of the cartilage; many of them show mitoses (fig.105). After 6 days' further cultivation, the continuity of the bone of the shaft is restored. The osteoblasts which have wandered into the gap between the cut ends sometimes form osteoid

tissue; often, however, they become compressed and do not do so. While repair is taking place, the rudiments increase in length, just as if they had not been cut.

Repair of rudiments isolated from 5-day embryos and cultivated for 24 hours in vitro before being cut.

Since it had been found that the repair of rudiments isolated from 5½-day embryos differs markedly from that of 5-day rudiments (in which repair takes place by cartilage), it was decided to cultivate 5-day rudiments for 24 hours in vitro before injury in order to determine if such a period of cultivation outside the animal body would render the cartilage incapable of repair. After this cultivation, the rudiments have increased in length and the cartilage shows differentiation into three zones. The chondroblasts of the central area are larger than in the control specimen but do not at this stage show the vacuolation which is such a prominent feature of hypertrophic chondroblasts. When such rudiments are cut and the cut ends placed in close apposition, union of the fragments takes place within 24 hours but the site of injury can be seen by transmitted light as a black transverse line in the cartilage. Histological examination

of such rudiments 4 days after the injury shows that union of the fragments takes place in a manner different from that described for 5-day rudiments cut prior to explantation (fig.108). The rudiments increase in length and continue their usual histological development. The cartilage cells above and below the site of fracture become hypertrophic and vacuolated, and the amount of matrix between them increases. By their rapid growth in all directions, the hypertrophic cartilage cells assist in bringing about fusion of the fragments in a purely mechanical way. The site of fracture is clearly indicated by the presence of a zone of necrotic chondroblasts across the centre of the shaft which appears as a dark line in the explants before fixation; the significance of this will be referred to later. The fibrous periosteum and the osteoblastic layer which have developed by differentiation of the rudimentary perichondrium already described are continuous at the site of fracture, and the growth of these layers and of the surrounding connective tissue also helps to fuse the fragments together. Cells from the osteoblastic layer fill up the gap round the margins of the dead cartilage and encroach upon it to a varying extent depending on the degree of separation of the cut surfaces and the rapidity

of development of the uninjured cartilage. These cells, however, never become transformed into chondroblasts. It appears, therefore, that the cartilage of the centre of the shaft of a long bone rudiment isolated from a 5-day fowl embryo is no longer capable of direct repair by cartilage after 24 hours' cultivation in vitro. The chondroblasts themselves are very sensitive and those injured by the trauma die; repair is then brought about by the progressive formation of matrix by the uninjured cartilage cells and by the agency of the developing layer of osteoblasts, the fibrous periosteum and the surrounding connective tissue. Differentiation of the osteogenic tissue is not inhibited by the injury, and a continuous sheath of periosteal bone covering the site of fracture is formed.

Repair of the terminal zones of cartilage in rudiments isolated from 6-day embryos.

The repair of the cartilage in the less differentiated parts of rudiments isolated from 6-day embryos was then investigated by making vertical and oblique as well as transverse cuts in the future epiphyseal region and in the zone between it and the centre of the shaft. This part of the investigation was done during

the winter, when development is retarded, and the rudiments used correspond to those isolated from 5½-day embryos already described. The cartilage was not completely severed as it is very difficult to obtain correct apposition of a small and a large piece of tissue on the surface of a plasma clot.

The results vary according to the position of the injury. (a) When a vertical cut involves only the small-celled cartilage and not that zone which after 2 days' cultivation becomes the flattened layer, complete repair by cartilage takes place and the articular surfaces develop their usual form. (b) When a transverse cut is made at the junction of the proximal or distal and the two middle fourths, repair again takes place by cartilage but constriction of the shaft develops at the site of injury. This is due to the rapidity with which the cartilage cells in that region become transformed into the flattened type, and to the corresponding diminution of proliferative activity associated with this differentiation. (c) When an oblique cut extends from the diaphyseal limit of the future epiphyses through that part of the cartilage which after 2 days' cultivation becomes the flattened layer, an area of necrosis is found at the inner end

surrounded by healthy flattened chondroblasts. The more superficial and less differentiated cartilage repairs itself completely by multiplication of the chondroblasts themselves. (d) When transverse cuts are made at the junction of the upper (or lower) and middle thirds, no repair by cartilage takes place and the injured area is easily located by the presence of a zone of necrotic chondroblasts. The necrosis is not so extensive as that which occurs in the centre of the shaft after a similar degree of trauma. When such rudiments are examined after 2 days' cultivation, the area of necrosis is surrounded by healthy flattened chondroblasts; after 4 days' cultivation, the surrounding healthy cells are hypertrophic. Union is brought about by the growth of the uninjured tissue on each side which approximates the cut surfaces, and, when the gap is large, by the ingrowth of cells from the tissue investing the cartilage at the point of injury. After 8 days' cultivation, osteogenesis has extended up the shaft beyond the site of injury and the cells which have grown into the gap also show osteogenic activity.

The repair of the epiphyseal cartilage, derived from 6-day rudiments, which has subsequently become differentiated as a result of cultivation in vitro.

Femora and tibiae from 6-day embryos were cultivated for 4 days in vitro, before being injured. During this period of cultivation, the extremities enlarge and develop their characteristic form. The centre of the shaft is composed of hypertrophic cells bounded on each side by broad zones of flattened cells. The epiphyses consist of small chondroblasts which on the diaphyseal side merge into the flattened zones and are separated by a gradually increasing amount of matrix as this area is approached. The articular surfaces are covered by a thin compact perichondrium which has marked chondrogenic capacity; the cells immediately underlying it are arranged parallel to the surface. When vertical cuts are made in the epiphyses, healing of the perichondrium takes place within 24 hours. After 4 days' cultivation, small areas of necrosis are present in the deeper parts of the epiphyseal cartilage adjoining the flattened zone, where the cells are separated by a fairly abundant matrix (fig. 109). The adjacent chondroblasts are

multiplying by mitosis which is a normal occurrence in this area during cultivation *in vitro*, and so has probably no direct relation to the injury. The perichondrium shows marked activity in the region where the cut was made. If the cut surfaces are not closely approximated after the injury, cells derived from the perichondrium wander into and fill the gap, in the superficial part of which they multiply by mitosis and continue to give rise to chondroblasts. In the deeper part of the cut, on the other hand, they fail to form cartilage matrix and come to resemble fibroblasts. The explanation of this striking difference is not yet apparent.

Repair of femora and tibiae isolated from 7-day fowl embryos.

At this stage of development the cartilage is arranged in three zones and the central area of hypertrophy, which consists of large vacuolated chondroblasts separated by a fairly abundant matrix, occupies about two-fifths of the total length of the rudiments. The epiphyses consist of small rounded cells and are separated from the hypertrophic area by two broad zones of flattened cells. The periosteal layers are well-

developed in the region of hypertrophic cartilage and consist of an outer fibrous coat sharply demarcated from the surrounding mesenchyme, and an inner layer of osteoblasts. Between the osteoblastic layer and the cartilage lies a thin sheet of early bone which is generally calcified (fig. 110).

Appearances after repair in vitro. - When 7-day rudiments are cut, the injury produces immediately a marked change in the cartilage in its proximity. The cartilage appears black by transmitted light and the change is more marked than in the case of 5½-day rudiments, in which the intercellular material is much less abundant. The change is apparently due to some alteration in the matrix, as it appears before degeneration and necrosis have had time to occur in the cells, and it varies in intensity according to the amount of matrix present. At first the altered matrix stains more intensely than the uninjured matrix with light green, safranin or the aniline blue of Mallory's triple stain; later, however, it may lose almost entirely its staining capacity, particularly in large explants.

Union of 7-day rudiments takes place less rapidly than in 5- to 6-day rudiments. After 48 hours' cultivation, however, the union feels quite firm when the

explants are transferred to fresh medium. But the site of fracture is always indicated by an opaque area in the cartilage. Union is brought about by the emigration of cells from the osteoblastic layer into the gap between the cut ends, by the growth and development of the cartilage above and below the fracture, which helps to approximate the fragments, and by the growth of the surrounding connective tissue. The extent to which the latter process takes place depends on the degree of separation of the cut surfaces and the rapidity of development of the cartilage. If the separation is relatively great the cells of the osteoblastic layer are able to grow into the gap before apposition of the cartilage occurs. Subsequently they form osteoid tissue in this region (fig. III/3). (Fig. 112) If the apposition is very close, the osteoblasts which wander in become compressed at right angles to the long axis and do not give rise to osteoid tissue. Areas of necrotic chondroblasts are present at the site of fracture above and below the line of fusion, and no evidence of proliferation of the hypertrophic cells has been seen. The osteoblastic and fibrous layers become continuous once more and the continuity of the investing layer of bone is also restored by the agency

of the osteoblasts. The deposition of periosteal bone continues and the rudiments increase in length. During cultivation, the emigration of fibroblasts and macrophages from the tissue surrounding the explant continues and after repeated subculture this emigration may involve the fibrous layer of the periosteum. When this occurs, ossification extends outwards into the looser connective tissue, and a small exostosis forms (fig.114). It appears, therefore, that the fibrous layer of the periosteum during development and during cultivation in vitro serves a similar function as a limiting membrane to that which it exercises in vivo, viz., it prevents the excessive formation of bone and limits its distribution. The 7-day rudiments do not tend to become so distorted as those from 5½-day embryos, probably owing to their lessened rate of growth.

The repair of femora and tibiae isolated from 9-day embryos.

At this stage of development the femora and tibiae are larger and considerably more advanced in histological development than at 7 days. The bone of the shaft has assumed a trabecular character and the osteoblastic

layer is vascularised (fig.115). As a rule, invasion of the cartilage by capillary blood-vessels, osteoblasts and fibroblasts does not appear until 9½ days after incubation, but sometimes this occurs early on the 9th day. It is necessary to separate the developing muscles as much as possible from the bones so as to allow the maximum amount of diffusion of nutritive materials into the large explants, and it is often difficult to do this without stripping the periosteal layers. The bones have to be cut carefully with a heavy and very sharp knife so as to avoid excessive compression of the cartilage. The clot on which they are placed should be as flat as possible to allow of accurate apposition of the fragments. Union of the fragments is generally fairly firm after 2 days' cultivation and the fractured bones can be transferred to fresh medium without difficulty.

Appearances after repair in vitro. - If the bones are examined histologically at this stage the amount of injury to the cartilage due to the fracture can be determined. Subsequently the cartilage degenerates, probably owing to the insufficient nutrition of such large explants under the conditions of cultivation in vitro. The bone and the periosteal

layers remain healthy for a much longer time. The cartilage does not play such an important part in bringing about union of the fragments as it does in younger rudiments, because in the central area it is already fully differentiated and has ceased to produce matrix. The growth of the fibrous layer of the periosteum and of the surrounding connective tissue unites them, and the osteoblasts accumulate at the site of fracture and fill up the margins between the divided cylinder of bone, where they multiply by mitosis. After 8 days' cultivation in vitro newly formed bone can be seen in many places and this becomes continuous with the existing bone (fig. 117.). After 14 days' cultivation the repair of the bony cylinder is generally complete all round the shaft. Cells from the osteoblastic layer also emigrate into the gap between the cut surfaces of cartilage. There they become spindle-shaped, resemble fibroblasts and multiply by mitosis but do not form osteoid tissue in this situation as they do in 7-day rudiments undergoing repair. They grow into the cartilage and occupy the lacunae of the damaged cartilage cells, where they can sometimes be seen lying beside necrotic chondroblasts and can be easily recognised in well-fixed preparations by their healthy appearance

and the character of their cytoplasm, which is more voluminous and less vacuolated than that of hypertrophic chondroblasts (Fig.116). The cartilage cells and matrix disappear during cultivation in the region of osteoblastic invasion, this area being eventually occupied only by spindle-shaped cells. Fibroblasts from the fibrous layer of the periosteum do not participate in this invasion of the cartilage and there is no development of capillary blood-vessels. Disappearance of the cartilage of the shaft occurs in normal development in vivo and has been fully described by Fell (1925), who found that capillary blood-vessels penetrate the bony cylinder between the 9th and 10th days of incubation, carrying with them osteoblasts and strands of fine connective tissue. The cartilage ultimately disappears from the centre of the shaft and is replaced by the developing marrow. Endochondral ossification similar to that which occurs throughout the developing diaphysis of a mammalian long bone occurs only at the epiphyseal end of the diaphysis. Up to the first day after hatching, no bone trabeculae are present in the centre of the shaft, i.e. in the region corresponding to the site of fracture, even although many osteoblasts have invaded this area. The mechanism

of resorption of cartilage is unknown, but it is probable, as suggested by Shipley (1928), that the osteoblasts play an important part. That it can occur without the agency of blood-vessels has been shown by Fell (1928), who described excavation of cartilage in cover-glass cultures of 8-day embryonic limb cartilage which had undergone ossification in vitro. In cultures of entire long bone rudiments, however, no excavation of cartilage has been seen by Fell, and I have seen it only in fractured 9-day bones and in 10-day bones in which penetration of the bony cylinder had taken place before explantation. The behaviour of the osteoblasts in the repair of 9-day bones is probably due to their possessing at that stage the capacity not only for forming bone but also for eroding cartilage. In relation to preformed bone, they retain the former property, but when they gain access to the cartilage, as they do after fracture, the latter property becomes predominant. It is not implied, of course, that in normal development osteoblasts are alone responsible for resorption of cartilage.

THE REPAIR in vitro OF MECKEL'S CARTILAGE AFTER INJURY.

Normal appearances. - During normal growth

in vivo Meckel's cartilage, which forms part of the embryonic skeletal rudiment of the lower jaw, does not undergo periosteal ossification except at the proximal end, where it gives rise to a typical cartilage bone, the os articulare. The rest of the cartilage becomes surrounded by typical membrane bone which develops in the adjacent mesenchyme. At the 6th day of incubation, the distal end consists of a rod of cartilage composed of small chondroblasts, lying in a scanty matrix surrounded by a cellular perichondrium. The rod increases in length mainly by the formation of intercellular material and to some extent by the activity of the perichondrium. After the 8th day of incubation, however, the perichondrium becomes less cellular, the outer cells becoming indistinguishable from the surrounding connective tissue. The inner cells give rise to a thin fibrous membrane which invests the cartilage. The chondroblasts do not undergo the hypertrophy and vacuolation found in the cartilage of developing long bones associated with periosteal ossification. The investigation of the repair of the distal end of Meckel's cartilage after injury provides useful material for comparison with fractured long bone rudiments. As it was not desired to include the articular ends of the

cartilages, they were cut off and the distal part of Meckel's cartilage was isolated as cleanly as possible from the surrounding tissue in order to prevent inclusion in the explant of osteogenic tissue. The apposition of the cut surfaces of such thin rods of cartilage was found to be very difficult and distortion was very liable to occur. Cuts were therefore made in the cartilage but the mesenchyme surrounding it was generally left intact at one point in order to connect the divided cartilage.

Appearances after repair in vitro of Meckel's cartilages isolated from 6-day embryos. - Union of the cut surfaces takes place quickly and the site of injury can be recognised only with difficulty in the explant after 24 hours' cultivation. Histological examination at this stage shows at the site of injury an accumulation of chondroblasts, in which mitotic figures are present, and these are separated by less matrix than the surrounding cartilage cells. No zone of necrotic tissue is present such as is found in the shaft of injured 5½-day long bone rudiments. The perichondrium is very active and is giving rise to chondroblasts. Repair is brought about by the activity of the perichondrium and the proliferation of the

chondroblasts themselves. After 8 days' cultivation in vitro the site of injury can only be recognised in such explants by the presence of a slight constriction of the cartilage. At this stage the perichondrial activity has ceased as a rule and the cartilage is bounded by a thin fibrous membrane. The rod has also increased in length and the matrix between the cells is much more abundant.

Normal appearances of Meckel's cartilage isolated from 8-day embryos. - At the 8th day of incubation the distal end of Meckel's cartilage is composed of chondroblasts which are larger and are separated by more intercellular material than at the 6th day. They do not, however, resemble the hypertrophic vacuolated cells found in the diaphysis of a developing long bone. The perichondrial activity has usually ceased and the cartilage, except at the proximal end, is invested by a thin fibrous sheath.

Appearances after repair in vitro of Meckel's cartilages isolated from 8-day embryos. - Transverse cuts were made in such cartilages and after different periods of cultivation they were fixed and examined histologically. Union of the cut surfaces takes place, but the site of injury can always be seen in the explant

by transmitted light as an opaque, blackish area, similar in origin and appearance to that already described in long bone rudiments. On histological examination the presence of a zone of necrotic cells is seen where the cartilage has been cut, and union appears to be brought about by the pressure of the increasing amount of matrix formed by the cartilage above and below the injured area, and by the rapid healing of the investing connective tissue. At the margins of the necrotic area, immediately under the fibrous sheath, there is often an accumulation of chondroblasts; but whether these arise by proliferation of the peripheral chondroblasts or by resumption of the chondrogenic activity of the perichondrium is uncertain: the latter process has been observed in some explants at places remote from the injury. If the gap between the cut surfaces is relatively large, however, it becomes filled with cells derived from the surrounding connective tissue, but these cells do not, even after 14 days' cultivation, become transformed into chondroblasts.

THE REPAIR OF MAMMALIAN EMBRYONIC BONES *in vitro* AFTER EXPERIMENTAL FRACTURE.

The repair of femora and tibiae of embryo mice removed from the uterus almost at full time was investigated in the same way. One femur and one tibia were fixed as histological controls; these controls, which were respectively 4·3 mm. and 4·2 mm. in length, were well-developed and showed the following structure (fig. 20). The middle three-fifths of the total length was invested by a layer of calcified bone, thicker in the centre of the shaft, where it was vascularised and had a trabecular structure, than at each end. The cartilage had disappeared from the centre of the shaft and was replaced by the developing marrow, capillary blood-vessels, thin bone lamellae and osteoblasts. Farther up the shaft, active endochondral bone formation was taking place and small areas of degenerating cartilage surrounded by osteoid tissue were present. The epiphyses consisted of small irregularly disposed chondroblasts. Between the epiphyses and the zone of provisional calcification were two zones of flattened chondroblasts which merged on the one hand into the small-celled cartilage of the epiphyses and on the other into the hypertrophic

chondroblasts of the zone of provisional calcification.

The experimental bones were severed through the centre of the shaft and the fragments placed with the cut ends in apposition on the surface of a plasma clot consisting of equal parts of fowl plasma and fowl embryo extract. After 2 days' cultivation, fibroblasts and wandering cells had emigrated on to the surface of the plasma clot from the connective tissue surrounding the explants, and this tissue had become continuous above and below the fracture. The union, however, was by no means firm and was broken during the process of separation of the explant from the clot in subculture, so that the fragments had once more to be placed in apposition. After a further 2 days' cultivation, the union was much firmer and no further difficulty was experienced in subculture. In specimens examined after 4 days' cultivation there were present between the cut ends of the bone numerous osteoblasts, separated by a fine fibrillar substance and easily distinguishable by their polygonal shape and bulky cytoplasm from the developing marrow cells and fibroblasts. Mitoses were observed amongst them. These cells were apparently derived from the endosteum, the peripheral osteoblasts and the osteoblasts of the

marrow-cavity. Bone cells liberated from their lacunae by the injury also appeared to be taking part in the repair. After 8 days' cultivation, areas of newly-formed osteoid tissue could be seen between the cut ends, continuous with the pre-existing bone. The matrix was less dense than that of the pre-existing bone and stained less intensely (fig. 12). This newly-formed bone was not uniform in amount all round the shaft, probably owing to nutritional differences in the various parts of such a relatively large explant. In a bone fixed after 22 days' cultivation, the shaft was completely invested with bone round three-fifths of its circumference and the site of fracture could only be recognised by the fact that repair was not complete all round. This bone was of a compact type, containing typical bone cells, and resembled that seen in cultures of femora from 5½-day fowl embryos which have been cultivated for 18 days.

During cultivation the bones increased in length, the maximum increase being about 33 per cent. The epiphyses also enlarged and retained their characteristic shape. Their histological differentiation continued and was similar to that which occurs in vivo. At explantation, the epiphyses consisted of small-

celled cartilage similar to that seen in developing chick epiphyses (fig.120). During cultivation the amount of intercellular material increased, and after 16 days in vitro the chondroblasts showed typical hypertrophy and vacuolation; this area of hypertrophy was separated from the hypertrophic cartilage of the diaphysis by a broad zone of flattened chondroblasts (fig.119). This formation of an independent centre of hypertrophy in the epiphysis, which is a feature of mammalian development, does not occur in the epiphysis of the fowl, which consists of small-celled cartilage throughout development both in vivo and in vitro. The mammalian epiphysis, therefore, is capable of continuing its characteristic histological differentiation even when deprived of nerve and blood supply and cultivated in a heterologous medium outside the animal body.

The replacement of cartilage by osteoid tissue continued in the epiphyseal regions of the diaphysis, but in the centre of the shaft many of the bone lamellae and the haemopoietic tissue present before explantation gradually disappeared and became replaced by fibrous tissue. This phenomenon occurred simultaneously with the transformation of the originally trabecular bone of the shaft into that of a compact type. The

significance of the fibrous replacement and the means by which it is brought about are not clear.

SUMMARY.

1. The part played by the various constituents of rudiments of long bones in bringing about repair has been studied by fracturing and incising them at various stages of development and then studying the behaviour of the fragments after cultivation in vitro. For this purpose fowl embryonic rudiments have been used chiefly, and to a less extent mouse embryonic material.

2. The results depend greatly on the period of embryonic life at which the injury is made.

3. In 5-day long bone rudiments of the fowl, after the cartilaginous shaft has been cut through and the fragments have been brought into close apposition, repair rapidly occurs by proliferation of the chondroblasts, so that the fragments fuse and no trace of the injury persists.

4. At a very slightly later stage, in rudiments from 5½-day embryos, repair is brought about by a series of processes, prominent among which are:-
(a) Rapid increase in the adjoining cartilage matrix, there being no proliferation of cartilage cells;
(b) restoration of continuity of the surrounding

osteogenic tissue and undifferentiated mesenchyme; and (c) the frequent ingrowth of osteogenic cells into the gap between the fragments, which sometimes results in the formation of osteoid tissue in this situation. The amount of osteoid tissue depends on the closeness of apposition of the fragments, being minimal when the distance between them is small.

5. When a 5-day embryonic femur or tibia is cultivated for 24 hours in vitro and then incised, the process of repair under these conditions is essentially similar to that described for 5½-day rudiments, incised before cultivation.

6. During and after repair in vitro development of the rudiments as a whole continues to progress normally, i.e. increase in length and breadth takes place and the epiphyses acquire their characteristic form. The cartilage and bone also show normal histogenesis.

7. If the rudiments are fractured at a later stage, when the formation of bone has begun (femora and tibiae from 7-day fowl embryos), repair is effected, after restoration of continuity of the osteoblastic layer and fibrous periosteum has occurred, by the deposition of bone between the fragments. Here again, when there

is an appreciable gap between the fragments, osteoblasts pass in and continue their histogenesis as in the 5½-day rudiments. The cartilage does not participate in the process of local repair. 5½-day rudiments which have been cultivated for 6 to 9 days before being cut behave similarly to 7-day rudiments fractured before explantation.

8. When rudiments from 9-day embryos are fractured, the processes of repair are similar to those described for rudiments from 7-day embryos, except that the cells from the osteoblastic layer which fill up the gap between the cut surfaces bring about resorption of cartilage.

9. At the earliest stage studied (5-day rudiments) the cartilage cells are as little susceptible to injury and as capable of proliferation as the surrounding mesenchyme. Later (from 5½ days onwards) they are distinctly more vulnerable and no longer show regenerative proliferation; these characters increase as histological development of the tissue progresses.

10. Meckel's cartilage exhibits somewhat different behaviour from that of long bone rudiments. At a comparable stage of development, to judge by histological appearances, the cells of Meckel's cartilage possess

the capacity for multiplication when the central part of the long bone rudiments has lost that property. Eventually they, too, become more susceptible to necrosis after injury. Thus, the behaviour of Meckel's cartilage resembles that of the deeper layers of the developing epiphyseal cartilage of long bones.

11. After fracture of embryonic mouse bones, examined in embryos approaching maturity, repair of the cortical bone occurs by the agency of osteoblasts and resembles generally that seen in the bony rudiment of the embryonic fowl. In the mouse bones the deposit of bone was thicker than in the fowl rudiments.

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THE DEVELOPMENT in vivo AND in vitro OF THE AVIAN PATELLA.

1. Introduction.

2. Histogenesis of the avian patella.

3. Methods.

4. Development in vitro of the avian patella.

5. Discussion.

6. Summary.

Introduction.

The part played by extrinsic and intrinsic factors in the development and maintenance of skeletal form is still undetermined, and while most authors agree that extrinsic factors such as movement, muscular activity, tension in the limb are essential to the maintenance of skeletal form, there is considerable difference of opinion as to the relative importance of these factors in early development. Murray and Huxley (1925) and Murray (1926) showed, by means of chorio-allantoic grafts, that the limb-bud of the chick embryo from the end of the third day onwards is a self-differentiating mosaic, and that the form of the cartilaginous elements of the developing limb, including that of the articular surfaces, self-differentiates and does not depend on the presence of contiguous elements, the nervous system, functional activity, muscular pull, etc. Murray (1926) described the results of a large number of experiments in which the graft consisted of limb-buds of avian embryos or fragments of limb-buds, from embryos 3 or more days old onwards. He found that the grafts increased in size and that cartilaginous structures were formed which were very like the normal. He showed

conclusively that in the limb-bud isolated from a 4-day embryo, all the segments of the limb skeleton can differentiate independently. Fell and Robison (1929) described the development in vitro of femora isolated from 5½-day and 6-day embryos and found that the femora increased in size and that the articular structures continued to develop in the absence of adjacent parts. Murray and Selby (1930) grafted femora isolated from 6-day embryos on to the chorio-allantois of other embryos. Their results were in general similar to those of Fell and Robison. In this paper, the authors review in considerable detail the literature of the subject and discuss the various factors concerned in the development of the cartilaginous skeleton, with particular reference to the femur. They conclude that while the gross form of the shaft and articular surfaces is brought into existence mainly by the action of forces intrinsic in the femur itself, which demand for their action a certain approach to normality of the surrounding conditions, an important part is played by extrinsic factors of a mechanical nature in modelling the finer details of skeletal form. On the other hand, they state that the form of the bony skeleton in the case of replacing bones depends entirely on conditions

extrinsic to the bone; it is primarily dependent on the form of the cartilaginous framework and is very liable to alteration if the mechanical conditions be changed.

As already stated, all observers do not agree that the factors responsible for the primary form of the cartilaginous skeleton are intrinsic in the limb-bud itself. Carey (1922) considers that the primary form of the cartilaginous skeleton depends entirely on mechanical conditions brought about by differential growth-rates of contiguous parts, the passive pull of soft parts retarded in growth, active muscular pull and the weight of the free part of the limb; in support of which view he adduces evidence derived from a study of the normal development. In a later paper, Carey, Zeit and McGrath (1927) describe the regeneration of the patella. These workers removed the patellas from puppies 6 to 8 weeks old and found that regeneration took place if movement of the knee-joint was permitted, the new patella passing through cartilaginous and bony stages. When arthrodesis of the knee-joint was performed, no regeneration of the patella occurred. They adduce these experiments in support of the mechanical theory of the origin of embryonic and adult bone

substance. They conclude that bone and presumably cartilage are formed by modification of a polyvalent mesenchyme cell, both the modification itself and the shape of the resulting element being determined by the mechanical conditions existing in the part.

As Murray and Selby (1930) have pointed out, however, there are at least two problems connected with skeletal development, (1) the development of the skeletal tissue, and (2) the development of the skeletal form. Carey and his co-workers appear to consider that the same forces act in both cases. In pathological conditions, cartilage and bone are certainly found to arise from mesenchyme which does not normally produce either cartilage or bone. Levi (1930) has produced cartilage experimentally in the region of the phalanges in guinea-pigs and fowls by intermittent pressure. Recently, Fischer (1931) has found in vitro that fibroblasts isolated from the heart produce cartilage when in contact with skeletal cartilage which has been freed entirely from perichondrium, the skeletal cartilage taking no part in the production of the newly-formed cartilage. It does not necessarily follow, however, that the factors which operate in regeneration, in the formation of ectopic bone, or in experimental conditions

such as described by Levi or Fischer act also in normal development. Although there is no direct experimental evidence pointing to the existence, in the embryo, of cells specifically and intrinsically determined for chondrogenesis, there is a certain amount of suggestive indirect evidence such as the experiments of Fell (1928), who found that cartilage differentiated in cultures of 3-day avian limb-bud, and those reported by Murray (1926) in which cartilage of all stages of histological development was found in grafts of 2-day limb-bud regions. Concerning the development of skeletal form, it is apparent, as already stated, from the work of Murray, that the primary cartilaginous form of an element such as the femur 'self-differentiates' when it is in conditions which are not too remote from the normal.

In the experiments recorded by Murray (1926) the appearance in grafts of structures resembling the patella is described, but later Murray and Selby (1930) state that the piece of cartilage which most resembled the patella was probably a detached piece of fibula. The patella does not normally begin to develop until the end of the 10th or the beginning of the 11th day, at which time movement is active in the embryo and the muscles

are well-developed and arranged in definite groups, that is to say, at a stage when the conditions postulated by Carey are thoroughly established. Accordingly, it was decided to ascertain whether or not presumptive patellar mesenchyme would differentiate *in vitro* when isolated from the rest of the limb skeleton, and to determine as far as possible the factors responsible for its final form.

HISTOGENESIS OF THE AVIAN PATELLA.

Since no adequate data are available, the normal development of the avian patella was investigated. It is convenient to describe the degree of development of the future knee-joint region and the bones in relation to it.at the beginning of the 9th day of incubation. The femur, tibia and fibula are well-developed and exhibit the main characteristics of form which they possess in later life. The shafts of both femur and tibia are ossified, the ossified area occupying at least one third of the total length of the bones. The fibula also shows advancing ossification; it has a well-developed head, but ends distally in a fine, tapering bony point. The muscular tissue consists of long thin fibres, arranged in definite bundles, corresponding to their future distribution in the limb.

Arising from the pelvic region and passing down on to the anterior surface of the femur is a well-defined mass of muscle which represents the great extensor group of the thigh. This ends at the upper limits of the condyles of the femur in a mass of mesenchyme which is continuous with the mesenchyme on the anterior surface of the tibia and with that lying between the ends of the bones where the knee-joint subsequently develops. Staining with thionin blue at this stage (Fig. 106), however, shows no trace of chondrogenesis. Round the epiphyseal ends of the bones, the mesenchyme has become condensed to form an actively chondrogenic perichondrium. When the ends of the bones are pulled apart, the mesenchyme often tends to separate from the perichondrium, and in fixed and stained preparations, an artificial line of cleavage is often seen between them. Histologically, this mesenchyme consists of fibroblasts separated by a very small amount of intercellular material. On the outer side of the limb, however, the tissue is less cellular and contains fine collagen fibrils arranged parallel to the long axis of the limb.

Towards the end of the 10th and the beginning of the 11th day, the epiphyseal perichondrium of both femur and tibia has become much thicker and is separated from

the surrounding mesenchyme and the developing intra-articular ligaments by well-defined spaces which represent the cavity of the knee-joint. An area of precartilage in which mitoses are numerous appears in the mesenchyme just below the termination of the extensor muscle. By the end of the 11th day, the mesenchyme surrounding this area of chondrification has become condensed to form a rudimentary perichondrium, ~~(Fig. ——)~~. By the end of the 12th day, the cartilage cells are separated by narrow but deeply staining partitions of matrix, and the chondroblasts are still multiplying by mitotic division. The whole mass is surrounded by a definitive perichondrium.

Between the 11th and 12th day (Fig. 137) the developing patella is roughly triangular in shape when looked at from the anterior or posterior aspects. The two sides are unequal in length, the outer or lateral side being shorter than the medial. They meet in a rounded apex which becomes slightly flattened out as development proceeds at the expense of the longer side. The rudiment increases rapidly in size in all directions, partly by perichondral activity and also by the formation of cartilage matrix. In the early stages of development, the anterior and posterior surfaces of the patellar

rudiment are convex, but during the 16th day of incubation two concavities develop on the posterior surface for articulation with the condyles of the femur. These are separated by a ridge which terminates below at the somewhat flattened apex of the patella. The articular surfaces are well-marked at the end of the 17th day and at this stage capillary blood-vessels and fibroblasts begin to penetrate the perichondrium and invade the cartilage. Simultaneously the development of the knee-joint and of the muscular system continues. A band of tough fibrous tissue develops between the lower part of the anterior surface of the patella and the upper anterior surface of the tibia and a mass of fatty tissue intervenes between this and the synovial membrane of the knee-joint. On the outer side, another ligament appears, which stretches to the head of the fibula.

Histologically, at this stage the chondroblasts of the patellar cartilage are of the small-celled type characteristic of hyaline cartilage and separated by a very abundant matrix. The perichondrium remains chondrogenic, and the connective tissue cells which accompany the invading blood vessels also form cartilage. As development proceeds, the patella increases in size.

The formation of deeply staining cartilage matrix continues, but the chondroblasts retain their small-celled character. At the 11th week after hatching, ossification begins. The perivascular fibroblasts cease giving rise to cartilage cells, and in the centre of the patella the cartilage cells around the blood vessels become hypertrophic and vacuolated. Calcium salts demonstrable with alizarin and silver nitrate are deposited in the matrix in these hypertrophic areas. The fibroblasts surrounding the blood vessels now assume the character of osteoblasts and lay down bone. Erosion of the cartilage takes place rapidly, and soon the whole patella, with the exception of the articular surfaces, becomes replaced by trabecular bone in the interstices of which haematopoiesis is active. This calcification of the hypertrophic cartilage is interesting in view of the fact that in normal osteogenesis of the long bones of the fowl (femur, tibia, fibula, humerus) calcification of the hypertrophic cartilage does not occur. In mammalian long bone development, it is a constant feature. In both birds and mammals, the hypertrophic cartilage cells appear identical; but, as judged by staining reactions, the quantity and possibly the quality of the cartilage matrix varies. The cartilage

of the long bones of mammals differs from those of birds in the greater quantity of matrix and in the fact that it stains more intensely with aniline dyes; the avian patella, on the other hand, has a very abundant matrix which stains deeply with aniline dyes.

