The Marcus Beck Laboratory reports.

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(2) Some Further Researches on the Cultivation of Tissues *in vitro*

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(3) Some Researches on Spirochætes occurring in the Alimentary Tract of Man and some of the Lower Animals

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(4) Controlled Growth *en masse* (Somatic Growth) of Embryonic Chick Tissue *in vitro*

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(5) Some Observations on the Development of Red Blood Cells as seen during the Growth of Embryonic Chick Tissue *in vitro*

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The Royal Society of Medicine.

Marcus Beck Laboratory Reports.—No. 1.

The Cultivation of Human Tumour Tissue *in vitro*.

By David Thomson, M.B., Ch.B.Edin., D.P.H.Cantab.,[1] and John Gordon Thomson, M.A., M.B., Ch.B.Edin.[2]

Read before a Meeting of the Society on May 11, 1914.

The President, Sir Francis H. Champneys, Bt., M.D., in the Chair.

Mr. President and Gentlemen,—

These researches on the Cultivation of Human and other Tissues were carried on in the Marcus Beck Laboratory, in this building, by Dr. J. G. Thomson, as Beit Memorial Research Fellow, and by myself, as Grocers' Research Scholar, under the directorship of Sir Ronald Ross. The work represents the first-fruits of the Laboratory, and it is only right that on this account we should make a brief reference to the memory of Marcus Beck. It would be indeed ungracious not to take special notice of the fact that the facilities we have enjoyed are due to the respect which he inspired, and which led to the generous donation to which the Laboratory owes its existence. A portrait of Marcus Beck hangs in the Laboratory, and underneath are his favourite verses, one of which reads as follows :—

> "I need not be missed if another succeed me,
> To reap down those fields which in Spring I have sown,
> He who ploughed and who sowed is not missed by the reaper,
> He is only remembered by what he has done."

[1] Grocers' Research Scholar.

[2] Beit Memorial Research Fellow.

Those among us who did not know Marcus Beck do not miss him, but we are now reaping the advantage from the Laboratory which he has inspired, and on account of which he will always be remembered. Before proceeding with my lecture I wish also to thank the President, the Council, and Mr. MacAlister for the facilities that they have given us in connexion with our work in this building.

(I) Introduction.

The discovery of the method of cultivating tissues *in vitro* by Harrison (1910) has opened up a wide field for research. It is unwise to prophesy how far this new power will develop, but undoubtedly it will lead to discoveries of great importance. In June, 1913, we attempted to cultivate human tumour tissue in the medium employed for cultivating the malarial parasite *in vitro*—viz., in fresh defribrinated human blood containing 0·5 per cent. of glucose. Small pieces of breast carcinoma were placed in test-tubes containing this medium and incubated at blood heat. Smears of the surface of these portions of tumour before inoculation, and again after one to two weeks' incubation, showed that new elongated cells had developed in the medium on the surface of the tissue. These were probably new connective tissue cells. This medium and method were, however, unsatisfactory. In September, 1913, one of us (D. Thomson) visited the Rockefeller Institute in New York and through the kindness of Dr. Carrel, whose excellent work on this subject is well known, was able to acquire the technique necessary for this research. Carrel has worked chiefly with the tissues of the chick embryo, and we have attempted the cultivation of human tissue on similar lines. The human tissue necessary was obtained at the Middlesex Hospital from operations performed by Sir John Bland-Sutton, to whom we are much indebted. As soon as the tumour or other human tissue was removed from the patient it was placed in a sterile bowl. A small piece was immediately cut out with aseptic precautions and transferred to a test-tube containing 5 to 10 c.c. of sterile Ringer's solution. This was placed in a Thermos flask to which some water about blood heat was added. The tissue was in this way transferred to the laboratory as quickly as possible and then planted into the necessary medium and incubated at 37·5° C. The space of time which elapsed between the removal of the tissue from the patient and its transference in the culture medium to the incubator was as a rule about two hours.

(II) The Culture Medium.

It seems to be generally believed that the tissue of a given animal can only be cultivated in the plasma of that species of animal, so that to grow chick tissue the plasma of a fowl is employed. The tissues of the frog, rabbit and guinea-pig have been grown always in the plasma of the frog, rabbit and guinea-pig respectively. With this idea it seemed necessary to employ human plasma for the cultivation of human tissues. We commenced our work, therefore, by using human blood plasma for the medium, but found this inconvenient and troublesome, chiefly on account of the rapidity with which human plasma clots. It was found later by one of us (D. Thomson) that human tissues could be grown satisfactorily in fowl plasma, and as this plasma can be kept fluid in paraffined tubes in the ice-chest for several months, it formed a much more convenient medium for our work. In this research the total number of inoculations of pieces of human tissue into various modifications of plasmatic media was fifty. Nine different tissues were employed—viz., liver, uterine fibroma, papillomatous tumour of the ovary, ovary, skin epithelium, scirrhous cancer of the breast, cancerous lymphatic gland, voluntary muscle, and scrapings of epithelial cells from the inner surface of the Fallopian tube. Of these nine species of tissue, only three showed distinct and definite growth in the medium—viz., the papilloma of the ovary, the ovarian tissue itself and the cancerous lymphatic gland. Slight growth took place in the case of liver and fibroma. It seemed to us that extract of the tissue itself should be added to the fowl plasma medium in order to get good growth. No extract was added in the case of the liver, and this possibly explains the slight growth in this case. Also in the case of the fibroid scirrhous cancer of the breast and the fibroma of the uterus no extract was added, because the tissue was so hard that we were unable to get an extract from it. Hard fibrous tissues did not grow well, but whether this was due entirely to the lack of extract we are unable to state. Soft tissues, such as could be easily mashed up for the purpose of getting an extract grew best, but whether this was due to the addition of the extract to the medium we are not prepared to state. Later we found that the best growth of human tissue which we have so far obtained occurred in a medium composed of fowl plasma one part plus chick embryo extract one part. The growth was more successful in this medium than in the medium composed of fowl plasma one part plus

extract of the given tissue one part, so that it would seem that extract of chick embryo can take the place of the human tissue extract.

It would appear to us, therefore, that for tissue growth one should employ plasma plus extract, and that the plasma and extract do not require to be specific. There would seem to be no reason, therefore, why certain human tissues and tumours should not be successfully transplanted into a fowl, since the tissues of certain mammals, including man, grow successfully in fowl plasma plus extract of chick embryo.

(III) Technique.

The medium is transferred by means of sterile Wright's pipettes to a sterile cover-slip. A tiny piece of the tissue is then placed in this medium and the cover-slip inverted over a hollow in a thick glass slide. The cover-slip is hermetically sealed to the slide by painting melted paraffin around the margin of the former. The preparation is then placed in the incubator at 37·5° C. A culture of this kind can be examined at any time by the low powers of the microscope, and sometimes even the oil-immersion lens can be focused on the growing cells.

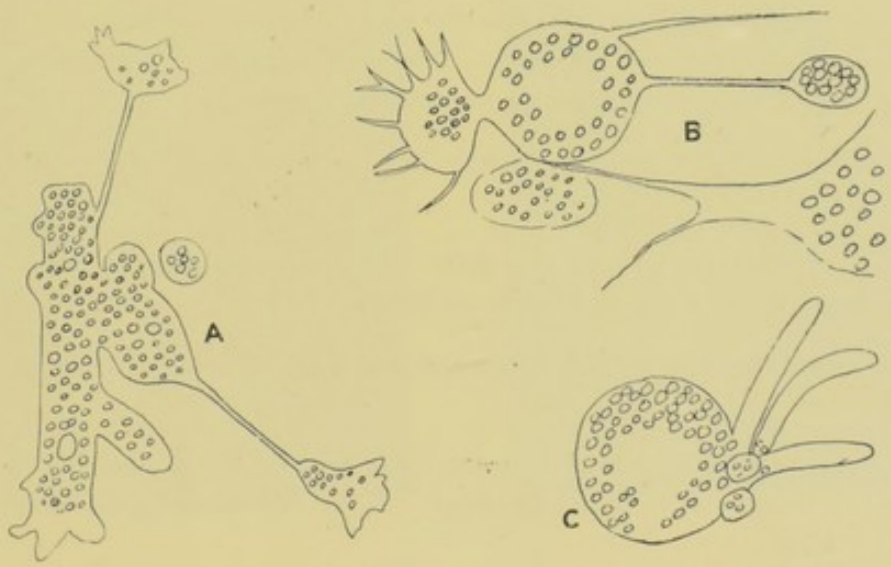
(*N.B.*—The plasma medium clots after some time so as to form a transparent jelly. This clotting occurs much more quickly when extract of chick embryo is added.)

(IV) The Growth of the Tissues.

We have attempted to illustrate this by means of successive photomicrographs of the live tissue.

(*a*) *Papilloma of the Ovary.*—This was a very soft tumour about the size of a child's head. It was described by the pathologist as an intracystic papilloma not truly malignant. The tissue of this tumour was apparently composed entirely of round cells, there being apparently no fibrous stroma present. A tiny portion, about 1 mm. in square area, was placed in a medium composed of fowl plasma one part, Ringer's solution (containing 0·5 per cent. of glucose) one part, and extract of the tumour in Ringer's solution one part. On the third day of incubation definite buds of growing tissue appeared. On the fifth day these were quite distinct, as shown in fig. 1, Plate I; and on the eighth day the amount of growth had considerably increased (fig. 2). This growth consisted of a solid extension of epithelial cells. As the growth extended it caused some liquefaction of the medium, which was of a gelatinous consistence,

and in the more liquefied parts the new growing cells were more scattered (fig. 3), but as a rule they remained in contact with each other by means of long, fine protoplasmic connexions (figs. 7, 8 and 9, Plate II). The new actively proliferating cells varied markedly from the cells of the original tissue planted in the medium. The appearance of the original cells is seen in fig. 4; while fig. 6 shows the original cells on the left and the new growing cells on the right. The latter are also seen in figs. 7, 8, 9 and 10. It is distinctly noticeable, therefore, that the new actively growing cells are large and amœboid, with long processes which communicate with each other, and they also contain large highly refractile granules. The original cells, on the other



Papilloma of ovary. Growth *in vitro*. Diagrams A, B and C represent the remarkable amœboid shapes of the growing cells. (× 600.)

hand, are much smaller. They show no amœboid processes and do not exhibit amœboid movement, and they contain few or no large refractile granules. The amœboid movement of the larger growing cells was very remarkable, as illustrated in the diagrams A, B and C. On the fourteenth day of incubation the new cells began to show signs of degeneration (fig. 11), and on the fifteenth day almost complete autolysis took place (fig. 12). There was no bacterial infection to account for this autolysis. It is possible that the cells, after having lost their vitality, were dissolved by their own ferments. We were unable to determine the exact nature of the highly refractile granules; most probably they were fat droplets. The smaller granules were seen to run

EXPLANATION OF PLATE I.

FIG. 1.—*Tissue :* Intracystic papilloma of human ovary (bordering on malignancy). *Medium :* Fowl plasma one part, Ringer's solution plus glucose one part, extract of the tissue one part. Photomicrograph of live tissue taken on the fifth day of incubation shows commencing growth. (× 40.)

FIG. 2.—The same specimen, eighth day of incubation, showing further growth. (× 40.)

FIG. 3.—Another portion of the same specimen, tenth day of incubation, showing outgrowth of large epithelial cells. (× 75.)

FIG. 4.—Photomicrograph of unstained wet film of original tumour cells. (× 400.)

FIG. 5.—Photomicrograph of unstained wet film of the tumour cells after incubating for thirty days in ascitic fluid. Note the star-shaped masses of crystals. (× 400.)

FIG. 6.—Photomicrograph of growing cells (same specimen as figs. 1, 2 and 3), tenth day of incubation. Original cells on left, growing cells on the right. Note the increase in size of the growing cells and also the appearance of large granules. (× 400.)

N.B.—Figs. 4, 5 and 6 are identically magnified. (× 400.)

Plate I.

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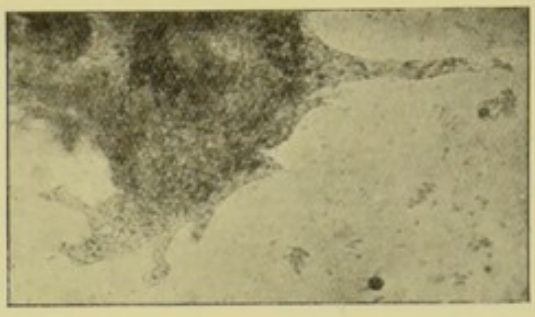

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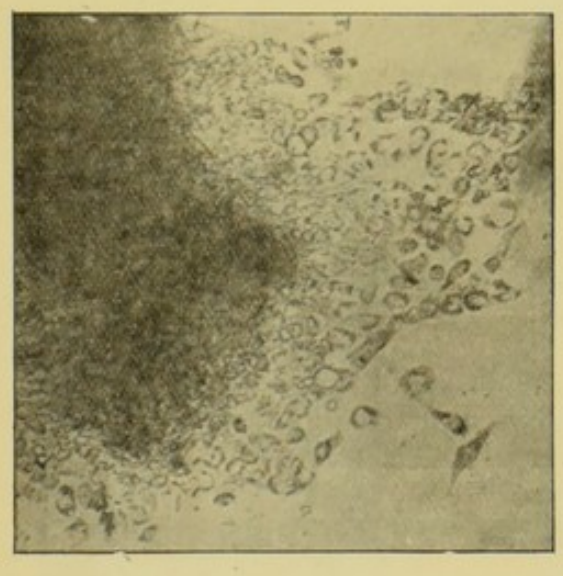

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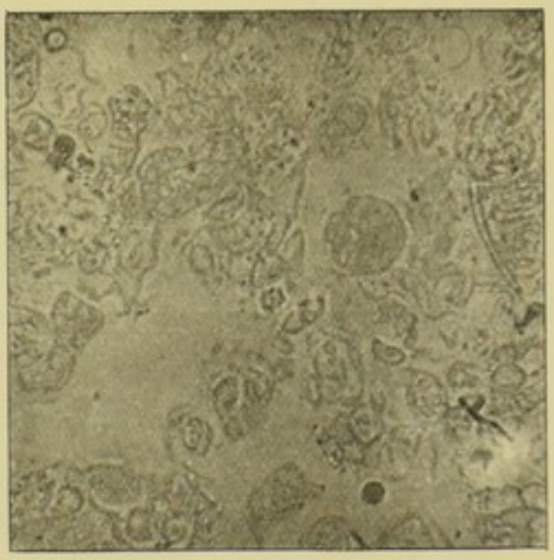

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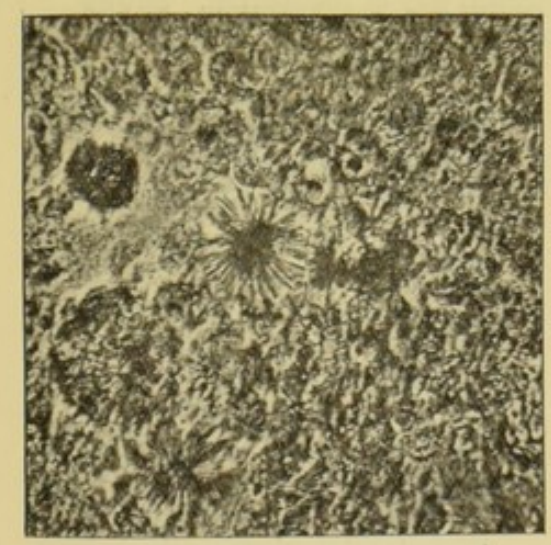

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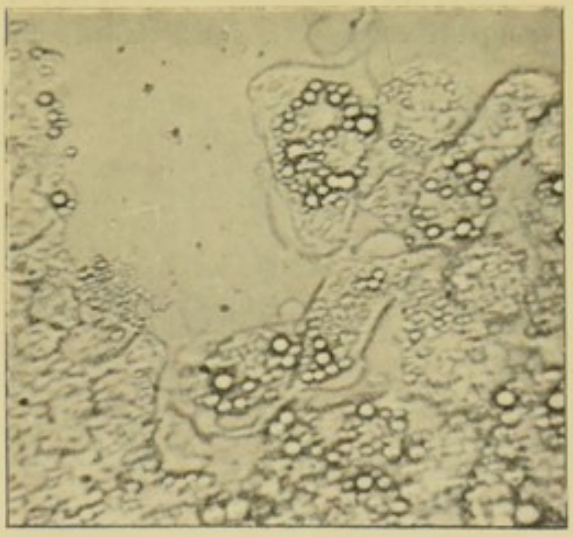

(D. Thomson, photo.)