METHODS.

The methods employed in this investigation consisted essentially (1) in the isolation from the limb skeleton and cultivation *in vitro* of the tissue in which the patella subsequently develops, and (2) in the cultivation of the entire knee-joint region. The tissue was taken from embryos at the beginning of the 7th, 8th and 9th days of incubation.

Perichondrium of developing articular surfaces gives rise to cartilage when cultivated *in vitro*, and so great care must be taken to prevent its inclusion in the explant. At the beginning of the 9th day of incubation, this perichondrium is fairly well-defined, and it is possible to remove the mass of tissue lying between the condyles of the femur without injuring or tearing it. With embryos of this age, the following procedure was adopted. The limbs were removed from the pelvic girdle and the skin dissected off. A transverse incision was made at the lower end of the extensor

group of muscles, avoiding injury to the perichondrium, and another in the region between the femur and the tibia and the fibula. The mass of tissue between these two cuts was carefully separated from the long bones and from the surrounding tissue. The future patellar tissue from 7- and 8-day embryos was removed in the same way, but greater difficulty was experienced in preventing injury to the epiphyses. Explants of the whole knee-joint were obtained by removing the skin and cutting through the muscles and bones a little beyond the epiphyses. The tissue thus removed, which was about 1½ mm. square in size in the case of 9-day embryos, less in those younger, was then explanted on large coverslips (1¼ in. square) so that either the anterior or posterior surface was uppermost. The medium used consisted of equal parts of fowl plasma and embryo extract prepared from a 9-day embryo. After the plasma had coagulated, the coverslips were inverted over large hollow-ground slides and sealed with paraffin in the usual way. The cultures were transferred to fresh medium every 48 hours. The zone of outgrowth was removed, the centre of the explant being kept intact. In some cases, the tissue was cultivated by the method already described which was used by Fell and Robison

(1929) for the cultivation of isolated femoral rudiments. The explants were placed on the surface of a clot, contained in a small watch-glass, consisting of equal parts of plasma and embryo extract. The watch-glass was enclosed in a Petri dish and was surrounded by cotton-wool saturated with sterile water. As before, the cultures were transferred to fresh medium every 48 hours, the zone of outgrowth being removed while the centre of the explant was kept intact. Whole knee-joint cultures were always cultivated on watch-glasses. The cultures were fixed in Zenker's fluid plus 2 per cent. acetic acid for quarter to half an hour after varying periods of cultivation in vitro. In the case of coverslip cultures, some of the cultures were sectioned serially either at right angles to, or parallel with, the plane of the coverslip, i.e. in either the coronal or the sagittal plane, and the sections stained with Mallory's triple stain or safranin picro-indigo-carmine. Others were mounted whole and stained with thionin blue. Watch-glass cultures were always sectioned serially in the sagittal plane.

The knee-joint region from which the tissue was taken was fixed either in Bouin's fluid or in Zenker's fluid plus 2 per cent. acetic acid, and serial sections

were examined histologically in order to make sure that chondrogenesis had not begun before explantation. The sections were in many cases stained with thionin blue, a stain which demonstrates chondrogenesis in the very earliest stages. Thus every culture had two controls. Wax reconstructions of cultures and of stages in the normal development of the patella were also made.

THE DEVELOPMENT in vitro OF THE AVIAN PATELLA.

Since the most interesting results were obtained from cultures of presumptive patellar tissue from embryos at the beginning of the 9th day of incubation, these will be described first.

Appearances of Cultures from 9-day Embryos.

The tissue when first explanted consists of a roughly rectangular mass of mesenchyme about 1¼ mm. square in size, in the centre of which no structure can be made out. Attached to one side are a few strands of muscle which represent the lower end of the extensor group. After 24 hours' cultivation in vitro the tissue has spread out slightly on the coverslip and there is a considerable emigration of fibroblasts and mononuclear wandering cells into the medium; this emigration increases during the subsequent 24 hours.

The muscle grows out in the form of long thin multi-nucleated strands which show no trace of cross-striation, although the tissue from which it has arisen is already striated. No trace of contraction of muscle strands was ever seen, and after three or four subcultures the muscle is no longer present in the zone of outgrowth. A number of degenerate cells are often present during the first three or four days' cultivation both in the explant and in the zone of outgrowth. These cells have probably been injured during the process of dissection; as a rule they disappear after the second or third sub-culture. On the third day of cultivation, a translucent area is generally seen at that end of the explant to which the muscle is attached. This indicates the commencement of chondrogenesis, but the details of this cannot be made out owing to the size and thickness of the explant. As cultivation proceeds, this area increases in size and translucency, and by the sixth day of cultivation it has a very definite roughly triangular form (Fig.25). The shape of the cartilage is well seen in 'whole mount' preparations stained with thionin blue (Fig.25). The base of the triangle is slightly concave, and to it are attached the remains of the muscle removed during the original dissection. The other two

sides are unequal in length, the short one being about one half the length of the longer. They meet in a rounded apex. The longer side is often slightly concave near the base. The cartilage increases in size and the shape of the mass persists as a rule for at least twenty days in vitro. After that time, however, it tends to become oval in shape and loses its typical outline, although some cultures have retained their triangular form for at least seven weeks. The cartilage mass is very precisely demarcated from the rest of the explant and does not extend in a diffuse manner into it. Both upper and lower surfaces of the cartilage are slightly convex and show no irregularity of contour during the culture period.

In some cultures, two separate areas of chondrification were found which were quite distinct from one another. One was always triangular in shape and was generally larger than the other which was rounded or oval in form. The triangular mass was always in direct relation to the muscle of the explant and probably represented the patellar rudiment, while the other had no constant position in the explant. In such a culture, the control usually showed that the epiphyseal perichondrium had been injured to a considerable extent. Where the injury was slight, second centres of chondrification were never found.

In such cases, the cartilage-forming cells probably emigrated into the medium and formed part of the zone of outgrowth where multiplication was active and the conditions unfavourable for differentiation. As the zone of outgrowth was always removed at each sub-culture and was not transplanted with the original explant, they did not become incorporated in the central mass where the circumstances are favourable for chondrogenesis.

Histological Appearances.

As already described, the cultures were fixed after varying periods of cultivation, and examined histologically either as whole mounts or in serial sections. After three days' cultivation, an area of early cartilage is generally to be seen. This consists of a zone of compactly arranged cells multiplying by mitotic division and separated by a small amount of intercellular material. It is roughly triangular in shape, the base of the triangle being always in close relation to the muscle which represents the lower end of the extensor group. At this stage the margins of the area of chondrogenesis are not sharply demarcated by a perichondrium from the surrounding tissue. (Fig. 30). A section through the knee-joint region from which this tissue was taken is shown in Fig. 28. As will be seen, the femoral and

tibial perichondria are intact, and examination of the whole series of sections reveals no injury at any point. Fig. 130 shows the knee-joint region of the other side from which no tissue was taken. The section is through the centre of the situation of the future patella and no trace of cartilage is present. The cartilage, therefore, has apparently differentiated in the culture, from the undifferentiated presumptive patellar tissue.

In a culture examined after six days' cultivation, the cartilage cells are separated by a fairly abundant matrix and the whole area is surrounded by a perichondrium which contains white fibres. From this stage onwards, increase in size of the cartilage mass is brought about mainly by the activity of the perichondrium and by the production of cartilage matrix. The chondroblasts themselves only show occasional mitoses. As already mentioned, the cartilage always appears in a definite position in the explant immediately below the muscle. The perichondrium consists of two or three layers of cells and is well demarcated from the rest of the tissue. On the longer side, however, the perichondrium is somewhat diffuse externally and tends to fade into the surrounding tissue, which consists of fibroblasts separated by fine collagen fibrils, and amongst which

fine thin-walled vessels persist for a considerable period. The limits of the cartilage, however, are always well defined. The muscular tissue of the original explant does not continue to develop. The muscle substance apparently undergoes some form of degeneration resulting in the formation of bundles of multinucleated cells with somewhat bulky cytoplasm, which are separated by fairly large spaces. The exact nature of this change is unknown, but the condition is quite obvious after the eighth day of cultivation. If the presumptive patellar tissue is explanted on a plasma clot in a watch-glass, the arrangement of muscle, developing patella and the sharp demarcation of the latter from the surrounding tissue is well seen after 6 days' cultivation owing to the manner in which the tissue spreads out on the surface of the clot. In some cultures, branched pigment cells appeared in the explant after several days' cultivation. These were not present at explantation and represent a differentiation of previously unpigmented elements.

The cartilage remains healthy for at least three weeks <u>in vitro</u>. After that time, areas of necrosis are often found in the centre of the cartilage, although in some cases it has been found healthy after 38 days. The

degeneration of the cartilage is probably due to the large size of the explants and the formation of a thick connective tissue capsule round the explant, which prevents diffusion of nutritive substances from the medium.

A series of cultures was maintained for eight weeks *in vitro*. The zone of outgrowth remained healthy and was derived from the thick connective tissue capsule which developed round the explant. In most of the cultures, the cartilage mass became oval in shape and was partially or completely necrotic at the end of the culture period, the surviving cartilage cells being of the small-celled type. No invasion of the cartilage by fibroblasts occurred, and it is presumed that the presence of blood vessels is necessary for the invasion of the cartilage. No trace of ossification was present; this was to be expected, as the length of the period of cultivation was less than that which normally elapses before ossification of the patella begins. The development of fat between the lower part of the posterior aspect of the patella and the synovial membrane of the knee-joint *in vivo* has been mentioned. In the cultures, the region corresponding in part to this area remained very cellular throughout the period of cultivation, but no transformation of fibroblasts into fat cells occurred.

In these cultures in which two centres of chondrification appeared, the smaller centre consisted of small-celled cartilage and was always surrounded by a perichondrium which was uniform in arrangement round the nodule, and no connection was ever found between it and the larger triangular mass.

The Form of the Patellar Cartilage which differentiates in vitro.

The triangular form of the cartilage mass which differentiated in vitro has been described. In all cultures of tissue isolated from 9-day embryos (81 specimens) the shape of the cartilage was more or less the same at least up to the twentieth day of cultivation. Variations in size were sometimes found, the mass remaining small in some cases. The concavity of the upper margin was slightly variable, being deeper in some cases than in others. Occasionally the difference in length between the two sides was diminished. (see fig. 134.) Apart from these minor differences, however, the form was remarkably constant.

As already mentioned, each culture had two controls, the knee-joint region from which the tissue was taken and the intact knee-joint region of the other side. All these controls were examined histologically. In the

intact knee-joints no trace of chondrogenesis at the site of the future patella was ever observed, showing that chondrogenesis must have taken place during cultivation in vitro. In the knee-joints of the side from which the tissue was taken fairly extensive tearing of the epiphyseal perichondrium of the femur or tibia was present in 18 specimens, out of 81. In only 5 cultures, however, were additional nodules of cartilage present. In 13 specimens, the femoral or tibial perichondrium had been separated but not removed from the cartilage. In none of the corresponding cultures were additional nodules of cartilage found. It is, therefore, to be concluded that the differentiated cartilage represents the patellar rudiment and has not arisen from epiphyseal perichondrium.

In normal development, as already stated, the patellar cartilage remains triangular in shape until the end of the 15th day of incubation. (See fig. 151.) The anterior and posterior surfaces are convex, the former being rather more rounded than the latter. Between the 16th and the 17th day, two well-marked concavities appear on the upper part of the posterior surface. Wax reconstructions were made from serial sections of the normal patella taken at the end of eleven and of fourteen and a half

days' incubation. Photographs of these are shown in Figs. 137, 138, ~~139 and 140.~~ The anterior surface is uppermost, and to the upper concave margin the extensor muscle was attached. The inequality of the two sides is obvious and in the older rudiment the smaller (lateral) side is slightly concave. Reconstructions were also made of the patella from 17- and 19-day embryos. These showed the presence of the concavities for articulation with the condyles of the femur. Figures 139, 140 are photographs of reconstructions made from serial sections of cultures after 14 and 20 days' incubation which were chosen at random from the series. The anterior and posterior surfaces of both are convex, and as will be seen, they are similar in shape and resemble also the reconstructions of the patella from 11- and 14½-day embryos. The most obvious difference between them and the patella from the 14½-day embryo is the absence in the cultures of a small concavity on the shorter or lateral side. They differ, however, from the reconstruction of the patella from the 17-day embryo in that they show no trace of articular surfaces. In a reconstruction of a 38-day culture, two projections were seen on one surface. Between them was a shallow depression, the depth of which was 70 μ. as estimated

from the number and thickness of the sections. In this culture, considerable necrosis of cartilage was present. The structures did not correspond in position to the situation of the articular surfaces, and it was decided that the condition was due to fixation shrinkage of the necrotic cartilage. The presence of some form of irregularity was detected in the sections by the presence of two areas of cartilage. In no other culture, except when an additional nodule of cartilage was present, was a similar appearance found in section.

From these observations, it is apparent that the cartilage has arisen from mesenchyme destined to form the patella and that the shape of the cartilage mass resembles that seen in the primary stages of the development of the patella. The resemblance ceases, however, when the articular surfaces begin to form.

Cultivation of entire knee-joints from 9-day embryos.

Since articular surfaces for the condyles of the femur do not develop in the patellar rudiment which has differentiated in vitro, the entire knee-joints from a series of embryos at the beginning of the 9th day were cultivated on watch-glasses in order to ascertain if the presence of the condyles of the femur would bring about the formation of concavities. Degeneration of the

explants took place very rapidly, however, owing to their large size and no differentiation of the patella occurred.

Appearances of cultures of tissue from 7- and 8-day embryos.

It was then decided to investigate the earliest stage at which the patellar cartilage would differentiate under the conditions of cultivation in vitro. For this purpose, the presumptive patellar tissue was removed from embryos at the beginning of the 7th and 8th days of incubation. In all cases, cartilage appeared in the explants from 8-day embryos, generally on the 3rd or 4th day of explantation. The shape of the cartilage mass was often, however, irregular, sometimes round, sometimes oval. In 50 per cent. of the cultures, however, it resembled that seen in the cultures from 9-day embryos. Two centres of chondrification which were quite distinct from one another often developed. This was due partly to the greater difficulty of removing the tissue at this stage and also to the fact that the epiphyseal perichondrium is not very sharply marked off from the surrounding tissue, and the small-celled cartilage of the epiphyses merges imperceptibly into the tissue intervening between the ends of the bones. Thus potentially epiphyseal

cartilage-forming tissue was probably removed along with future patellar tissue. It was thus not always possible to say whether or not the cartilage had arisen from future patellar tissue. The triangular form of the patella, in the case of tissue from 8-day embryos, certainly does not differentiate invariably, as it does in the case of tissue removed from 9-day embryos.

In the tissue isolated from the inter-condylar region of 7-day embryos, cartilage always appeared after 2 or 3 days' cultivation. The mass never resembled the shape of the patellar cartilage. Histological examination of the knee-joint region at this stage revealed the fact that the small-celled cartilage of the epiphyses merged imperceptibly into the surrounding tissue, and it was impossible to determine the limit of the tissue destined to form epiphyseal cartilage.

Since indubitable patellar cartilage does not differentiate in the tissue removed from the patellar site of 7-day embryos, the whole future knee-joint region from such embryos was cultivated in vitro in order to see if patellar cartilage would differentiate itself from the mass of chondrogenic tissue surrounding the developing epiphyses. The tissue was explanted on watch-glasses so that the anterior surface was upwards, and this position was preserved throughout the culture period. The explants

grew rapidly in size, but separation of the developing articular surfaces did not take place. The epiphyses fused, the patella did not differentiate, and the whole knee-joint, after 8 days' cultivation was represented by an irregular mass of cartilage. Degeneration of the cartilage took place very rapidly owing to the large size of the explants.

Cultivation of part of the Patellar Mesenchyme.

Murray (1926), found that in a number of grafts derived from transverse halves of limb-buds, the cartilaginous shafts are incomplete, ending usually in a blunt point. Histologically the cartilage was of the hypertrophic and vacuolated type found in the centre of the developing skeletal rudiment. He obtained a similar result in a graft of the posterior limb regions of a 2-day embryo, and considers that this incompleteness is due to the cut which divided the original bud or tissue having traversed the anlage of the skeletal element concerned. He is of the opinion that the anlagen of the cartilaginous elements of the long bones of the limb-bud are each a mosaic with respect to the kind of cartilage to be produced from each part, and that, accordingly, no one part is capable of regenerating the whole. Since, however, the patella develops relatively late in embryonic

life and the cartilage remains of the small-celled type for at least 12 weeks after chondrogenesis has begun, it was of interest to determine whether or not in embryos at the beginning of the 9th day of incubation the patella is also a mosaic system.

The future patellar tissue was removed from both limbs of embryos at the beginning of the 9th day, the knee-joint regions being kept as controls. Only those cultures which had controls showing no trace of injury to the epiphyseal perichondrium were studied. The tissue from one side was kept intact and explanted on coverslips in the usual way, and that from the other was divided longitudinally into two parts as nearly equal as possible. These parts were explanted separately. In the divided explants, the zone of outgrowth was preserved at subculture for the first three passages. In the entire explants, the cartilage differentiated as usual, forming a triangular mass. In the divided explants, a centre of chondrification usually appeared in each. One of these was always larger than the other and was generally oval in shape, while the smaller was generally rounded (Figs. 135, 136). The larger covered an area at least four times that of the smaller. In no case did the form of the cartilage mass which developed in a

divided explant resemble the shape of that which appeared in the undivided explant (Fig. 134). The sum of the total amount of cartilage obtained in the divided explants was always less than that obtained in the undivided explant from the other side of the embryo. This is probably accounted for by the injury to the cartilage-forming tissue during the cutting. In addition, some of the cartilage-forming cells probably emigrated into the medium during the first passage and may not have become incorporated in the part of the explant where differentiation takes place. The inequality in size may be explained by the division of the tissue into two parts which, though approximately equal in size, are unequal as far as cartilage production is concerned. In some cases, a centre of chondrification appeared in one explant only. This was always larger than was the case when two centres were found, but was never so large as the cartilage in the undivided explant, nor had it the triangular shape of the other. In such cases it was always noticed at the time of explantation that the parts were unequal in size.

It seems likely in those cases in which cartilage appeared in both explants that the smaller nodule represents that part of the patella medial to a line drawn at

right angles to, and bisecting, the upper margin; while the larger mass represents that part of the patella lateral to that line. Since, however, the two pieces when placed together have not the form of the normal patellar cartilage in vitro, it cannot be definitely stated that the patella is a mosaic. The only conclusion to be drawn from this experiment is that the tissue in which the patella develops is, as a whole, a mosaic in which the non-cartilage forming part cannot replace any of the cartilage-forming tissue.

DISCUSSION.

The patella is still regarded by many workers as a sesamoid bone which develops in tendon and is comparable to the structure found in the neighbourhood of the interphalangeal joints of many animals. The work of de Vries (1909)*, who made a comparative study of the development of the patella in different animal species, showed that the cartilaginous rudiment of the patella develops in a cellular mesenchyme in a manner histologically similar to that of the cartilage of the rest of the limb-skeleton This finding has been confirmed for the avian patella.

As mentioned in the introduction, Murray and Selby (1930) consider that the gross form of the cartilaginous

* Quoted by Keith, Human Embryology. Reference not available.

skeleton and of the articular structures self-differentiates and they consider that these principles are probably applicable to the rest of the limb-skeleton. Concerning the self-differentiation of cartilaginous forms they state, however, that their data deal mainly with the femur, and they have no evidence concerning the influences which regulate the development of 'negative' (concave) articular surfaces. In addition, these workers show that there is no direct evidence which demonstrates conclusively that cartilage as a tissue differentiates in the absence of extrinsic conditions.

Carey (1922), from experimental and morphological investigations of a different kind, concludes that the development of cartilage as a tissue and the development of the primary form of the cartilaginous skeleton depends entirely on mechanical conditions present in the part. The results of the present investigations do not give any additional information concerning the conditions determining the differentiation of cartilage. They show, however, that the tissue in which the patella develops is of such a nature that cartilage develops in it when it is isolated from the limb-skeleton and from the mechanical conditions existing therein. It might be considered that the presence of muscle in the explant

by exercising tension on the mesenchyme would favour the formation of cartilage. The muscle, however, was scanty in amount and much of it grew out into the medium and was removed at the first subculture: it did not preserve its histological identity and showed no functional activity. In some cultures the muscle was almost completely removed and the cartilage differentiated in the usual way.

The shape of the patellar cartilage which develops in vitro resembles closely that seen in the early stages of normal development in vivo. Such an almost geometrical form is never seen in nodules of cartilage which develop in vitro in cultures of 3-day limb-buds or in cultures of perichondrium. It is apparent, therefore, from the observations recorded that the primary form of the patella 'self-differentiates' in vitro in the sense stated by Murray and Selby (1930).

A significant feature was the absence of the development of the concave articular structure for articulation with the condyles of the femur. The non-appearance may have been due to the slowing down of the developmental rate which occurs in vitro. Even after 7 weeks' cultivation both on coverslips and in watch glasses, however, no trace of articular surfaces was seen in patellar cartilages which had

preserved their primary form *in vitro*. It appears, therefore, that in the case of the patella the presence of contiguous elements, in this case the condyles of the femur, is necessary for the development of the articular surface. The 'negative' surface thus possibly differs from a 'positive' surface like the head of the femur in that it requires for its development the presence of the corresponding surface.

The mesenchyme destined to form the patellar cartilage seems to be isolated from the surrounding tissue at a relatively late stage of development. Only after the formation of epiphyseal cartilage begins to be limited to a definite zone of cells can indubitable patellar-forming mesenchyme be obtained. The development of the patella *in vivo* does not appear to take place until almost three days after this has occurred.

SUMMARY.

1. The normal development of the avian patella has been described. Chondrogenesis begins at the end of the tenth and the beginning of the eleventh day of incubation. The articular surfaces for the femur do not begin to appear until the sixteenth day. Shortly after this, the cartilage is invaded by capillary blood-vessels and fibroblasts. Many of these perivascular fibroblasts

become transformed into chondroblasts and give rise to cartilage. About the twelfth week after hatching, the perivascular formation of cartilage ceases and the cartilage round the blood-vessels hypertrophies: in the matrix of this hypertrophic cartilage calcification takes place. Simultaneously, bone is formed around the blood-vessels by the perivascular fibroblasts which have become transformed into osteoblasts.

2. The mesenchyme from 7-, 8- and 9-day avian embryos from the site in which the patella develops was cultivated in vitro. Cartilage appeared in the explants in all cases. In tissue from 9-day and from some 8-day embryos the form of the cartilage mass resembled that seen in the early stages of normal development. This was not the case with the tissue from 7- and some 8-day embryos.

3. Articular surfaces did not appear in the cultures even after 7 weeks' cultivation.

4. In cultures of the knee-joint region, including the ends of the long bones, from 7-day and 9-day embryos, the patella did not differentiate. This is considered to be due to the large size of the explants which prevented adequate nutrition of the tissue.

5. In cultures of parts of patellar mesenchyme, cartilage appeared in vitro, but the size of the cartilage mass was smaller, and it did not resemble that which developed in cultures of the undivided patellar mesenchyme. The tissue in which the patella develops is a mosaic of cartilage-forming and non-cartilage-forming tissue, and no one part is capable of regenerating the whole.

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THE FORMATION OF HAEMATOIDIN in vitro AND in vivo.

During a preliminary study of the appearances found in cultures of newly-born mouse liver, the phagocytosis of red blood corpuscles derived from the explant by cells of the macrophage type was frequently observed. When the cultures were maintained for periods up to 10 days, changes sometimes took place in the phagocytosed corpuscles, resulting in the formation of crystals of haematoidin inside the cells. The behaviour of macrophages derived from the spleen and subcutaneous tissue towards red blood corpuscles was then investigated and the whole process, from the stage of phagocytosis to the appearances of crystals of haematoidin, was studied. The formation of haematoidin in vivo was also studied.

Methods.

Cultures of liver, spleen and subcutaneous tissues of newly-born mice up to one week after birth were used. As it was essential that the cultures should remain undisturbed during the culture period, and, in order to provide optimum conditions for examining the cells during life, the cultures were prepared in hanging-drop preparations, a fluid medium, consisting of equal parts of rat serum and mouse embryo extract, being employed. After the first two days of cultivation, the medium was renewed either every day or every second

day, as appeared necessary. To this medium at explantation, a small drop of a suspension of mouse red blood corpuscles was added. The suspension was obtained by washing defibrinated mouse blood free of serum and suspending in buffered saline, the volume of saline added being about one half of the total volume of the blood from which the corpuscles were derived. In other experiments, lysed corpuscles (haemoglobin in solution) were added to the medium in the proportion of 2 drops of medium to 1 drop of lysed corpuscles.

Appearances seen in Cultures of Liver.

The cultivation of liver tissue has been described on p.195. As will be remembered, wandering cells of the macrophage type emigrate from the liver in large numbers. They show the typical segregation apparatus when stained with neutral red, and during cultivation they undergo hypertrophy, and the intra-cellular granules and vacuoles in the cells increase in number. In all probability, they are derived mainly from the Kupffer cells of the liver as in cultures of liver derived from young mice, in which the Kupffer cells were stained intravitam with trypan blue, numerous dye-containing macrophages were found: the macrophages of the portal tracts

probably also migrate into the medium. The red blood corpuscles soon become entangled in the processes of the macrophages and come to lie inside the cytoplasm. Surrounding the now intra-cellular corpuscle a very narrow ring of clear cytoplasm is often seen. At the end of the second day of cultivation, most of the cells contain phagocytosed red blood corpuscles, as many as ten being sometimes seen in one cell. Dead polymorpho-nuclear leucocytes and lymphocytes are also ingested by the macrophages.

Changes in the intra-cellular corpuscles then begin, usually towards the end of the second day of cultivation, sometimes later, sometimes earlier. These changes may take place in one of two ways. In some cells, the corpuscles appear to dissolve in the cytoplasm resulting in a diffuse golden-yellow coloration of the cytoplasm. In other cells, the corpuscles may shrink, assume bizarre shapes and become slightly brownish in colour. Whatever the preliminary stages, golden-brown crystals of haematoidin appear in the cytoplasm from the 4th day of cultivation onwards. (Fig. 124). The appearance of haematoidin crystals has been seen as early as the 3rd day, and as late as the 9th day, of cultivation. The crystals do not occupy a constant

position in the cell and vary considerably in size. The smallest ones are usually rhomboidal, the largest, which may be 5 μ in length, have the shape of parallelograms. As a rule, they remain intra-cellular but occasionally I have observed small crystals being extruded from the cell.

If such a culture is fixed, dehydrated and cleared in xylol, the crystals are dissolved out immediately by the clearing agent. Attempts were made to apply the van den Bergh reaction in order to identify the golden-yellow pigment which often appears in the cells before the crystals. These, however, were completely unsuccessful.

Crystals of haematoidin were occasionally observed in the liver fragment itself. They generally appeared after crystal formation had begun in the zone of out-growth. Owing to the thickness of the explant, the process could not be followed in detail but the yellowish coloration sometimes seen in the emigrated cells was often observed there. The crystals in the explant are probably derived either from material already present in the Kupffer cells before explantation, or from red blood corpuscles remaining in the capillaries which were phagocytosed by persisting Kupffer cells.

Haematoidin formation did not take place in all liver cultures, although phagocytosis of the red blood corpuscles always occurred, and the conditions determining its occurrence were not determined.

Appearances in fixed and stained preparations.

Cultures were fixed after varying periods of cultivation and the prussian blue reaction was applied to the cells. Various stages in the formation of haemosiderin were found within the cells. After 3 to 4 days' cultivation, many of the macrophages showed a diffuse blue coloration of the cytoplasm. In such cells the red blood corpuscles could no longer be detected in the cytoplasm. In others, those in which the corpuscles had become shrunken, deeply coloured granular masses appeared (see fig. 123) and many of these contained darker granules in their interior. Localised blueing of the cytoplasm was also seen in such cells. After haematoidin crystals had appeared, haemosiderin could be demonstrated in the form of granules. Occasionally, in cells in which it was known that crystals had just appeared before fixation, the application of the prussian blue reaction resulted in the formation of a coating of iron-containing substance round the crystals. This appearance was found in cells in which shrinkage of the intra-cellular corpuscles had preceded the appearance of haematoidin.

Outgrowths of liver epithelium, fibroblasts, mesothelium and endothelium occurred in many of the cultures. None of these types of cells phagocytosed red blood corpuscles, and in none of them did haematoidin appear.

Appearances in cultures of Spleen.

The growth characteristics of splenic cultures are described on p. 211. The cultures were cultivated in the same way, red blood corpuscles being added to the medium. Macrophages emigrated in large numbers and the phenomenon of phagocytosis, resulting in the appearance of haematoidin crystals, took place. All the cells in which haematoidin appeared gave the typical macrophage reaction with neutral red. In the central fragments, as in the liver cultures, haematoidin crystals sometimes appeared after 6 to 8 days' cultivation, and these, as in the case of the liver, were probably formed either from material present before explantation, or from red blood corpuscles which were phagocytosed by the splenic macrophages. As in liver cultures, haematoidin formation did not always occur although phagocytosis was always marked.

Subcutaneous Tissue.

Although the subcutaneous tissue of the mouse contains a large number of histocytes, they are often scanty in cultures of subcutaneous tissue, and many cultures had to be prepared before typical haematoidin

formation was observed. The formation of haematoidin was similar to that described in liver and spleen cultures. Haemosiderin could also be demonstrated in the cells. Fig. 123 shows two macrophages each containing haematoidin and haemosiderin. As will be seen, the cells are separating after mitotic division. The fibroblasts did not participate in the formation of haematoidin, and haemosiderin was not found in the cytoplasm.

A suspension of red blood corpuscles was then lysed by freezing and added to the culture medium in order to ascertain whether or not the macrophages would form haematoidin intra-cellularly from haemoglobin in solution. In none of the cultures, out of a series of 170, were haematoidin crystals observed in the macrophages, except in cells which had phagocytosed red blood corpuscles derived from the explant .

No accurate information is available concerning the time which usually elapses between the occurrence of a haemorrhage and the appearance of haematoidin crystals. The only observations so far found are those of Durck (1892). Durck produced cerebral injuries

in guinea-pigs and rabbits and examined the sites of injury after varying periods. Haemosiderin began to appear a few days after the injury, but haematoidin crystals were not detected until about 50 days later. By this time, the haemosiderin had disappeared. The time taken before the appearance of haematoidin was thus very much longer than in my experiments in vivo. Since the subcutaneous tissue of mice contains a large number of macrophages, a series of animals was injected with 0.5 c.c. of washed corpuscles and killed at varying intervals after the injection, in order to determine the time elapsing between the injection and the appearance of haematoidin in vivo. Spreads of the subcutaneous tissue were examined in the fresh state, and after fixation and staining. The cells were also tested for iron with the prussian blue reaction. 3 days after inoculation, many cells at the site of the injection contained phagocytosed red blood corpuscles, and these cells reacted like macrophages with neutral red. 6 days after inoculation, the macrophages showed a diffuse iron reaction. 2 days later, crystals of haematoidin were found in the cells and these, though larger, were similar in shape to those found in macrophages in vitro. All these haematoidin-containing cells showed small granules of haemosiderin as well as a diffuse blue coloration of the cytoplasm.

No extra-cellular haematoidin crystals were found, and the iron reaction was limited strictly to the cells. 18 days after the commencement of the experiment, haematoidin crystals were found lying free in the tissue between the cells and haemosiderin granules were also demonstrable in this situation. Whether these substances were formed extra-cellularly or were released from dead macrophages could not be ascertained.

The process of formation of intra-cellular haematoidin, up to the time of appearance of the crystals, is thus similar *in vivo* and *in vitro*, and takes approximately the same time.

Since haematoidin did not occur *in vitro* when lysed corpuscles were added to the medium, a series of mice was injected with lysed whole blood, lysed corpuscles, and haemoglobin from which the stromata of the corpuscles had been removed by centrifuging. In no case did haematoidin appear in the macrophages, although after varying lengths of time, haemosiderin, first in a diffuse, and later in a granular, form could be demonstrated. It is apparent, therefore, that the conditions of cultivation of macrophages *in vitro* are not solely responsible for the absence of haematoidin formation when the cells are in the presence of haemoglobin in solution.

The description given above of the phagocytosis of red blood corpuscles by cells of the macrophage type, and the subsequent production within the cell of haematoidin, and an iron-containing moiety, confirms the results of Rich, (1924). Rich observed the formation of bile pigment in avian subcutaneous tissue macrophages which had phagocytosed red blood corpuscles. The bile pigment was present either in the form of 'bilirubin' = 'haematoidin', or as biliverdin, which coloured the cell diffusely. An iron-containing portion was also present. Rich never observed phagocytosis of red blood corpuscles by ectodermal or endodermal cells and concluded, so far as his evidence goes, that only the cells of the reticulo-endothelial system are concerned in the production of bile pigment, or its isomere, haematoidin. He did not observe any indication of extra-cellular bilirubin or haematoidin formation.

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CYTOLOGICAL CHANGES FOLLOWING MILD TOXIC ACTION in vitro WITH SPECIAL REFERENCE TO THE MITOCHONDRIA.

A healthy living cell growing in vitro shows the structure which has been detailed on pages 121 to 125. The nucleus appears homogeneous and contains one or more nucleoli. The cytoplasm is also homogeneous and in it are embedded the mitochondria and one or more fat globules which are of the nature of neutral fat. The centriole may or may not be present. In the case of epithelial cells, cell boundaries cannot be made out in the living state. If a healthy kidney epithelial cell is stained supravitally (p.87) with a 1/25,000 solution of Janus Green B, the mitochondria take up the dye and an appearance similar to that seen in Fig. 155 is produced. The mitochondria are long and filamentous, only a few rod-shaped, and one or two granular, forms being present. When neutral red is applied to an absolutely healthy kidney epithelial cell, no vitally stained granules are to be seen. If a similar healthy cell is fixed in osmic acid and stained, after appropriate treatment, with Altmann's fuchsin and methyl green (p.85) the appearances seen in Fig. 160 are produced. The mitochondria, which, as in Fig. 155, consist mainly of slender filaments and a few rod-shaped forms, are stained pink with the fuchsin. The nucleus

is stained a greenish-pink colour, the nucleolus standing out prominently. Spaces in the cytoplasm where fatty droplets have been dissolved out by the clearing agent are to be seen. When fixed in formalin and stained with Nile Blue sulphate A, the fatty droplets stain bright pink and consist of neutral fats only, (Fig. 161).

During cultivation in vitro, degenerative changes of varying degree of severity are liable to take place (p. 129). These degenerative changes have been described by W.H. Lewis, (1919) and consist in the appearance of granules and vacuoles which stain intensely with neutral red. According to Lewis, the granules and vacuoles are derived from the cytoplasm and are not formed by transformation of the mitochondria. The mitochondria also undergo degenerative changes but these are of a different nature, and have no relation to the granules and vacuoles aforementioned. During a study of kidney epithelial cells, it was found that, in contrast to fibroblasts, vacuolation of the cytoplasm did not often occur, the degenerative changes consisting mainly in the appearance of granules of varying size. These granules appeared to accompany, and result from, mitochondrial changes. Kidney epithelium thus provides suitable material for the investigation of the origin and nature of ~~the~~ granules which appear during degeneration.

It has the additional advantage that it can be cultivated readily in fluid media on coverslips, and so can be easily examined during life. The spread out character of the cells is very suitable for subsequent cytological staining. Accordingly, the effect of a mild toxic agent on kidney epithelial cells was studied, and the resulting cytological changes investigated.

Methods.

Cultures of fragments of kidney of newly-born rats were prepared in a fluid medium (p.72). After 16 to 18 hours' growth, fairly extensive epithelial sheets had formed in many of the cultures. These were examined carefully and only those which appeared healthy as judged by the standards laid down on p.365, were chosen for experiment. In order to produce slight toxic effects over a prolonged period, it was found convenient to expose the cells to the action of volatile substances placed upon the hollow of the hollow-ground slide upon which the culture rested. Phosphorous dissolved in olive oil was first used. The P_2O_5 given off by the phosphorous was dissolved in the medium,

however, rendering it more acid, and this produced too rapid degeneration of the cells. Chloroform and carbon tetrachloride dissolved in olive oil were then tried and the latter proved to be the most convenient in use. The cultures were examined in the living state, after supravital staining with Janus Green B in a dilution of 1/25,000 and neutral red in a dilution of 1/40,000, and after fixation and staining by the usual methods.

Appearances in Cells exposed to Mild Toxic Action in vitro

The first observed effect was a slight swelling of the mitochondria which rendered them more visible under ordinary illumination. This occurred about 2 hours after the beginning of the experiment.

After about 4 hours, they break up into short rods and granules by a process of transverse fission. At this stage, they stain intensely with Janus Green B (Fig.156). After this state has been reached, variations in the individual mitochondria in a single cell take place, provided the toxic action is not too intense. Some may persist in the condition described above for a considerable time, others undergo further changes. These changes consist of (1) swelling and fusion of the altered mitochondria, resulting in the formation of "granules", (2) alteration in staining reaction to

Janus Green B, and neutral red, and (3) the appearance of fatty globules inside the fused mitochondria. The extent of fusion of the mitochondria varies in different parts of the same cell, so that small and large "granules" are formed. If such a cell is stained with a mixture of Janus Green B and neutral red, both rod-shaped and granular mitochondria are stained by the Janus Green. The "granules", however, which were formed by fusion and swelling of the mitochondria stain intensely with neutral red. Fig. 158. If a similar cell is stained with Janus Green B only, the granules acquire a bluish colour, less intense than that of the mitochondria, thus showing that they are still capable of being stained with Janus Green B, to a certain extent, although they have a much greater affinity for neutral red, as evidenced by the staining which takes place when a mixture of the dyes is supplied.

As degeneration proceeds, these "granules" enlarge and 12 hours after the beginning of the experiment, a highly refractile globule resembling fat can be seen in the interior of some of them. When such cells are stained with neutral red, these fatty globules can be seen within the red "granules". Fig 159. When stained with Janus Green B, which still stains the "granules" slightly,

the fatty globule is again visible within the dye-stained mass (Fig. ~~152~~ 157). At this stage, the affinity of the granules" for neutral red is once more evidenced by their capacity for staining intensely with this dye when a mixture of Janus Green B and neutral red is supplied. (Fig. 158.) The small mitochondrial rods and granules, in which degeneration has not advanced, still continue to stain intensely with the Janus Green B. In Fig. 159 a cell containing neutral red masses of varying size, some of which contain fatty globules, is seen. As degeneration proceeds, the fatty globules increase in size and the neutral-red staining, non-fat-containing part diminishes in amount. Staining with Janus Green B becomes fainter until only a pale, bluish purple coloration is found. Occasionally, however, a bright blue staining dot may remain.

APPEARANCES IN FIXED AND STAINED PREPARATIONS.

Supravitally stained preparations with neutral red and Janus Green B last only a few hours and no really satisfactory method has yet been elaborated which fixes these stains in the cytoplasm in order that permanent preparations may be obtained. Cultures at stages corresponding with those described above were fixed and stained with Altmann's fuchsin and methyl green, and with iron haematoxylin. As the former method is more specific than the latter, and is also capable of demonstrating very slight qualitative changes in the mitochondria, it was used extensively in these investigations.

The earliest stages in mitochondrial injury is demonstrated by an apparent increase in thickness and/increased intensity of staining both with fuchsin and iron haematoxylin, so that with fuchsin they appear bright red, and with haematoxylin, almost black: in addition these stains are less easily removed by the differentiating fluids. This corresponds with their increased visibility in the living state and increased capacity for staining with Janus Green. When the filamentous forms break up into rods and granules as previously described in the living cells, these rods and granules stain with an even greater

intensity than in the earlier stages of degeneration. (Fig. 162). This intensity of staining persists in those mitochondria which do not undergo further change. In those mitochondria, however, which become swollen, fuse with one another, and acquire an affinity for staining with neutral red, the capacity for staining with fuchsin disappears, and they take on a greyish green colour with the methyl green. Occasionally, however, a bright red dot may be seen at one part of the greyish green granule; since a corresponding appearance has been seen with Janus green, one may justifiably infer that this represents the remains of mitochondrial substance. Iron haematoxylin, at this stage, continues to stain both the mitochondria and the granules intensely. In cells in which the fatty droplets which appear within the "granules" have attained a relatively large size, spaces can be seen in the greyish green granules which have been apparently left by the dissolving-out of a fatty substance. In iron haematoxylin preparations, similar spaces can be found.

When such cultures are stained with Sudan III, staining of the intra-"granular" fat did not occur. Since mitochondria are destroyed by acid fixation

and since the material surrounding the fatty globules has been seen to be derived from the mitochondria, a series of preparations was fixed in Zenker plus 4% acetic acid, in the hope of destroying the "granules" as well as the mitochondria, thus rendering the intra-"granular" fat penetrable by fat stains. The "granules", however, resisted the acid of the fixative to a great extent, thus showing that they had lost one of the most marked characteristics of mitochondria. The aldol method of Lorrain Smith was also used, but the results obtained were uncertain. With Ciaccio's method, small globules of stained material were found in a few cases in some of the "granules" but the results were not uniform. When preparations were fixed in formalin and stained with Nile Blue sulphate A, the result shown in Fig. 163 was obtained in many cases. In the cytoplasm neutral fat globules (stained pink) are seen. Small bright blue granules, probably of fatty acids are also present. The "granules", however, show the most interesting features. In the cell figured, they are purplish in colour, and many of them contain bright blue droplets which probably consist of fatty acid. The droplet of fatty acid represents in, all probability,

the intra-"granular" fatty droplet. The purplish staining material represents the remains of the degenerate mitochondria from which the granule was derived. In most of the "granules" found in degenerating cells, similar droplets of fatty acid are present. In very degenerate cells, however, the granule may be bright purplish in colour and surrounded only by a thin rim of faint staining material, thus showing that the original character of the fatty droplet, as judged by staining reactions, is not maintained.

In cultures which have been allowed to degenerate spontaneously, similar mitochondrial changes take place but these occur less quickly and uniformly. Accordingly the mitochondrial changes above described are not a specific reaction to the effect of carbon tetrachloride but are evidently an expression of the effect of general toxic changes which may occur in various unfavourable conditions.

As mentioned in the introduction, W.H. Lewis considers that the "granules" and vacuoles, possessing a marked affinity for neutral red, which appear in mesenchymal cells undergoing degeneration in vitro, are derived from the cytoplasm, the mitochondria playing no part in their production. From direct observation, I have found that in the epithelial cells of kidney cultures, "granules" having a marked affinity for neutral red appear under mild toxic conditions. Since W.H. Lewis did not apparently observe further changes in the "granules" of mesenchymal cells leading to the appearance of fatty droplets, the possibility arises that two types of 'degeneration' granule may occur, one derived from the mitochondria, the other from the cytoplasm. In support of this view, is the fact that, in kidney epithelial cells, vacuolation which seems of constant occurrence in Lewis' cultures was rare in my epithelial cultures.

Alterations in mitochondria in disease and in experimental conditions have been described by various workers and the changes which occur are regarded as amongst the earliest indications of cell injury. Scott, (1916) has described the changes in the pancreas

pancreas following phosphorus poisoning. He observed the breaking up of the mitochondria into short, thick forms. Agglutination and fusion then took place and lipoid droplets appeared in the agglutinated masses. Lorrain Smith and Rettie (1925) found that in cloudy swelling and fatty degeneration, mitochondria were destroyed and lipoids were released.

The appearances found in my cultures resemble, to some extent, the description given by Scott. He regarded the droplets which developed in the agglutinated and fused mitochondria as lipoids. In my cultures, the fatty droplets which appeared inside the "granules" produced by swelling and fusion of the mitochondria, reacted, with Nile Blue sulphate, like fatty acid. Since, however, the "granules" alter in character as degeneration progresses, these droplets may only represent a stage in the series of changes which occur in the mitochondria in toxic conditions.

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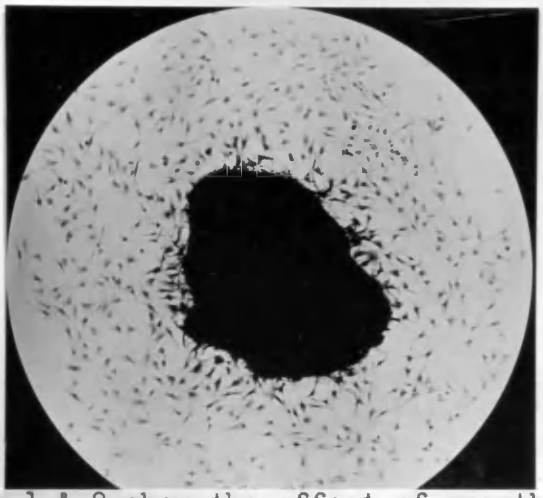
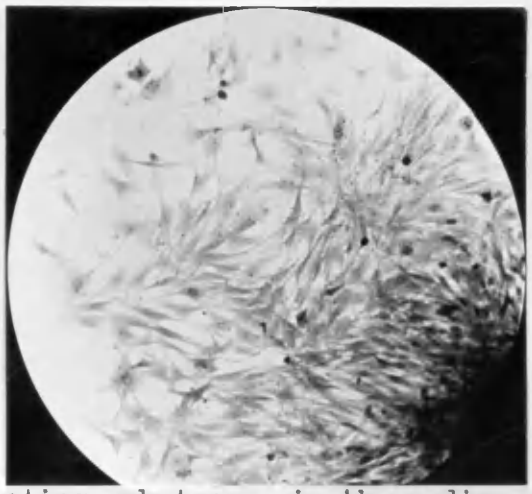
Lewis, W.H., Johns. Hopk, Hosp. Bull., 1919, 30, 81.

Scott, W.J.M., Amer. J. Anat., 1916, 20, 237.

Smith, J. Lorrain, and Kettie, T., J. Path. Bact., 1925, 28, 627.





Figs.1 & 2 show the effect of growth promoting substances in the medium.

Fig.1 is a culture of heart from a newly-born mouse fixed after 48 hours' growth in a normal serum. The cells are fibroblasts. Bouin: iron haematoxylin. x 50.

Fig.2 is part of a culture prepared at the same time which was cultivated in equal parts of mouse serum and mouse embryo extract and fixed after 44 hours' growth. The outgrowth, which consists of fibroblasts, is much more abundant, and mitotic figures are numerous. Bouin: iron haematoxylin. x 80

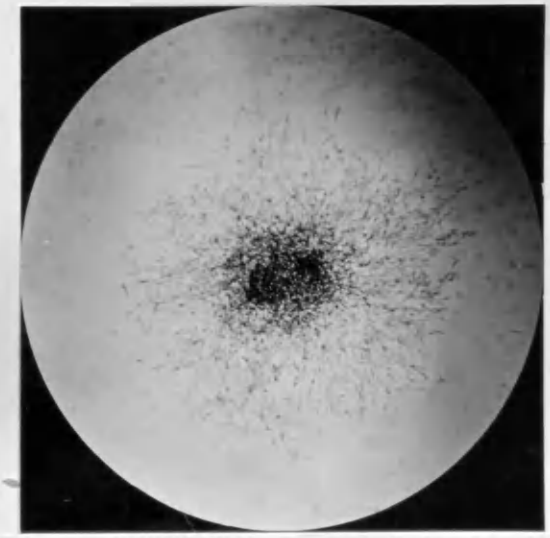


Fig.3.- Subcutaneous tissue from a 9-day avian embryo cultivated for 24 hours in equal parts of plasma and embryo extract. The culture shows the usual radial type of outgrowth of fibroblasts. Zenker: Weigert's haematoxylin. x 20.

Fig.4.- Culture of fibroblasts from the sclerotic of an 8-day avian embryo fixed after 44 hours' growth in equal parts of plasma and embryo extract. Lying close to the fragment of sclerotic is a piece of neural ectoderm from which no outgrowth took place. The fibroblasts emigrated in a radial direction from the piece of sclerotic, surrounded the piece of neural ectoderm, and thereafter, as seen in the figure, resumed the radial direction. Zenker formol: Harris' haematoxylin. x 35.

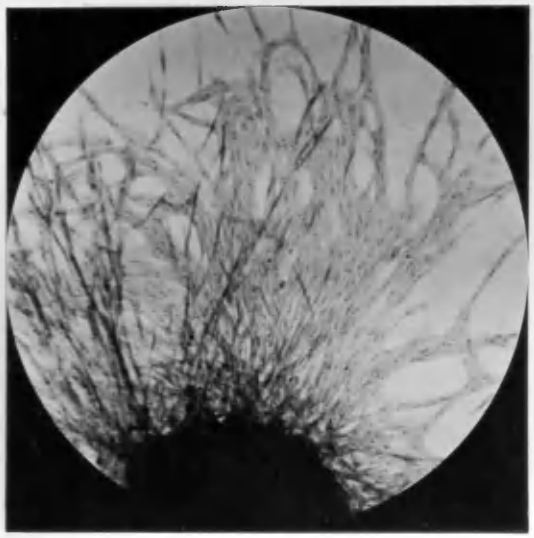


Fig.5.- Endothelium from liver showing strand-like outgrowth. Some of the strands are migrating radially; others are growing round the explant. Sprouts of endothelium are penetrating the coagulum from above. Zenker formol: Weigert's iron haematoxylin. x 75.

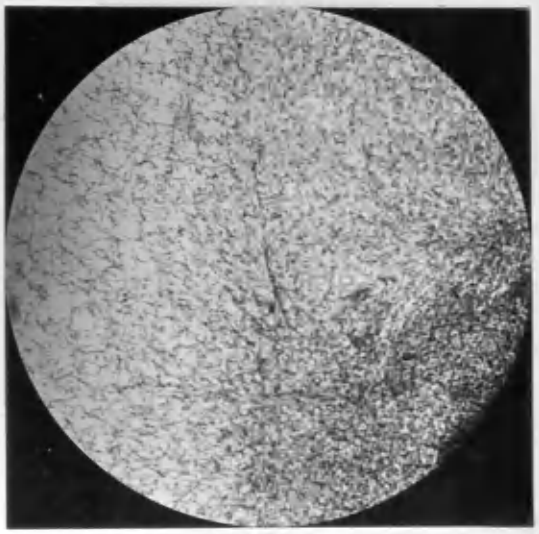


Fig.6.- Part of zone of outgrowth of culture of choroid and sclerotic from 9-day avian embryo after 48 hours' cultivation. The cells are mainly fibroblasts. Zenker: Harris' haematoxylin. x 35.

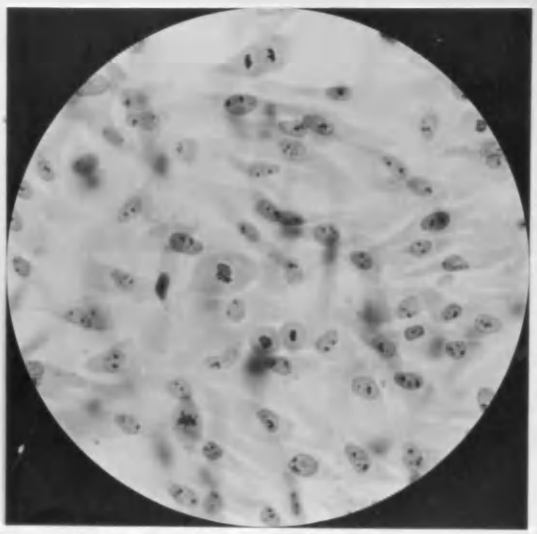


Fig.7.- Fibroblasts from subcutaneous tissue of 8-day avian embryo. Cells are forming an adherent reticulum. Several mitoses are present. Zenker formol: Harris' haematoxylin. x 300.

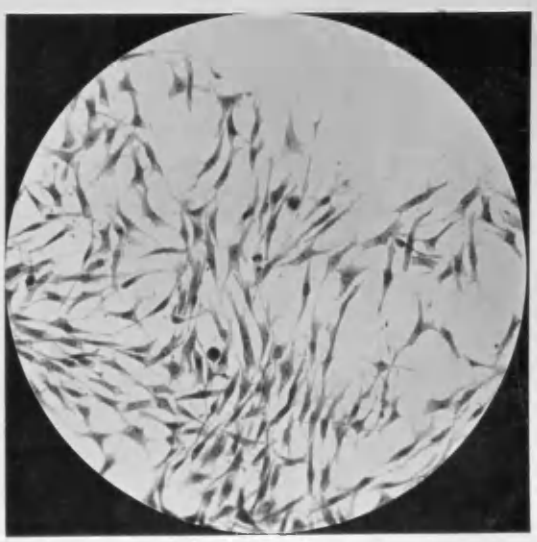


Fig.8.- Fibroblasts from subcutaneous tissue of a young mouse after 24 hours' growth *in vitro*. Most of the cells are spindle-shaped and are in contact with one another by fine elongated processes. Fleming's fixative: iron haematoxylin. x 120.

Fig.9.- Fibroblasts from the heart of a young mouse after 48 hours' cultivation in serum and embryo extract. The cells are elongated and spindle-shaped and possess long fine processes which adhere to neighbouring cells. Flemming's fixative minus acetic acid: iron haematoxylin. x 300.

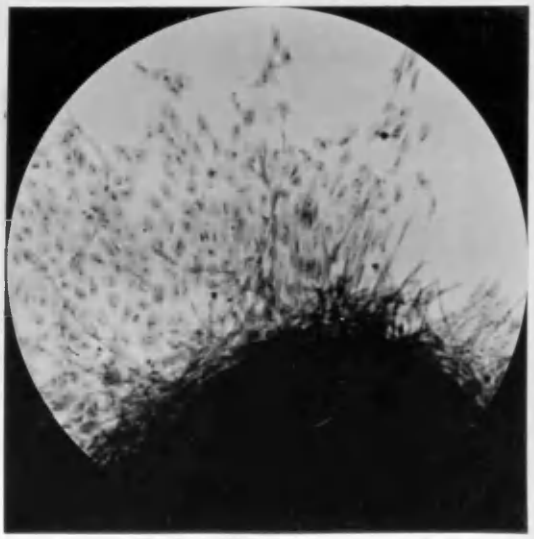


Fig.10.- Part of a mesothelial membrane from the heart of a 9-day avian embryo after 18 hours' cultivation. Fibroblasts are also seen in the zone of outgrowth. At the periphery of the membrane, the cells are becoming isolated and resemble fibroblasts. Zenker: Weigert's haematoxylin. x 75.

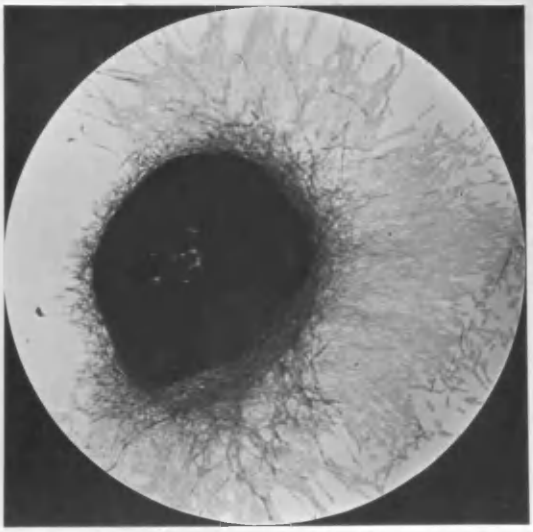


Fig.11.- Culture of liver from a 7-day embryo cultivated for 48 hours in plasma and embryo extract. The fragment was taken from the surface of the liver and from the uncut peritoneal aspect only a few cells have emigrated. On the other side, a wide outgrowth of endothelial cells in the form of strands is seen. Endothelial sprouts are also present. Zenker: Weigert's haematoxylin. x 30.

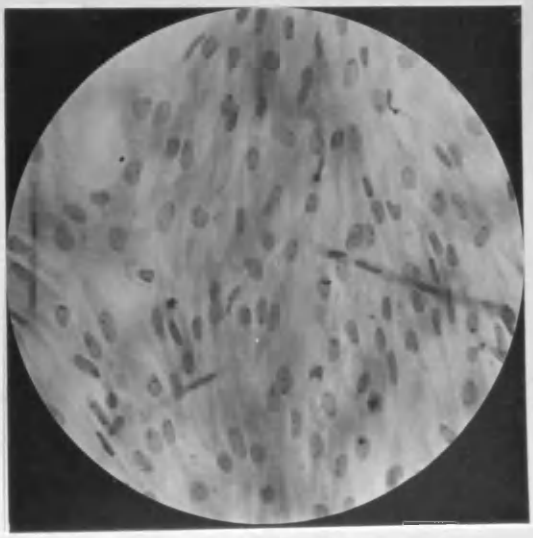


Fig.12.- Part of fig.11 under a higher power showing the arrangement of the endothelial cells. The narrow strand of cells crossing the field is penetrating the coagulum from a higher level. Harris' haematoxylin. x 250.

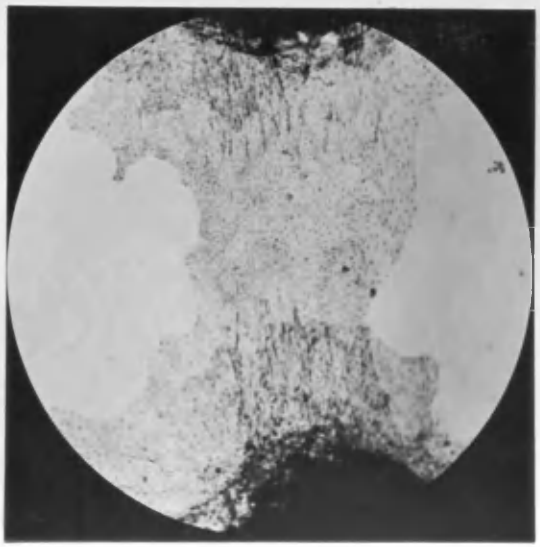


Fig.13.- Culture showing the union of two epithelial carpets; one derived from the cortex, the other from the medulla of newly-born rat kidney. Outgrowth of fibroblasts is also present. (First subculture.) Zenker plus acetic acid: Harris' haematoxylin. x 30.

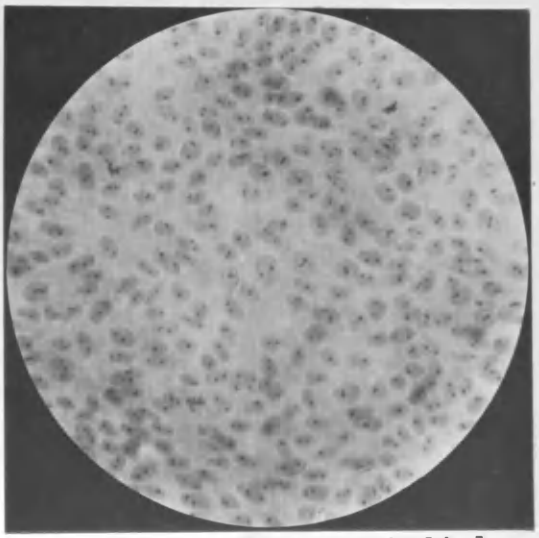


Fig.14.- Part of an epithelial membrane derived from a culture of stomach from a 12-day avian embryo after 48 hours' incubation. The cells are in contact with one another and no intercellular spaces are seen. Several mitoses are present. Weigert's iron haematoxylin. x 300.

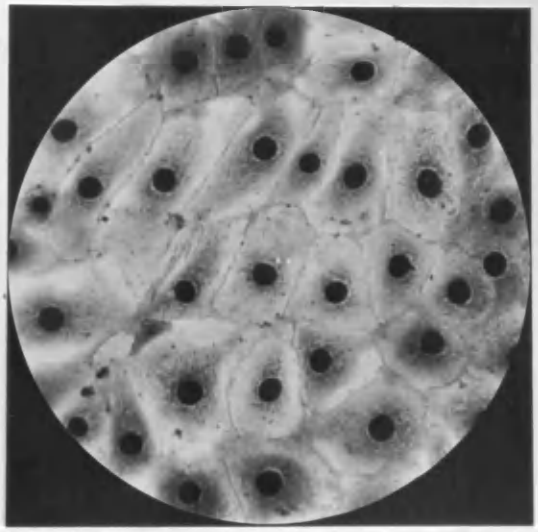


Fig.15.- Epithelial cells from culture of newly-born rat kidney cultivated in fluid medium. The cells are spread out in a thin layer on the cover-slip and differ in shape. Cell outlines are well seen. No intercellular spaces are present. Osmic acid: iron haematoxylin. x 300.

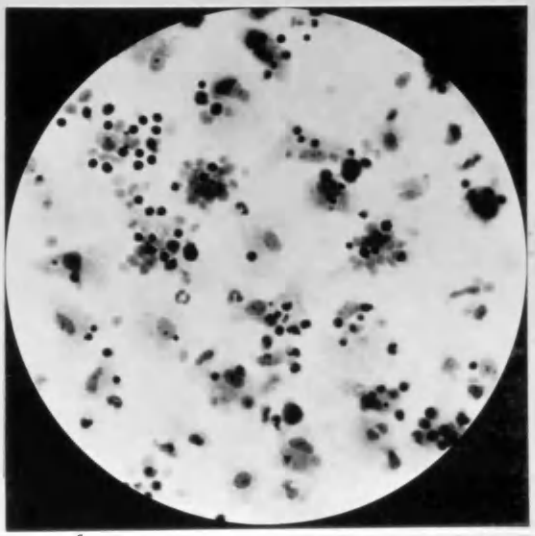


Fig.16.- Macrophages from culture of spleen of young rat after 48 hours' incubation. The cells contain ingested red blood corpuscles and leucocytes. Zenker formol: Harris' haematoxylin. x 300.

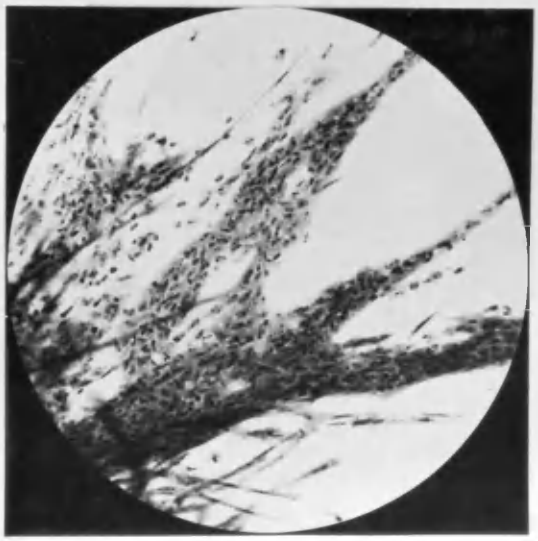


Fig.17.- Culture of heart from 8-day avian embryo fixed after 3 days' cultivation. A reticular outgrowth of intensely-staining heart muscle cells is seen. This showed contraction during life. Bouin: iron haematoxylin. x 100.

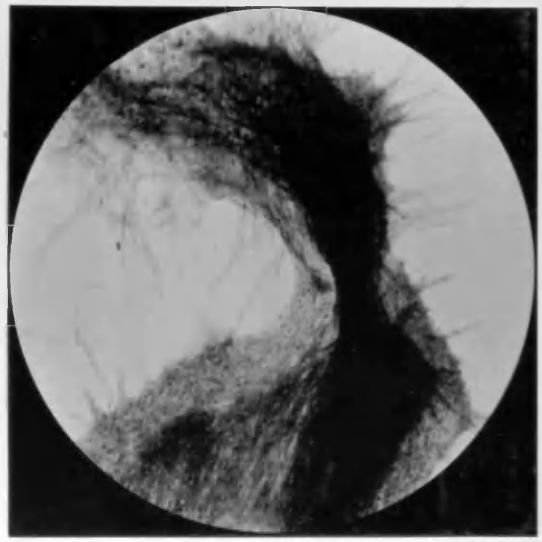
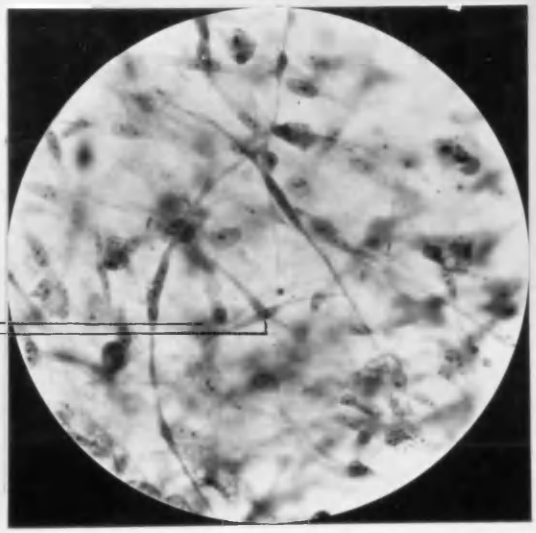
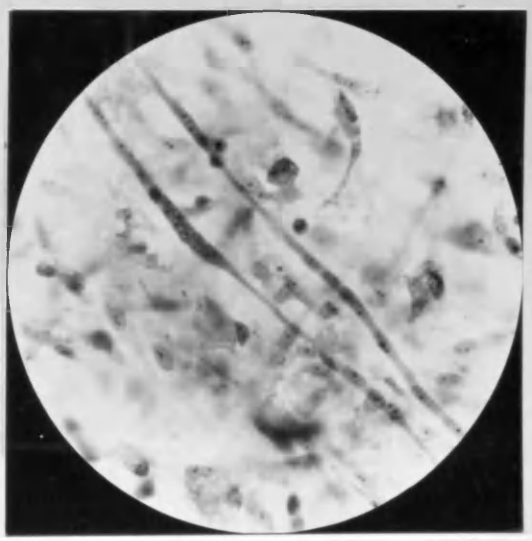


Fig.18.- Culture of amnion from 6-day avian embryo after 24 hours' incubation showing outgrowth of amniotic ectoderm in the form of an epithelial layer. Smooth muscle strands are also seen projecting from the explant. Zenker: Weigert's iron haematoxylin. x 65.





Figs.19 & 20 show multinucleated muscle cells which have become isolated from the strands of origin in the explant derived from a 12-day avian embryo, and have migrated into the zone of outgrowth where they lie amongst fibroblasts. In Fig.19 two mononucleated myoblasts lie in the centre of the field (M - myoblasts). Zenker formol: Weigert's iron haematoxylin. x 300.

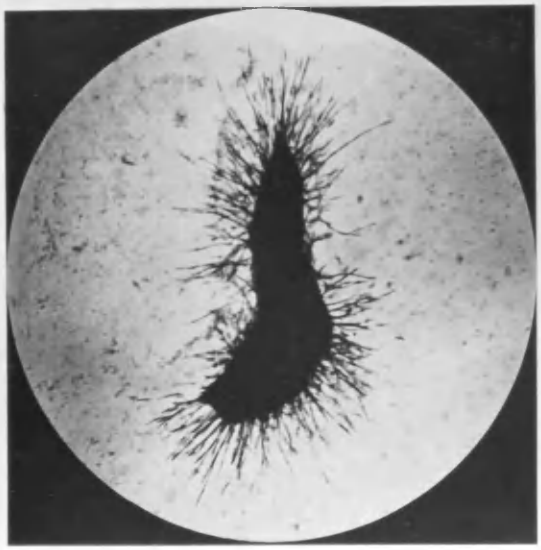


Fig.21.- Culture of muscle (2nd passage) from 10-day avian embryo after 24 hours' growth in plasma and embryo extract. The muscle has grown out in the form of thick sprouts which stain intensely. Zenker formol: Weigert's haematoxylin. x 40.