EXPLANATION OF PLATE II.

FIG. 7.—Same specimen of live tissue, ninth day of incubation. Shows amœboid processes in the growing tumour cells. Fine granules are seen at the terminal ends of these processes. (× 400.)

FIG. 8.—Same specimen of live tissue, ninth day of incubation. Shows amœboid processes of the larger tumour cells. (× 720.)

FIG. 9.—Same specimen of live tissue, ninth day of incubation. Shows similar processes; one process ends in a mass of granules. (× 720.)

FIG. 10.—Same specimen of live tissue, ninth day of incubation. Shows the large size of the growing cells which always contain granules. (× 720.)

FIG. 11.—Same specimen of live tissue, fourteenth day of incubation. Shows commencing autolysis of the growing cells. (× 400.)

FIG. 12.—Same specimen of live tissue, fifteenth day of incubation. Shows complete autolysis of the growing cells. (× 400.)

PLATE II.

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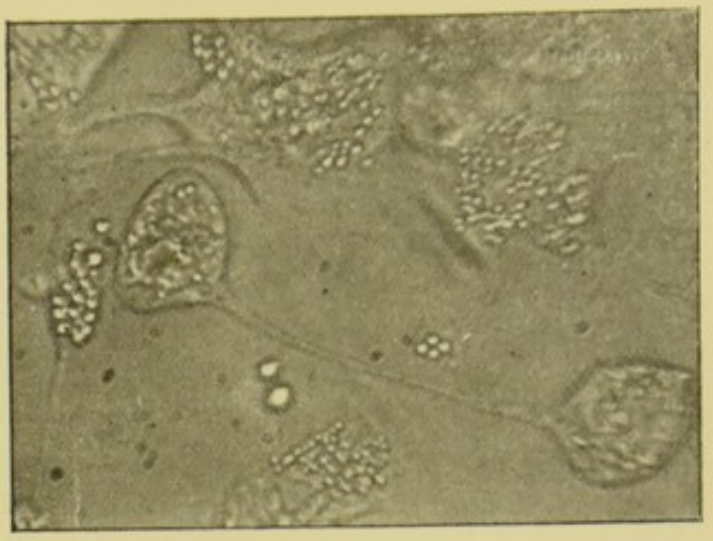

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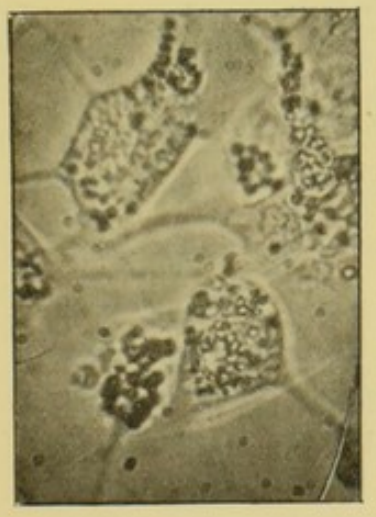

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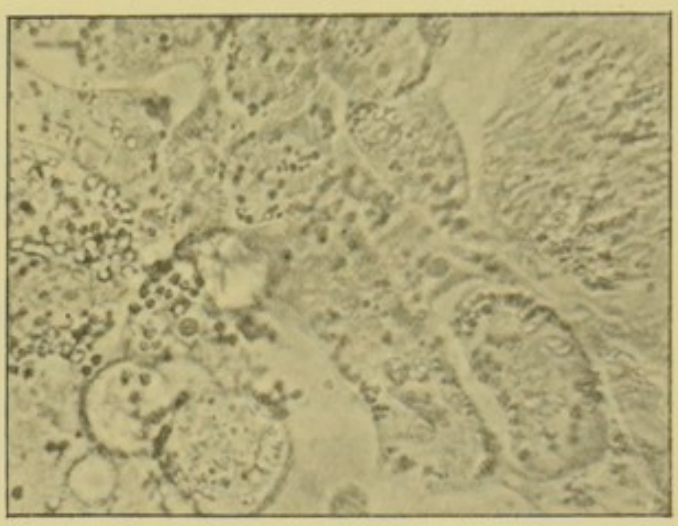

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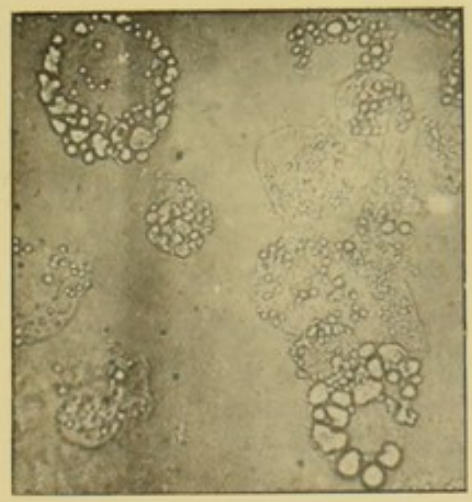

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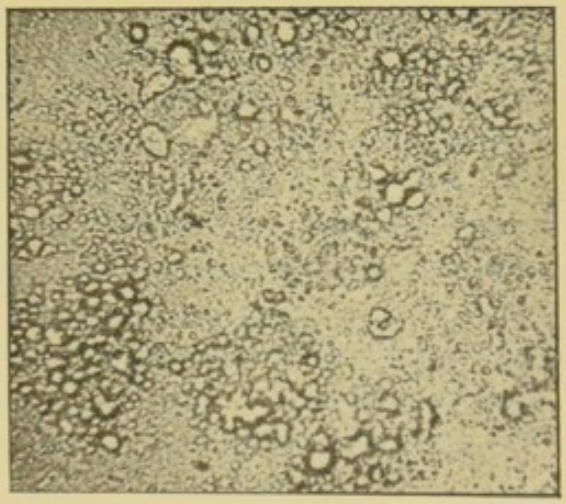

(D. Thomson, photo.)

along the pseudopodia of the cells at times, and were frequently extruded from the ends of those processes into the surrounding medium. Fig. 7 shows the granules in the fine pseudopodia very clearly. Another point of interest in connexion with this tumour tissue was the development of stars of fine crystals (fig. 5, Plate I) after the culture preparation had been kept for about four weeks. These stars also developed in the tubes of ascitic fluid to which pieces of the original tumour had been added, and incubated for a month or more. They appeared to resemble tyrosine crystals in some respects, and evidently they indicate the presence of some chemical substance in the tumour.

(*b*) *Carcinomatous gland from the neck*, secondary to carcinoma of the floor of the mouth. Small pieces of this tissue were inoculated into six variations of fowl plasma medium as follows:—

(1) Fowl plasma one part plus extract of the cancer gland one part.
(2) Fowl plasma one part plus extract of the cancer gland one part and of chick embryo one part.
(3) Fowl plasma one part plus extract of chick embryo one part.
(4) Fowl plasma one part plus Ringer's solution two parts plus cancer gland extract two parts.
(5) Fowl plasma one part plus glucose Ringer's solution one part and cancer extract one part.
(6) Fowl plasma one part plus ascitic fluid one part plus cancer gland extract one part.

Successful growth was only obtained in No. (3) medium, and very slight growths occurred in (2), (4) and (5), and no growth was observed in (1) and (6). It appeared, therefore, that fowl plasma plus extract of chick embryo formed the most suitable soil. In this medium an outgrowth of branching cells from the original tissue was seen after forty hours' incubation at 37·5° C., as seen in fig. 18, Plate III. After five days' incubation there appeared, in several places, solid buds composed of epithelial cells, and these increased in size day by day (figs. 13, 14, and 15). Fig. 16 shows an outgrowth of new epithelial cells (presumably cancer cells) after nine days' incubation, and fig. 17 shows the further extension of the same portion four days later—i.e., after thirteen days' incubation. At the same time the long branching cells continued to extend further into the medium (fig. 19). The latter most probably represent the growth of the stroma cells of the cancerous gland tissue. It is interesting to note that no such branching cells grew from the epithelioma of the ovary already described, and we have already pointed out that that tumour consisted only of epithelial cells, and apparently contained no stroma. It was a soft, friable tumour,

whereas the cancerous gland was more tough and fibrous. The growing epithelial cells of the cancerous gland were, as in the case of the papilloma of the ovary, larger and more granular than the original cells; also they were amœboid and irregular in shape. The growing cancer cells were packed with fine refractile granules, finer than in the case of the growing papilloma cells of the ovary. The growth of the cancer began to flag after the thirteenth day, and I made a permanent preparation of the specimen on the fifteenth day. The stained specimen showed a slight infection with bacilli, most probably an extraneous or secondary infection of the original tissue.

(*c*) *The Ovarian Tissue.*—This grew successfully in a medium composed of fowl plasma one part plus Ringer's solution one part plus extract of ovary (in Ringer) two parts. A definite outgrowth of long, branching cells appeared after forty-two hours' incubation. This growth increased for six days when, unfortunately, the cover-glass was broken in an attempt to focus the oil-immersion lens on the tissue. The outgrowth of long, branching cells in this case was presumably only connective tissue stroma cells. No outgrowth of epithelial cells appeared.

(V) Further Observations.

Parallel with these researches in the cultivation of human tissues we have investigated the growth *in vitro* of the tissues of chick embryos, &c. A special article has been devoted to this work, with a detailed description of the technique. Plasma medium is more or less solid in consistence, and it would appear to us that, in this medium, cells of all kinds have a tendency to elongate and throw out protoplasmic processes, whether they be epithelial cells or cells of connective tissue. But the cells of each species of tissue retain their characteristic appearance in many respects. The connective tissue cells elongate and branch very markedly, whereas the epithelial cells do not elongate so much, and although they tend to send out long processes they do not branch in the true sense of the word, and, unlike the connective tissue cells, they tend to keep together in a solid, advancing growth. So far we have been unable to get any proliferation of true muscle tissue, but liver and kidney epithelium have been grown, also spleen, bone-marrow, the tissue of young feathers, and also the tissue of the brain.

With regard to the scraping of the inner surface of the human Fallopian tubes, we were unable to find any definite proliferation of the epithelial cells, but the cilia of these cells kept in active motion for forty

EXPLANATION OF PLATE III.

FIG. 13.—*Tissue:* Carcinomatous gland removed from neck by Sir J. Bland-Sutton. *Medium:* Fowl plasma one part, extract of chick embryo one part. Photomicrograph of the live tissue after five days' incubation. Shows outgrowth of elongated branching cells (probably stroma cells), and also commencing budding of the epithelial cancer cells. (× 66.)

FIG. 14.—The same portion of tissue after six days' incubation. Note appearance of a new epithelial bud. (× 66.)

FIG. 15.—The same portion after nine days' incubation. Note further extension of epithelial cells, and increase of branching cells. (× 66.)

FIG. 16.—Another portion of same specimen after nine days' incubation. Note marked outgrowth of epithelial cancer cells. (× 66.)

FIG. 17.—The same portion after thirteen days' incubation. Showing marked increase of epithelial cancerous growth. (× 66.)

FIG. 18.—Another portion of the same specimen after four days' incubation. Showing commencing outgrowth of branching stroma cells. (× 66.)

FIG. 19.—The same portion after ten days' incubation. Shows marked increase of branching stroma cells. (× 66.)

Plate III.

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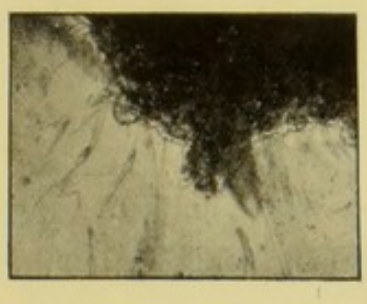

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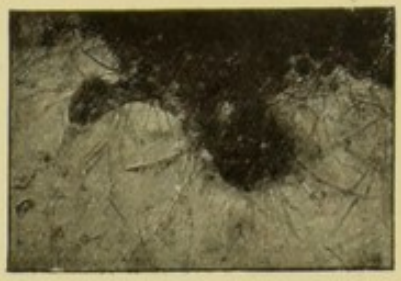

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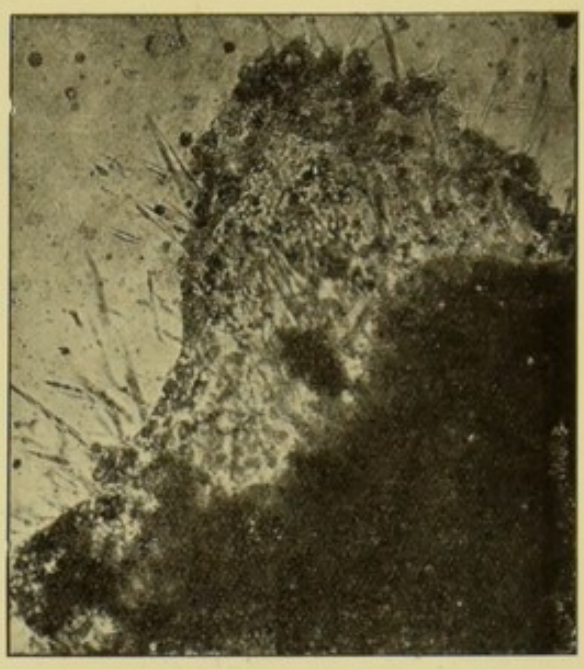

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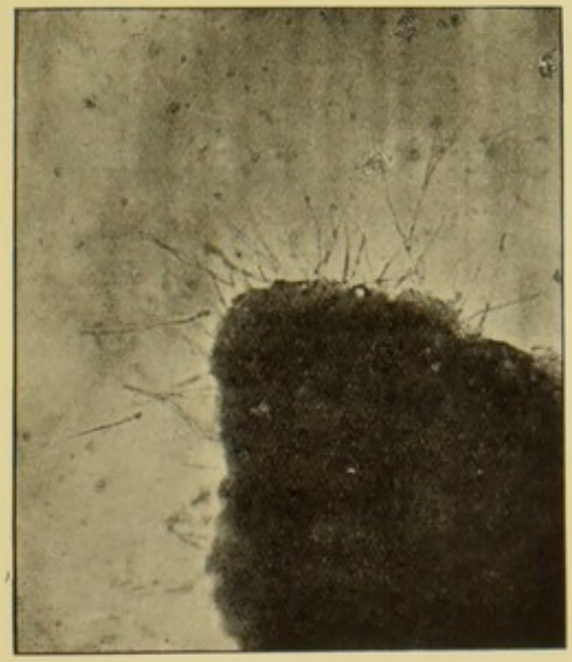

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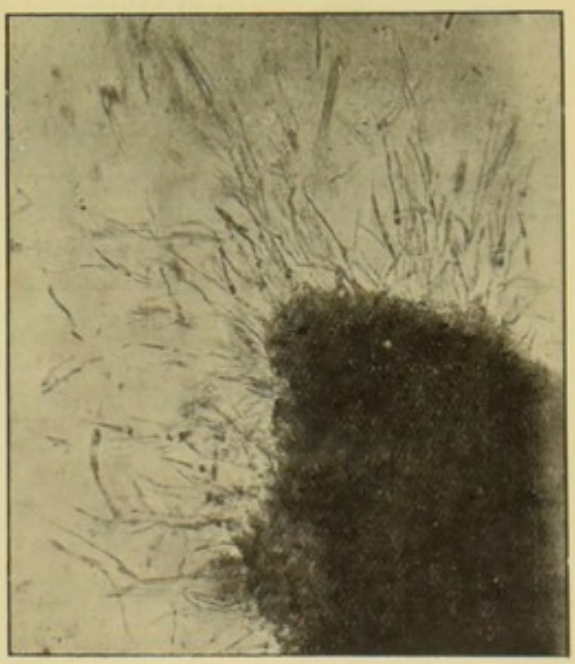

(D. Thomson, photo.)

hours after they had been transferred into culture medium. The growth of human and other mammalian tissue was much slower than that of chick embryo tissue. In the latter, definite growth took place in twenty-four hours, and it continued to grow for three to five days, after which time the growth ceased and degenerative changes occurred, unless it was transplanted into fresh medium. With human and other mammalian tissue, on the other hand, no very definite growth occurred until about the fifth day, and this continued to increase till about the twelfth or thirteenth day, when it ceased and began to degenerate. We have not attempted so far to transplant growing human tissue into fresh media to prolong its life.