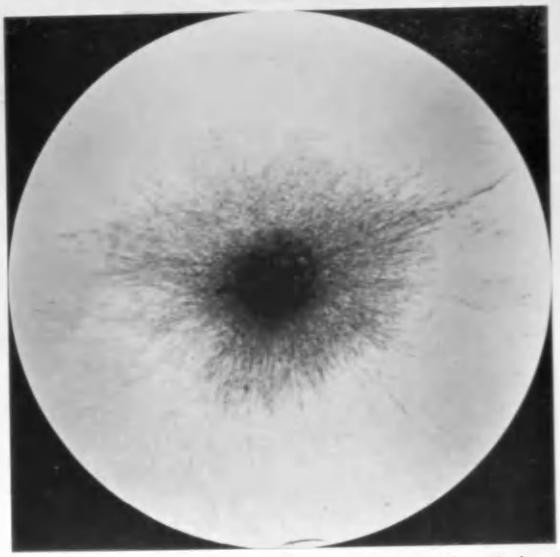


Fig.22.- Radial outgrowth of thin muscle strands from a 7-day embryo. Numerous mesenchyme cells are also present. Zenker plus acetic acid: Harris' haematoxylin. x 20.

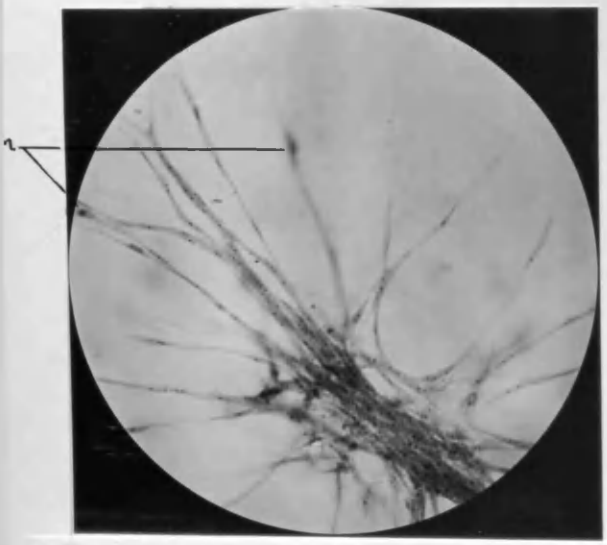


Fig.23.- Muscle strands from 9-day embryo showing mitoses and anastomoses between the strands. Zenker formol: iron haematoxylin. x 125. (M = mitosis).

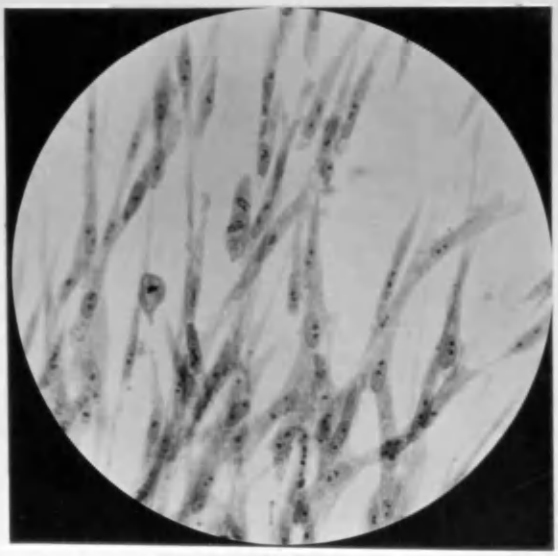


Fig.24.- Culture in plasma and embryo extract diluted with distilled water. Muscle strands from 10-day embryo showing lateral branches and mononucleated myoblasts, some in mitosis. Zenker formol: Weigert's haematoxylin. x 250.

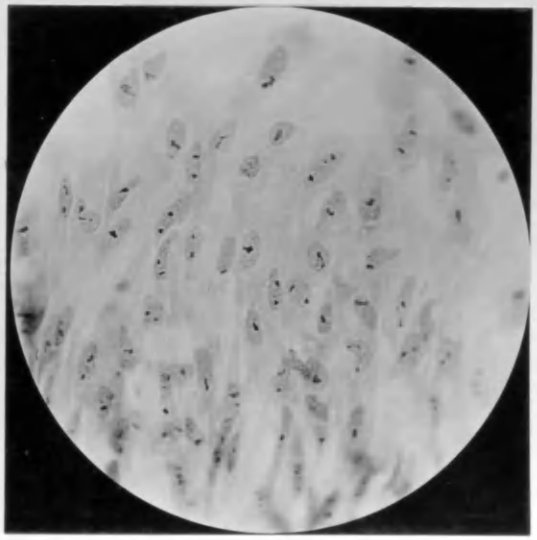


Fig.25.- Endothelial cells from heart of a 9-day avian embryo. The cells form an adherent reticulum. Cell outlines can be distinguished. Zenker: iron haematoxylin. x 250.

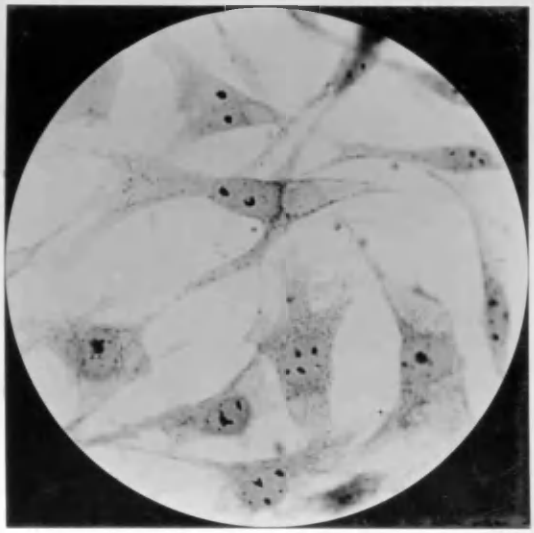


Fig.26.- Fibroblasts from subcutaneous tissue of newly-born mouse. The cells are polygonal in shape and in contact with one another by long fine processes which contain mitochondria. Osmic acid: iron haematoxylin. x 500.

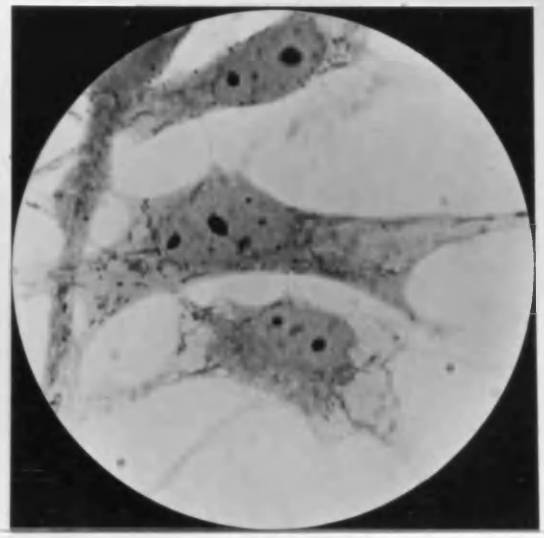


Fig.27.- High power view of Fig.26, showing mitochondria in a fibroblast. Osmic acid: iron haematoxylin. x 1,000.

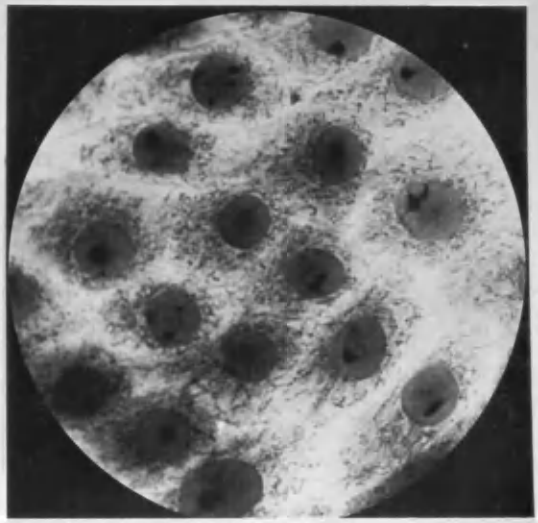


Fig.28.- Epithelial cells from kidney culture in fluid medium. The nuclei, which contain one or more intensely staining nucleoli, show the almost homogeneous character obtained after osmic acid fixation. Surrounding the nuclei and lying embedded in the cytoplasm are numerous fine filamentous and broad-shaped mitochondria. Fat droplets are also seen. Osmic acid: iron haematoxylin and Altmann's fuchsin. x 800.

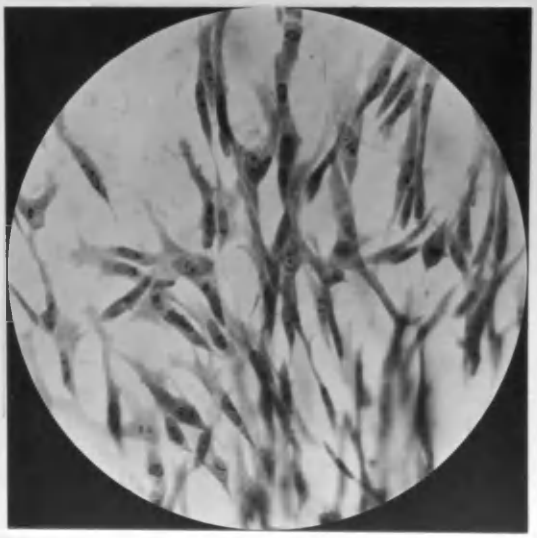


Fig.29.- Cartilage cells from epiphysis of 8-day avian embryo after 18 hours' growth in vitro. The cells are elongated and spindle-shaped. Several mitoses are present. Zenker formol: Weigert's iron haematoxylin. x 300.

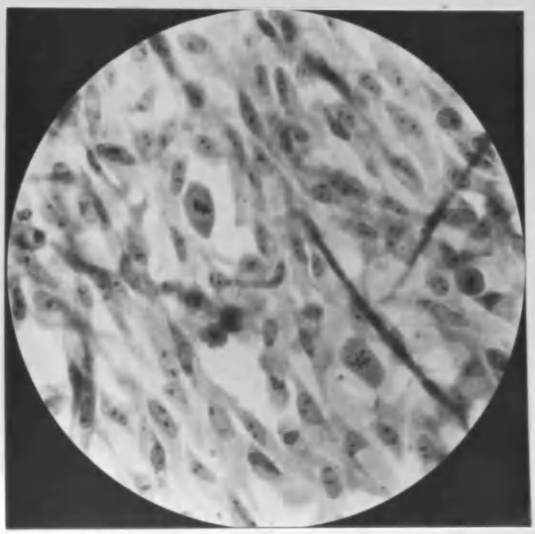


Fig.30.- Cartilage cells from epiphysis of 7-day avian embryo after 48 hours' cultivation. The cells are larger and less intensely stained than in Fig.29. Numerous cells in various stages of mitosis are seen. Zenker formol: Weigert's iron haematoxylin. x 300.

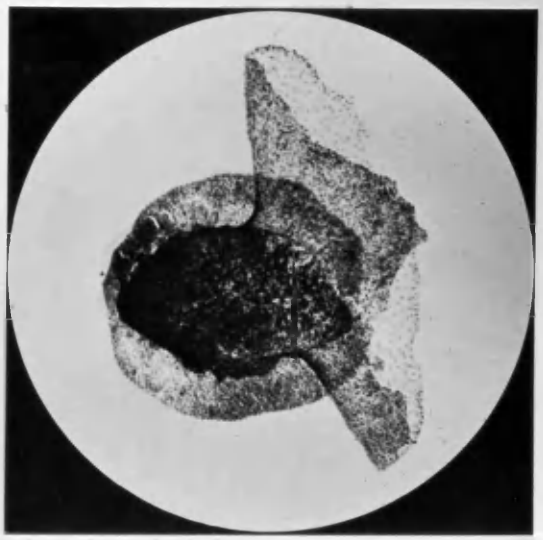


Fig.31.- Culture of fragment of cortex of rat kidney (48 hours' cultivation). The zone of outgrowth consists of several sheets of epithelial cells which have grown out at different levels in the coagulum. Zenker plus acetic acid: Harris' haematoxylin. x 35.

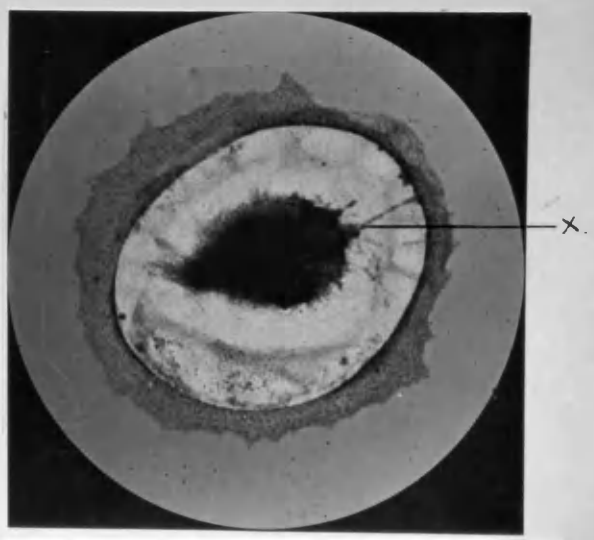
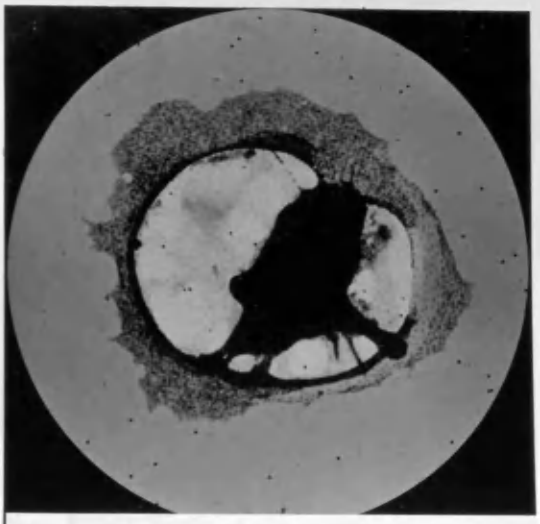


Fig.32.- The effect of liquefaction of the plasma on epithelial culture is shown. The primary sheet of epithelium, which completely surrounded the explant, became detached within 30 hours except at one point (X). Later the detached cells grew out into the unliquefied area in two planes. A fresh carpet emigrated on the surface of the cover-slip in a liquefied area. Zenker plus acetic acid: Harris' haematoxylin. x 35.



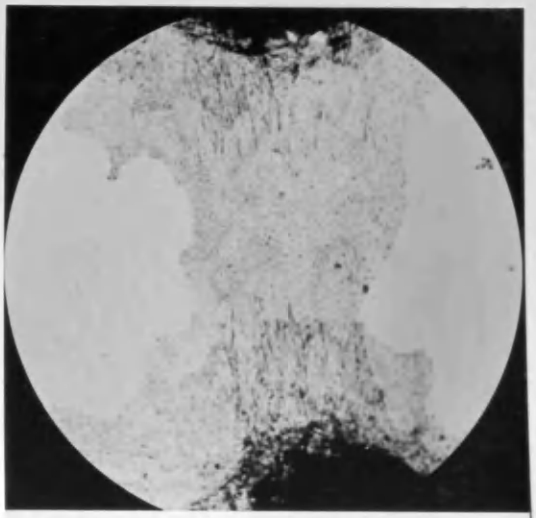


Fig.33 also shows the effect of liquefaction of the medium on a kidney culture, the primary epithelial membrane remaining attached to the explant in several places. The projections from the explant are not newly-formed structures of tubular character but represent contracted masses of epithelial cells. Zenker formol: Harris' haematoxylin. x 35.

Fig.34 shows the membranous appearance of kidney epithelium similar in character from both cortex and medulla. Migration of fibroblasts is also seen but their presence has not influenced the character of the epithelial outgrowth. Zenker plus acetic acid: Harris' haematoxylin. x 30.

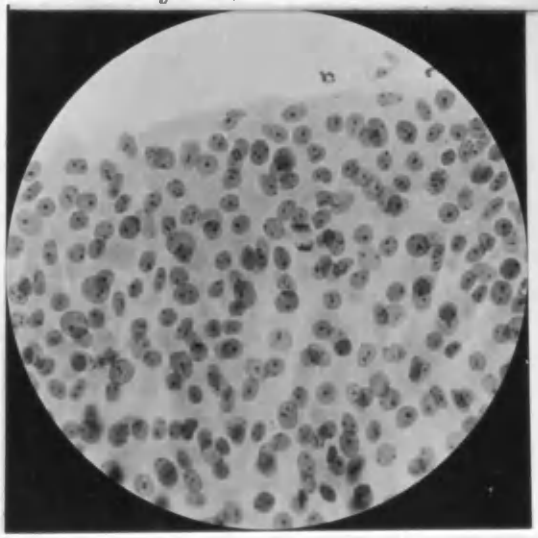


Fig.35.- Growing edge of kidney epithelial culture in plasma and embryo extract from a young rat. The membrane consists of more than one layer of cells and numerous mitoses are present. Zenker formol: Weigert's iron haematoxylin. x 250.

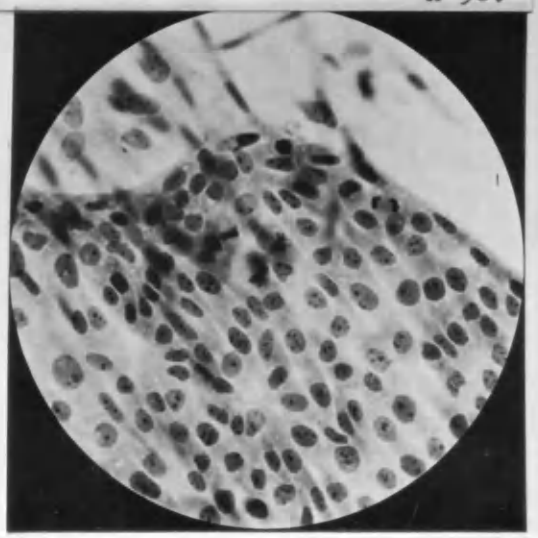


Fig.36.- Growing edge of another kidney epithelial carpet which is only one cell thick, cultivated in plasma and embryo extract. The cells are in general elongated at right angles to the surface of the explant. Several mitoses are present. Fibroblasts are also seen. Zenker plus acetic acid: iron haematoxylin. x 250.

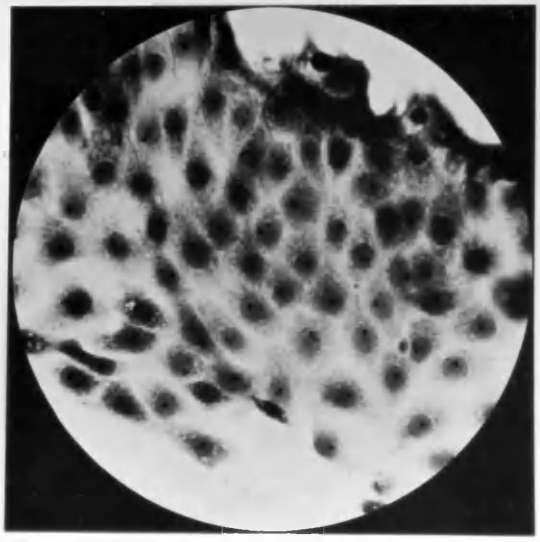


Fig.37.- Part of kidney epithelial membrane in fluid medium, showing the variation in extent to which the cells may stretch themselves out. Osmic acid: Altmann's fuchsin. x 400.

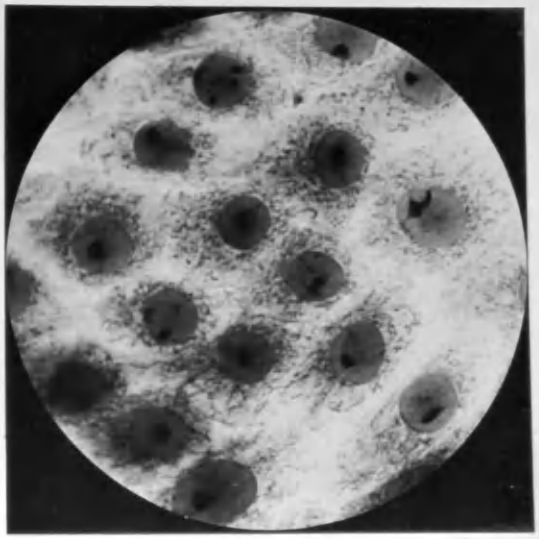


Fig.38.- High power view of Fig.37, showing mitochondria in kidney epithelial cells. x 800.

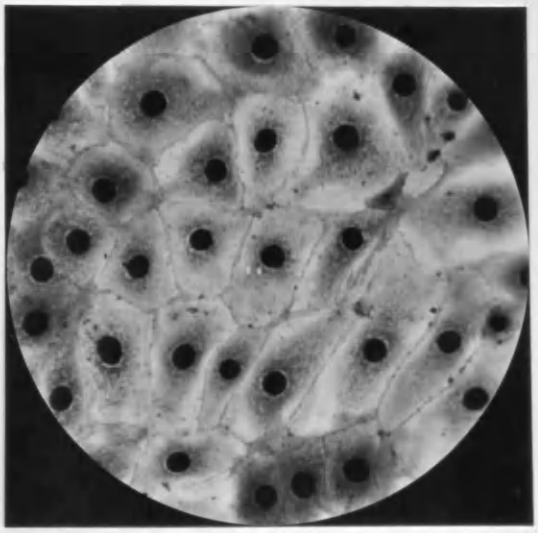


Fig.39.- In this culture no trace of cell outlines was seen during life. During fixation in osmic acid cell boundaries appeared. Osmic acid: iron haematoxylin. x 300.

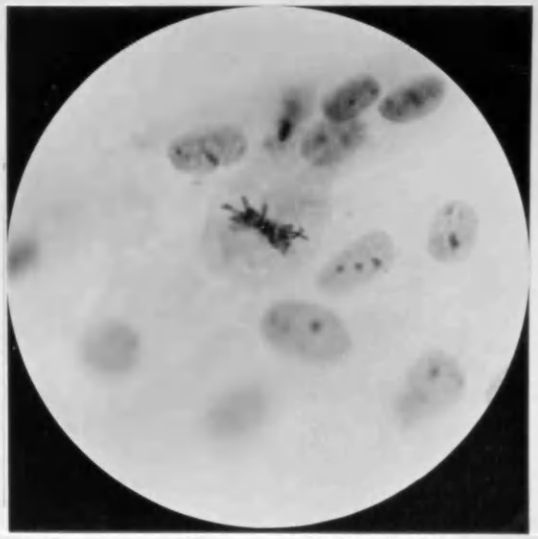


Fig.40.- Epithelial cell from kidney carpet in metaphase stage of mitosis. Longitudinal division of the chromosomes is seen. Zenker formol: Harris' haematoxylin. x 700.

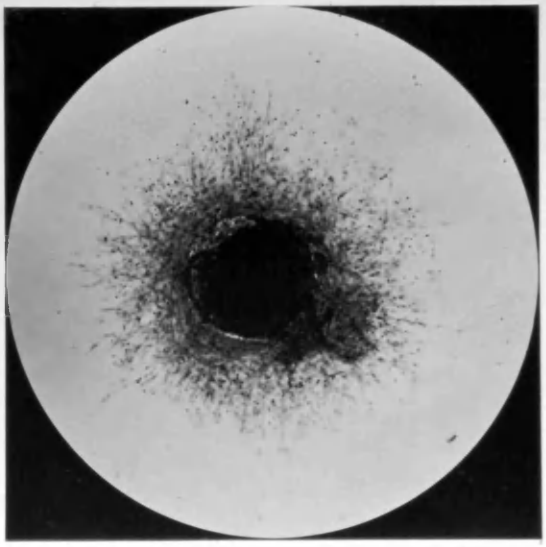
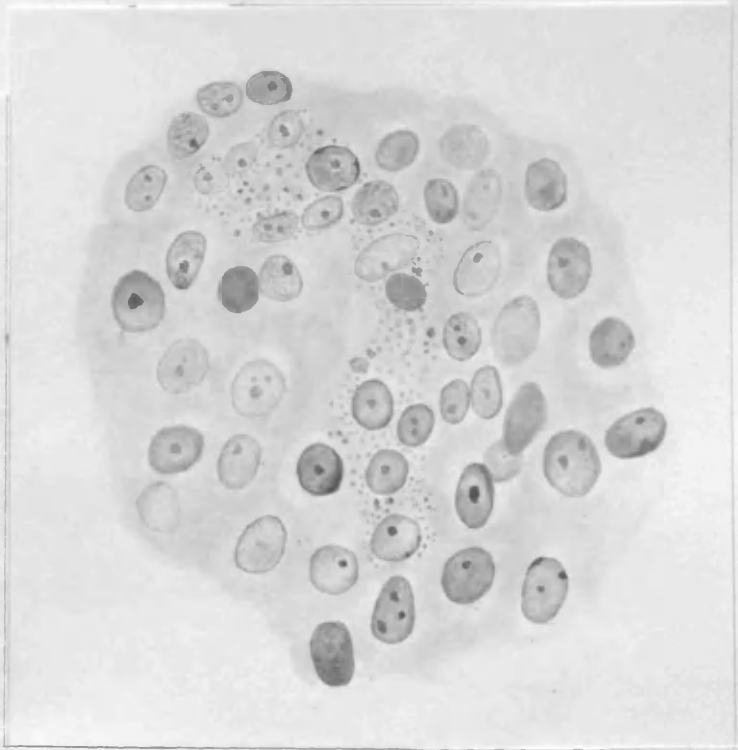
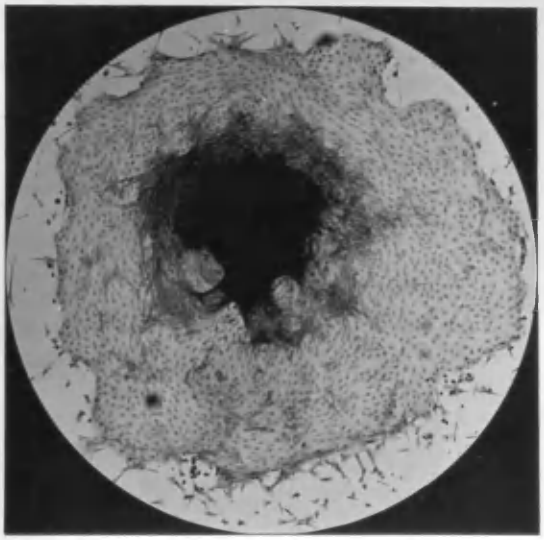


Fig.41.- Culture of kidney fixed at the end of the 4th.passage. During the first two passages outgrowth of epithelium was abundant. After the 2nd. subculture fibroblastic outgrowth took place. During the 3rd. passage numerous fibroblasts migrated and prevented the outgrowth of epithelium after the 3rd. subculture. In the zone of outgrowth fibroblasts and wandering cells are seen. No epithelium is present. Zenker: Harris' haematoxylin. x 20.



ig.42.- Epithelial cells from kidney of 14-day rat which was injected intra vitam with vital new red. The proximal convoluted tubules contained granules of the dye. Dye-containing cells have migrated into the medium participating in the formation of the epithelial membrane. These dye-containing cells are in contact on all sides with non-dye-containing cells. Corrosive sublimate: Harris' haematoxylin.



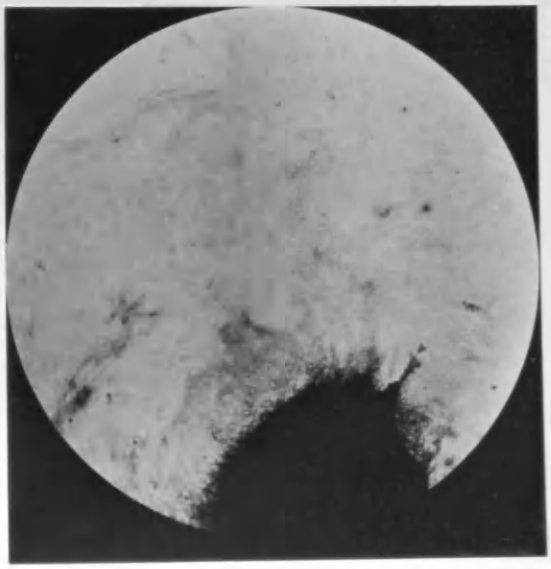


Fig.43.- Culture of intestine from 14-day avian embryo, showing epithelial outgrowth completely surrounding the explant. Smooth muscle cells are present round the central fragment and wandering cells have also migrated into the medium. Zenker formol: Weigert's iron haematoxylin: Scharlach R. x 35.

Fig.44.- Part of epithelial membrane from culture of 14-day intestine which was stained with thionin blue. (Second subculture.) Mucin-containing cells can be seen round the central fragment. Zenker formol. x 75.

Fig.45.- High power view of Fig.44, showing cells containing mucin granules round the explant. x 250.

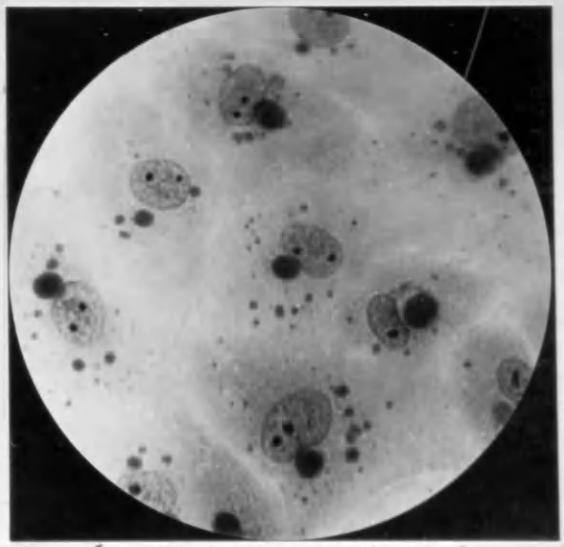


Fig.46.- High power view of epithelial cells at periphery of Fig.43, showing presence of large and small fat globules. x 700.

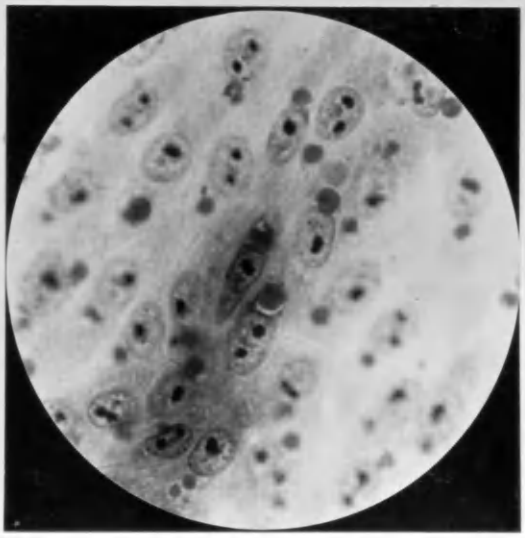


Fig.47.- High power view of cells near central fragment of Fig.43. The epithelial cells contain only large fat globules. The cells are not so spread out as those in Fig.46, which are at the periphery of the culture. x 700.

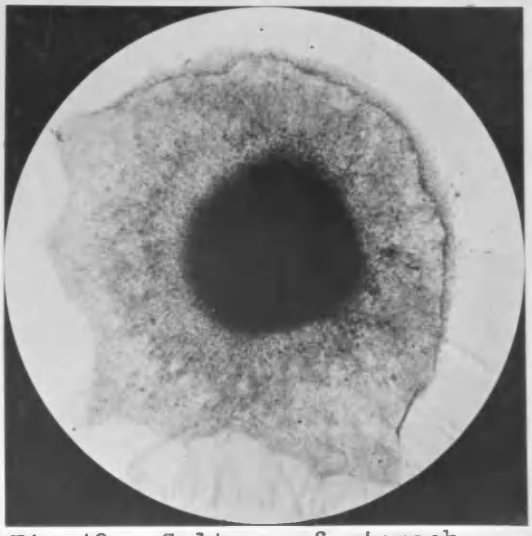


Fig.48.- Culture of stomach from 9-day avian embryo, showing outgrowth of epithelium. 30 hours' cultivation in plasma and embryo extract. Zenker formol: Weigert's iron haematoxylin. x 30.

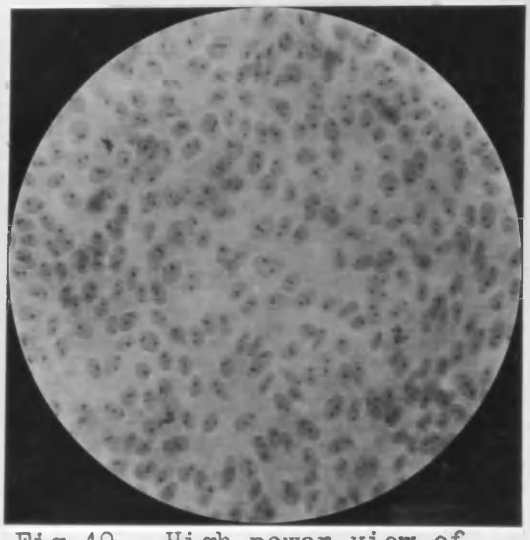


Fig.49.- High power view of epithelial cells in Fig.48. Mitoses are present. x 150.

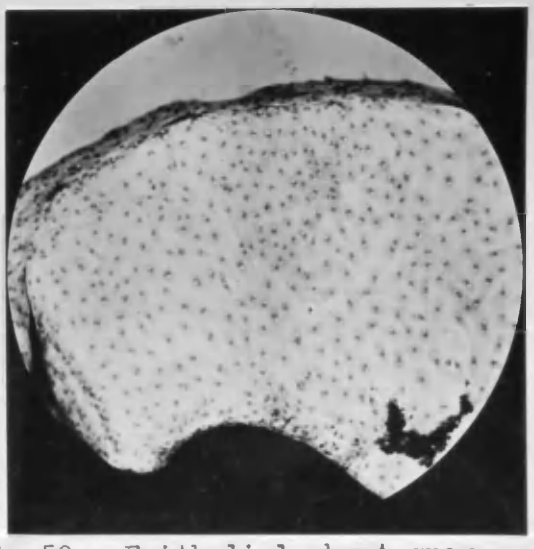


Fig.50.- Epithelial sheet, growing on cover-slip, derived from culture of 12-day avian gall-bladder. Note the spaces in the cells produced by the dissolving out of large globules of fat. The appearances are similar to those found in cultures of stomach and intestine. Zenker formol: Weigert's haematoxylin. x 75.

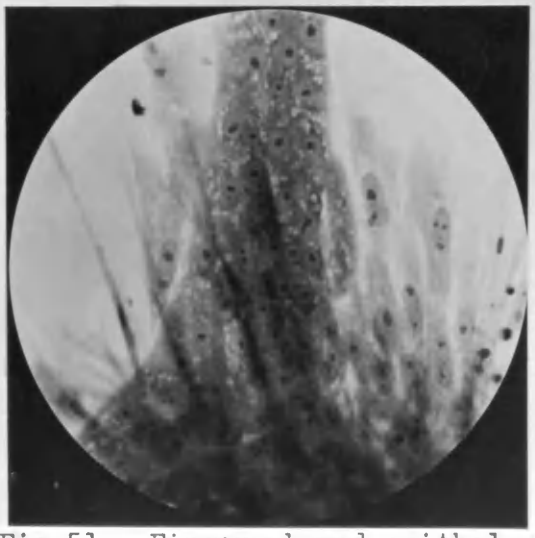


Fig.51.- Finger-shaped epithelial outgrowth from liver of 10-day avian embryo. The cells show spaces out of which fatty droplets have been dissolved. Endothelial cells are also present. Zenker formol: Weigert's iron haematoxylin. x 300.

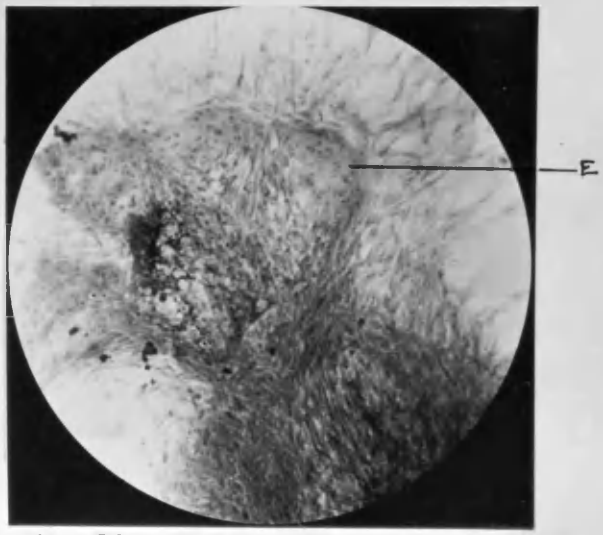


Fig.52.- Outgrowth of epithelium and endothelium from liver of 8-day avian embryo. (Second subculture.) Zenker formol: Weigert's iron haematoxylin. x 60. (E = epithelium).

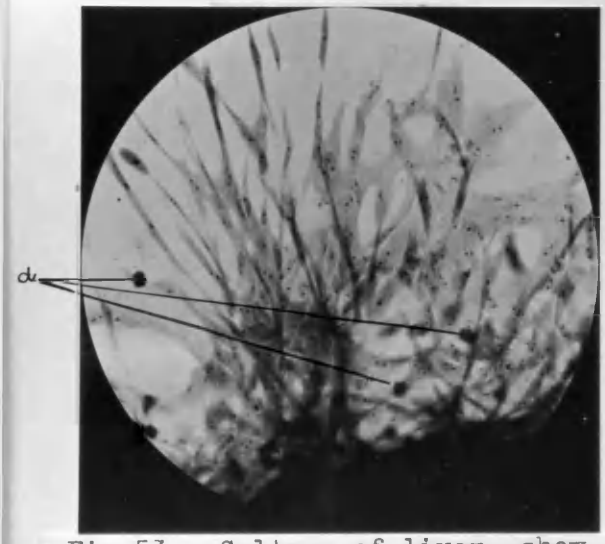


Fig.53.- Culture of liver, showing endothelial cells, from which no epithelial outgrowth took place. A few intensely-staining degenerate liver cells, in which no structure can be made out, are seen. d = degenerate liver cells. Zenker formol: Weigert's iron haematoxylin. x 150.

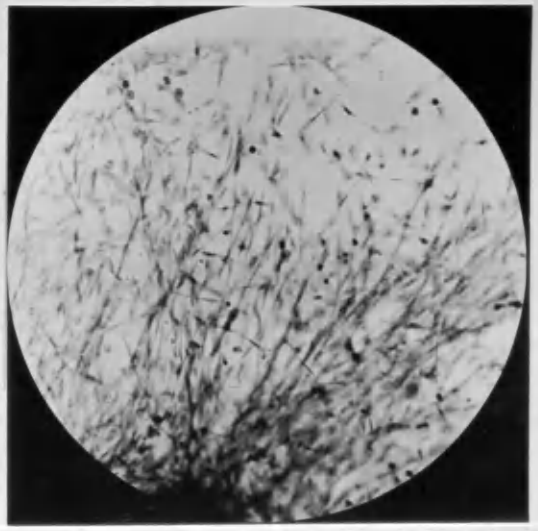
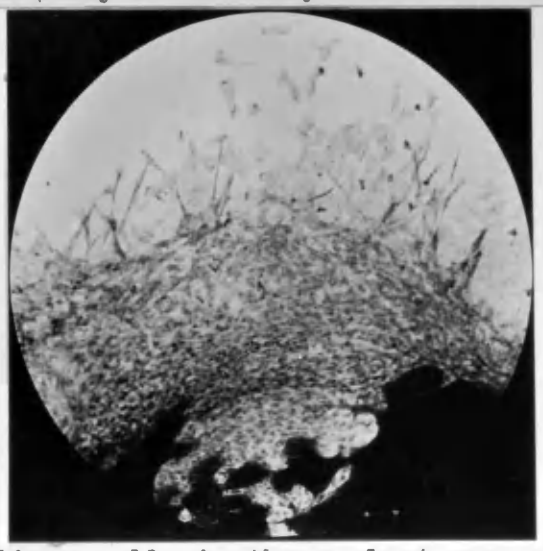


Fig.54.- Culture of liver from 16-day avian embryo. In the zone of outgrowth fibroblasts and wandering cells, mainly macrophages, are seen. A few lymphocytes and monocytes are present. Note the elongation of the macrophages characteristic of actively migrating cells of this type in a coagulated medium. Zenker formol: Weigert's iron haematoxylin. x 54.

Fig.55.- Periphery of liver culture from 7-day avian embryo. The columns



of liver cells in the explant are very intensely stained and no endothelial outgrowth has taken place. Between them can be seen the sinusoidal endothelium which is continuous with the cells in the zone of outgrowth. Note the tendency of the endothelium to grow round the explant. Zenker: Weigert's iron haematoxylin. x 75.

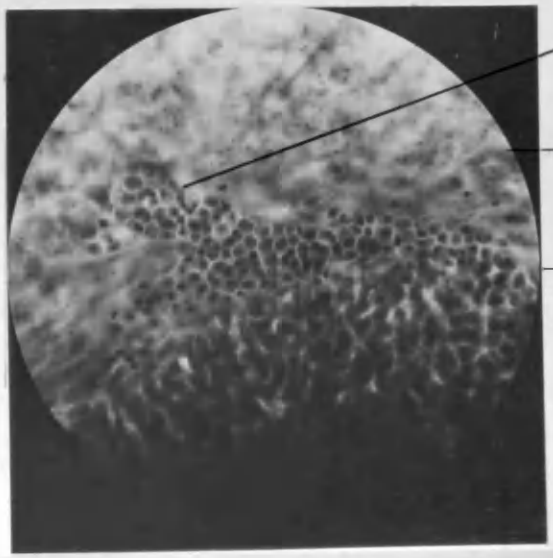


Fig.56.- Periphery of explant of culture of 5-day avian liver showing a sinusoid in which haemopoiesis is progressing. L = limit of liver cell zone: S = sinusoid: E.E = limits of thick zone of endothelial cells which have grown round the fragment, preventing emigration of liver parenchyma. Zenker: Harris' haematoxylin. x 300.

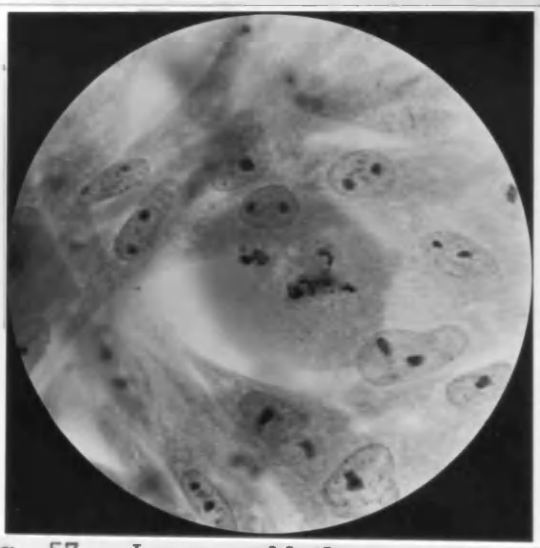


Fig. 57.- Large cell from culture of liver showing abnormal mitosis, the chromosomes being divided into two unequal groups. Zenker: Weigert's iron haematoxylin. x 750.

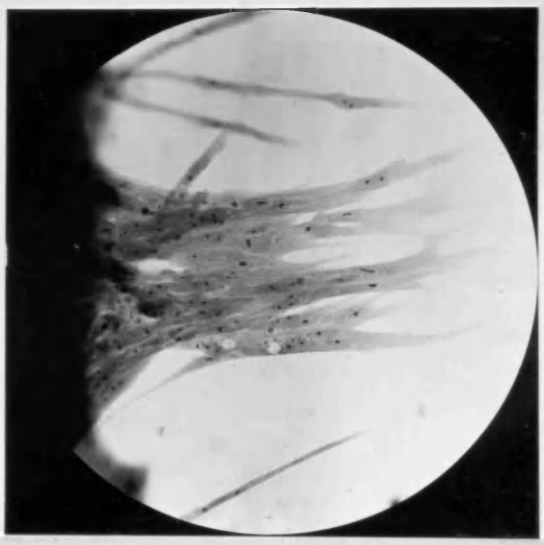


Fig. 58.- Strands of elongated band-like smooth muscle cells from culture of amnion. Longitudinal fibrillae are to be seen in the cells. Zenker formol: Weigert's iron haematoxylin. x 250.

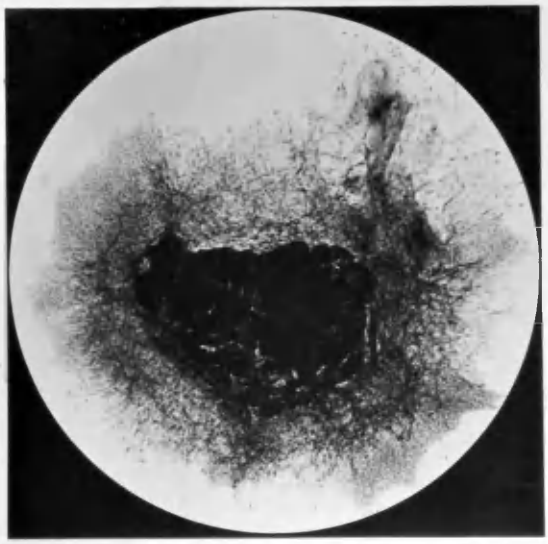


Fig. 59.- Culture of lung from 14-day avian embryo (3rd. sub-culture). The zone of outgrowth shows broad sheets of bronchial epithelium, fibroblasts and wandering cells. Bouin: Harris' haematoxylin. x 30.

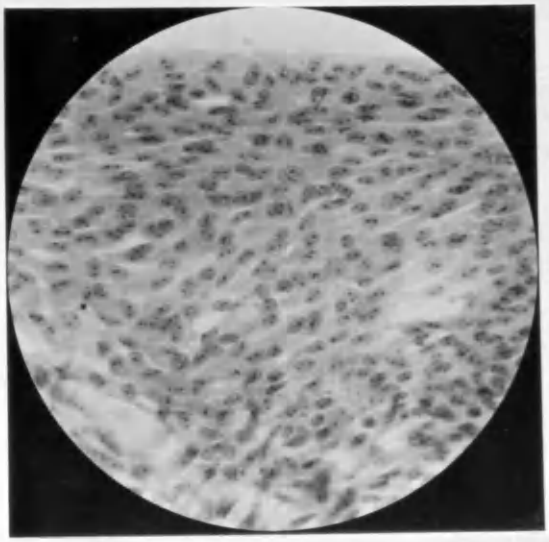


Fig.60.- High power view of epithelium from fig.59. The epithelial sheet resembles that seen in cultures of skin. Bouin: Harris' haematoxylin. x 30.

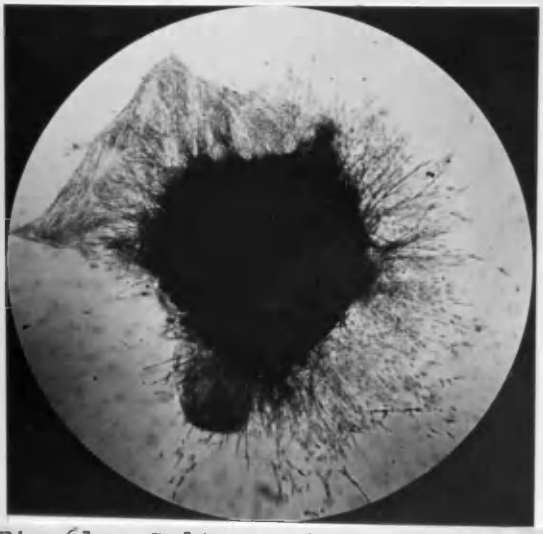


Fig.61.- Culture of thyroid of 14-day avian embryo after 2 days' cultivation. A sheet of epithelial cells showing variation in shape is present. Endothelial sprouts, wandering cells and fibroblasts are also seen. Zenker formol: Weigert's iron haematoxylin. x 30.

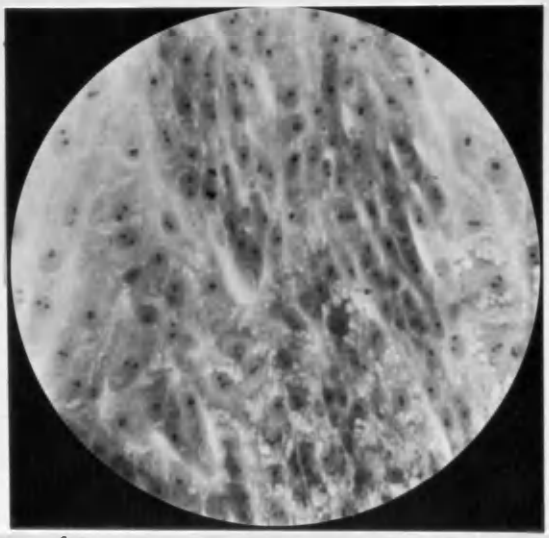


Fig.62.- Epithelium from culture of thyroid showing variation in shape of epithelial cells. Spaces produced by the dissolving out of fatty and lipoid globules which often appear in large numbers of cultures of thyroid epithelium are seen. Zenker formol: Weigert's iron haematoxylin. x 300.

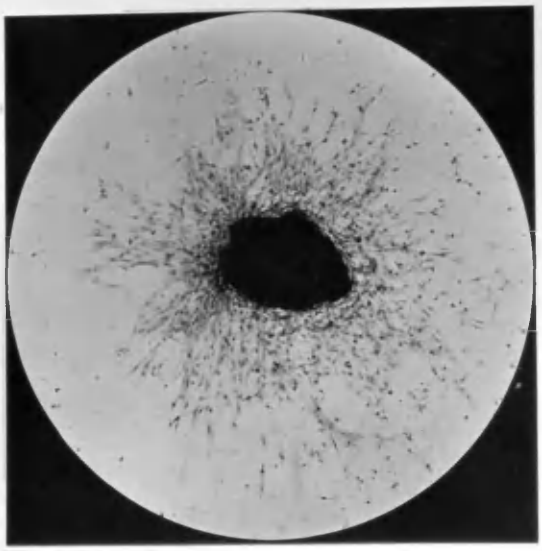


Fig.63.- 3rd.subculture of newly-born mouse thyroid. During the first two passages fibroblasts and epithelial cells were found. Later excessive fibroblastic outgrowth inhibited emigration of the epithelium and in this culture only fibroblasts and macrophages are present in the zone of outgrowth. Zenker: Harris' haematoxylin. x 40.

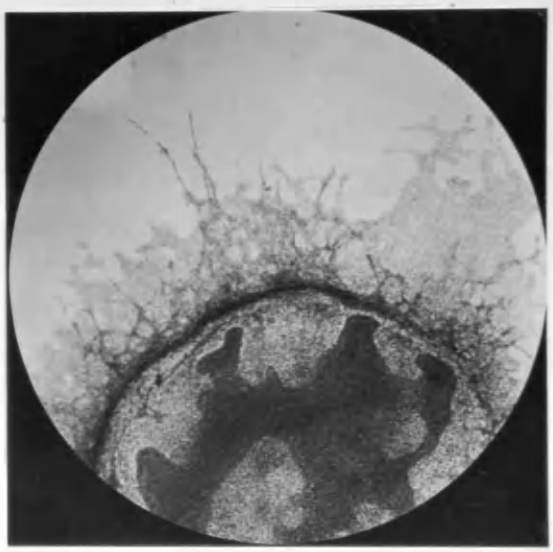


Fig.64.- Culture of allantois from 7-day avian embryo showing outgrowth of epithelium and sprouts of endothelium. Zenker: Harris' haematoxylin. x 30.

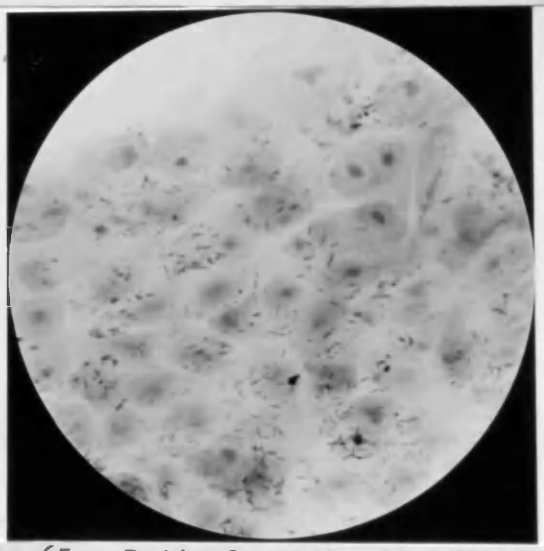


Fig.65.- Retinal epithelium from 6-day avian embryo. Fine pigment rods are seen in epithelial cells and these have increased in amount during cultivation. Zenker: Harris' haematoxylin. x 700.

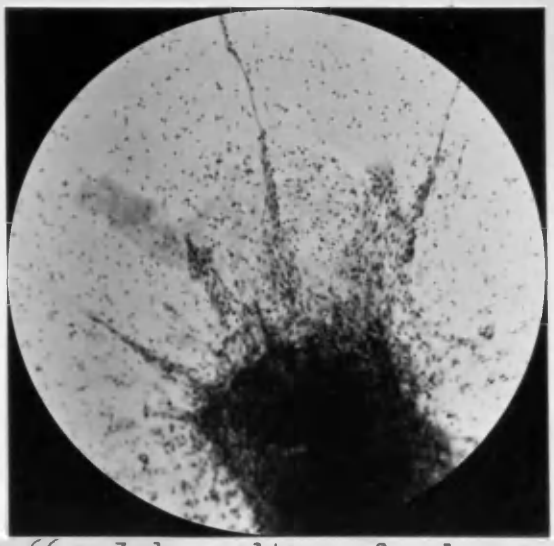


Fig.66.- 3-day culture of spleen from young rat, stained intra vitam with vital new red, showing numerous wandering cells consisting mainly of macrophages and monocytes and long and somewhat flattened strands of sinusoidal epithelium. The endothelial cells contain fine granules of the dye which cannot be seen in the photograph. Corrosive sublimate: Harris' haematoxylin. x 60.

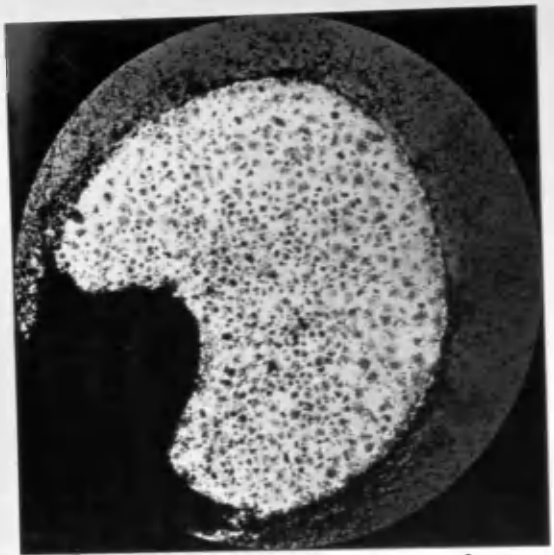


Fig.67.- Culture of spleen from newly-born rat after 2 days' cultivation. Extensive liquefaction has taken place in the area figured. In the liquefied zone numerous spread-out macrophages containing ingested material are present. Zenker: Harris' haematoxylin. x 40.

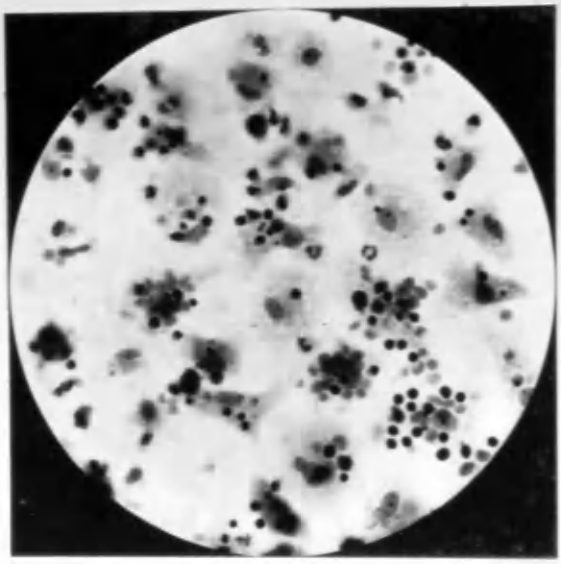


Fig.68.- High power view of macrophages from culture of spleen showing numerous phagocytosed red blood corpuscles, lymphocytes and polymorphs. Zenker: Harris' haematoxylin. x 300.

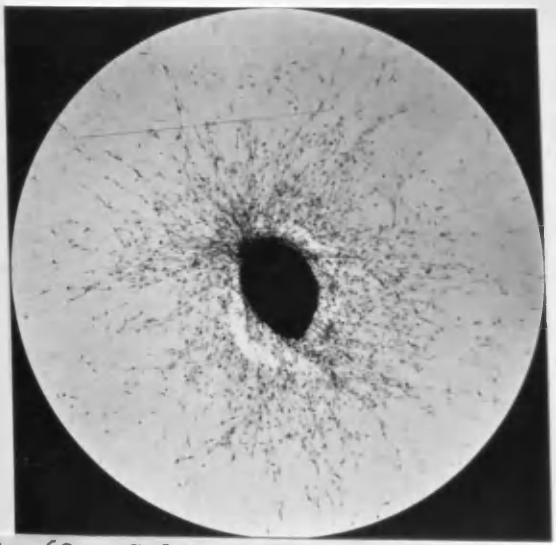


Fig.69.- Culture of pancreas from 14-day rat showing fibroblasts and macrophages. A small epithelial outgrowth was obtained in the primary culture. During the second passage the fibroblasts, however, grew round the explant, preventing further epithelial outgrowth. Zenker formol: Harris' haematoxylin. x 300.

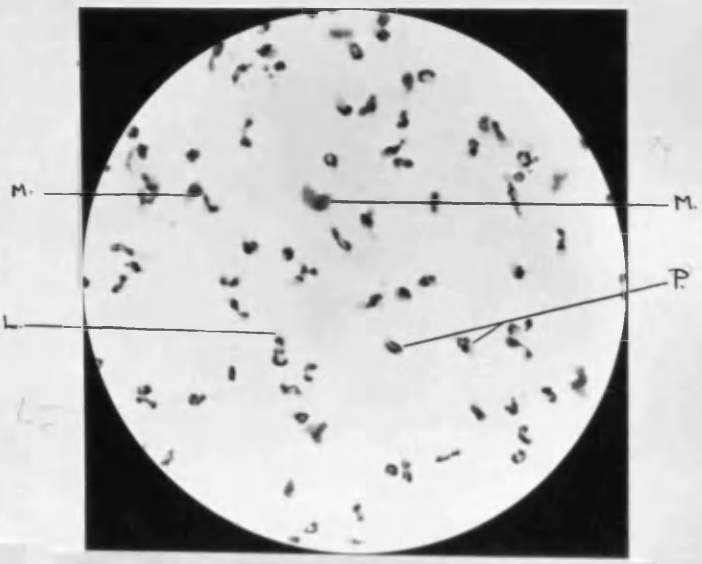


Fig.70.- Polymorphonuclear leucocytes, lymphocytes, monocytes which have emigrated from cultures of spleen.
P = polymorphs: L = lymphocytes: M = monocytes. Zenker: Harris' haematoxylin. x 300.

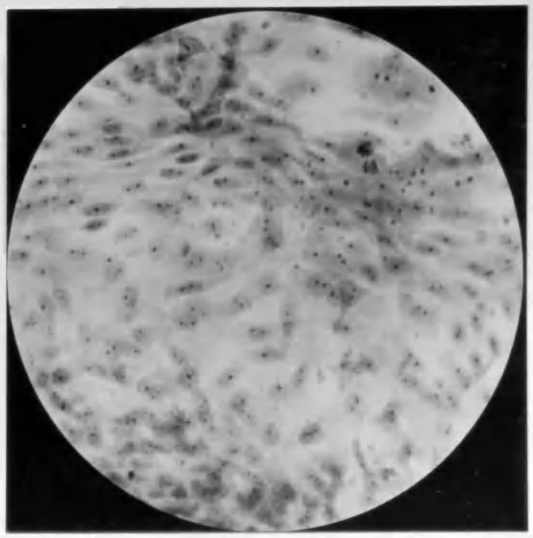


Fig.71.- Part of epithelial membrane from culture of skin of 5-day embryo. During life no spaces could be observed between the cells. The reticular appearance of the cells seen was produced by fixation. Zenker: Weigert's iron haematoxylin. x 250.

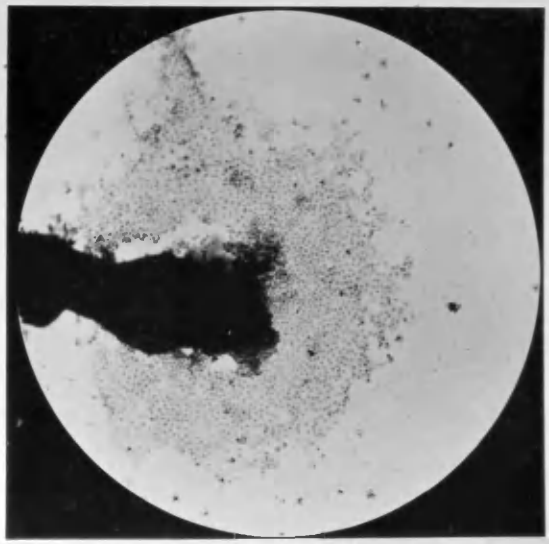


Fig.72.- 4-day culture of spontaneous mouse tumour (adenocarcinoma) showing typical epithelial outgrowth resembling that seen in cultures of normal organs. A few wandering cells are present in the zone of outgrowth. Zenker: carbol fuchsin. x 72.

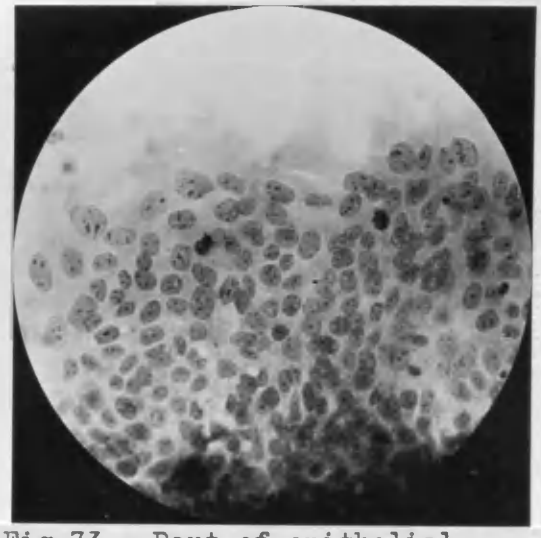


Fig.73.- Part of epithelial membrane from another spontaneous mouse tumour cultivated in plasma and embryo extract. The cells show typical epithelial arrangement similar to that seen in cultures of normal organs. Several mitoses are present. Zenker plus acetic acid: Harris' haematoxylin. x 250.

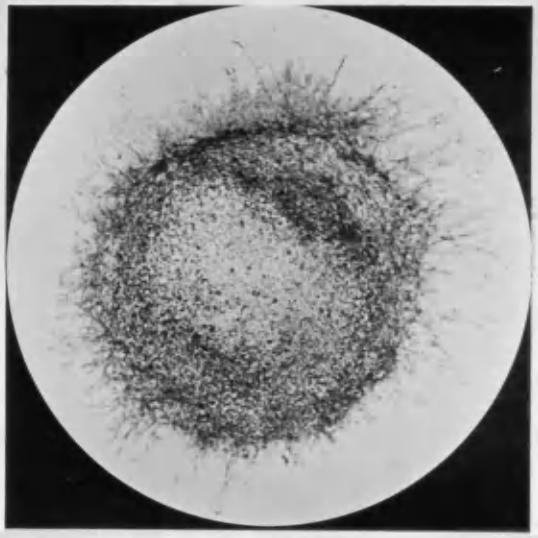


Fig.74.- Mesenchyme from 4-day avian embryo. The explant has spread out in a thin sheet and is surrounded by a narrow fringe of cells. Zenker: Harris' haematoxylin. x 30.

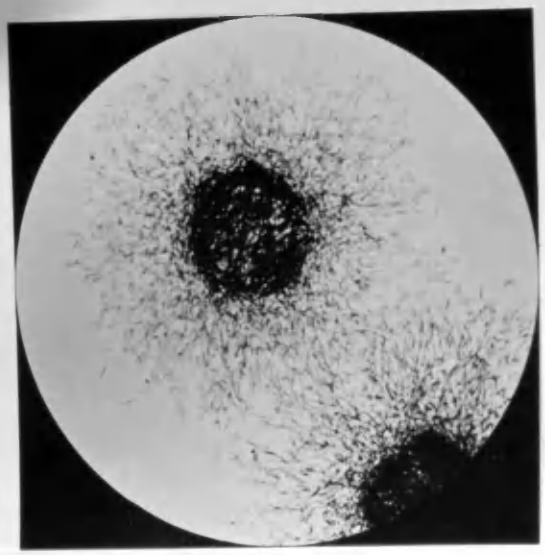


Fig.75.- In this preparation two small fragments of subcutaneous tissue from 8-day avian embryo were cultivated close to one another (24 hours' cultivation). The fibroblasts show the usual radial arrangement and those from one culture have approached and are mingling with those from the other. Zenker formol: Weigert's iron haematoxylin. x 35.

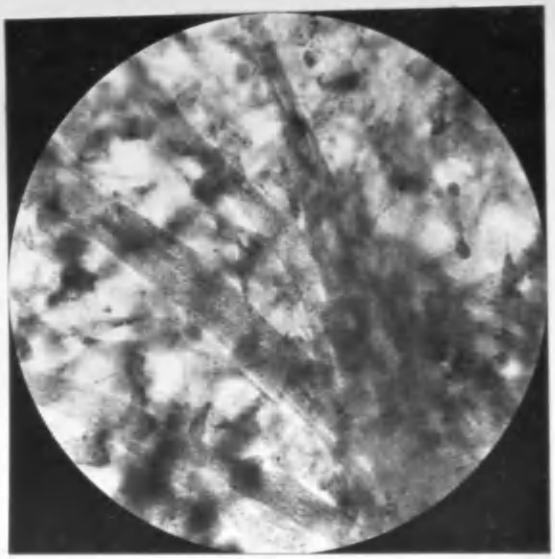


Fig.76.- Part of culture of muscle from 12-day avian embryo. Broad muscle strands containing fibrillae and numerous nuclei arranged in longitudinal rows are seen. Zenker: Nile blue sulphate A. x 300.

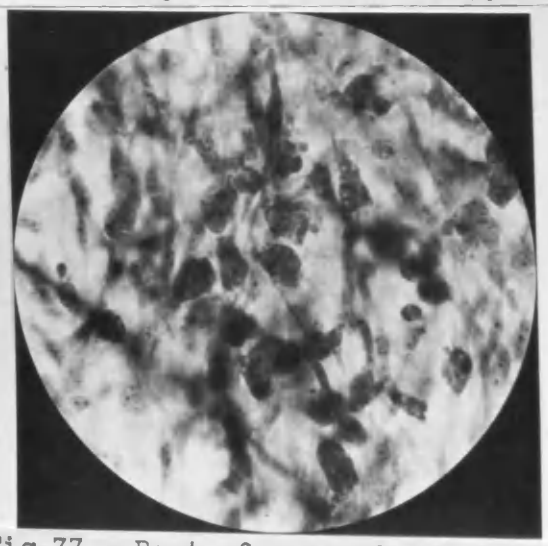


Fig.77.- Part of zone of outgrowth of culture of fatty tissue from 12-day avian embryo which has been stained with Nile blue sulphate A. Fibroblasts and intensely stained, rounded, fat cells are seen. Formalin. x 300.