(VI) Summary and Conclusions.

(*a*) Growth of human tumour tissue and normal human tissue can be obtained *in vitro* in a medium composed of fowl plasma to which extract of the human tissue, or extract of chick embryo, has been added.

(*b*) The growth of epithelial cells differs from that of connective tissue cells. The former grow out in the form of buds or in a solid, advancing mass, whereas the latter radiate out in long, branching lines.

(*c*) The new growing cells are much larger than the original cells from which they grow. They also contain numerous highly refractile granules and are markedly amœboid, and tend to throw out long, protoplasmic processes into the medium.

(*d*) The growing tissue tends to liquefy the medium, more especially the solid outgrowth of epithelial cell.

(*e*) Connective tissue would appear to grow more readily than specialized epithelial tissue.

(*f*) The growth of human and other mammalian tissue *in vitro* is much slower than that of embryonic chick tissue *in vitro*, but the former tissues continue to proliferate in the original medium for a longer time before degeneration occurs.

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The Royal Society of Medicine.

Marcus Beck Laboratory Reports.—No. 2.

Some Further Researches on the Cultivation of Tissues *in vitro*.

By David Thomson, M.B., Ch.B.Edin., D.P.H.Cantab.[1]

Read before a Meeting of the Society on May 11, 1914.

The President, Sir Francis H. Champneys, Bt., M.D., in the Chair.

(I) Technique.

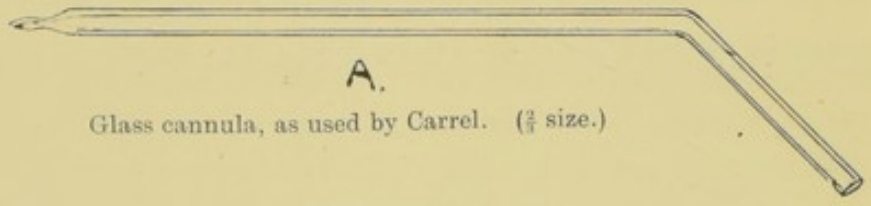
The technique for the cultivation of tissues *in vitro* has been developed chiefly by Carrel, *vide* Carrel and Burrows (1911), Carrel (1912), Carrel and Ingebrigtsen (1912), Abderhalden (1912), Oppel (1912). Carrel works in a specially prepared aseptic room like an operating theatre with a staff of trained assistants, chiefly because he also carries on surgical work with regard to the grafting of organs. For ordinary tissue cultivation, however, I have been able to reduce the complications so that it can be successfully carried on in an ordinary laboratory with the help of one assistant. In fact, the work of tissue inoculation and subculture *in vitro* can be carried on almost as easily as any ordinary bacteriological work.

(*a*) *The Preparation of the Medium.*—The substances necessary for the medium are blood plasma, Ringer's solution, and tissue extract. So far as I can ascertain, it was generally believed that the tissue of a given animal can only be cultivated in a medium containing the blood

[1] Grocers' Research Scholar. From the Marcus Beck Laboratory, Royal Society of Medicine, London.

plasma of that species of animal, but in the paper by my brother and myself which has just been read it is pointed out that the tissue of mammals, including man, may be successfully cultivated in fowl blood plasma. Mammalian blood plasma clots much more rapidly than fowl plasma, so the plasma of the latter is much the best for a general tissue culture medium, since it can be kept in a fluid condition for some months in an ice incubator.

(1) The fowl plasma is obtained in the following manner: Sterilize by dry heat about one dozen centrifugal tubes and immerse these in a vessel containing molten paraffin (52° hardness) in a dry heat sterilizer capable of maintaining a temperature of about 200° C. This is done in order to coat the tubes with a sterile layer of paraffin. The tubes are corked with sterile paraffin-coated corks and are then placed in a vessel in the ice incubator to cool them to about 0° C. It is now necessary to prepare for the bleeding of the fowl. A large quantity of blood can be



A.

Glass cannula, as used by Carrel. ($\frac{2}{3}$ size.)

obtained by withdrawing it from one of the carotid arteries by inserting a glass cannula similar to that depicted in diagram A. About 60 c.c. of blood can be obtained without killing the fowl, and if it is allowed to bleed to death even more can be drained away. The cannula should be boiled in a solution of sodium citrate and kept in the vessel ready for use. The other materials required for this operation are ether for anæsthesia, a tin vessel containing cotton-wool for the anæsthetic mask, a board with four pegs for tying down the fowl, sterile scalpels, scissors, dissecting and clamping forceps, needles, silk thread or catgut, and some dilute antiseptic lotion with swabs, and also some sterile cloths or towels with which to cover up the animal, leaving a small open space for the operation on the neck. The animal is anæsthetized and then placed on its back on the board and its legs and wings are securely fastened to the four pegs. A block should be placed under the neck to keep it up. The feathers are plucked from the neck and the skin cleansed with antiseptic lotion, and the sterile towels are placed over

the animal, exposing only the prepared skin. An incision about 2 to 3 in. long is made a little to one side of the middle line and the tissues are separated until the carotid is found. A ligature is tied round the vessel at its distal end and another loose ligature is placed about ½ in. proximal to this for the purpose of tying on the neck of the cannula when inserted into the vessel. Clamp the vessel proximal to the loose ligature, then make a small cut in the vessel (between the ligatures) with fine scissors. Insert the cannula and tie the loose ligature firmly around the neck of the former, release the clamp and allow the blood to flow into the paraffined and cooled centrifugal tubes. The fowl should not be anæsthetized too deeply, since no blood will be obtained if it dies from an overdose. If the carotid is ligatured proximally to the wound, and the skin stitched up, the fowl may live, and can be used again on a future occasion. The blood obtained in this way is rapidly centrifugalized to drive down the corpuscles and is then stored in the ice incubator. It is not necessary to pipette off the supernatant plasma and to transfer it to other tubes free from corpuscles.

(2) *Ringer's Solution.*—The following is the formula of the solution employed by Carrel: Distilled water, 1,000 c.c.; sodium chloride, 9 grm.; calcium chloride, 0·25 grm.; potassium chloride, 0·42 grm. It might be advisable to add sodium bicarbonate, 0·3 grm., since tissues while growing produce acid products (Rous, 1913).

(*b*) *Tissue Extract.*—Carrel (1913) has shown that tissues grow much more rapidly and profusely if certain extracts are added to the medium. He found that extract of chick embryo, extract of adult spleen, and Rous sarcoma extract, were most active in stimulating the growth of the connective tissue of an embryonic chick heart. Tissue extracts, of which, in my opinion embryonic chick extract is the best for all-round purposes, are prepared as follows: The embryonic chick (about eight days old) is removed from the egg with aseptic precautions and placed in a sterile watch-glass in a sterile Petri dish. Three to five drops of sterile Ringer's solution are added, and the embryo is then easily clipped up into an emulsion by means of a fine pair of sterile scissors (curved blades). The emulsion is transferred by a Wright's pipette with teat to a sterile tube, centrifugalized, and kept indefinitely in the ice incubator. The clear supernatant fluid is the extract used. Extracts of other tissues are made in a similar manner.

(*c*) *The Culture Preparations.*—I have found that very satisfactory aseptic results can be obtained by working on a polished oak table in a

clean laboratory. The room should be as free from dust as possible. The table is scrubbed with dilute lysol and the necessary apparatus placed upon it. The following apparatus is required: Petri dishes, some of these containing a watch-glass; closed vessels, of glass or metal, containing cataract knives, forceps and scissors with straight and curved blades; also a few all-metal needles (all the instruments should be as fine as possible); closed vessels (preferably large Petri dishes) containing cover-slips and special glass slides; a sloping rack on which are placed plugged test-tubes containing Wright's pipettes, one of which should be paraffined; a vessel containing rubber teats, a bottle of vaseline and a fine glass rod; a large test-tube of Ringer's solution; a vessel of melted paraffin (52° hardness), a brush, and a Bunsen burner, complete the outfit. All the apparatus should be sterilized and dry. I find a dry sterilizer most useful. The rubber teats should be boiled. The cover-slips necessary for this work should be 50 mm. long by 22 mm. broad. I prefer No. 1 slips, so that a high microscopic magnification may be used if necessary. The glass slides required measure about 3 in. long by 1 in. broad, and $\frac{1}{5}$ in. in thickness. They contain a hollow depression in the centre about 18 mm. in diameter and about 2 mm. deep. In other words, these slides are similar to those used for Widal work, but are thicker, so as to obtain a deeper hollow. In addition, it is advisable to have a few ordinary slides made of black glass. When all is ready the sterile Wright's pipettes should be fitted with sterile teats and replaced in the sterile test-tubes on the sloping rack. The paraffined pipette should be used for holding the fowl plasma. Other pipettes should contain Ringer's solution, and extract of chick embryo, and any other solution which may be desired for modifying the medium. Take a black glass slide and cover it with the lid of a Petri dish. Place on this a sterile cover-slip. A drop of fowl plasma is transferred to this cover-slip, and then the small portion of tissue, 1 mm. square or less, is transferred on the point of a needle or cataract knife into the plasma on the cover-slip. (The tissue is kept in Ringer's solution contained in the hollow slide before being cut to the required size and transferred to the medium.) A drop of chick embryo extract is then added to the drop of plasma and mixed and spread out with the point of the cataract knife. The addition of this extract causes the plasma to clot quickly and also improves the medium. When the plasma is clotted, take a glass slide and place a spot of vaseline on each side of the hollow depression, invert this over the cover-slip, which sticks by means of the vaseline. We

now have the tissue in a sterile hanging-drop medium. The cover-slip is hermetically sealed to the slide by painting melted paraffin around the margin of the former. Fig. 1, Plate I, illustrates the process just described. A black glass slide is used as a background for the cover-slip so that the small piece of whitish tissue can be seen more easily. One need not hurry unduly in the above technique, since the tissue in Ringer's solution can survive a considerable exposure to room temperature and still grow successfully. The influence of temperature and fluid medium on the survival of embryonic tissues *in vitro* has been investigated by Lambert (1913).

(*c*) *Transplantation or Subculture of Tissues.* — Carrel has subcultured tissue with great success. In his laboratory the same tissue has been subcultured for one and half years, having during that time undergone over 130 passages into fresh media. He recommends subculturing every two or three days for embryonic chick tissue. The tissues of adult mammals, including man (Thomson, D., and Thomson, J. G., 1914), however, grow much more slowly than embryonic chick tissue, so that in the case of the former it would be sufficient to subculture every eight to ten days. Carrel subcultures as follows: Remove the cover-slip from the culture preparation and cut out the piece of growing tissue from the medium and transfer it to sterile Ringer's solution in the hollowed-out slide. It is allowed to soak in this solution for about thirty seconds, and if it has markedly increased in size it should be divided before transferring it into the new medium. After the tissue has grown extensively, a yellowish necrotic area sometimes develops in the centre; this necrotic area should be removed before transplanting into the fresh medium.

(II) Observations on the Growth of various Tissues in vitro.

The total number of culture preparations made in this research was 200. In very young chick embryos about four to eight days old, it is rather difficult to isolate different species of tissue, and at this early age the cells seem to be nearly always round cells. Fig. 2 shows a portion of embryonic chick tissue composed of round cells in which little growth has occurred. In the solid jelly-like medium, however, the growth which occurs is always of a branching nature, in which the new cells appear to be entirely different from the original cells. Thus cells which were originally small and round show an outgrowth of

EXPLANATION OF PLATES.

All the photographs were taken while the tissue was alive, except in figs. 13, 26, 27, 28, and 32, which were taken from stained permanent preparations.

PLATE I.

FIG. 1.—Illustrates the method of making a culture preparation. A, cataract knife used for cutting the tissue. D, Wright's pipette used for transferring culture medium to the cover-slip lying upon the black glass slide, B. Note the small piece of tissue (white) lying in the culture medium. C, thick glass slide with hollow depression, with two drops of vaseline on either side. The vaseline causes the cover-slip to adhere to the slide in the position required, and melted paraffin is then painted around the margin to hermetically seal the preparation. (About $\frac{1}{2}$ nat. size.)

FIG. 2.—A portion of embryonic chick tissue, with clean-cut edges, showing practically no growth, six days' incubation. Medium: Fowl plasma one part, extract of human papilloma of ovary one part. (× 75.)

FIG. 3.—New growth from embryonic chick tissue, twenty-four hours' incubation. Note that the original consists of round cells, whereas the growth is composed of elongated branching cells. Medium: Fowl plasma one part, Ringer's two parts, extract of embryo one part. (× 66.)

FIG. 4.—Same preparation as fig. 3, eight days' incubation. Showing bubble-like degenerative changes. (× 66.)

FIG. 5.—Similar preparation, six days' incubation. Showing the highly refractile granules within the cells. (× 400.)

FIG. 6.—Similar preparation, forty-eight hours' incubation. Shows the granular and branching character of the growing cells. (× 290.)

Plate I.

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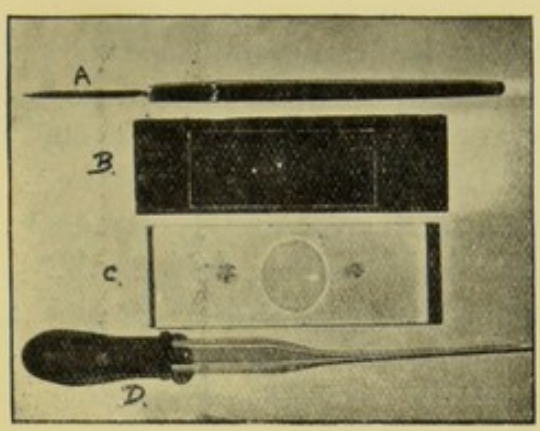

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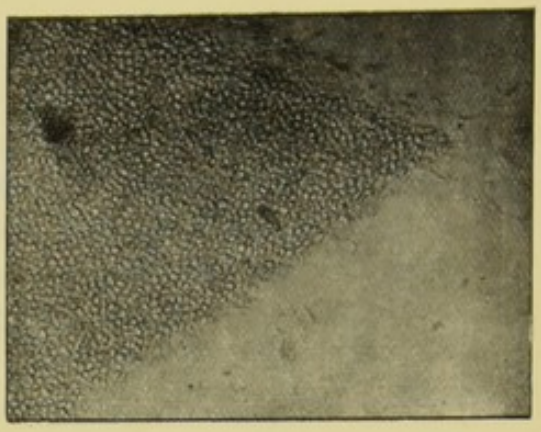

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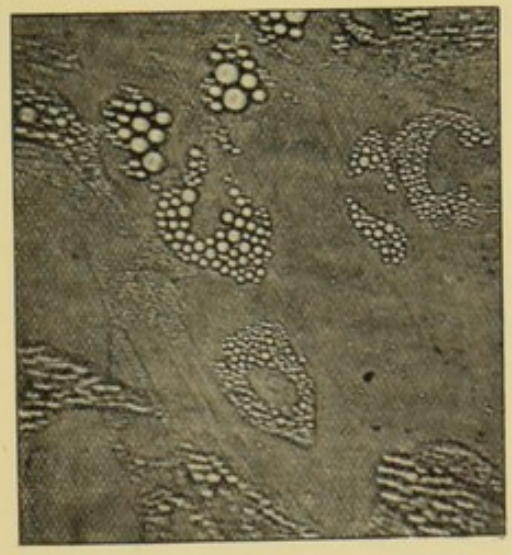

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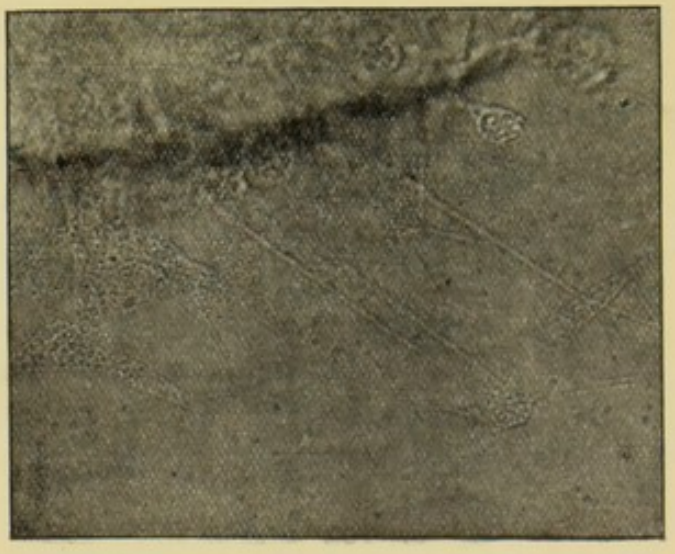


(D. Thomson, photo.)

large, elongated, branching cells filled with highly refractile granules (figs. 3, 4, 5 and 6). It may be maintained by some that these new cells are merely cells which have emigrated from the original tissue, but this is not the case. Carrel has proved beyond all doubt that this is real growth, since the same piece of tissue cultivated in this manner has grown for eighteen months and during that time has been divided and subdivided more than one hundred times (Ebeling, 1913). On one occasion the tissue augmented more than fifty times in six days (Carrel, 1913). No one who has actually watched the behaviour of tissue in culture can ever question the fact that true proliferation is occurring. The most puzzling phenomenon to my mind is the constant tendency of the growing cells to elongate and throw out processes. This tendency occurs not only with connective tissue but also in the case of epithelium.

Figs. 3 and 6 show the production of elongated branching cells from a mass composed apparently of round cells only. Figs. 7, 8, 9, and 10, Plate II, represent successive stages in the growth of a piece of epithelium from the leg of an embryonic chick, and again the new growing cells show a distinct tendency to elongate. Fig. 11 shows what appears to me to be an outgrowth of epithelial cells from the kidney of an adult rabbit. In the accompanying paper (Thomson, D., and Thomson, J. G., 1914) it has been shown, however, that in the case of human tumour tissue and normal human tissue the growth *in vitro* in plasmatic medium varies according to the nature of the tissue. The connective tissue branches very markedly, whereas the epithelium grows in the form of buds or in a solid advancing line, nevertheless the cells of the new growth of epithelium tend to be elongate and throughout long processes, whereas the original cells from which they have sprung are round with few or no processes. Fig. 12 shows a more or less compact extension of epithelial cells from a portion of chick embryo tissue, which again shows the tendency to elongation, especially at the growing margin.

Lambert and Hanes (1913) have shown that some growing tissue cells show different appearances due to the various different mechanical factors of the medium, and it appears to me that this remarkable difference between the original cells and the new cells which arise from these is due to a difference in the osmotic tension of their new surroundings. I have endeavoured therefore to vary the medium in order to see if different forms of cell proliferation could be produced in this way. Varying amounts of Ringer's solution and even distilled water

were added to the plasma basis, but so long as the medium clotted to form a solid jelly-like mass the new cells continued to show the tendency to elongate, as shown in figs. 13 and 14, Plate III. I then tried various fluid media, composed of (*a*) white of the egg from which the embryo was removed, plus extract of embryo; (*b*) ascitic fluid plus extract of embryo; (*c*) extract of embryo alone. In all cases where a fluid medium was employed, on inverting the cover-slip over the hollow in the slide the fluid ran towards the margin of the hollow, leaving an air-bubble in the centre, and the best growth or apparent growth always occurred in the thin layer of fluid medium which remained on the cover-slip in contact with the air of the bubble, while, on the other hand, no growth occurred from the tissue immersed in the ring of fluid medium around the air-bubble. Figs. 15 and 16 show this very clearly. Fig. 16 shows the remarkable extension or apparent growth which took place in the air-bubble from the original portions of tissue A and B, whereas the portions of tissue C, D and E in the ring of fluid medium around show no growth whatsoever. The fluid medium in this case consisted of fowl plasma one part, egg white one part, Ringer's solution two parts, and embryo extract one part. The solid advancing extension consisted entirely of round cells exactly like the cells of the implanted tissue. Fig. 17 shows a similar extension of round cells (higher magnification) in the air-bubble in a fluid medium composed of egg white one part plus embryo extract one part. In the fluid medium composed of ascitic fluid one part plus extract of embryo one part, and extract of embryo alone, a very slight extension of round cells occurred, with a slight tendency to branching in places where the medium had dried. Again, fig. 18 shows a solid extension of round cells (A) from the darker original portion of tissue, and beyond the margin of the round-cell extension the cells have commenced to branch (B). In this case the medium consisted of chick serum one part, chick plasma one part, plus extract of embryo one part. The tissue was lying in a thin layer of semi-fluid medium in the air-bubble. The medium separated into two layers—an inspissated solid layer next the glass of the cover-slip and a deeper fluid layer. The round-cell extension seen in the photograph occurred in the fluid layer, whereas the branching cells seen out of focus occurred in the solid layer. I have attempted therefore to prove that the cause of the branching phenomenon is the semi-solid or jelly consistence of the plasma medium, and that where the medium remains fluid the cells retain their original round shape. I will criticize these experiments,

PLATE II.

FIG. 7.—Piece of epithelium from leg of embryonic chick after six hours' incubation. Medium : Fowl plasma one part, Ringer's one part, extract of embryo one part. (× 115.)

FIG. 8.—Same preparation after twenty-four hours' incubation. (× 115.)

FIG. 9.—Same preparation after forty-eight hours' incubation. (× 115).

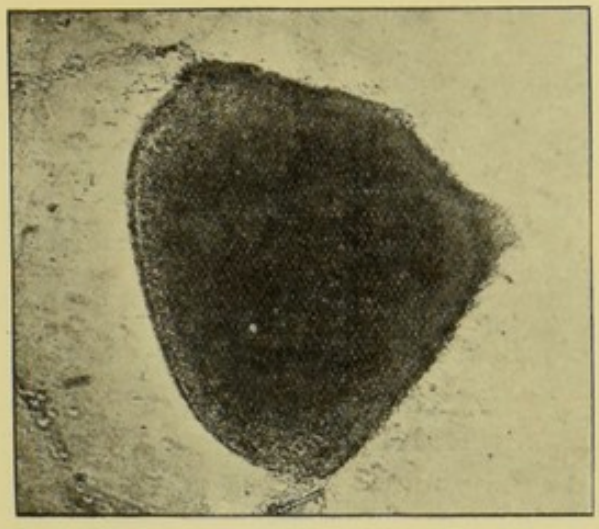
FIG. 10.—Same preparation after three days' incubation. (× 115). N.B.—The tissue has grown beyond the limits of the photographic field.

FIG. 11.—Shows outgrowth of new cells (epithelium ?) from a piece of adult rabbit kidney, ten days' incubation. Medium : Fowl plasma one part, extract of chick embryo one part, extract of the kidney one part. (× 115.)

FIG. 12.—Shows more or less solid advancing growth from a piece of epithelium of an embryonic chick, four days' incubation. Medium : Fowl plasma one part, extract of embryo one part. (× 66.)

Plate II.

7



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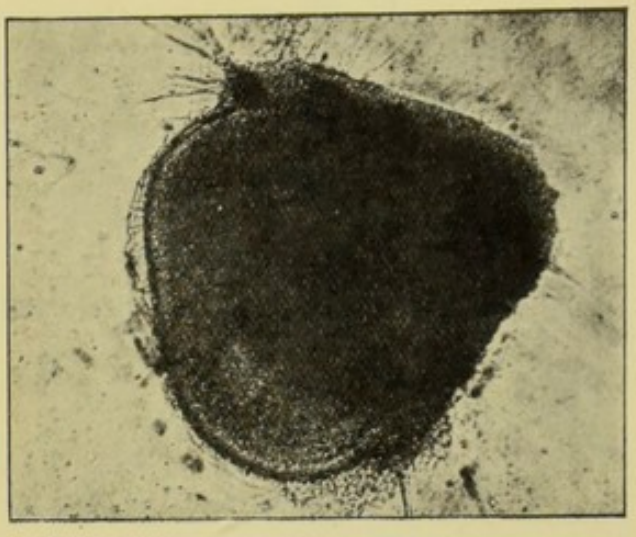

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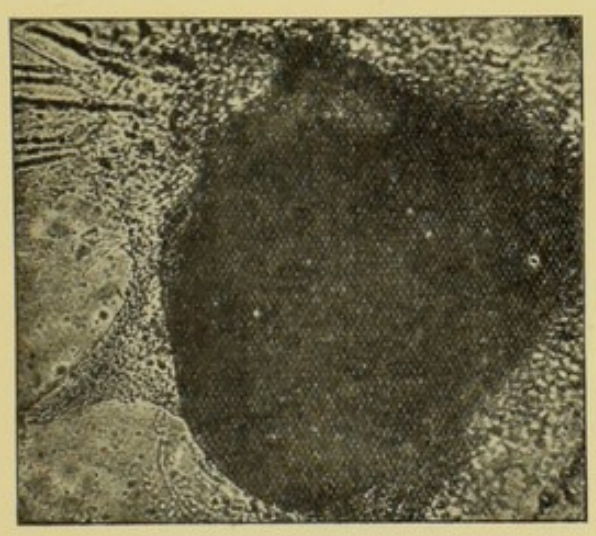

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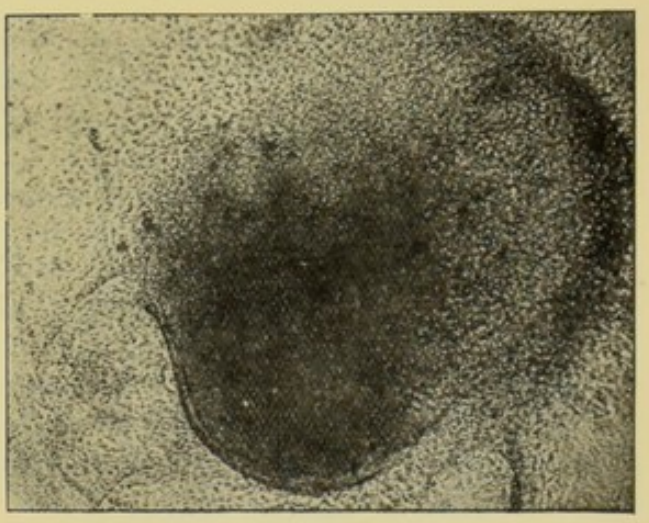

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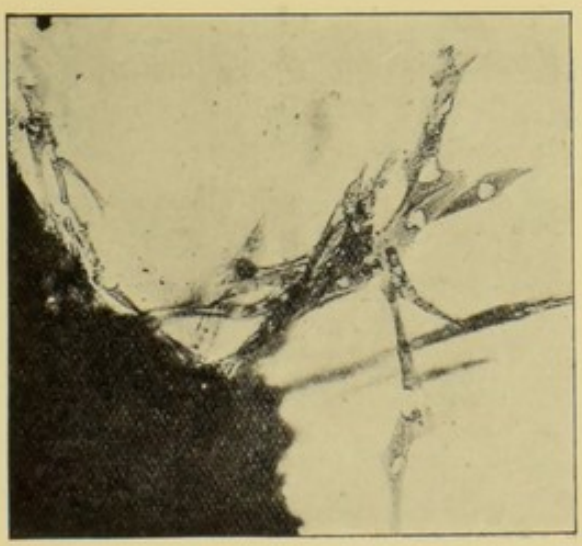

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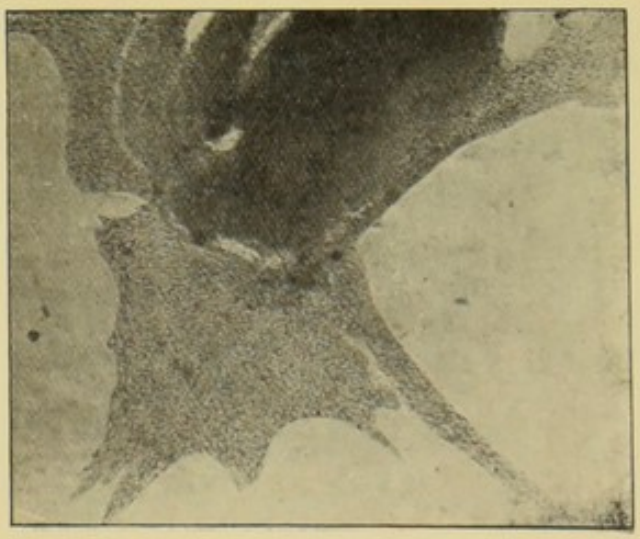

(D. Thomson, photo.)

PLATE III.

FIG. 13.—Shows an extension of large branching cells which grew out from a pulsating heart of a chick embryo, tenth day of incubation. The heart continued to pulsate during the ten days, during which time it was transplanted three times into fresh medium. Medium : Fowl plasma one part, extract of brain of chick embryo one part. (× 66.)

FIG. 14.—Shows a colony of new growth occurring from original central portion, fourth day of incubation. Medium : Fowl plasma one part, Ringer's one part, extract of embryo one part. (× 66.)

FIG. 15.—Shows absence of growth in liquid medium outside air-bubble, and apparently extensive growth in air-bubble from original portions of embryonic chick tissue, A and B. Medium : Fowl plasma one part, egg white one part, Ringer's two parts, embryo extract one part. (× 10.)

FIG. 16.—Same specimen showing the full extent of the apparent growth from portions A and B, after five days' incubation. (× 10.) N.B.—The new extension consisted of round cells similar to those of the original tissue.

FIG. 17.—Shows extension of round cells from embryonic chick tissue in fluid medium (egg white one part, embryo extract one part) in air-bubble, after forty-eight hours' incubation. (× 290.)

FIG. 18.—Shows two kinds of growth from original embryonic chick tissue : A, round-cell extension in fluid layer of the medium ; B, branching cell extension in the solid layer of the medium, sixth day of incubation. Medium : Fowl plasma one part, fowl serum one part, extract of embryo one part. (× 66.)

PLATE III.