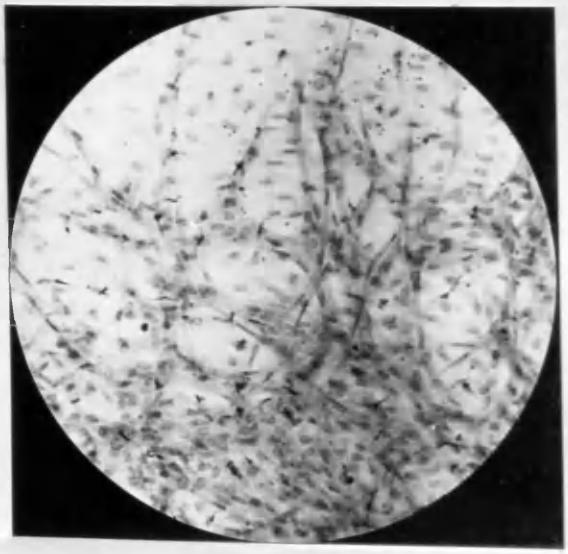


Fig.78.- Endothelium from 9-day avian embryo showing both capillary sprouts and membranous arrangement of the endothelial cells. Zenker: Harris' haematoxylin. x 100.

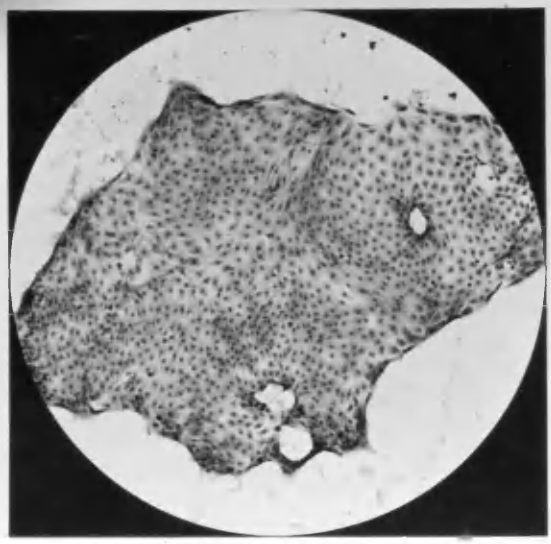


Fig.79.- Newly-born mouse kidney: two hours in normal rabbit serum. Typical epithelial growth one cell thick. Some cells at margin show evidence of injury. Zenker minus acetic acid; iron haematoxylin. x 80.

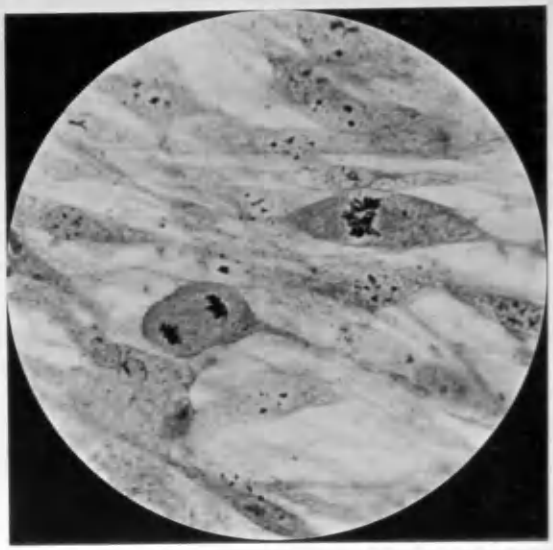
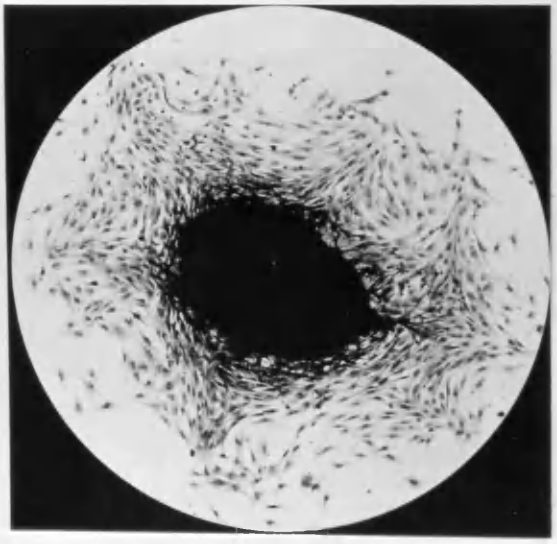
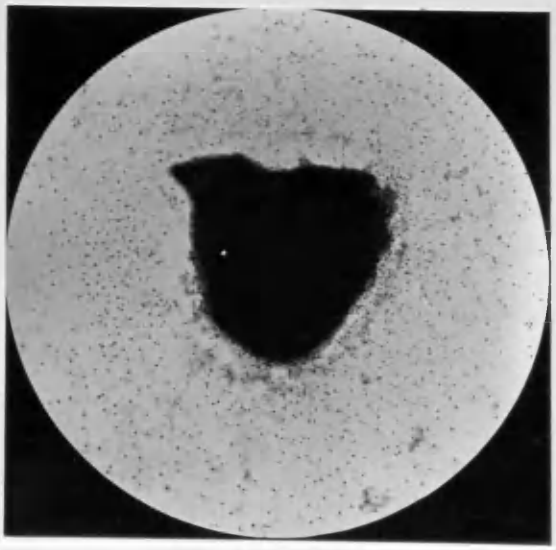


Fig.80.- Fibroblasts from newly-born mouse heart after two hours in normal rabbit serum. Numerous normal mitotic figures. Zenker minus acetic acid, iron haematoxylin. x 750.





Figs.81 & 82.- Embryo mouse heart, good growth of fibroblasts after forty-eight hours. Medium then removed, cultures washed and serum added for two hours. Zenker minus acetic acid, iron haematoxylin.

Fig.81.- Normal rabbit serum. Cells normal, sharply defined nuclear structure and long cytoplasmic processes. x 80.

Fig.82.- Cytotoxic serum. Cells greatly shrunken, outlines difficult to distinguish. x 80.

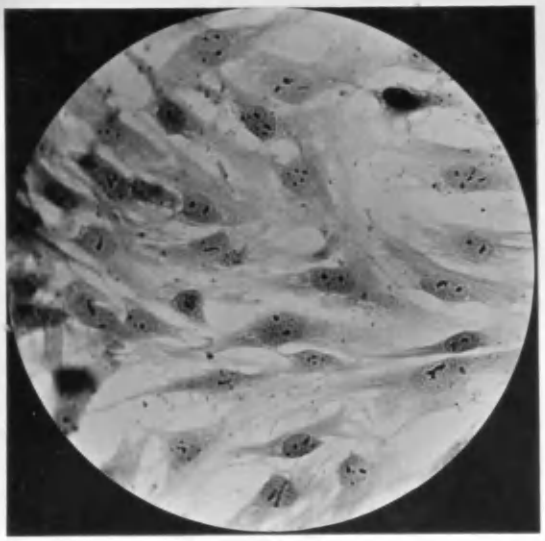


Fig.83.- High power of fig.81. Cells healthy; cell in early prophase in centre. x 300.

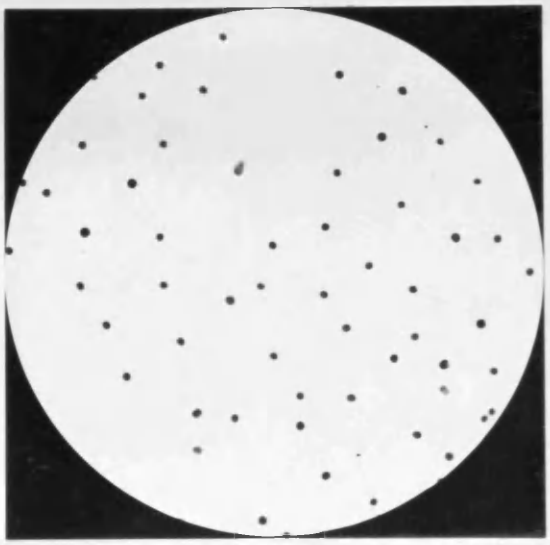


Fig.84.- High power of fig.82. Extreme pyknosis and loss of cytoplasmic structure. x 300.

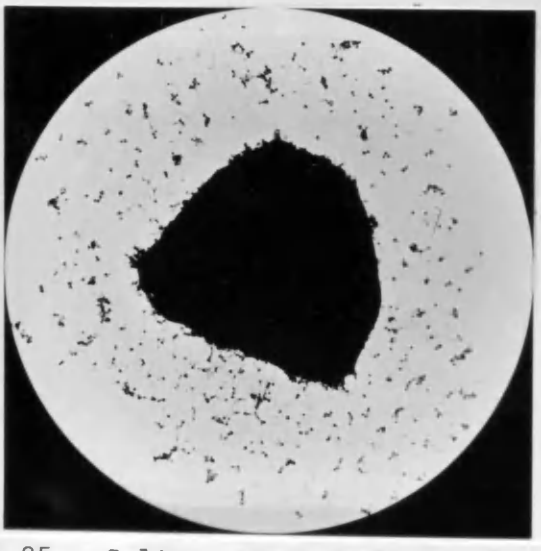
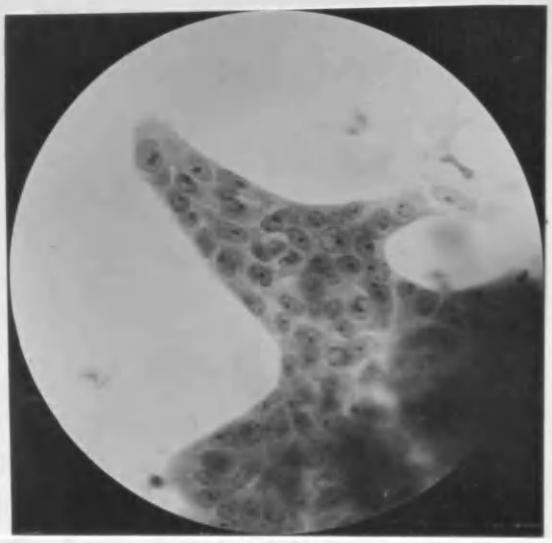
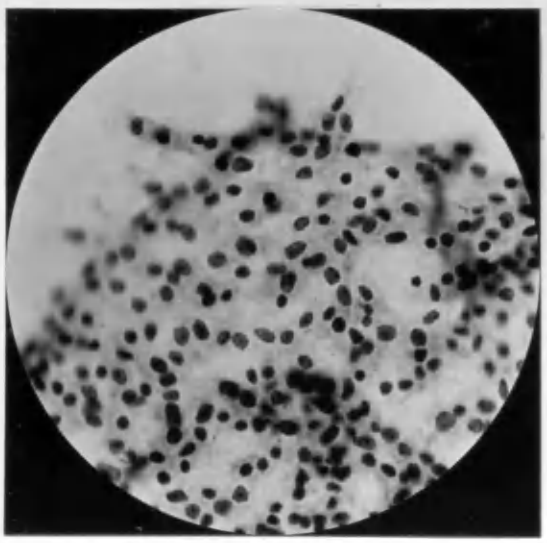


Fig.85.- Culture of fibroblasts from mouse heart treated with heated antiserum for 30 hours. All the cells, as evidenced by supravital staining with neutral red, were dead before fixation. They are rounded up and have contracted individually and in clumps. Zenker formol, iron haematoxylin. x 45.



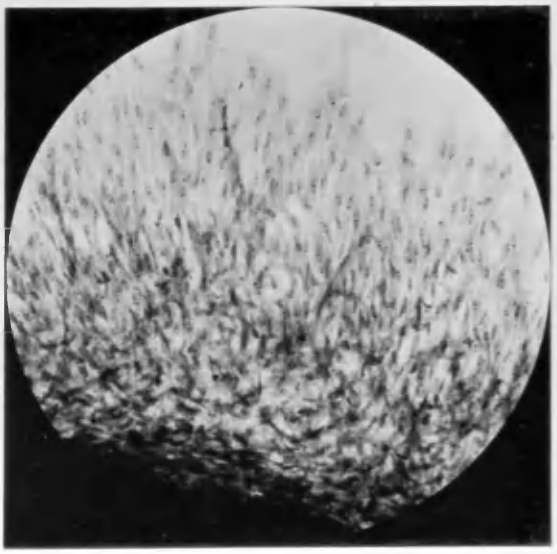
Figs.86 & 87.- Cultures of carcinoma M. 63 after forty-eight hours' incubation. Treated as before with serum for two hours. Zenker-formol. Harris' haematoxylin.

Fig.86.- Normal rabbit serum. Epithelial arrangement of cells with finger-like processes at periphery. x 350.



Figs.86 & 87.- Cultures of carcinoma M. 63 after forty-eight hours' incubation. Treated as before with serum for two hours. Zenker-formol. Harris' haematoxylin.

Fig.87.- Cytotoxic serum. The nuclei are pyknotic and the cytoplasm is unstained. x 400.



Figs.88 & 89.- Embryo chicken heart fibroblasts after twenty-four hours' incubation. Treated for two hours with fresh antiserum and antiserum absorbed with guinea-pig organs. Zenker-formol. Harris' haematoxylin.

Fig.88.- Absorbed antiserum. Normal appearance of fibroblasts. x 125.

Fig.89.- Cytotoxic serum. Cells shrunken, nuclei pyknotic and cytoplasm faintly stained. x 125.

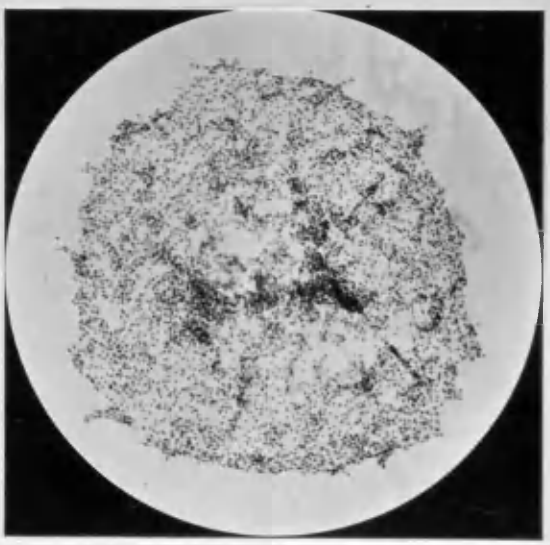


Fig.90.- Sheet of tumour-epithelium treated for 2 hours with antiserum. The nuclei are pyknotic and cytoplasm unstained. Zenker formol, Harris' haematoxylin. x 60.

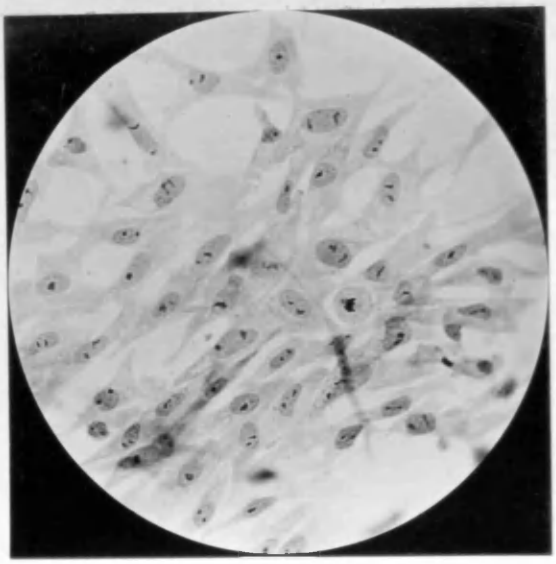


Fig.91.- High power of fig. 88. Nuclei and cytoplasm stain normally: mitotic figure in centre. x 350.

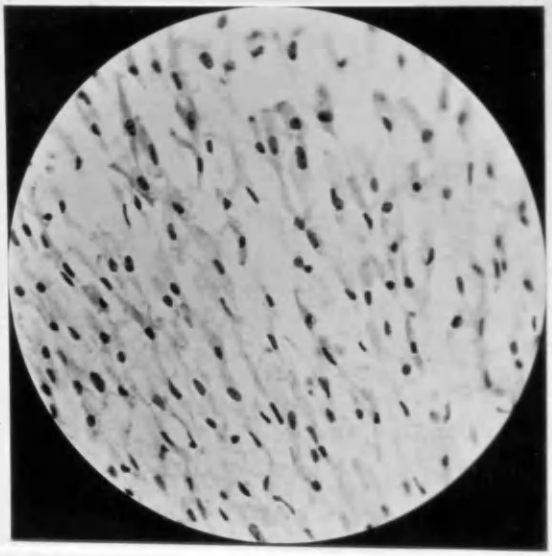


Fig.92.- High power of fig. 89. Nuclei are markedly pyknotic, cytoplasm vacuolated and faintly stained. x 350.

Figs. 91 & 92 are high power photographs of figs. 88 & 89 respectively.

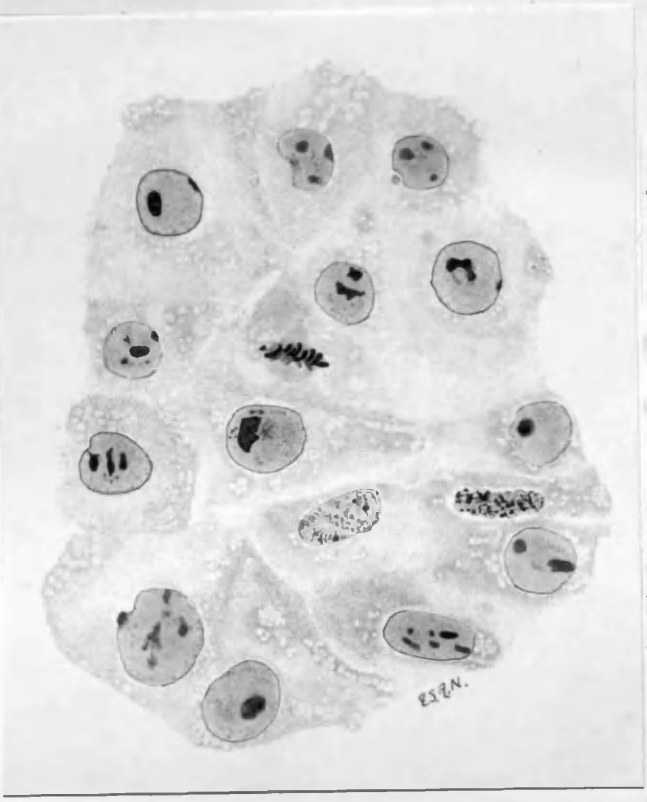


Fig.93.- Normal rabbit serum. Typical epithelial growth. Cell outlines plain and spaces previously occupied by lipoid droplets are seen. Two cells in early prophase and one in metaphase stage of mitotic division are present. × 900.

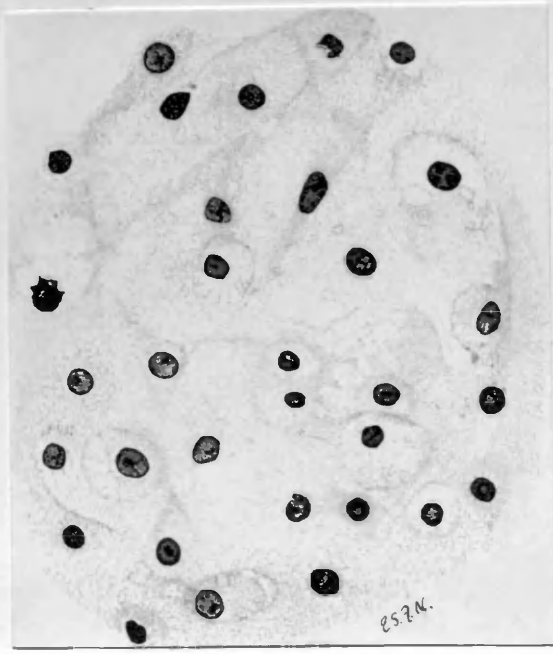


Fig.94.- Cytotoxic serum. Cells greatly shrunken, but attached to one another. The nuclei are much smaller than in fig.93, and are pyknotic and crenated. The cytoplasm is granular and the usual structure has disappeared. ×900.

Figs.93 & 94.- Newly-born rat kidney epithelium after incubation for forty-eight hours. Treated with serum for one hour. Zenker-formol. Harris' haematoxylin.

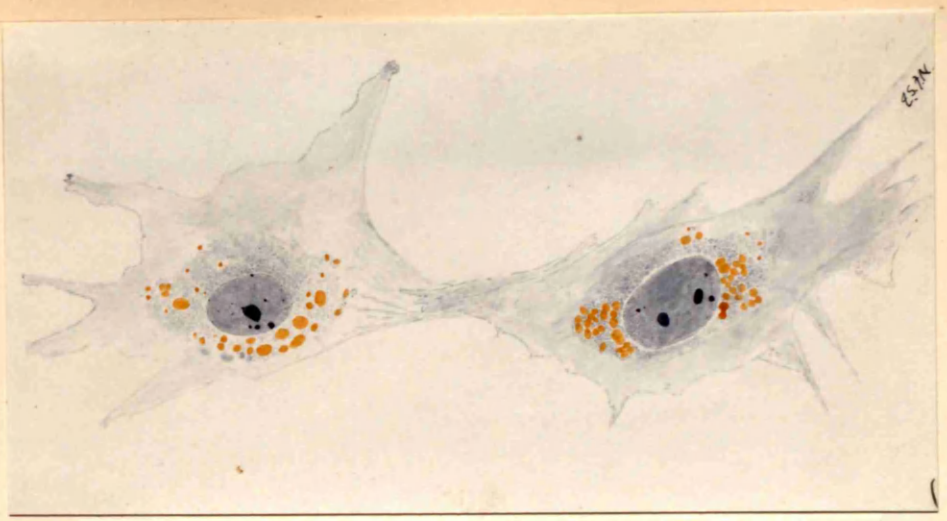


Fig.95.- Normal rabbit serum. The cells are healthy and the normal arrangement of lipoid droplets and fibrillae is seen. x720.

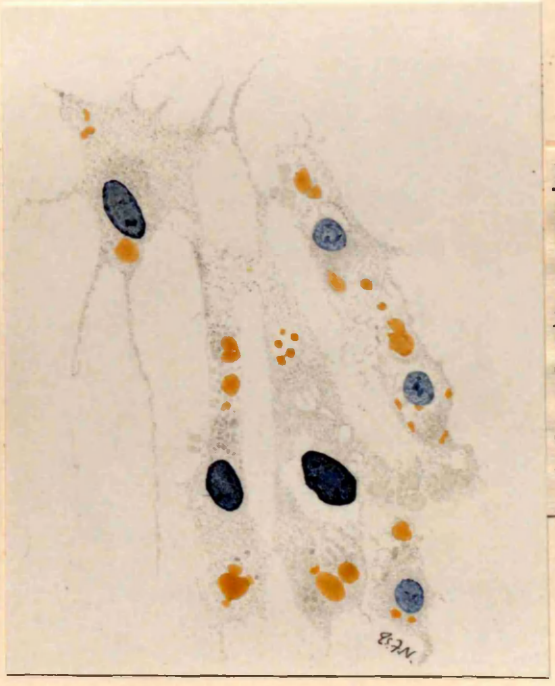
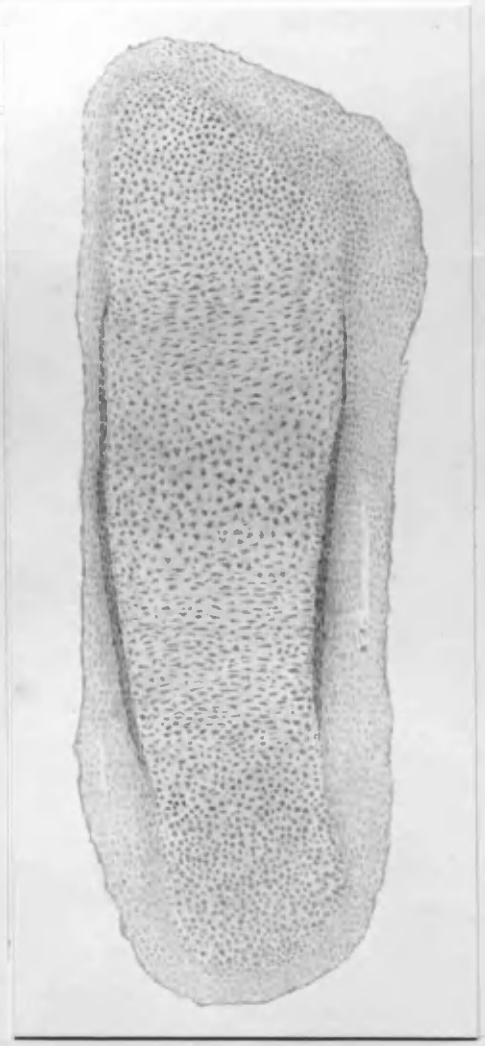


Fig.96.- Cytotoxic serum. The cells are shrunken but remain in contact by fine granular cytoplasmic bridges. Lipoid droplets have fused together in places, and no fibrillae are present. x720.

Figs.95 & 96.- Mesothelial cells from embryo mouse heart after incubation for forty-eight hours. Treated with serum for two hours. Zenker minus acetic acid, iron haematoxylin and sudan III.

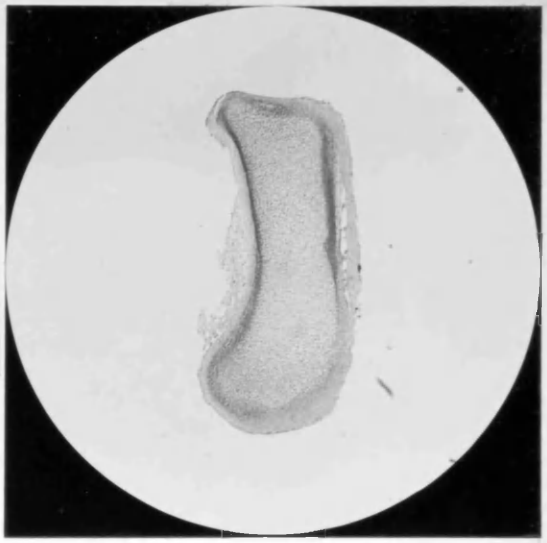
x35 97

Fig. 97. - Section through centre of control tibia from a 5-day fowl embryo. The tibia is composed of a simple type of cartilage and shows no differentiation into epiphyses and diaphysis. Mallory's triple stain. x 35.



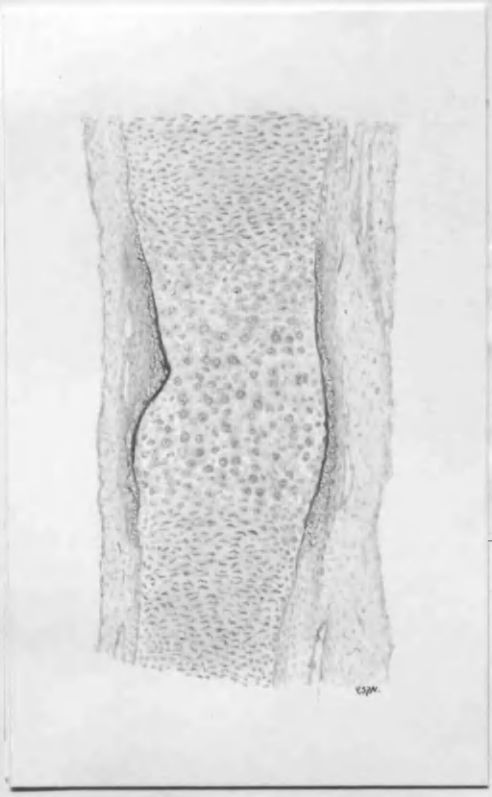
98

Fig. 98. - Section through centre of a tibia from 5-day fowl embryo, cut through centre of shaft and allowed to repair for 24 hours in vitro. No trace of the injury persists. Normal histogenesis of cartilage has begun. Masson's iron haematoxylin, ponceau 2R, light green. x 112



x30 99

Fig. 99. - Section through centre of control tibia from 5-day fowl embryo cultivated for 24 hours in vitro (cf. fig. 2). Mallory's triple stain. x 30.



100

Fig. 100. - Section through centre of a tibia from 5-day fowl embryo, cut through centre of shaft and allowed to repair for 6 days in vitro. No trace of injury can be seen and normal histogenesis of cartilage and bone is continuing. Masson's iron haematoxylin, ponceau 2R, light green. x 80.

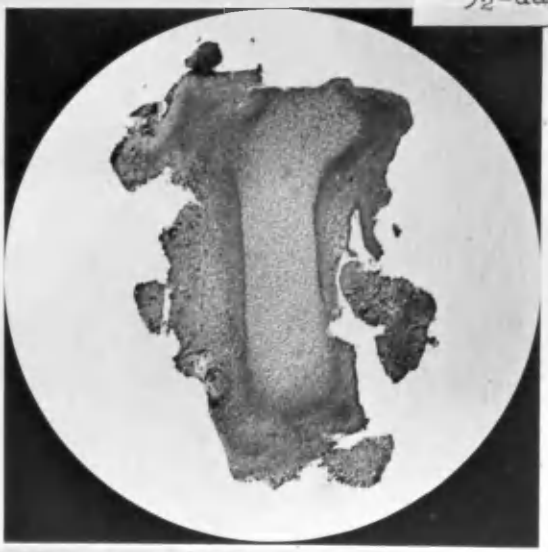
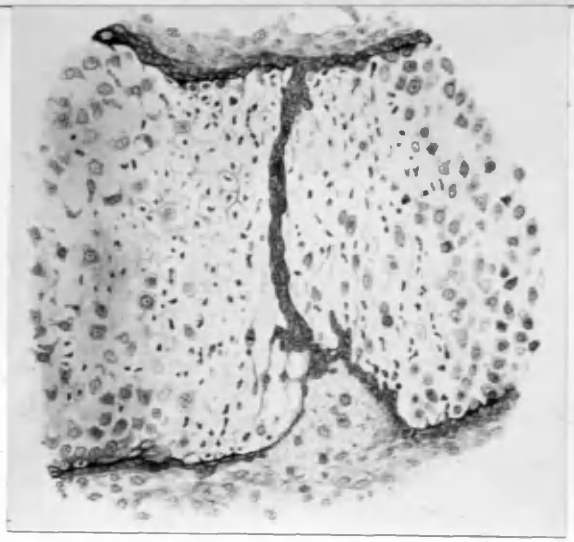


Fig.101.- Section through centre of control femur from 5½-day fowl embryo. Cartilage shows no differentiation into epiphyses and diaphysis but cells of centre of shaft are larger and separated by more matrix than in fig. 97. Mallory's triple stain. x 35.

Fig.102.- Section thro' centre of femur from 5½-day fowl embryo which was cut thro' centre



of shaft and allowed to repair for 6 days in vitro. Union has occurred and normal histogenesis of uninjured cartilage and of the osteogenic tissue is progressing. Osteoblasts, continuous with those of the shaft, form a bridge across the cartilage. The adjoining chondroblasts are necrotic. On each side of the necrotic areas hypertrophic chondroblasts are seen. Mallory's triple stain. x

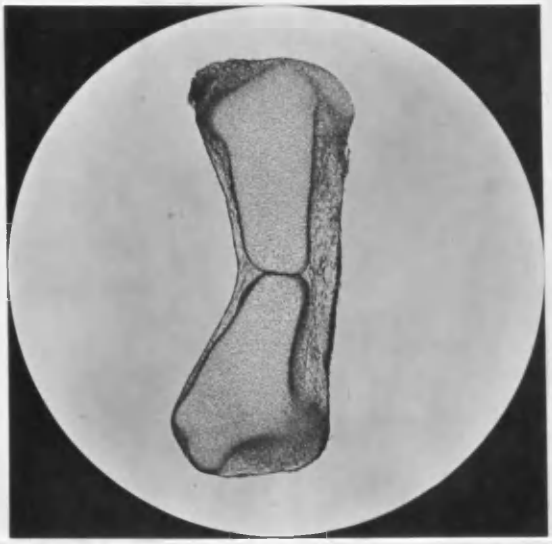


Fig.103.- Section thro' centre of femur from 5½-day fowl embryo which was cut thro' centre of shaft and allowed to repair for 3 days. Union has been brought about by developing osteogenic tissue which is being compressed by the developing cartilage. Masson's iron haematoxylin, ponceau 2R, light green. x 30.

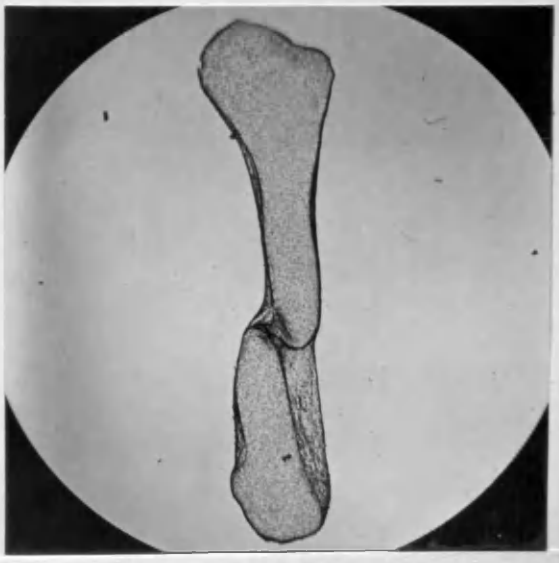


Fig.104.- Section through centre of tibia from 5½-day fowl embryo which was cut through centre of shaft and allowed to repair for 9 days. Apposition of cut ends was faulty: union of cut ends brought about by osteogenic layers and surrounding tissue and formation of osteoid tissue between the fragments is proceeding. Safranin picro-indigo-carmine. x 25.

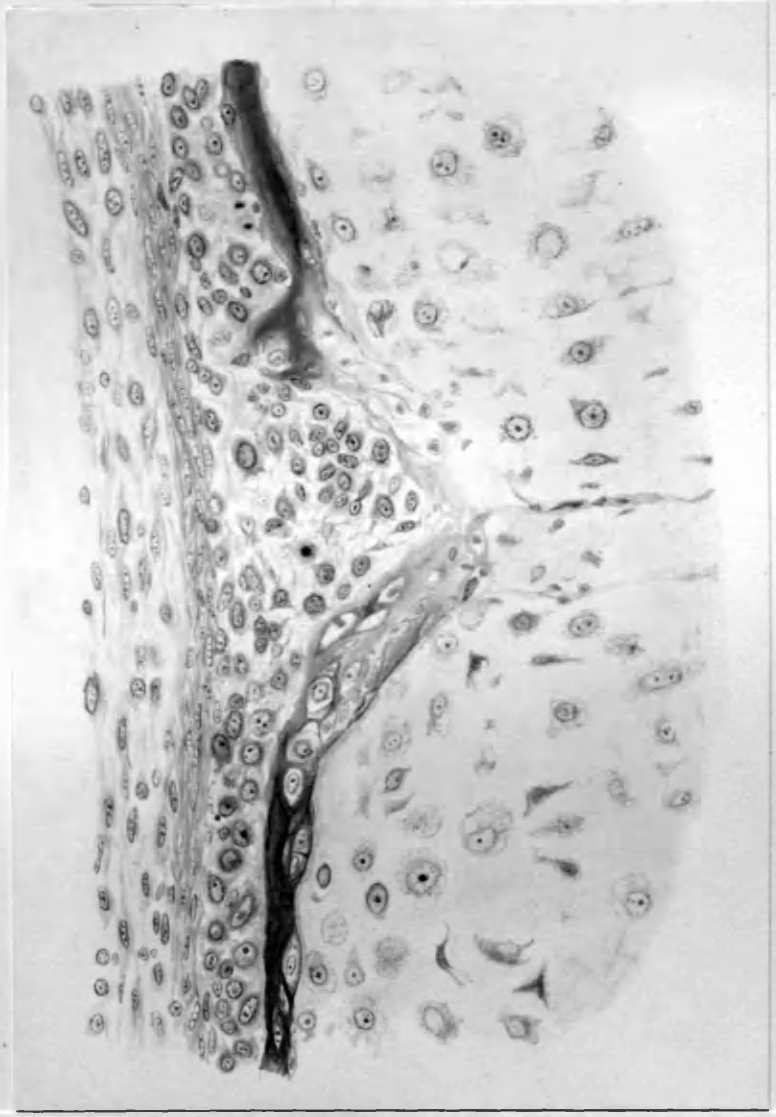


Fig. 105. - Part of a section through centre of a tibia from 5½-day fowl embryo cultivated for 6 days *in vitro*, then cut through centre of shaft and allowed to repair for 2 days *in vitro*. The osteoblastic layer and the fibrous periosteum have repaired themselves and osteoblasts have accumulated round the margins of the necrotic cartilage where they show mitoses. Masson's iron haematoxylin, ponceau 2R, light green. x 430.

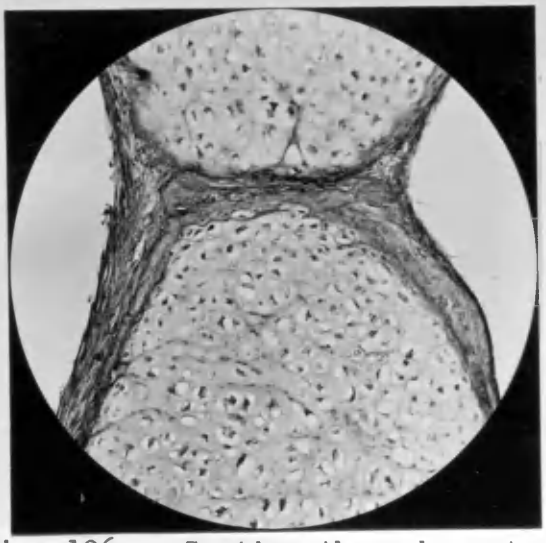


Fig. 106. - Section through centre of shaft of fractured tibia from 5½-day fowl embryo which was cultivated for 12 days in vitro. A zone of osteoid tissue continuous with that of the shaft has been formed by the osteoblasts which have grown in between the cut surfaces of cartilage. Safranin picro-indigo-carmine. x 150.

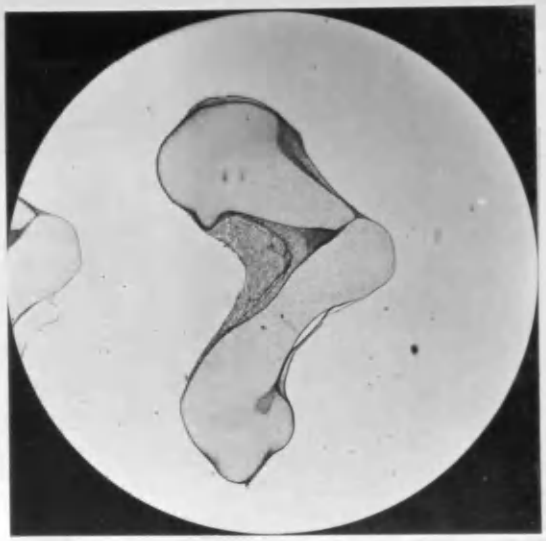
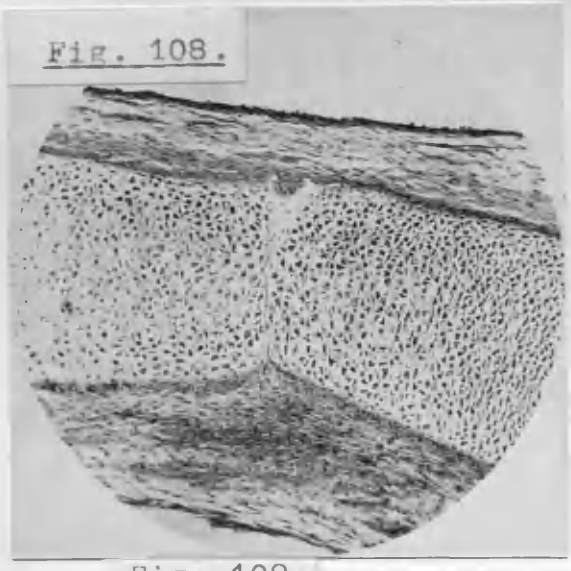


Fig.107.- Section thro' centre of tibia from 5½-day fowl embryo which was cut and allowed to repair for 9 days. Marked distortion has occurred. Over convexity of the cartilage, the osteoblastic layers have almost disappeared, in the concavity, formation of osteoid tissue is proceeding. Mallory's triple stain. x 17.



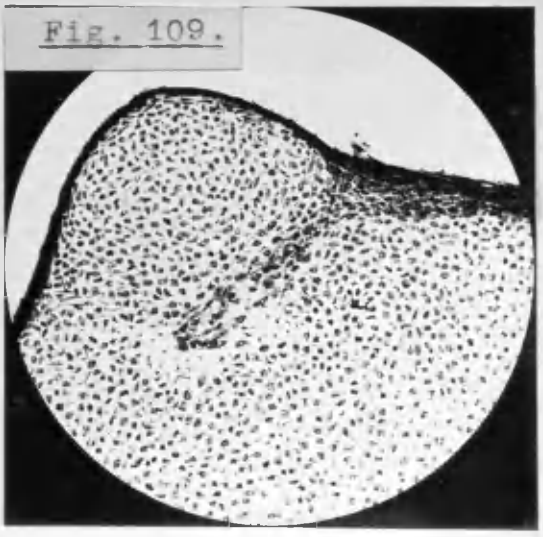


Fig. 109. Developing epiphysis of 6-day tibia which was cultivated for 4 days *in vitro* and then incised through the articular surface and cultivated for a further 4 days. Repair of the perichondrium has taken place and it is giving rise to cartilage cells which have emigrated into the gap. An area of necrotic cartilage is present at the inner end of the cut. Masson's iron hæmatoxylin, ponceau 2R, light green. × 175.

Fig. 108. 5-day tibia cultivated for 24 hours *in vitro* then incised at centre of shaft and cultivated for a further 4 days. Repair has occurred and normal histogenesis of cartilage and bone is taking place. The site of injury is indicated by an area of necrosis in the cartilage and a few osteoblastic cells have accumulated in the gap between the cut surfaces. Iron hæmatoxylin, ponceau 2R, light green. × 80.

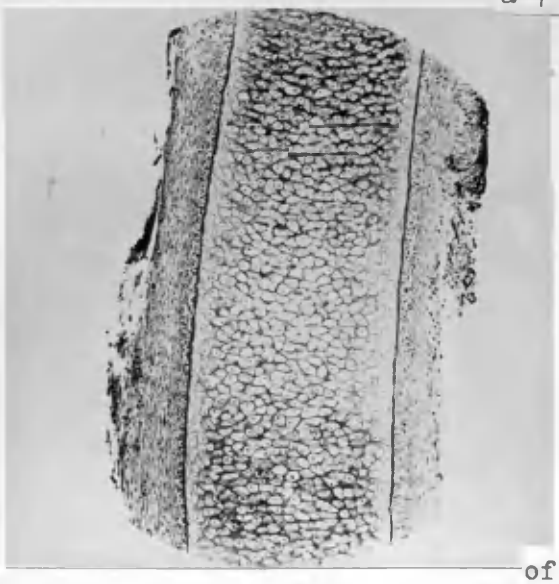
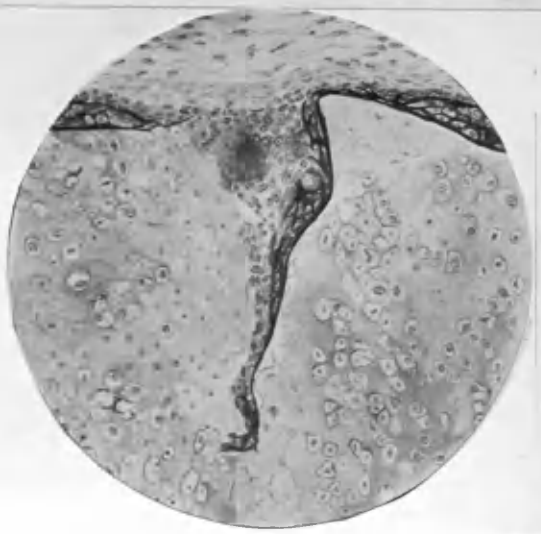
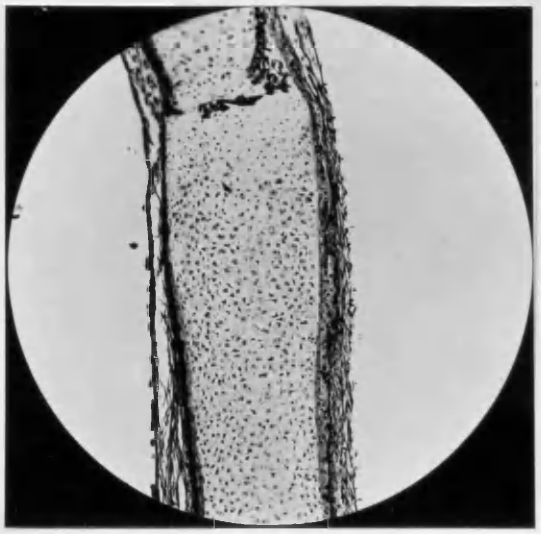


Fig. 110. - Section through centre of control femur from 7-day fowl embryo showing hypertrophic cartilage, developing bone and periosteal layers. Magenta picro-indigo-carmine. x 200.

Fig.111.- Part of a section thro' centre of a 7-day femur which was incised thro' centre



of shaft and allowed to repair for 9 days in vitro. The periosteal layers are continuous at the site of injury and continuity of the periosteal bone is being restored. Osteoblasts have grown into the gap between the cut surfaces of the now necrotic cartilage and have formed osteoid tissue continuous with the bone of the shaft. Magenta picro-indigo-carmine. x 145.



ig.112.- Part of section thro' centre of 7-ay tibia which was incised thro' centre of haft and allowed to repair for 2 days. steoblasts have migrated between the cut nds of cartilage and union has been brought bout by restoration of continuity of peri-steal layers and the fusion of cartilage ue to its progressive development above nd below the site of injury. Bouin:Masson's ron haematoxylin, ponceau 2R, light green. x 175.

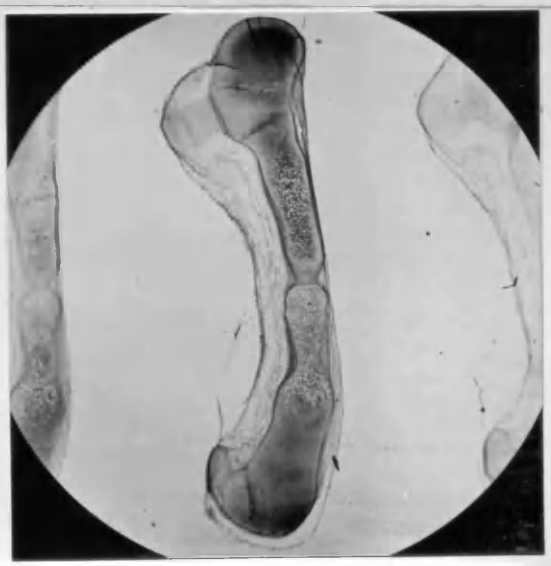


Fig.113.- Section thro' centre of shaft of 7-day femur which was incised thro' centre of shaft and allowed to repair for 16 days. The site of injury is occupied by a broad zone of osteoid tissue continuous with that of the shaft above and below the site of injury. Bouin: magenta picro-indigo-carmine. x 17.

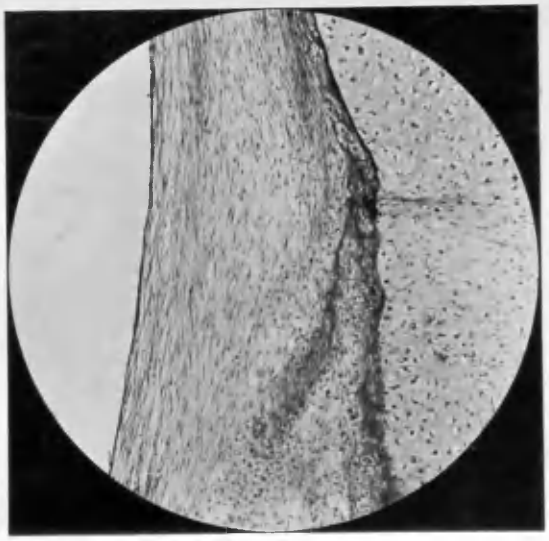


Fig.114.- Part of section of a 7-day tibia cultivated for 16 days in vitro showing extension of ossification into the surrounding connective tissue in a region where the fibrous periosteum has disappeared. Ossification is normal where the fibrous periosteum is intact. Safranin picro-indigo-carmine. x 80.

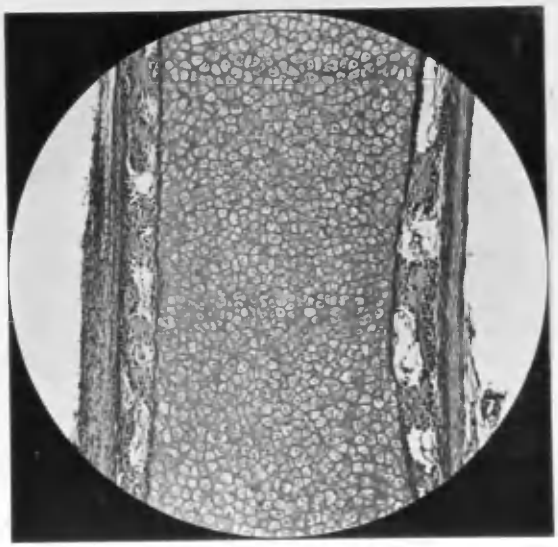


Fig.115.- Section through centre of shaft of 9-day (control) tibia showing hypertrophic cartilage, trabecular bone and periosteal layers. Masson's iron haematoxylin, ponceau 2R, light green. x 80.

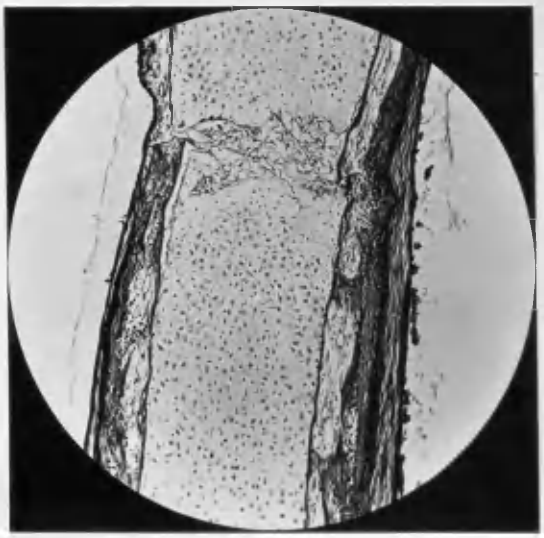


Fig.116.- Section through centre of shaft of 9-day tibia which was cut completely across and allowed to repair for 10 days in vitro. Continuity of the periosteal layers is complete all round but union of bony cylinder is complete on one side only. Masson's iron haematoxylin, ponceau 2R, light green. x 70.

Fig.117.- High power of 116: part of section through site of fracture of 9-day tibia which was cultivated for 10 days in vitro. The continuity of the periosteal layers and of the outer cylinder of bone has been restored. The gap between the cut surfaces of cartilage is occupied by spindle-shaped cells resembling fibroblasts, derived, however, from the osteoblastic layer, which are invading the cartilage and causing its resorption. Masson's iron haematoxylin, ponceau 2R, light green. x 200.

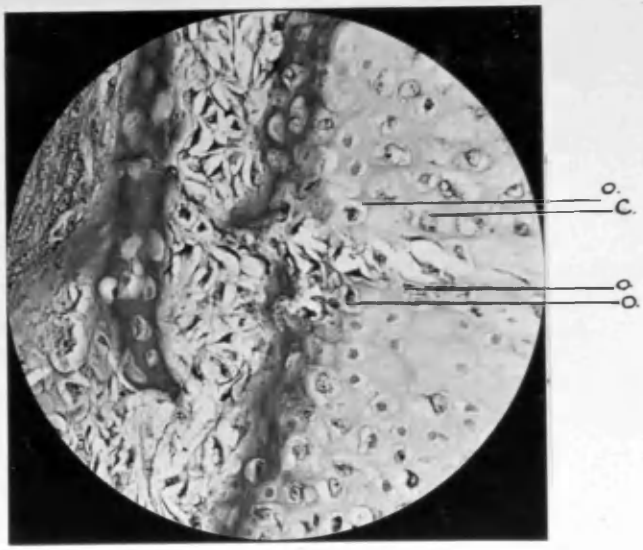


Fig.118.- Part of section thro' site of fracture of femur from 9-day embryo showing invasion of cartilage capsules by osteoblasts. These are distinguished by their polygonal or triangular shape and by their intensely staining cytoplasm. The surviving chondroblasts have faintly staining nuclei and vacuolated cytoplasm. o = osteoblasts, c = chondroblasts. Safranin picro-indigo-carmine. x 250.

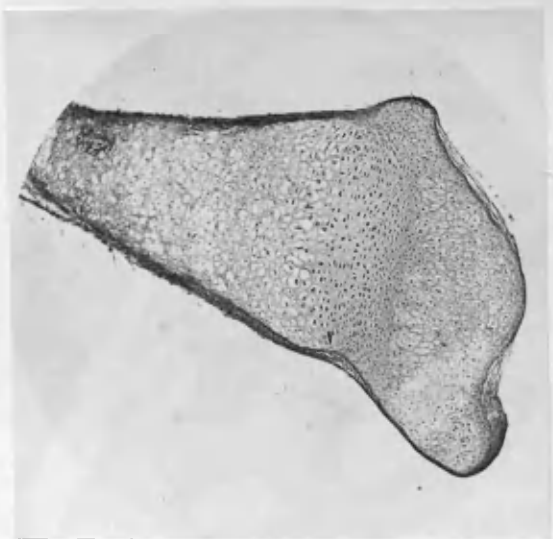


Fig.119.- Section through proximal epiphysis of embryonic mouse tibia cultivated for 16 days *in vitro*. The epiphyseal cartilage is hypertrophic and is separated from the cartilage of the diaphysis by a zone of flattened cells (cf.fig.120). Safranin picro-indigo-carmine. x 40.

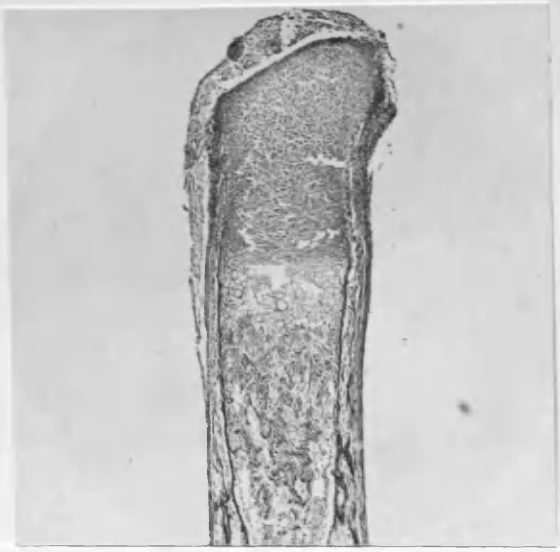


Fig.120.- Section through centre of control embryonic mouse tibia showing small-celled epiphyseal region, trabecular bone and developing marrow. Safranin picco-indigo-carmine. x 30.

Fig.121.- Site of fracture of embryonic mouse tibia after 8 days' cultivation showing newly-formed osteoid tissue continuous with the pre-existing and intensely staining bone. Safranin picro-indigo-carmine. x 570.

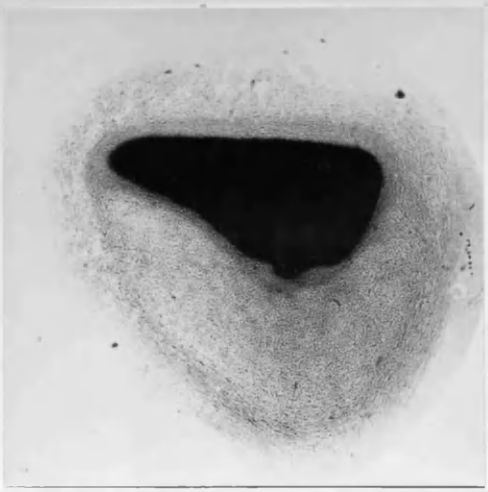


Fig.125.- Whole mount of patella culture 6 days after appearance of cartilage in the explant. Note the triangular shape of the cartilage mass which is sharply demarcated from the surrounding tissue. Zenker: thionin blue.

Fig.126.- Section through centre of knee-joint region of limb of other side of same embryo as that from which explant in fig.125 was derived. The section was stained with thionin blue which demonstrates specifically areas of early chondrogenesis. At the site (S) at which the patella develops, no trace of cartilage is seen. Zenker. x 36.

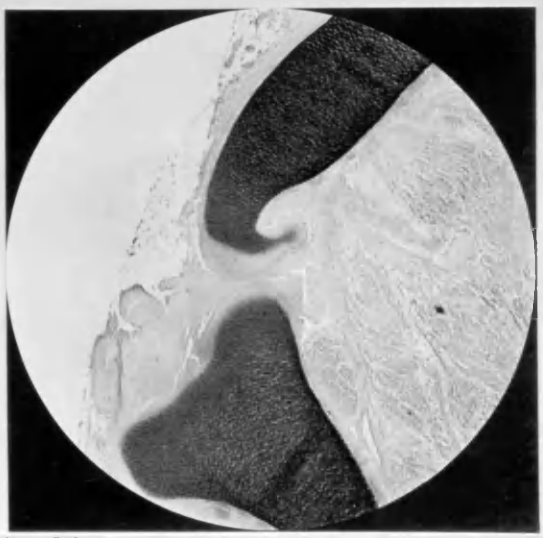


Fig.127.- Section through the middle of knee-joint region of limb from which presumptive patellar tissue was removed. In this mass of mesenchyme the cartilage in fig.125 developed. Epiphyses of the femur and tibia are uninjured and the perichondrium is intact. Zenker: thionin blue. x 36.

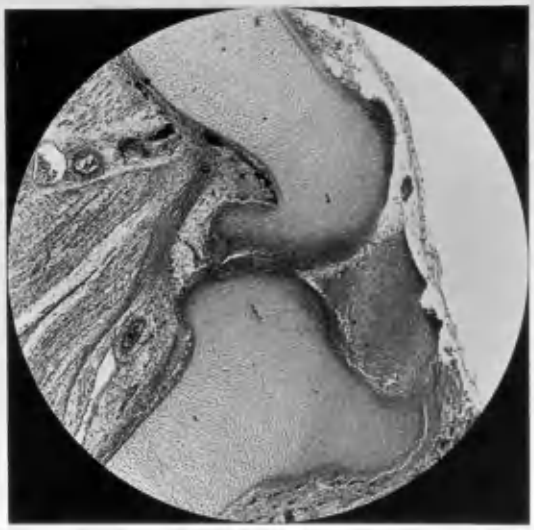


Fig. 128.- Section through centre of knee-joint from which tissue for culture shown in fig.130 was taken. The femoral and tibial epiphyses are undamaged and the perichondrium is intact. Bouin: Mallory's triple stain. x 40.

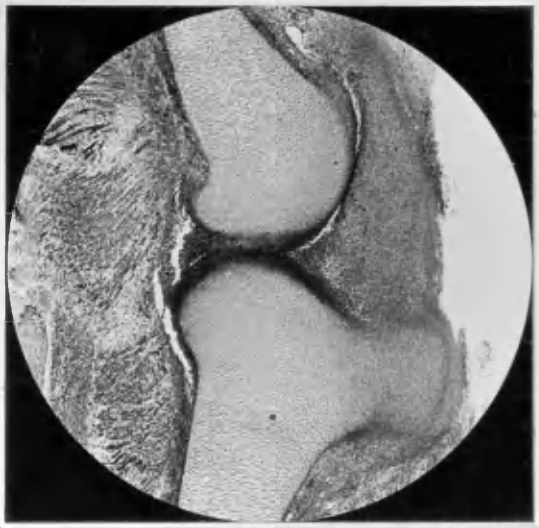


Fig.129.- Section through centre of knee-joint of limb from other side of same embryo as fig.128. In the region where the patella subsequently develops no cartilage is to be seen. Bouin: Mallory's triple stain. x 40.

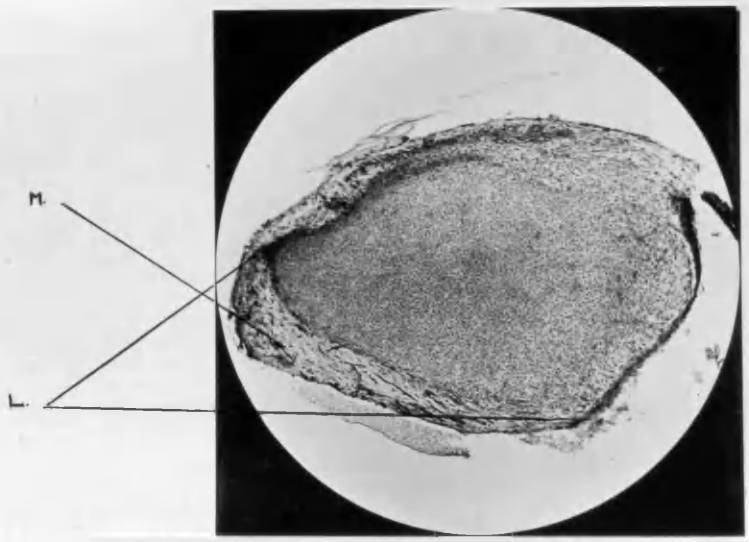


Fig.130.- Culture of presumptive patellar mesenchyme after 3 days' cultivation, showing commencing chondrogenesis. L = upper limit of future patella: M = muscle fibres derived from distal end of great extensor muscle of thigh. The area of commencing chondrogenesis is roughly triangular. Zenker plus acetic acid: Mallory's triple stain. x 40.

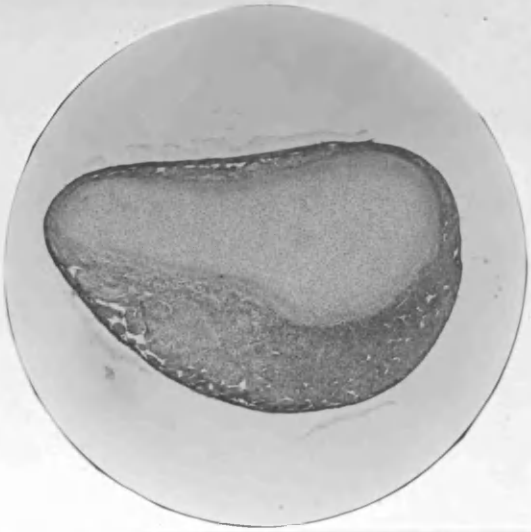


Fig.131.- Section through culture of patella developing in vitro after 12 days' cultivation. The cartilage mass, which consists of small-celled chondroblasts, is roughly triangular in shape and sharply demarcated from the surrounding tissue. Zenker: Mallory's triple stain. x 55.

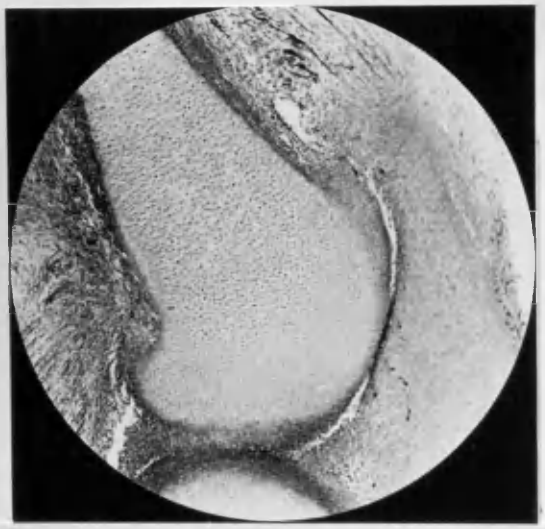


Fig.132.- Section through knee-joint of other side of same embryo as fig.131. Cartilage formation in the patellar region has not yet begun. Bouin: Mallory's triple stain. x 60.

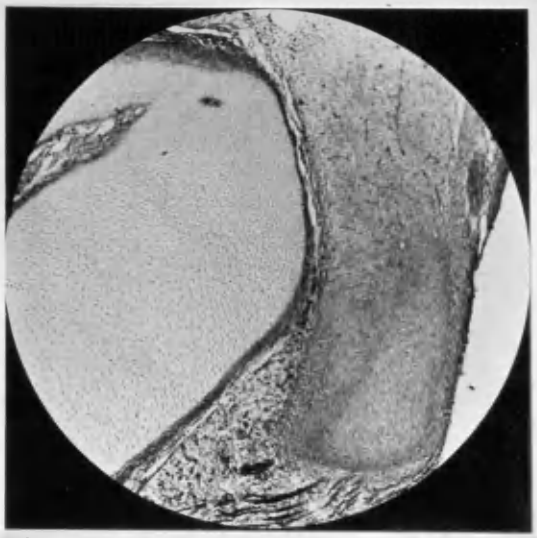


Fig.133.- Section through knee-joint region from 13-day avian embryo, showing formation of cartilaginous patella.
P = patella. Bouin: Mallory's triple stain. x 40.

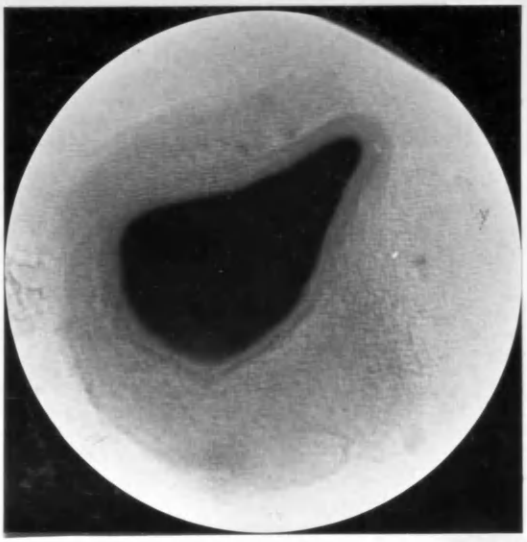
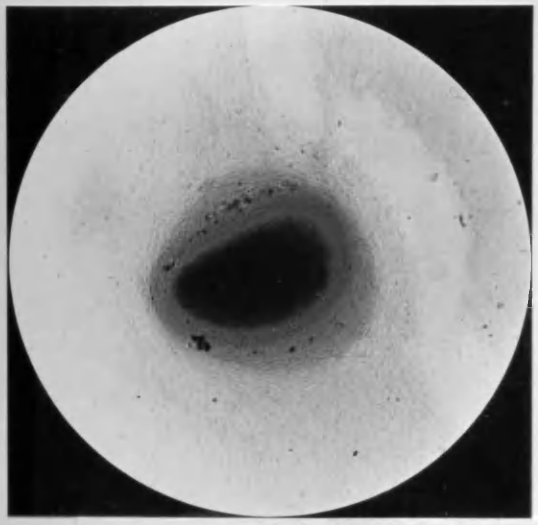
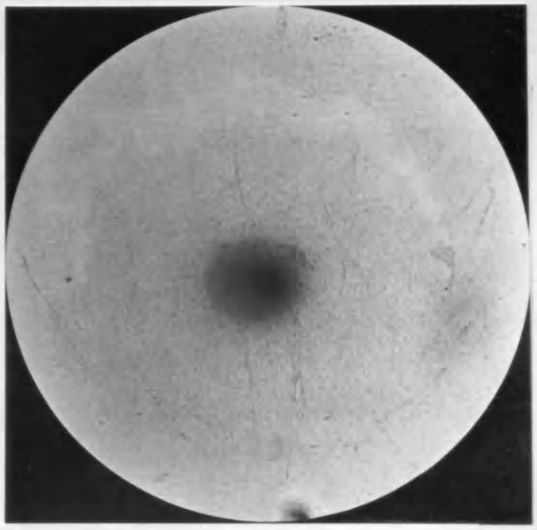


Fig.134.- Whole mount of avian patella which differentiated in vitro. 8 days' cultivation. Zenker: thionin blue. x 40.