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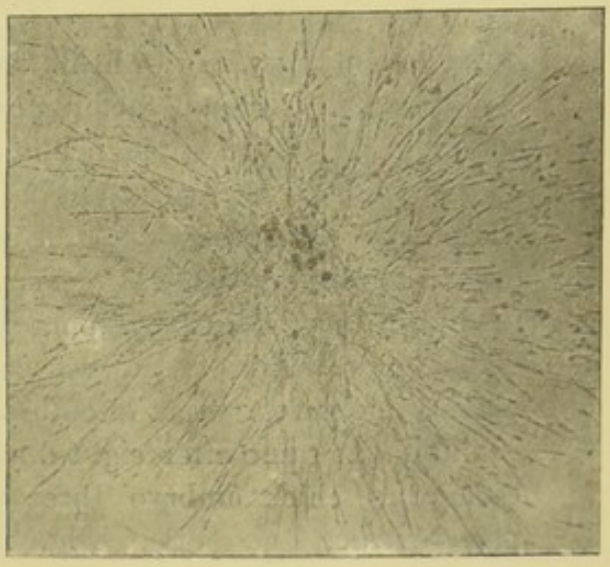

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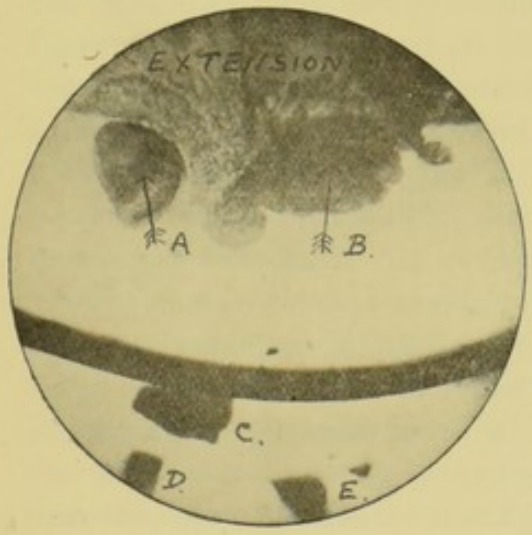

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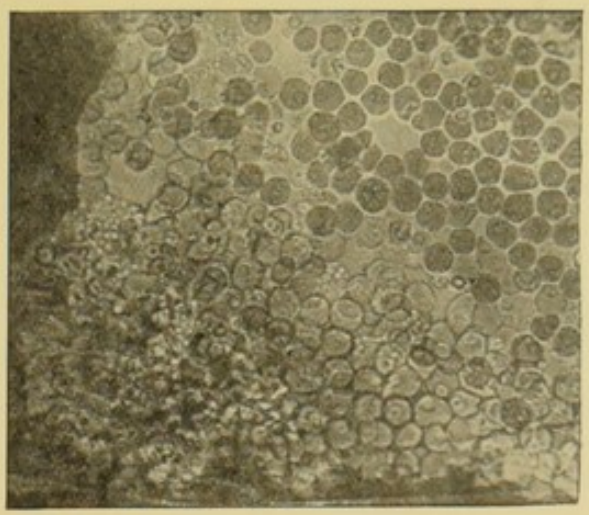

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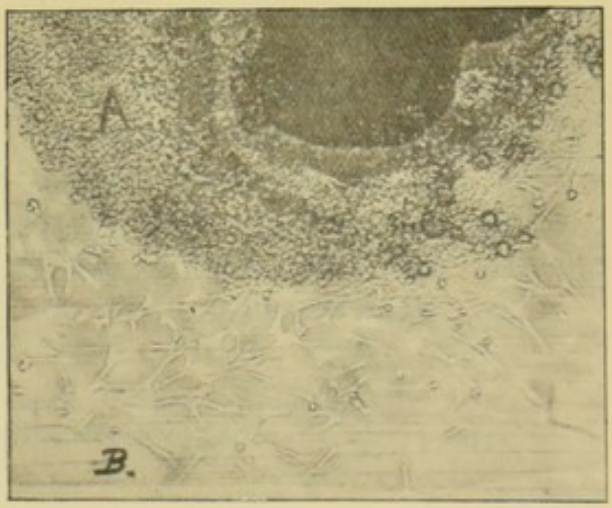

(D. Thomson, photo)

however, in that it is difficult to be satisfied whether the extension of round cells in the fluid medium is really a true proliferation or simply an exudation of the original cells.

I wish now to call attention to the photographs on Plate IV, since these have some bearing on this subject. I attempted to grow portions of the embryonic chick *en bloc*, so to speak, in the jelly-like plasma medium. Fig. 19 shows a photomicrograph of the toe of an embryonic chick, nine days old, newly placed in a medium composed of fowl plasma one part, extract of embryo two parts. Fig. 20 is a photomicrograph of the same after eight days' incubation. Fig. 21 shows the tail-end of a chick embryo three days old (medium, fowl plasma one part, extract one part). Fig. 22 shows the same after forty-eight hours' incubation. Fig. 23 shows a complete embryo, two days old (medium, fowl plasma one part, extract one part). The heart in this embryo kept beating actively in the medium, and after two days the embryo was removed, washed, and placed in fresh medium. The heart continued to beat actively, and on the fourth day it was again placed in fresh medium; during this process, however, the embryo, which was very friable, broke into two portions and was discarded. Fig. 24 is a photomicrograph taken on the fourth day of incubation. The second series of photographs shows in all three cases a distinct total increase in size over the corresponding three cases of the first series (the magnification in the respective pairs is identical). From these experiments it would appear that a total somatic growth had been obtained *in vitro*, and it is very important to note that in these cases no outgrowth of branching cells took place as occurs when small pieces of the tissue are cut away. It would appear, therefore, that a branching outgrowth will not occur in these cases due either to some somatic control or to some intact basement membrane.[1] If, however, the continuity of the mass of tissue is injured by a cut or an abrasion then a growth of branching cell would quickly occur at the injured or abraded part. Thus, if an entire embryo be cut in two and placed in the medium, branching growth will only occur from the cut surface, and none will take place from the uninjured surface. It is highly probable that this branching growth represents exactly what occurs in a live animal which has received a wound. Plasma is first poured out; this solidifies, and the cells, which are free and exposed, branch into the plasma clot and heal the wound.

[1] It is interesting to note that malignant tumours have no basement membrane, and this is probably the reason why they grow without limit and control.

(III) The Growth of Special Tissues.

(*a*) *Brain and Spinal Cord Tissue.* — The brain tissue of the embryonic chick grows readily *in vitro*. Branching cells, connective tissue (?) and long fibrils, axis cylinders, are produced. On one occasion a kind of plexus composed of long fibrils with ganglion-like swellings was produced. Excellent work in this subject has been done by Ingebrigtsen (1913); *see* also Harrison (1910) and Burrows (1911). On one occasion I attempted to cultivate brain tissue from a full-time human fœtus; but no growth was obtained, most likely because the fœtus had been dead for about twelve hours before the tissue was obtained.

(*b*) *Nerve-fibre and Ganglion Tissue.*—A piece of nerve (both ends cut) from a chick embryo placed in the usual medium showed no apparent extension of the axis cylinders, but there was a copious branching growth from the sheath, evidently connective tissue cells. *See* also Lewis and Lewis (1912) and Marinesco and Minea (1912).

(*c*) *Muscle Tissue.*—I have been unable so far to get a growth of true voluntary muscle-fibre cells. When a young embryonic chick heart is cultivated in the usual medium only branching cells are produced. These new cells are supposed to be connective tissue cells, since they have no resemblance to the original muscle cells. The young embryonic heart of the chick is composed of rounded cells. The heart continues to pulsate in the medium in spite of the fact that it becomes surrounded by a mass of growing branching cells. The cells of the new growth show no tendency to pulsate.

(*d*) *Connective Tissue.*—This species of tissue grows very readily in the form of long branching cells. It is very difficult to get specialized tissues entirely free from the former, so that all tissue growth is apt to be mixed, and it is often difficult to differentiate which are the special cells and which the connective tissue in the new proliferation. In some cases, however, the difference is quite distinct. I have noticed this difference most markedly in a piece of human cancer tissue cultivated in a medium composed of fowl plasma plus embryonic chick extract. In this case the connective tissue was the first to proliferate in the form of very long branching cells; later, the epithelial cancer cells grew out in buds or in the form of a solid advancing extension (Thomson, D., and Thomson, J. G., 1914). A considerable amount of work has been

PLATE IV.

FIG. 19.—Toe of embryonic chick immediately after its transference into medium composed of fowl plasma one part, embryo extract one part. (× 66.)

FIG. 20.—Same preparation, eighth day of incubation. (× 66.)

FIG. 21.—Tail of embryonic chick immediately after its transference into medium, fowl plasma one part, embryo extract one part. (× 66.)

FIG. 22.—Same preparation, after forty-eight hours' incubation. (× 66.)

FIG. 23.—Total embryo (two days hatched), twenty-four hours after it had been placed in medium, fowl plasma one part, embryo extract one part. (× 27.)

FIG. 24.—The same preparation, after three days' incubation. (The embryo was transferred into fresh medium on the second day. Note the increase in size with the appearance of embryonic eyes. The heart continued to beat throughout.) (× 27.)

PLATE IV.

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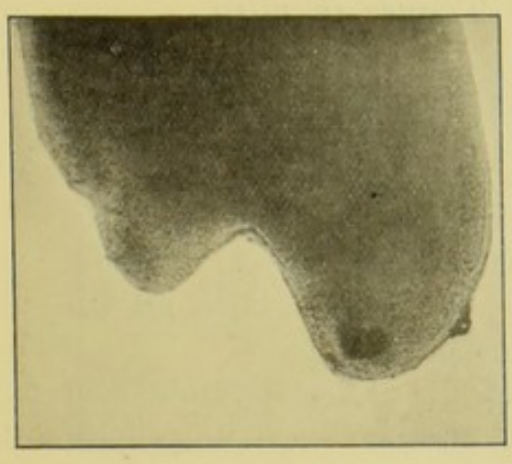

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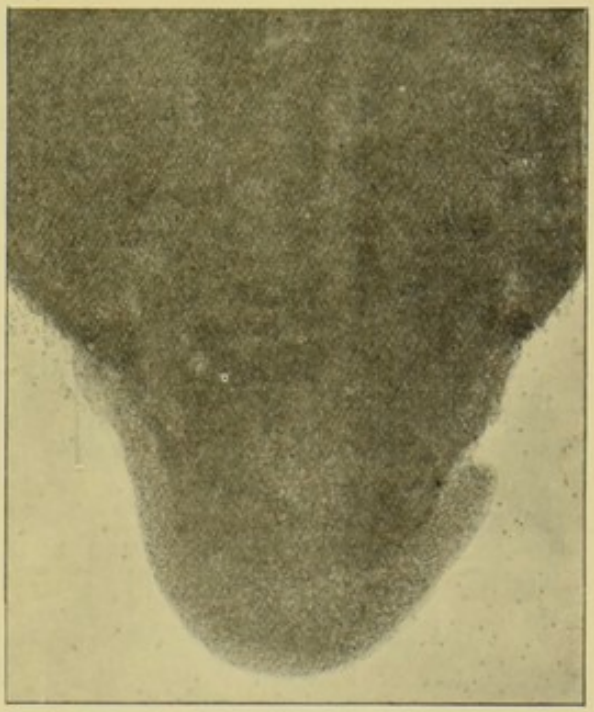

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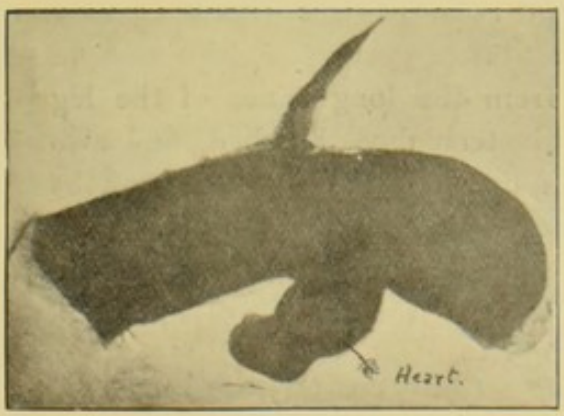

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(D. Thomson, photo.)

done on the growth of connective tissue by several investigators: Carrel (1913), Ebeling (1913), Pozzi (1912), and Lambert (1912-13).

(*e*) *Skin Epithelium.*—Figs. 7, 8, 9, and 10 represent successive stages in the growth of a shaving of epithelium from the leg of an embryonic chick. The new cells show a definite tendency to elongate. I have attempted, without success, on one occasion to obtain growth from epithelial cells scraped from the human skin.

(*f*) *Growth from Embryonic Chick Feathers.*—If a young embryonic chick feather is cut and placed in the usual medium a definite cellular proliferation occurs at the cut end, composed of round and branching cells. The cut end becomes rounded and bulbous, and branching cells extend for some distance into the medium (figs. 29 to 32). If the feather be a black one, blackish branching cells, containing a varying amount of pigment, grow out from the cut end (fig. 32).

(*g*) *Liver.*—On two occasions I have attempted to cultivate the liver tissue of embryonic chicks about twelve days hatched, and each time an outgrowth of elongated cells occurred. Whether or not these were liver cells I am unable to state. They looked more like connective tissue cells.

(*h*) *Kidney.*—Fig. 11 shows an outgrowth of elongated cells from a portion of adult rabbit kidney in a medium composed of fowl plasma one part, plus extract of kidney one part, plus extract of chick embryo one part. The new cells in this case, though elongated, do not resemble the usual appearance of connective tissue proliferation, and I am inclined to look upon them as epithelial kidney cells. See also the works of Walton (1914) on this subject.

(*i*) *Bone.*—On the few occasions in which I have planted this tissue from the embryonic chick, in the usual medium, a proliferation of long branching cells, accompanied with considerable numbers of large rounded or oblong cells, was produced. The latter cells contained numerous large refractile granules.

(*j*) *Bone-marrow.* — The growth *in vitro* of this tissue is very interesting. It differs markedly from all other growths except that of the spleen. The tissue was obtained from the long bones of the leg of embryonic chicks from twelve to nineteen days hatched, and also from the long bones of hatched chicks fourteen days old. The growths in all cases resembled each other, more or less. The growth is not a branching proliferation, nor is it a solid extension. It differs from both in that it consists for the most part of isolated, active, amœboid

cells of varying shape (fig. 25). About the third day of incubation, however, long radiating lines of cells appear, which are probably true connective tissue cells. Leucocytes, chiefly of the polymorphonuclear type, are produced, and also red cells in abundance. Foot (1912-13) has studied the cultivation of this tissue very carefully, but, strange to say, he did not find any production of red cells.

(*k*) *Spleen.*—The proliferation of this tissue resembles that of bone-marrow very markedly. I have, however, cultivated only the spleen tissue of the embryonic chick, so that I am not in a position to state that the same type of growth occurs in the case of adult spleen tissue. Walton (1914) obtained a similar growth from adult rabbit spleen.

Fig. 27 is a photomicrograph representing a twenty-four hours' growth *in vitro*, and fig. 28 shows a higher magnification of the same after three days' incubation. The latter shows polymorphonuclear leucocytes, and other large mononucleated cells. The growth of this tissue has been studied very carefully by watching it continuously under the microscope, the preparation being kept at the necessary temperature by means of a warm stage, or by having the microscope in a special warm chamber. I am convinced that the extension of cells is not of the nature of a migration from the original piece of tissue, since I have seen the new cells themselves subdividing into several other cells. The first cells to grow out from the original tissue are apparently leucocyte-producing cells. Later, about the second or third day, there is a new proliferation of cells, which produce red corpuscles; large numbers of the latter are produced during the days following. On the third day long radiating lines of connective tissue cells appear, as in the case of the bone-marrow. It seems to me that there is little doubt that the embryonic spleen at least produces nearly all the elements of which blood is composed. Red marrow does not appear in the bones of the developing chick until about the twelfth day of hatching, so possibly the spleen is largely responsible for the blood produced some time before the marrow has developed. The red corpuscles are produced from large actively amœboid cells, and when nearly fully developed they are discharged from these cells. At first the developing corpuscles are round, with large nucleus and bluish staining protoplasm, but gradually they become oval, while the nucleus becomes smaller and more compact, and a gradual transition from bluish to red occurs in the staining of the protoplasm around the nucleus. I hope, however, to give another paper devoted to this interesting process after a further study of the subject.

PLATE V.

FIG. 25.—Shows growth from bone-marrow of embryonic chick (nineteen days hatched), after three days' incubation. Medium : Fowl plasma one part, embryo extract one part. (× 110.)

FIG. 26.—Same preparation, higher magnification, showing the production of polymorphonuclear leucocytes. (× 600.)

FIG. 27.—Shows the growth from portion of spleen of an embryonic chick (nineteen days hatched), after twenty-four hours' incubation. Medium : Fowl plasma one part, embryo extract one part. (× 110.)

FIG. 28.—Same preparation, higher magnification, after three days' incubation. Note the large round cells, and the production of polymorphonuclear leucocytes. (× 600.)

FIG. 29.—Appearance of cut end of feather of embryonic chick (fifteen days hatched) when placed in the medium. (× 66.)

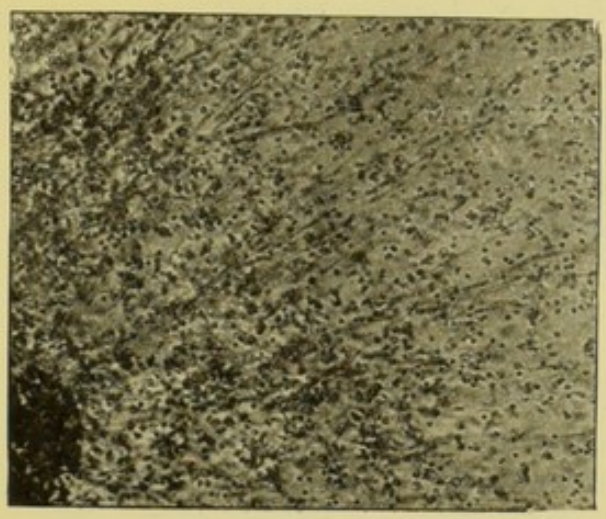
FIG. 30.—Appearance of cut end of similar feather, after three days' incubation in medium composed of fowl plasma one part, extract of human ovary one part. (× 66.)