Figs. 135 and 136 are cultures derived from the future patellar mesenchyme of the other side of the same embryo as fig. 134. The mesenchyme was divided into two equal parts which were explanted separately. The cartilage in culture shown in fig.135 is considerably greater in amount than that obtained in the culture shown in fig.136, but the sum of the cartilage in both cultures is less than that in fig.134. Zenker: thionin blue. x 40.

Fig. 134 should have been mounted with the upper margin of cartilage parallel to the upper edge of the plate.

Fig.137.- Wax reconstruction of patella isolated from avian embryo at the end of the 11th day.

Fig.138.- Wax reconstruction of patella isolated from avian embryo after 14½ days' incubation.

Fig.139.- Wax reconstruction of 20-day culture of avian patella which differentiated *in vitro*.

Fig.140.- Wax reconstruction of 14-day culture of avian patella which differentiated *in vitro*.

Note: All 4 photographs of the reconstructions should have been placed with the upper margin parallel with the top of the place.

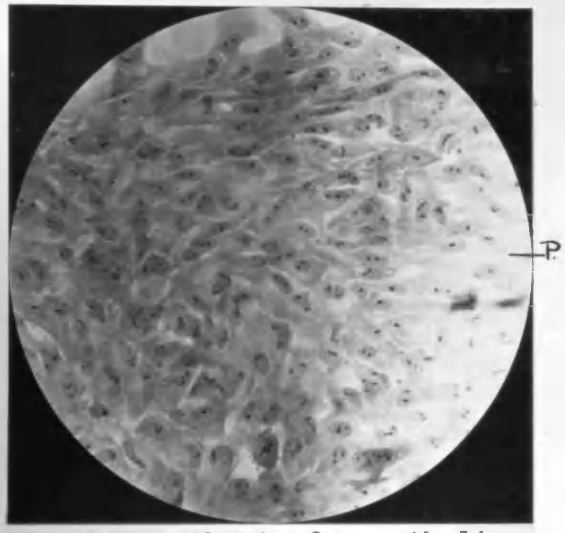


Fig.141.- Sheet of mesothelium from heart of 10-day avian embryo. Note the spread-out character of the cells towards the periphery of the culture, (P). Zenker: Weigert's iron haematoxylin. x 100.

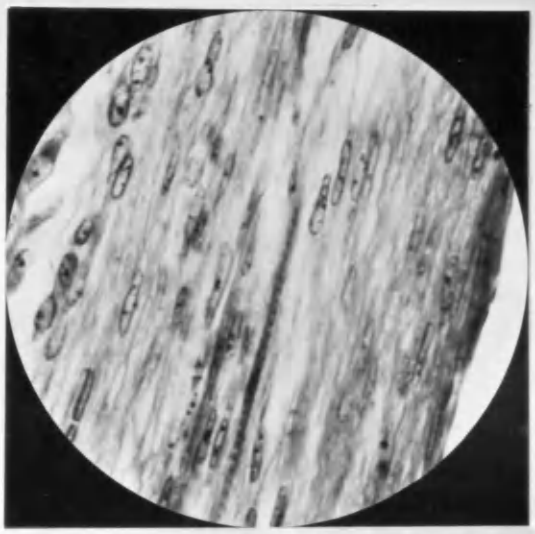


Fig.142.- Part of connective tissue surrounding skeletal rudiment from 5-day embryo cultivated for 12 days. Fine collagen fibres are seen separating the cells and in the centre are two thin cross-striated muscle fibres which have developed in vitro. Zenker plus acetic acid: Masson's iron haematoxylin: Ponceau's 2 R light green. x 650.

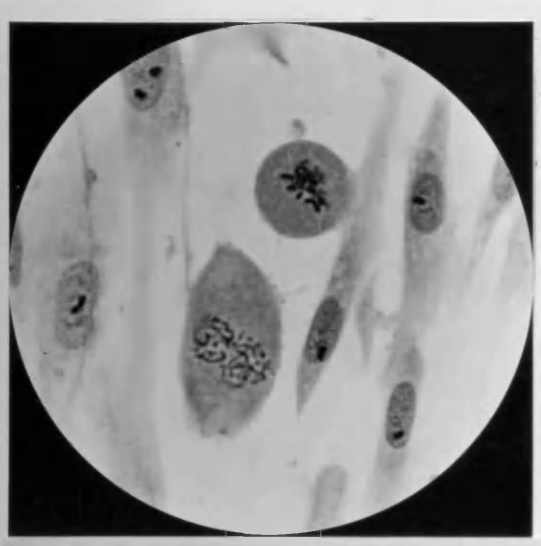


Fig.143.- Culture of subcutaneous tissue from choroid of 9-day embryo. A large cell containing two nuclei, both in late prophase stage of mitotic division is seen in the centre. Above it is a cell in metaphase stage. Zenker plus acetic acid: Harris' haematoxylin. x 650.

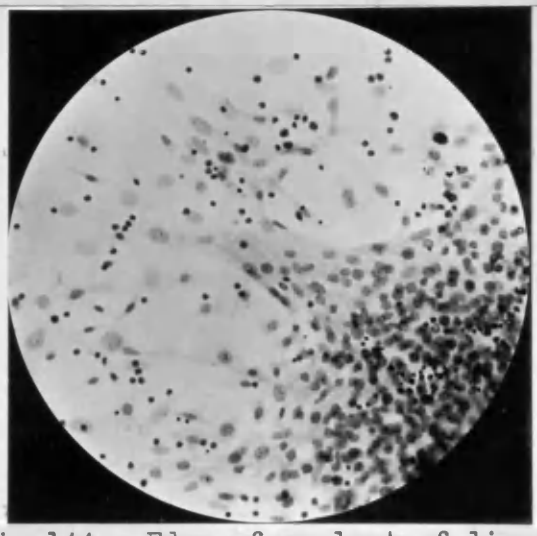


Fig.144.- Edge of explant of liver from newly-born mouse after 2 days' cultivation. Numerous fibroblasts, wandering cells and lymphocytes are seen. A small sheet of mesothelium is also present. Corrosive sublimate: Harris' haematoxylin. x 200.

Fig. 145.

Fig. 146.

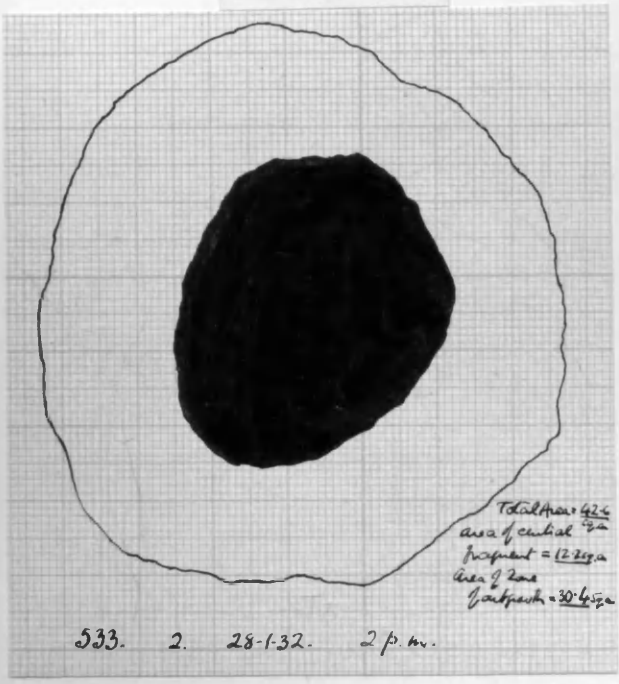


Fig. 147.

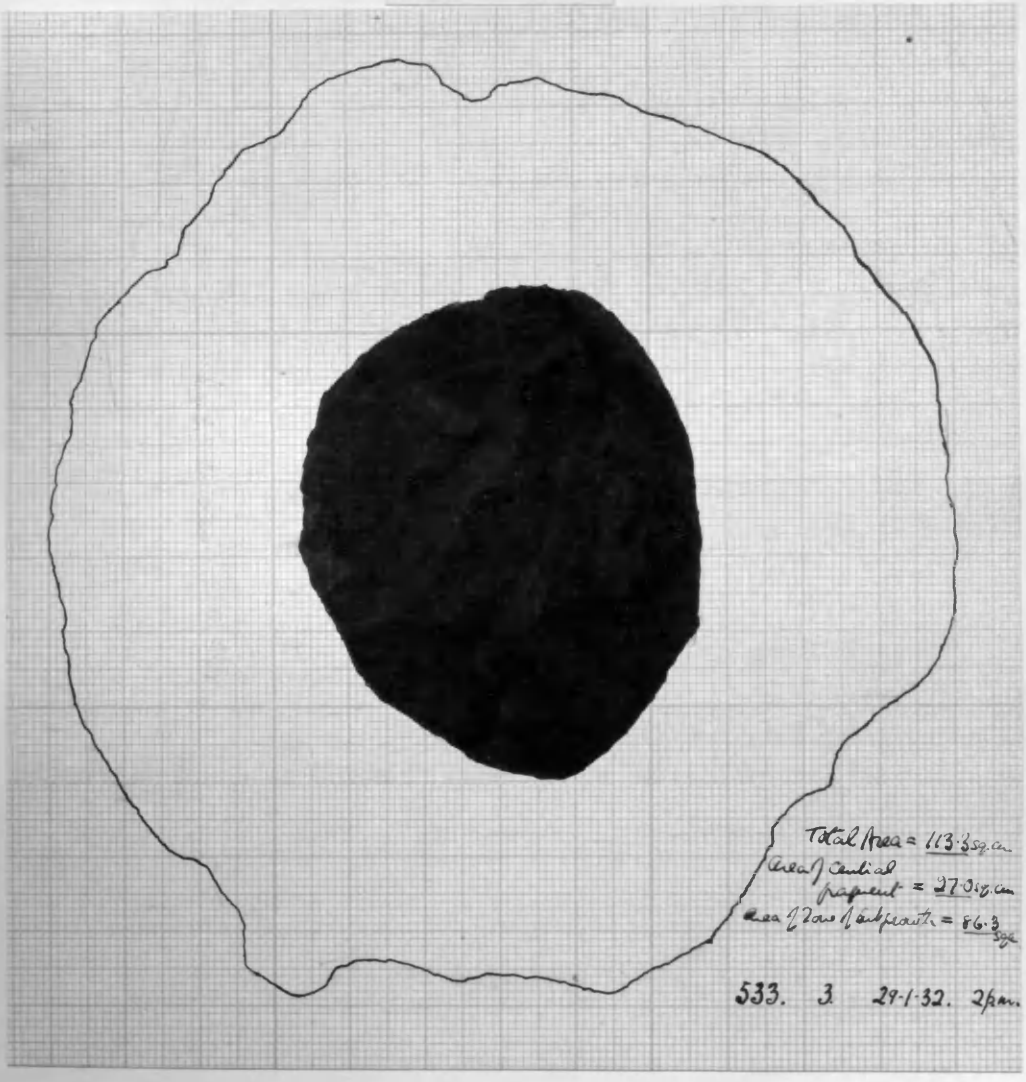

Total Area = 113.859 sq. cm.
Area of caudal fragment = 27.319 sq. cm.
Area of two of each growth = 86.3 sq. cm.
533. 3 29-1-32. 2 fm.

Fig. 148.

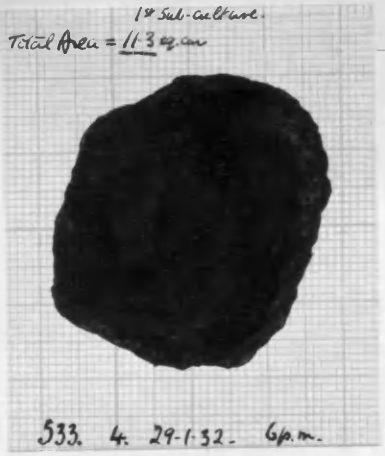


Fig. 149.

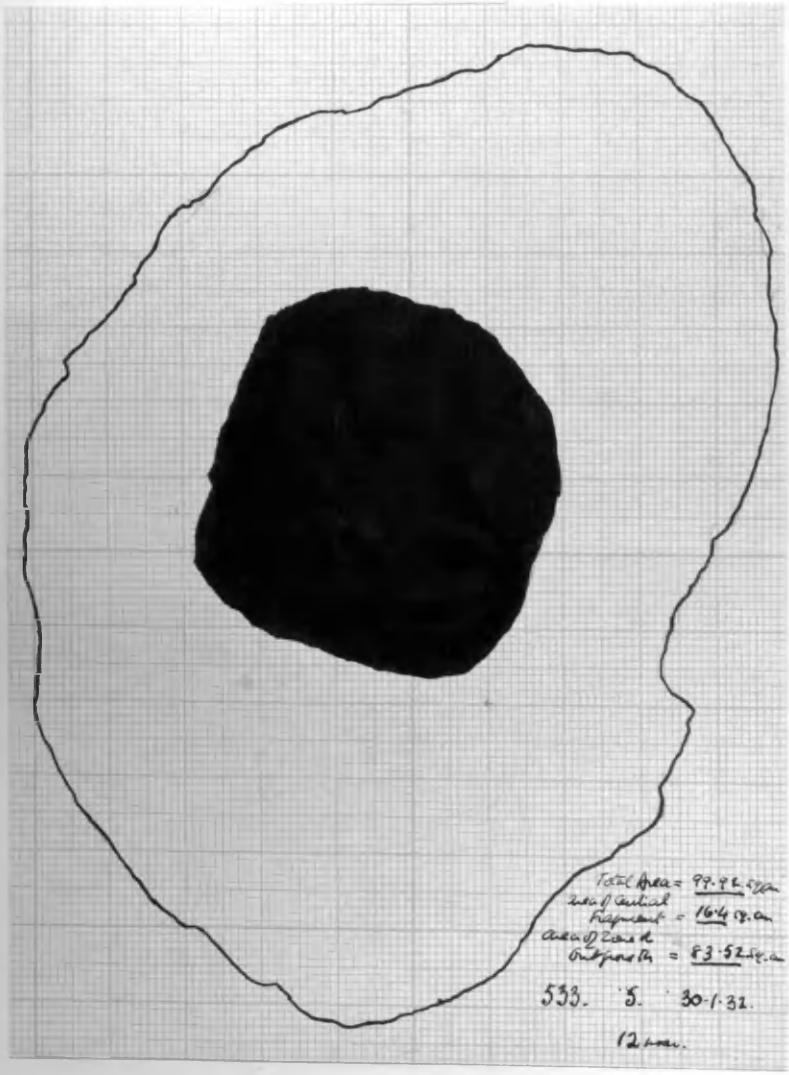


Fig. 150.

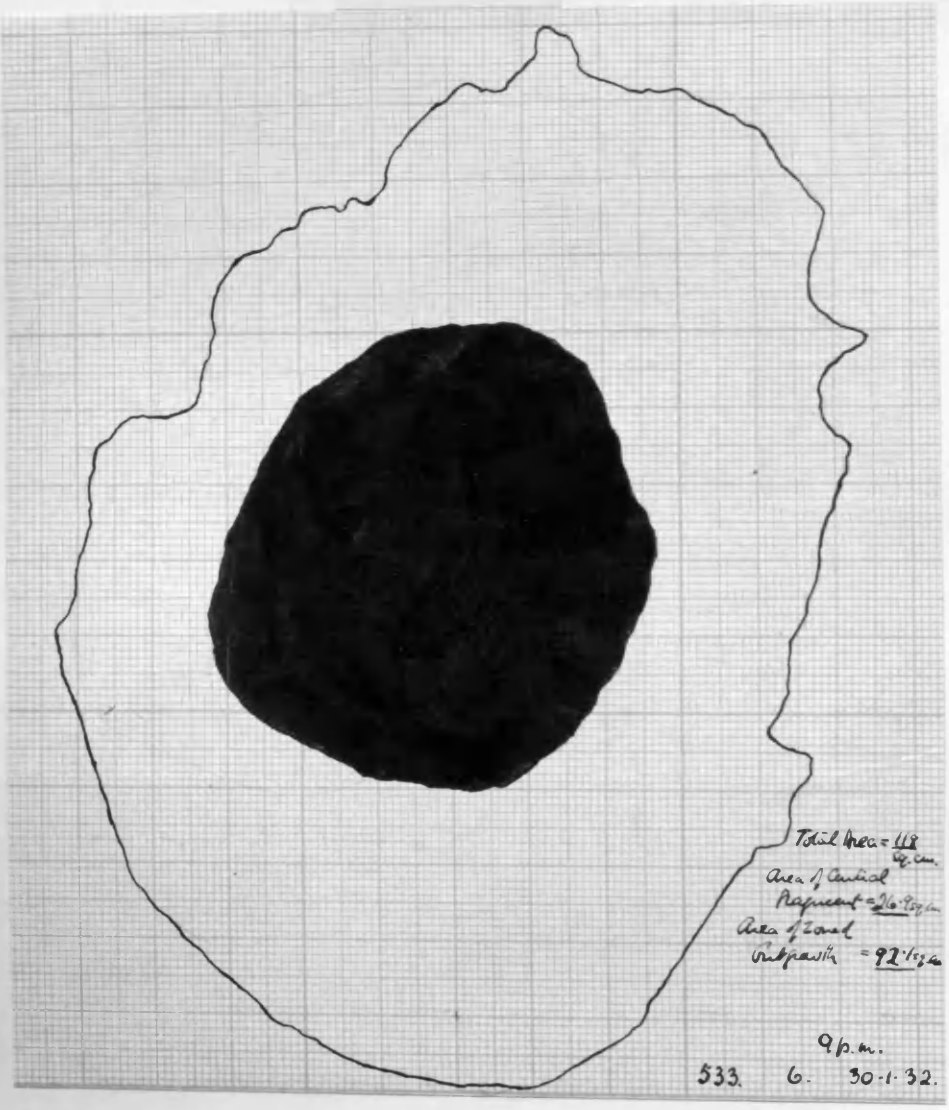

Total Area = 118 Sq. cm.
Area of Central Nucleus = 26·8 Sq. cm.
Area of Zonal Cathpart = 92·132 cm.
9 p.m.
533 6. 30·1·32.

Fig. 151.

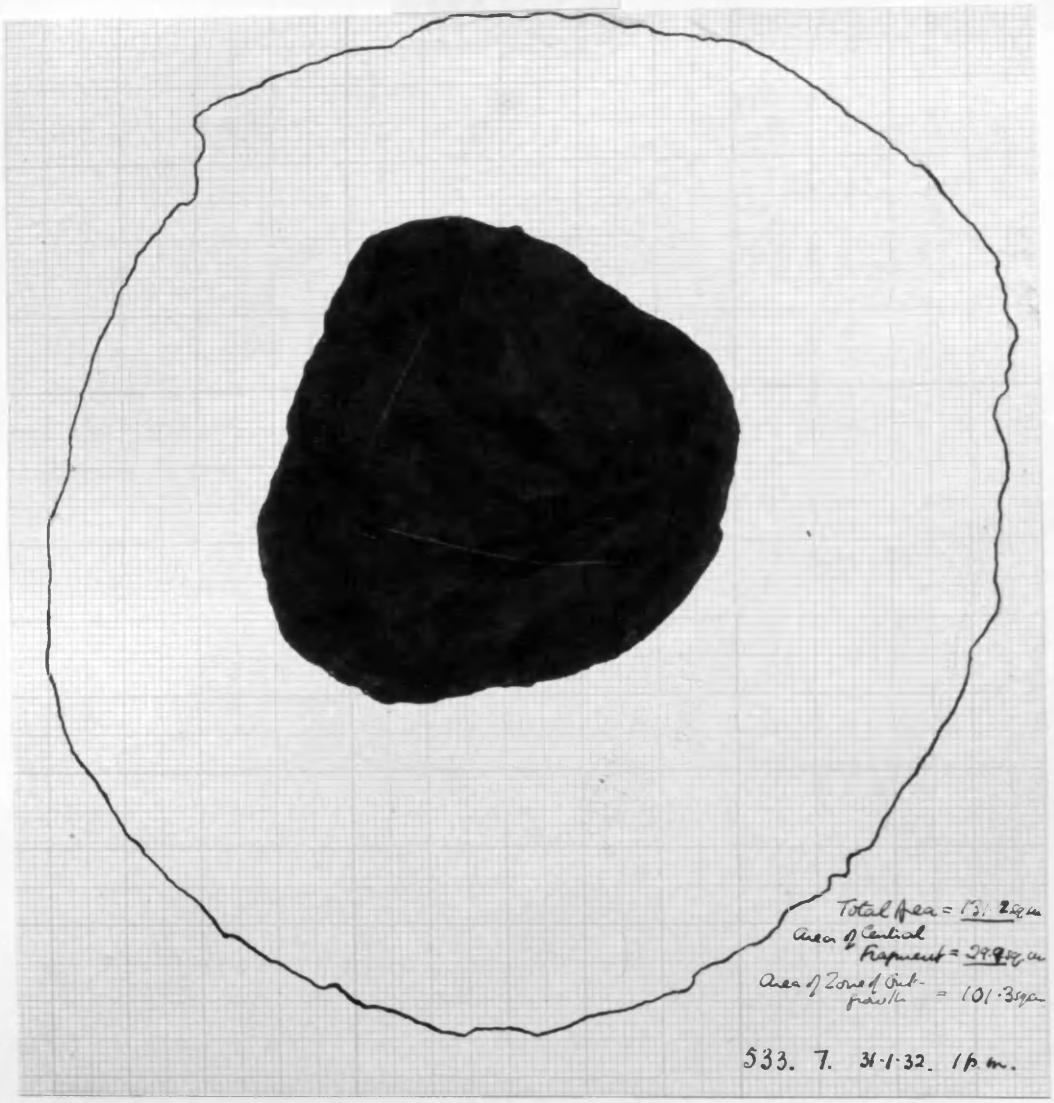

Total Area = 131.2 sq cm
Area of Central Fragment = 29.9 sq cm
Area of Zone of Out-growth = 101.3 sq cm
533. 7. 31·1·32. 1 p.m.

Fig. 152.

Fig. 153.

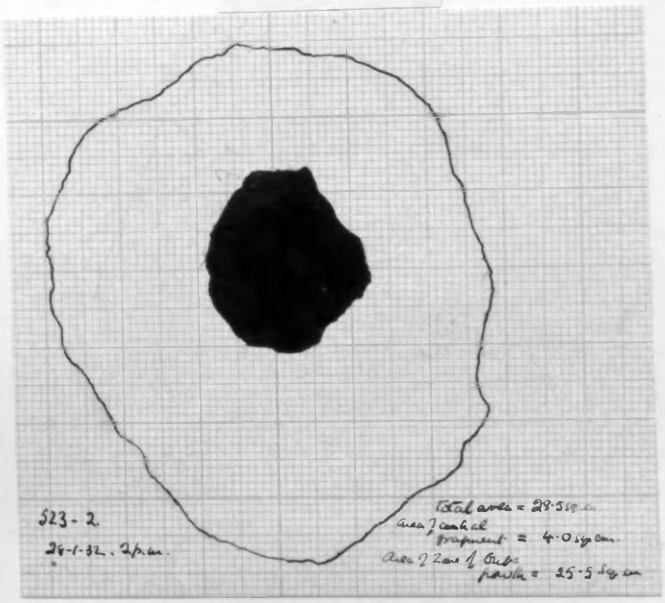


Fig. 154.

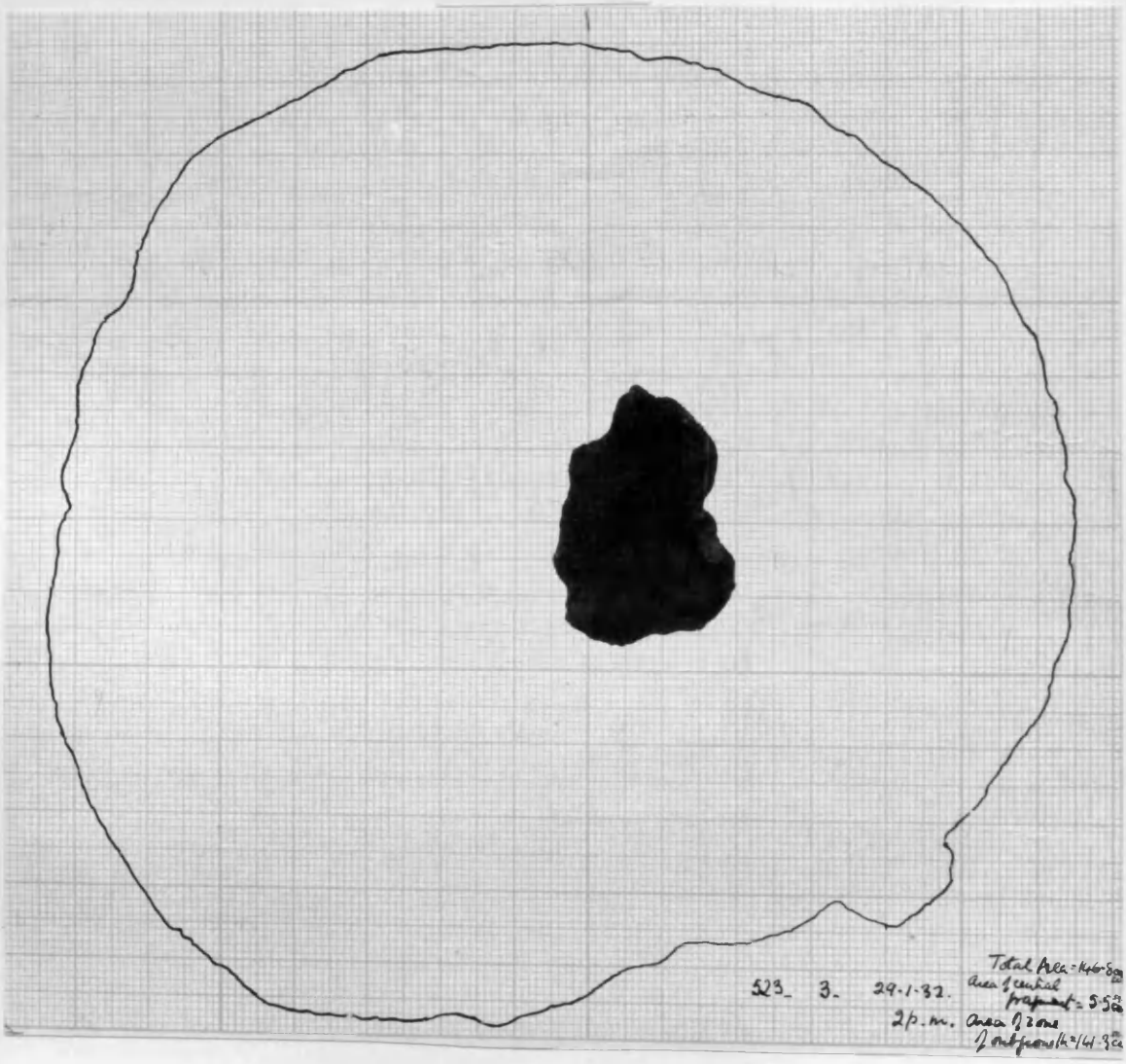


Fig.155.- Healthy kidney epithelial cell stained janus green B, showing fine filamentous and rod-shaped mitochondria. Two granular mitochondria are present. A few fat globules are also seen. × 1,200

Fig.156.- Kidney epithelial cell stained janus green B after 4 hours' exposure to carbon tetrachloride. The mitochondria consist of short, thick rods, and granules. Compare fig. 162. × 1,200

In figs. 155, 156, 157, 158 and 159 the unstained fatty globules are coloured pale green.

Fig.157.- Kidney epithelial cell stained janus green B, after 10 hours' exposure to carbon tetrachloride. The mitochondria consist of short, thick rods and granules. A few spherical forms are also seen, and several fatty globules containing small granules of dye-stained substance are present. In some of these, the dye-stained particles are purplish in colour. x 1,200

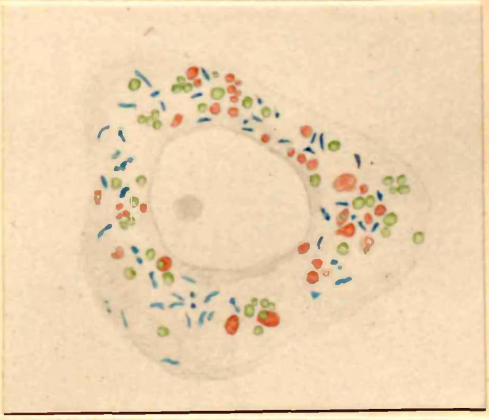


Fig.158.- Kidney epithelial cell stained with janus green B and neutral red, after 12 hours' exposure to carbon tetrachloride. The mitochondria are granular and rod-shaped. Numerous neutral red stained granules of varying sizes are present. Some of these contain small, fatty droplets. x 1,200

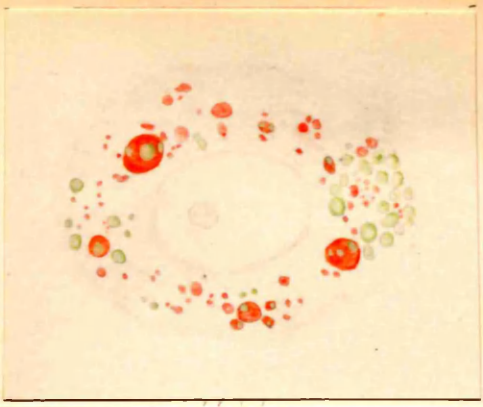


Fig.159.- Kidney epithelial cell stained with neutral red after 12 hours' exposure to carbon tetrachloride. Numerous vitally stained granules of varying sizes are present. Some of these contain globules of fatty material. Two large masses (m = large masses), formed by fusion of several neutral red granules, are seen. x 1,200

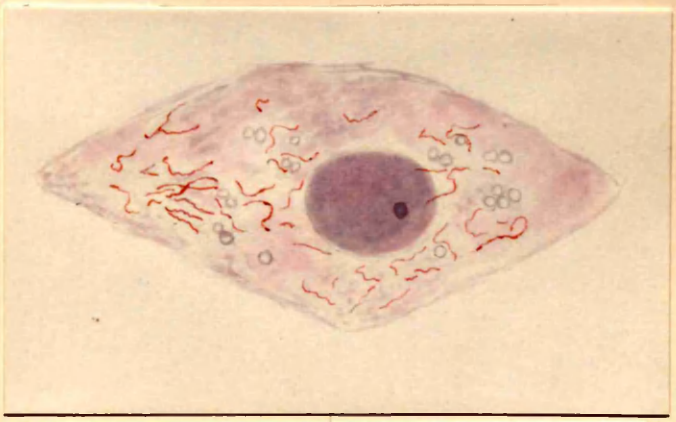


Fig.160.- Healthy kidney epithelial cell fixed in osmic acid and stained with Altmann's fuchsin and methyl green. Numerous fine filamentous mitochondria are present. x 1,200

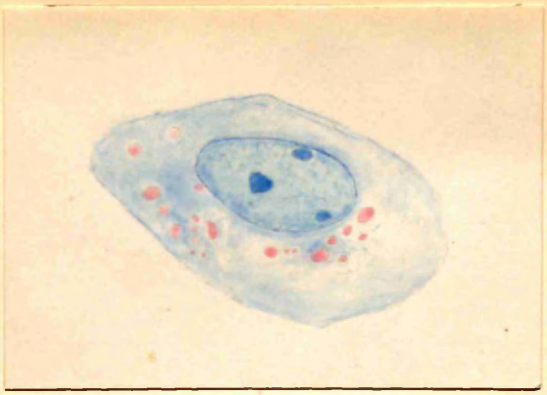


Fig.161.- Healthy kidney epithelial cell fixed in formalin and stained with Nile blue sulphate A. The fat globules are pink in colour and consist of neutral fat. ×1,200

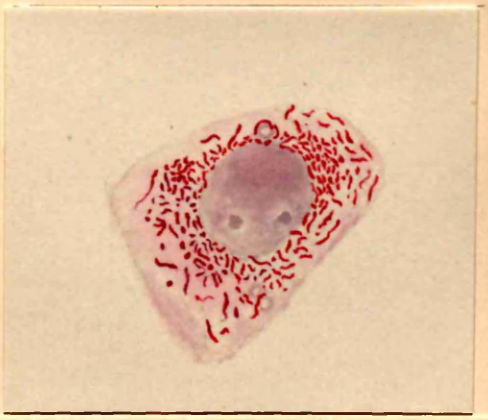


Fig.162.- Kidney epithelial cell fixed in osmic acid after 4 hours' exposure to carbon tetrachloride. The mitochondria consist of granules and short, thick rods. A few short filaments are present round the periphery of the cells. Compare fig. 156. ×1,200

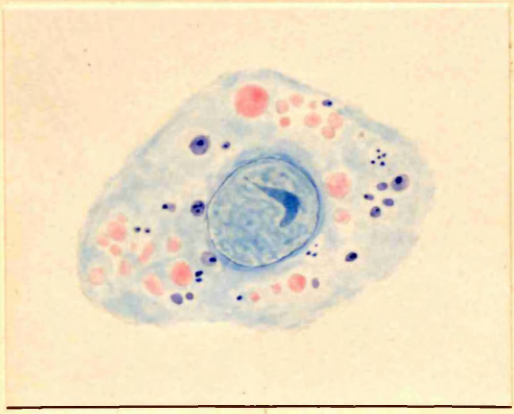


Fig.163.- Kidney epithelial cell fixed in formalin and stained with Nile blue sulphate A, after 8 hours' exposure to carbon tetrachloride. Pink globules of neutral fat are seen. Several small, bright blue granules of fatty acids and purplish masses, some of them containing small, bright blue droplets of fatty acids are also seen. ×1,200