FIG. 31.—Identical preparation as last, after four days' incubation. Note the appearance of new sprouting cell. (× 66.)

FIG. 32.—Illustrates the growth or healing process of cut end of feather of embryonic chick (eleven days hatched), after five days' incubation in a medium composed of fowl plasma one part, embryo extract one part. Note the formation of a large bulb at the cut end of the feather, also the new growing cells around. (× 66.)

PLATE V.

25



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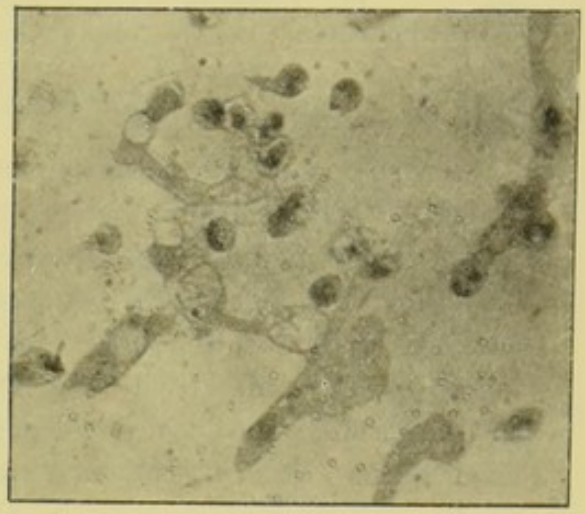

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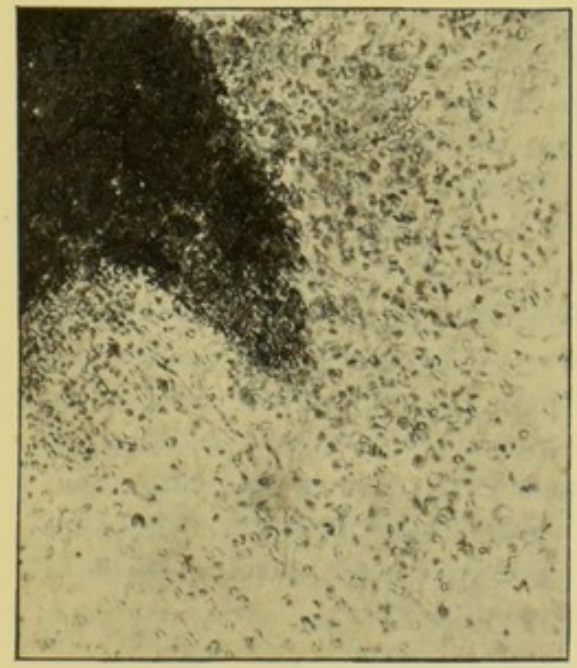

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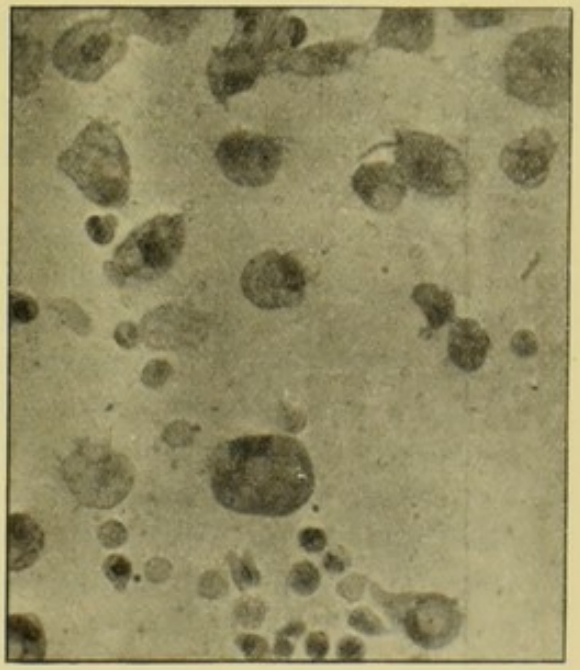

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(D. Thomson, photo.)

(IV) Further Remarks.

I wish to say something on the effect of various tissue extracts when added to the medium. Carrel has shown that certain extracts when added to the medium increase the rate of growth in the tissue as much as tenfold and more. He believed that the effect of extract is specific, but this is not quite correct, as I have found that the extract of the tissue of one animal will help the growth of the tissue of an animal of quite a different species to the first. In fact, I obtained better growth from human cancer tissue in a medium composed of fowl plasma plus chick embryo extract, than in a medium containing fowl plasma plus extract of the cancer tissue itself. On the other hand, it would appear that certain extracts inhibit the growth of certain tissues. Thus, I failed to get any growth in chick embryo tissue in a fowl plasma medium containing extract of a human papillomatous tumour of the ovary. Again, no growth was obtained in fowl plasma to which extract of Rous sarcoma was added (fig. 1). In the accompanying paper (Thomson, D., and Thomson, J. G., 1914) it is pointed out that the stars of crystals resembling tyrosine crystals developed in the media to which extract of the papillomatous tumour of the human ovary was added. Similar crystals occasionally develop in media containing extract of chick embryo, so that it possibly represents the presence of a certain chemical substance in these extracts. Fowl plasma clots after being kept for some time at room temperature or in the incubator at blood heat. If extract of chick embryo is added to it, it clots very much more rapidly (one minute or less). Extracts of the tissues of other animals (mammals) do not appear to cause this rapid clotting effect in fowl plasma.

(V) Summary and Conclusions.

(1) The tissue of various animals, including mammals and man, can be cultivated *in vitro* in a medium composed of fowl plasma one part, plus extract of chicken embryo one part.

(2) It would appear that all proliferating cells tend to elongate and throw out processes in a solid plasma medium, whereas in a fluid medium they retain their original shape and appearance. The cells of different tissues, however, retain certain distinguishing characteristics in the plasma medium, so that these cells evidently have some inherent

points of difference from each other which remain with them regardless of the medium or environment.

(3) Complete uninjured portions of chick embryonic tissue grow to some extent *in vitro* under somatic control.

(4) The chick spleen in embryonic life at least is capable of producing red corpuscles as well as various forms of leucocytes.

(5) The extract of one species of animal tissue appears in certain cases to stimulate the growth of tissue from an animal of different species, whereas in other cases the converse seems to hold good.

(6) Extracts of certain tissues deposit stars of crystals resembling tyrosine when kept in the incubator for a period of two weeks and upwards.

Addendum.

By permission of the President, at the conclusion of the foregoing report, Dr. H. D. McCulloch, demonstrated, by means of the kinematograph, cytogenesis and histogenesis in embryo chick's heart tissue and spleen tissue, including their accessory free cells, *in vitro*. Dr. McCulloch said:—

Last winter I suggested to Dr. David Thomson the importance of using the kinematograph in connexion with cytological research. On seeing the announcement of the reading of the extremely interesting report by Dr. J. G. Thomson, to which you have just listened, I thought this might be a good opportunity for again urging my plea before the Society. Having obtained the series of films from Dr. J. Comandon, of the Pasteur Institute, I decided to make an attempt to interpret some of the less conspicuous phenomena that are to be observed, and to indicate the direction in which further cytological research might with advantage be pursued. With your permission, and that of the author, I will proceed to do so. These tissues were photographed when several days old, after cultivation upon coagulated plasma by Harrison and Burrows's method, the same as is employed by Carrel.

The first series shows a fragment of the embryo chick's heart, in which energy is seen being expended by a continuance of the characteristic rhythmic contractions of that organ. How far do these contractions conduce to hypertrophy or growth of the muscle cells, and how far is the growth vegetative and embryonal? Again, there would

be some relationship, with a greater difference, between this growth *in vitro* and that observed in cells stimulated to activity by extrinsic chemical agencies that evoke protoplasmic irritability, and that which follows upon the influence on cells by the periodic hormonic secretions. There is also the field of radio-biological research, in which Mottram and Beckton have shown that the effect of radio-active agencies on nuclear activity is much more marked during mitosis than during the resting or vegetative periods of the cell.

The second series of films show a fragment of splenic tissue of the embryo chick, with its rudimentary Malpighian bodies and the cells which are presumably the progenitors of the blood cells encircling the fragment, in various stages of immaturity, and from which they appear to have emigrated within a certain radius of its centre. The majority of these cells are seen to be actively rotating and moving under some central influence, in the artificial conditions for which some allowance should be made. The two or three large germinal cells, like giant cells, might be detached Malpighian bodies moving to take up new situations in relation to the surrounding proliferation. The few mature leucocytes to be seen attract attention by their characteristic amœbism, and by the more active contortions when on the point of undergoing mitosis. The well-known nuclear phases of this process are, however, less clearly seen than when they are displayed in the metamorphosis of the newt's leucocyte or mononucleated white cell, into the far more inert nucleated red cell, during the erythrocytosis that follows upon high feeding.

In the next series, under still higher magnification of the cardiac tissue, marginal growth of the connective tissue stroma cells is seen to be proceeding. These cells appear as fixed radiating fusiform prolongations. In their cytoplasm small spherical granules are seen to stream to and fro in the direction of growth. The nuclei of these cells are obscured. Doubtless the absence of a capsule or limiting membrane influences the rate of peripheral proliferation.

In concluding these brief observations, seeing that the phenomenon of tactism in the impregnation of the ovum by spermatozoa has now been frequently demonstrated by this method, as well as that of the physiological mechanism of phagocytosis, the time seems opportune for inquiring into the origin and life-history of the leucocyte, the actual occurrence of diapedesis and its true significance. I refer to suggestions that I have ventured to make elsewhere—(1) that leucocytes are not essentially blood cells as generally accepted; (2) that polymorphism of

the nucleus is an indication of the extent of function, and is the result of the amœbic passage of these cells through variously dense tissue textures; (3) that the reason why the bone-marrow of adults is seen to be charged with masses of these polynuclear cells is not that they are generated in the myelin matrix as such, but that they are there for the purpose of undergoing secondary regeneration as mononuclear myelocytes. I submit that the lymphatic glands of the body are the primary source of mononuclear leucocytes. These are matters to which Dr. Comandon has kindly promised to give his earnest attention, but I see no reason why we should not be able to extend our researches in this direction, if facilities are afforded for carrying them out in this Marcus Beck Laboratory.

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The Royal Society of Medicine.

Marcus Beck Laboratory Reports.—No. 3.

Some Researches on Spirochætes occurring in the Alimentary Tract of Man and some of the Lower Animals.

By John Gordon Thomson, M.A., M.B., Ch.B.Edin.,[1] and David Thomson, M.B., Ch.B.Edin., D.P.H.Cantab.[2]

Read before a Meeting of the Society on July 14, 1914.

The President, Sir Francis H. Champneys, Bt., M.D., in the Chair.

Introduction.

This research has been carried out in the Marcus Beck Laboratory of the Royal Society of Medicine under the directorship of Sir Ronald Ross, K.C.B., F.R.S. In this paper it is intended to confine the attention to the spirochætes which occur in the mouth and intestines of man and some of the lower animals in health, and in certain pathological conditions. When we consider the important part played by *Spirochætæ* and *Treponemata* in the production of serious diseases in animals and man all over the world, in this country and in the tropics, it seems difficult to realize that the commoner spirochætes of the human mouth and alimentary canal are almost entirely neglected by the research students and medical men in this country. The neglect

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seems to be based on the assumption that these protozoa commonly occurring in apparently normal healthy individuals are in all probability harmless saprophytes. Although in our present communication we are unable to assign any real pathological importance to the spirochætes inhabiting the alimentary tract of man, it seems to us very important that these should be carefully studied. We have to thank Dr. H. B. Fantham and Dr. Annie Porter for the great assistance we have obtained in a difficult subject from the excellent work done by them in the study of the morphology of spirochætes. The bibliography given by Dr. Bosanquet in his book has been found to be very useful.

The help of the President, Sir Francis Champneys, the Secretary, Mr. MacAlister, and the Director, Sir Ronald Ross, in all matters connected with the Laboratory have made the work there most pleasant.

The Material used in this Research.

The material used was obtained from various sources through the kindness of Dr. Keats, of Camberwell Infirmary. As practically every human mouth harbours spirochætes they were easily obtained, and a large number of cases of pyorrhœa alveolaris chiefly associated with chronic rheumatoid arthritis received particular attention. The fæces of several normal individuals as well as those of thirty chronic rheumatoid arthritis patients were studied. The pus from ulcerated throats, cancrum oris, cancer of the tongue, myositis ossificans of the jaw, and the fæces of a large number of guinea-pigs, rats, dogs, and fowls were carefully studied.

The Method of Examination.

The method employed is a very simple and rapid one. A thin film is made from the suspected material on a glass slide. This is fixed by heat. A concentrated alcoholic solution of gentian violet is prepared and used as a stock solution. For staining the slide we use one or two drops of the stock solution with each cubic centimetre of ordinary tap-water. We then pour this over the film previously fixed by heat, and heat gently. In a few minutes the spirochætes and treponemata are intensely stained and the slide is washed in tap-water and dried. This technique used in conjunction with the dark-ground illuminator enables one to study the spirochætes rapidly both in fresh and stained speci-

mens. Giemsa stain was also used after wet fixation of the film. When stained with gentian violet it has been noted that often darker stained areas of the bodies of the spirochætes are demonstrated, clearly suggesting granule formation.

Nomenclature.

No subject is more confusing to the research student than the study of spirochætes. The immensity of the literature and the incomplete descriptions given by various authors at different times add to the difficulties. It is not intended here to enter into any discussion, but it is perfectly evident that many mistakes have been made by several investigators and new species have been named before there has been sufficient evidence for doing so.

Fantham (1911) says: "The spirochæte group as a whole (or various members of it) has received so many names that it seems to be a mania to rename it according to the individual fancy." Most protozoologists we feel sure agree with this statement.

The present state of our knowledge regarding spiral organisms enables us to distinguish three different genera.

(1) *Spirochæta.*—This protozoon has a flexible, wavy body with a corkscrew fin or flange which is called the membrane or crista. In the body there is a nucleus, consisting of a series of bars or rodlets ("granules") which is composed of chromatin. The formation of small ovoid bodies or spores which are resistant and capable of producing new spirochætes has been clearly proved by Fantham (1907, 1908, 1909, 1911) in his studies of the spirochætes of lamellibranchs, birds and mammals; Bosanquet (1911) also noted the formation of coccoid bodies in a preparation containing *Spirochæta anodontæ.*

(2) *Treponema.*—The bodies in these protozoa are extremely thin and are thrown into waves or coils which are said to be fixed or "preformed." There is no visible membrane and no visible nucleus. Balfour (1911), however, has recently stated that *Treponema pallidum* is a granule-shedder.

(3) *Spirillum.*—These are bacteria with a more or less flexible body. Flagella are present terminally and sometimes at the side. There is no membrane.

It would seem probable that the differences between *Spirochætæ* and *Treponemata* may be merely a question of size, and that this explains the invisibility of the membrane and nucleus in the *Treponemata.* That

there is a nucleus of a diffuse character in the *Treponema* seems certain, and the fact that Balfour has described granule-shedding in *Treponema pallidum* suggests that this organism may be really a member of the genus *Spirochæta*. We are aware that Zuelzer (1911) claims to have re-studied the type species, *Spirochæta plicatilis* Ehrenberg, and states that there is an axial rod therein. However, until further work on the subject has been done, we use the name "spirochætes" as above without prejudice. (Fantham, 1911.)

Spirillum as a name has been applied to many of the smaller spirochætes, but it is now recognized that this term should be applied only to bacteria with flexible bodies and flagella.

The Difficulty of Distinguishing various Species of Spirochætes by Morphology.

There are three named species of spirochætes, which in many respects are very similar in appearance when examined under the dark-ground illuminator or in stained films; these are *Spirochæta buccalis* (Cohn, 1877), *Spirochæta refringens* (Schaudinn and Hoffmann, 1905), and *Spirochæta balanitidis* (von Prowazek and Hoffmann, 1906). The resemblance of *Spirochæta vincenti* (Blanchard, 1906) to *Spirochæta buccalis* is also said to be very close, and some observers think they are identical. Noguchi (1912) has pointed out that it is quite impossible to distinguish *Spirochæta buccalis* from *Spirochæta refringens* by morphological appearances, and again Rille (1905) and Kraus (1906) believed that *Spirochæta refringens* is the same as *Spirochæta balanitidis*. Other observers, Eitner (1907) and Richards and Hunt (1906), declare that at least four distinct varieties have been described indiscriminately as *Spirochæta refringens*. It is quite evident, therefore, that in this subject there is hopeless confusion. However, in order to escape this confusion, it is satisfactory enough for the time being to regard types like *Spirochæta refringens* and *Spirochæta balanitidis*, which are found in the mouth, as belonging to the species *Spirochæta buccalis*. This latter is a fairly broad spirochæte $\frac{1}{2}$ μ to 1 μ in breadth, which has tapering points and long shallow undulations. In length it varies from 12 μ to 20 μ, and under the dark-ground illuminator it seems to be flat or ribbon-shaped. It is an active spirochæte and has a corkscrew motion from left to right. Again, in many specimens stained by us we were able to see clearly a series of dark and light staining areas along the bodies suggesting granule formation (figs. 1 to 7 and figs. 14

to 17, Plate II). These figures show that the type varies and figs. 14 to 16 (Plate II) suggest a species corresponding to *Spirochæta refringens* or *Spirochæta balanitidis.*

In our opinion *Spirochæta vincenti* (or what we consider to be *Spirochæta vincenti*) can be recognized as different from *Spirochæta buccalis*, as it is a thinner spirochæte, not ribbon-like, and the coils are more irregular owing to the great flexibility of the body. This flexibility is well seen under the dark-ground illuminator. We have found it quite simple to select this species, both in stained films and also in living specimens. The figures 8 to 13, Plate II (*Spirochæta vincenti*), when carefully compared with the type *Spirochæta buccalis*, show a marked difference. Veszpremi (1907) described a spirochæte which he found in the abscess of the jaw, and he called this a new species—namely, *Spirochæta gracilis.* Bosanquet (1911) thinks this spirochæte is probably identical with *Spirochæta vincenti.* Recently the excellent work of Noguchi (1912), in which he has been able to isolate by cultivation methods three different species of treponemata from the human mouth, has demonstrated to us a new method of isolating and differentiating different varieties. These treponemata are all new species and have been named *Treponema macrodentium*, *Treponema microdentium*, and *Treponema mucosum* (Noguchi, 1912). These three new species were probably all included under the old name, *Spirochæta dentium* (Koch, 1877). Castellani (1906 and 1909) has described a form of hæmorrhagic bronchitis, occurring in tropical countries, and in association with this disease he has described four varieties of spirochætes—namely: (*a*) Very thick individuals 18 μ to 39 μ in length with irregular coils which vary in length but are not very numerous. (*b*) Individuals resembling *Spirochæta refringens* (Schaudinn) and possessing a few graceful curves and pointed extremities. (*c*) Thin, delicate spirochætes with numerous uniform coils and tapering ends. Sometimes one of the extremities is blunted. This is perhaps the commonest type. (*d*) Extremely thin, delicate organisms with very few irregularly shaped coils. Whether these are new species or not it is difficult to state, as it seems that they are very like in some respects certain of the types found in the mouth in other conditions. Castellani has described as a new species *Spirochæta subtilis*, an organism which he found in the scrapings of the oral mucosa and the intestines.

EXPLANATION OF PHOTOMICROGRAPHS (PLATE I).

All the slides were stained with an alcoholic solution of gentian violet.

Magnification is the same in all the photographs, and is 1,300 diameters.

FIG. 1.—Spirochæte from the fæces of a fowl.

FIG. 2.—Photomicrograph from a slide prepared from a severe case of pyorrhœa alveolaris. Note the large clumps of spirochætes and fusiform bacilli. The spirochætes belong to the type *Spirochæta vincenti.*

FIG. 3.—Photomicrograph from a slide prepared from a specimen of human fæces which had been kept for several months in a bottle with water. Two varieties of spirochæte will be noted, a short thick form, very similar in character to that shown in fig. 4, and another type, very thin and with very fine coils, one end of which is attached to a round piece of debris. Fig. 7 is another photograph of this thin variety, showing a longer specimen, but the fine coils are not seen.

FIG. 4.—Spirochætes in the human fæces showing the type called by Werner *Spirochæta eurygyrata.* These fæces were from an apparently normal human being. This type of spirochæte was found to be fairly common in the fæces of chronic rheumatoid arthritic cases.

FIG. 5.—Spirochætes from the fæces of an apparently normal human being showing the type described by Werner, and named *Spirochæta stenogyrata.* This seems very similar to the minute forms of *Treponema microdentium.* Very numerous spirochætes of this type were found in the fæces of a case of profound anæmia.

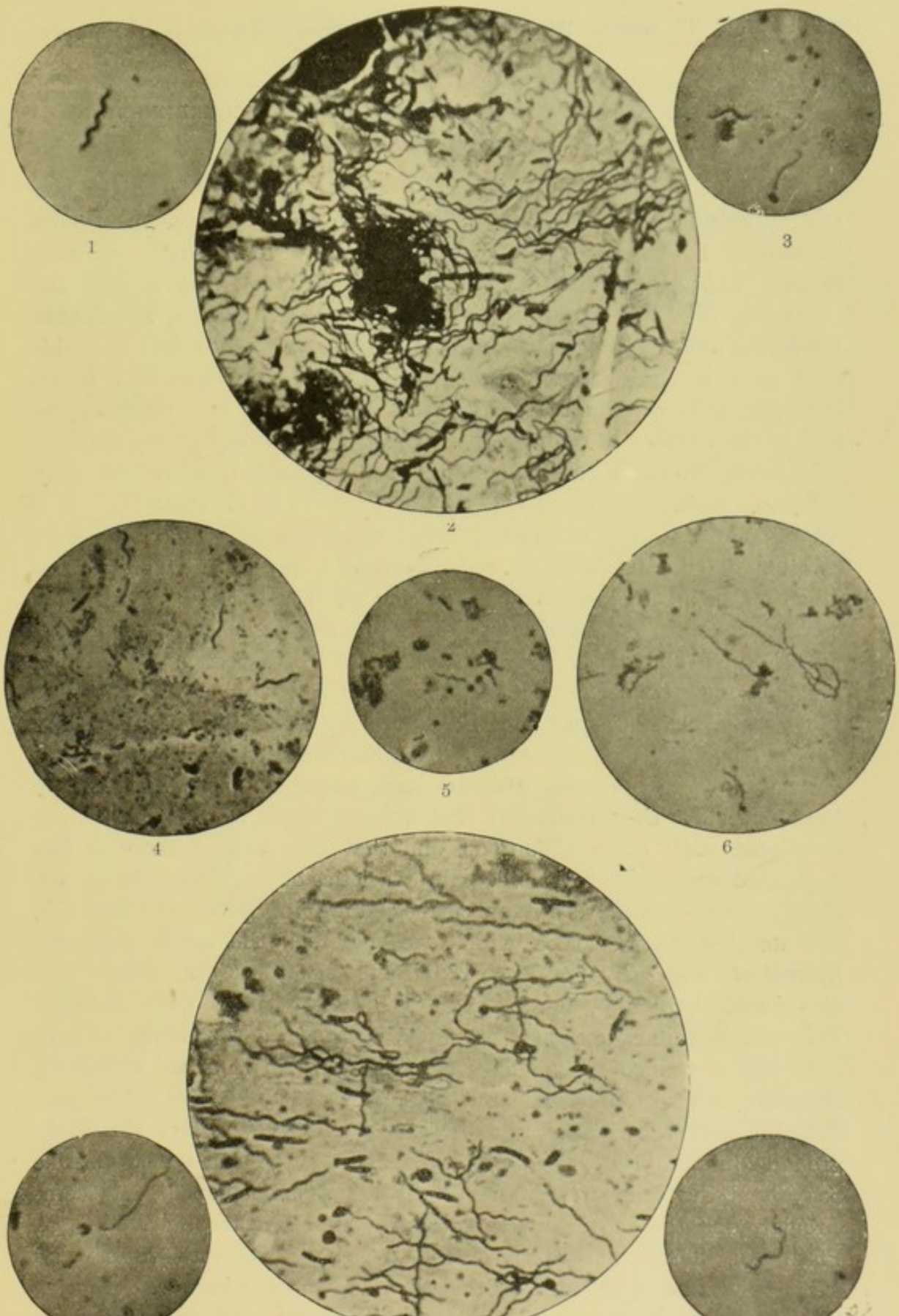
FIG. 6.—*Treponema microdentium* from a culture taken from the human mouth.

FIG. 7.—(See description under fig. 3.)

FIG. 8.—Photomicrograph taken from a preparation made from a case of Vincent's angina. The spirochætes vary greatly in size and thickness. At the upper margin two very long spirochætes with regular coils, other shorter spirochætes are seen, and some long, thin spirochætes with irregular coils. Fusiform bacilli are present.

FIG. 9.—Spirochæte from the fæces of a fowl (Plymouth Rock).

PLATE I.



(D. Thomson, photo.) Magnification = 1,300 diameters. 10 μ

Authors' Observations on Spirochætes occurring in the Human Mouth.

We have pointed out that, owing to the great variation in size and appearance of spirochætes belonging to a certain type, it is quite impossible by morphological characters alone to separate out new species, and as an example of this difficulty we have quoted the confusion that has arisen regarding *Spirochæta buccalis*, *Spirochæta refringens* and *Spirochæta balanitidis*. All these more or less resemble each other in being rather broad, ribbon-shaped organisms with tapering ends, wide coils and spiral movements, but they vary much in size and in the number of undulations. However, it is easy to separate out spirochætes in the mouth belonging to this general type, and we shall call these *Spirochæta buccalis* (figs. 1 to 7 and 14 to 17, Plate II). It is interesting to note that some of these when stained with an alcoholic solution of gentian violet in water showed a series of darker-stained areas in their bodies, strongly suggesting the formation of granules or coccoid bodies. We have not seen any previous mention of this in the literature on the subject (figs. 1 to 3, Plate II). Blanchard (1906) fixed the spirochæte found by Vincent (1898-99) as a new species, but unfortunately, as far as we can make out, he neglected to describe it. In our investigations, by careful examination of smears taken from ulcerations of the throat, cancrum oris, pyorrhœa alveolaris, &c., we have come to the conclusion that various types of spirochætes are associated with these conditions, and that they are all more or less associated with fusiform bacilli. Two varieties of large spirochætes are chiefly found—namely, a type corresponding to *Spirochæta buccalis*, and another distinct type which we may call the much discussed *Spirochæta vincenti* (figs. 13, 18 to 20, and 8 to 12, Plate II); also photomicrograph, fig. 2, Plate I). This type is quite definite. It is a long, thin spirochæte with wide, irregular coils, tapering at both ends, and in stained specimens it is noted that the coils are extremely irregular in size and shape and the bodies tend to curve round into different shapes owing to the extreme flexibility. Under the dark-ground illuminator it is to be noted that this type has a very flexible body, and the coils are not fixed. It is not band-shaped, but seems circular, and the movement is corkscrew in character (figs. 8 to 12, Plate II). These were derived from a case of Vincent's angina and were associated with fusiform bacilli. The photomicrograph (fig. 2, Plate I) shows similar spirochætes associated with fusiform bacilli from

a case of pyorrhœa alveolaris. The same type, *Spirochæta vincenti*, was found in a case of cancrum oris (fig. 13, Plate II) and in a case of abscess of the jaw complicating myositis ossificans (figs. 23 and 24, Plate II). Veszpremi (1907) described a spirochæte in an abscess of the jaw and he named it *Spirochæta gracilis*, but Bosanquet (1911) thinks this is identical with *Spirochæta vincenti*. Our drawings and observations would tend to prove this, but we must always guard against being definite about it, as Veszpremi may have been observing quite a different spirochæte. With respect to the confusion that has arisen by some observers considering *Spirochæta buccalis* as being the same as *Spirochæta vincenti*, we think this has probably arisen from their actually finding the type *Spirochæta buccalis* in Vincent's angina. The photomicrograph (fig. 8, Plate I) illustrates this very clearly. In this photograph from an ulcer of the fauces it will be noted there are numerous extremely large, thick, band-shaped organisms associated with fusiform bacilli (figs. 14 to 17, Plate II). From our studies, therefore, it seems quite clear that in ulcerations of the throat and severe pyorrhœas two types of spirochæte may occur separately or together—namely, *Spirochæta buccalis* (which may turn out to be composed of different species) and another type which we consider is *Spirochæta vincenti*. The fact that both these occur in certain definite pathological lesions seems to us clearly to explain the confusion that has arisen amongst different observers.

In specimens taken from the human mouth treponemata are common, and these greatly vary in size (figs. 25 to 27, Plate II). They have fixed coils and a rapid spiral movement. As pointed out by Noguchi, the only method of differentiating these is by cultivation.

Castellani (1906 and 1909) described a form of hæmorrhagic bronchitis occurring in the tropics and he found that this was associated with numerous spirochætes of different types. Branch (1907) in the West Indies and Jackson (1908) in the Philippine Islands also have described spirochætes associated with bronchitis. Recently Taylor (1914) has found spirochætes associated with bronchitis in Uganda; Chalmers, O'Farrell and Fantham (1913) have found them in bronchitic patients in the Sudan. Evidence is thus accumulating that these spirochætes are pathogenic in the tropics and give rise to severe bronchial symptoms.

EXPLANATION OF PLATE II.

All slides were stained with alcoholic solution of gentian violet.

Drawn with aid of camera lucida. Magnification = 1,500 diameters.

FIGS. 1 to 7.—*Spirochæta buccalis* from cases of severe pyorrhœa alveolaris of human mouth. Note figs. 1 to 3 show the dark-stained areas suggestive of granules.

FIGS. 8 to 12.—*Spirochæta vincenti* from cases of Vincent's angina associated with fusiform bacilli.

FIG. 13.—*Spirochæta vincenti* from a case of cancrum oris.

FIGS. 14 to 16.—These spirochætes were drawn from a case of Vincent's angina, and the type suggests that they are different from either *Spirochæta buccalis* or *Spirochæta vincenti*. In certain respects they resemble *Spirochæta refringens*.

FIG. 17.—*Spirochæta buccalis* from ulcer of throat.

FIGS. 18 to 20.—*Spirochæta vincenti* from cases of pyorrhœa alveolaris associated with chronic rheumatoid arthritis.

FIGS. 21 and 22.—*Spirochæta buccalis* from cases of pyorrhœa alveolaris associated with chronic rheumatoid arthritis.

FIGS. 23 and 24.—*Spirochæta vincenti* from a case of abscess of the jaw complicating a case of myositis ossificans.

FIGS. 25 and 26.—*Treponema microdentium* from pyorrhœa alveolaris.

FIG. 27.—*Treponema macrodentium*.

FIGS. 28 to 32.—Types of treponema found in cultures prepared from a case of pyorrhœa alveolaris.

FIGS. 33 to 35.—Spirochætes from apparently normal human fæces which clearly resemble the *Spirochæta stenogyrata* described by Werner. Compare figs. 75 and 76.

FIGS. 36 to 38.—Spirochætes found in normal human fæces, and also in cases of chronic rheumatoid arthritis which correspond to the type of *Spirochæta eurygyrata* described by Werner. Compare these with figs. 73 and 74 as depicted by Werner.

FIGS. 39 to 43.—New species of treponema hitherto undescribed. This was found in an old specimen of human fæces kept in a bottle at room temperature. Fig. 42 shows only a straight line. Figs. 42 and 43 show organism attached at one end to a rounded body.

FIG. 44.—Another variety of spirochæte, short and thick, with pointed ends. This was found in the same specimen of fæces kept in a bottle.

FIGS. 45 and 46.—Spirochætes found in the stool of an amœbic dysentery. Note these are larger and have irregular coils. These also tended to be blunted at the ends.

FIGS. 47 to 50.—Variety of spirochæte found in the fæces of guinea-pigs. Note the dark-stained areas in some suggesting granules.

FIGS. 51 and 52.—Another variety of spirochæte from the fæces of a guinea-pig. Note the dark-stained granules.

FIGS. 53 to 55.—Spirochætes from fæces of a dog, which was suffering from *Babesia canis*.

FIGS. 56 and 57.—Spirochætes from fæces of a rat.

FIGS. 58 to 61.—Spirochætes from fæces of a hen.

FIGS. 62 to 64.—Spirochætes from bronchitic sputum.

FIG. 65.—*Treponema pallidum* from a congenital syphilitic liver.

FIG. 66.—*Treponema microdentium* in culture to show the striking resemblance to *Treponema pallidum*. It would be impossible in this case to tell this from *Treponema pallidum* from morphological appearance only.

FIG. 67.—Spirochæte from a culture, probably *Spirochæta vincenti*.

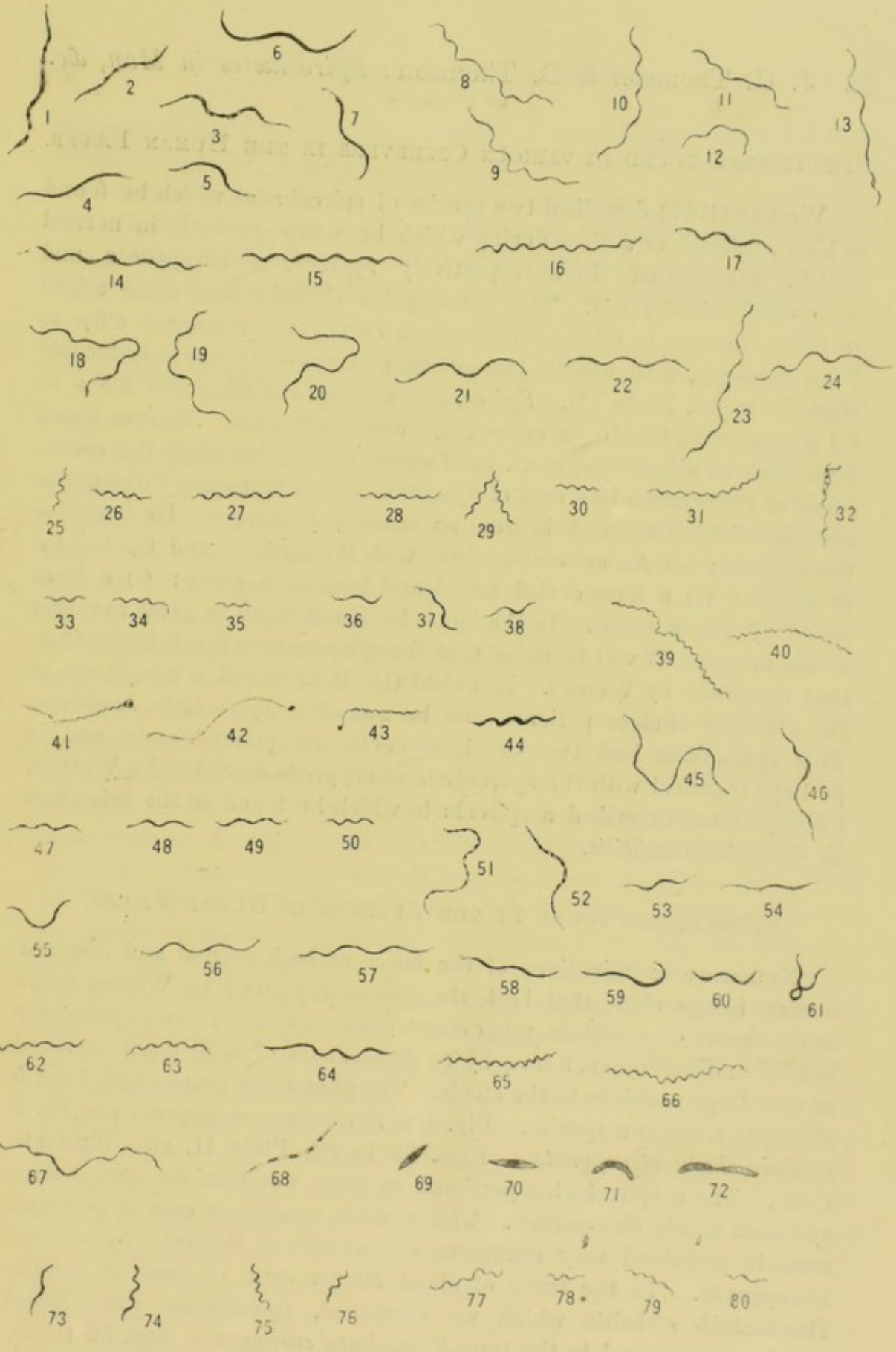
FIG. 68.—A cladothrix sometimes associated with *Spirochæta vincenti* in the mouth.

FIGS. 69 to 72.—Fusiform bacilli from the mouths of patients often associated with Vincent's angina, and also with severe pyorrhœa.

FIGS. 73 to 76.—Copies of the spirochætes as depicted by Werner. These are given to show the striking resemblance to those found by the authors in normal human fæces.

FIGS. 77 to 80.—Various spirochætes found in the fæces from a case affected with profound anæmia.

PLATE II.



J. G. Thomson del. Magnification = 1500 diameters

10 μ

Spirochætes found by various Observers in the Human Fæces.

Werner (1909) described two species of spirochætes which he found in his own fæces at a time during which he was apparently in normal health. He named these respectively *Spirochæta eurygyrata* and *Spirochæta stenogyrata*. This investigator thought that these might be fairly common in others. *Spirochæta eurygyrata* measured 4·6 μ to 7·3 μ in length, with usually two curves, and the body was very flexible (figs. 73 to 74, Plate II). *Spirochæta stenogyrata* measured 3·5 μ to 6·1 μ long, with two to six curves, and was less flexible. Werner found both these in a case of sprue. Le Dantec (1903) described the occurrence of spirochætes in a case of dysentery which he named "dysentérie spirillaire" to distinguish it from an amœbic dysentery. He describes three forms: (*a*) An undulating form with three curves and 6 μ to 14 μ in length; (*b*) a form curled round and forming a circle; (*c*) a form rolled up like a spiral. In the stools he found these in great numbers in the mucus. It will be noted that the spirochæte is much larger than that described by Werner. Kowalski (1894) described a spirochæte in the stools of cholera patients, and he named it *Spirochæta hachaizæ*. This spirochæte had two or three curls and pointed ends, and is perhaps identical with the *Spirochæta stenogyrata* described by Werner. Castellani has described a spirochæte which he found in the intestines as *Spirochæta subtilis*.

Spirochætes found by the Authors in Human Fæces.

Numerous examinations of the fæces of both normal and diseased human beings show that both the species described by Werner occur in the fæces of a certain proportion of individuals. In two apparently normal individuals examined by us spirochætes of both types occurred in very large numbers in the stools. The photomicrographs figs. 4 and 5 illustrate these two species. Fig. 4 is *Spirochæta eurygyrata* and fig. 5 is *Spirochæta stenogyrata*. Figs. 36 to 38, Plate II, also illustrate these. These spirochætes persisted in large numbers for several days and then finally disappeared. The fæces in an obscure case of profound anæmia contained very numerous spirochætes of the type *Spirochæta stenogyrata*. In the fæces of about 50 per cent. of cases of chronic rheumatoid arthritis which we examined, spirochætes were found which corresponded to the type *Spirochæta eurygyrata* (figs. 36 to 38, Plate II). These were never very numerous in the specimens examined.

In a case of amœbic dysentery we found a much larger spirochæte with irregular coils, which varied in length from 8 μ to 20 μ and which seems to correspond to a certain extent to the type described by Le Dantec (figs. 45 and 46). This is a large spirochæte with ends rather blunted, and large irregular curves showing great flexibility of the body. This certainly is quite a different species to those described by Werner.

A New Variety of Treponema found in an Old Specimen of Human Fæces.

A specimen of human fæces from an unknown source, after being kept in a bottle with some tap-water for several months, was found to contain active living specimens of two different kinds of spiral organisms. One form was a short, thick spirochæte with pointed extremities and two or three regular curves (fig. 1, Plate I, and fig. 44, Plate II). This spirochæte when compared with those described by Werner seems to be somewhat different, the coils tending to be deeper and slightly narrower as well as more regular.

The treponema was a very long and very fine spiral organism with extremely minute coils, so minute that we have failed to obtain a satisfactory photograph. This organism measured in length 10 μ, 15 μ and 20 μ or more, and there were twenty or more minute regular waves well seen in deeply stained specimens. These were drawn by the aid of the camera lucida, and several drawings are given (figs. 39 to 43, Plate II). It will be noted that in many cases the appearance of the organism in stained specimens was a straight line (fig. 42, Plate II), and it often happened that one end was attached to a round body. Under the dark-ground illuminator the motion of these was extremely rapid and was corkscrew-like in character. The minute coils were fixed. This seems to be quite a new variety of treponema which, so far, we have not seen described. It is one of the finest spiral organisms we have seen, and is certainly too thin to measure with accuracy. What the source of this protozoon was we are unfortunately unable to state. We have been unable to find them in stagnant water, and so far we have not again found them in other old specimens of human fæces kept in similar conditions. We regret we do not know why the fæces were kept or who the patient was. Specimens of fæces are being kept under similar conditions, but we shall be unable to state definitely whether these can again be found until several months have elapsed. It is curious that these spirochætes continued to live and multiply for weeks

in the original bottle, but we were quite unable to get them to live in any other medium. That this is a new species of treponema we feel quite sure, but at present it seems unwise to name it.

Spirochætes found by various Observers in the Fæces of Lower Animals.

Here a very wide field of research is open, and we must apologize for giving our attention to only a few animals commonly kept in the laboratory. We give so far as we are able a list of spirochætes found at different times in the intestines of animals.

Spirochætes in the gastric ulcers of a fox (Sambon).

Spirochætes in alimentary tract of cats, rats and dogs (Bizzozero and Salomon, 1896).

Spirilla in the intestines of pigs (Smith, 1894).

Spirochætes in the intestines of dogs and monkeys infected with trypanosomes (Balfour).

Spirochætes in the intestines of normal mice (Wenyon).

Spirochætes in the intestines of birds (Kent).

Spirochæta lovati, cæcum of a grouse (Fantham).

Spirochæta bufonis, intestine of a frog (Dobell).

Spirochæta of lamellibranchs—e.g., *Spirochæta balbianii*, *Spirochæta anodontæ*, *Spirochæta solenis* (Fantham), and *Spirochæta mytili* (Porter).

Spirochætes in the alimentary tract of insects—e.g., *Spirochæta glossinæ*, stomach of tsetse-flies (Novy and Knapp, 1906); *Spirochæta culicis*, in the gut and Malpighian tubules of *Culex pipiens*, and the larvæ of *Anopheles maculipennis* (Jaffé, 1907); *Spirochæta minei*, in the stomach of soldier and worker ants (Prowazek, 1910).

It is evident from this list that spirochætes occur in the intestines of most animals.

Spirochæte found by the Authors in the Fæces of Guinea-pigs.

While one of us was engaged in a research on *Trypanosoma rhodesiense* in the blood of guinea-pigs a blood-film stained by Giemsa's method was found to be filled with spirochætes. Several films were immediately prepared from the same animal and no spirochætes could be found. That the first film was simply a contamination was, there-

fore, perfectly obvious, and a search was accordingly made to find what was the source of these spirochætes. This accidental contamination of a blood-film with spirochætes shows the importance of being careful, as it might easily have been concluded that this was a new species of spirochæte infesting the blood of a guinea-pig. In this case the spirochæte was evidently from the mouth or intestines of a guinea-pig, as we have found them occurring in these animals.

Two varieties of spirochætes were found in the fæces of guinea-pigs. As no account of a spirochæte in the fæces of these animals has been given a full description of these seems necessary. The first variety is a small spirochæte with well-formed, regular, fixed coils and pointed at both ends. It measured from 3·75 μ to 7 μ as a maximum, but is usually about 5·5 μ in length and the waves are about three in number. When stained with gentian violet a series of little dark-staining granules may be seen suggesting a diffuse nucleus or the formation of granules (figs. 47 and 49, Plate II). These are not always seen, but in a great many slides they were beautifully demonstrated, as many as six dark-stained bodies being present. Under the dark-ground illuminator the motion of these is like a corkscrew, the rotation being from left to right. This is the commonest species of spirochæte found in the fæces of a guinea-pig. It occurred in a large percentage of normal guinea-pigs, usually in small numbers, but in the cæcum of several guinea-pigs which died of tuberculosis and trypanosomiasis it was found in very large numbers, so that it seemed that in conditions in which the health of the animal was affected and the vitality lowered this spirochæte was able to flourish in greater abundance.

The other variety of spirochæte found in the fæces of a guinea-pig was only found on one occasion in an animal which suffered from diarrhœa. This was a larger spirochæte, measuring from 10 μ to 14 μ in length, with large, irregular coils usually about two in number. The ends tapered to a point. In this case the coils were not fixed, and motion was more snake-like, but the movement also was spiral in character. Here again when successfully stained with gentian violet a series of dark-stained areas were distinctly seen lying close to one another, as many as twenty of these being counted in one spirochæte (figs. 51 to 52, Plate II).

Spirochætes in the Fæces of Wild Rats.

Salomon found spirilla in the intestines of dogs, cats and rats, and described three different varieties. The form found by us does not seem to correspond to any of those described by that observer, but of course several other varieties must exist in all probability in rats. The form found by us in the fæces of several wild rats caught in the Liverpool docks varied from 7 μ to 10 μ in length. The body was thin and tapered to a point at each end. The waves were flattened and irregular, and not fixed. In this case we did not see any differentiation in the staining of the body and no dark granules were seen (figs. 56 and 57, Plate II). In examining the fæces of animals it is necessary to be careful not to mistake flagella of *Trichomonas intestinalis* for spirochætes.

Spirochætes in the Fæces of a Dog.

In the fæces of a terrier infected with *Piroplasma canis* a short, thick spirochæte with tapering ends was found. They had only one or two curls, flat in character, and measured in length from 4 μ to 9 μ. They were actively motile with flexible bodies and exhibited spiral movements (figs. 53 to 55, Plate II). The organisms described by Bizzozero in the dog were 3 μ to 8 μ long and had three to seven waves.

Spirochætes in the Fæces of a Hen (Plymouth Rock).

This is a thick spirochæte with tapering ends and flat curves, usually about two in number. They vary in length from 5 μ to 10 μ. The curves are wide and flat and vary from two to four in number. Under the dark-ground illuminator these are seen to move actively with a spiral motion. Kent described spirochætes in the intestines of birds, and it is probable that they are common in the alimentary tract of most fowls (figs. 58 to 61, Plate II). It will be noted that in these the body is flexible and that the spirochæte may become twisted on itself. The photomicrograph (fig. 9, Plate I) illustrates this type of spirochæte very well.

The Cultivation of Spirochætes of the Mouth.

After the splendid and laborious work of Noguchi (1912) in the cultivation of spirochætes we feel inclined to apologize for having obtained very indifferent results in our experiments. In no case were we able to grow a pure culture, and the types of spirochætes which seemed to grow most easily by the methods we adopted were the *Treponema microdentium* and probably *Treponema mucosum*.

Method.

About 1 in. of pure sterile human ascitic fluid is put into a small test-tube. This is inoculated with a loopful of material from a case of pyorrhœa alveolaris. The tube is closed with a sterile cotton-wool plug and the top rendered air-tight by means of a thin coating of ordinary sealing wax. This is incubated at 37° C., and in about seven to ten days it will be noticed that there is a white deposit in the bottom of the test-tube. In this culture it is found that the spirochætes have multiplied to a great extent in the fluid and that they are growing in symbiosis with numerous other organisms. Examination under the dark-ground illuminator shows, if the culture is successful, numerous active small treponemata. Noguchi has cultivated these in fluid media and afterwards isolated them in pure cultures in solid media by using anaerobic precautions. No strict anaerobiosis was used by us, but since the tube is sealed, and various cocci and bacteria are growing in the tube, it is to be assumed that in a very short time all the available oxygen is used up and so the treponemata find favourable anaerobic conditions. We give a photomicrograph taken from a subculture and this shows the clumps of small treponemata. We have managed many times by this method to obtain impure cultures of treponema, and we have thought it probable that by this method a very good mixed vaccine might be obtained which might be useful in cases of pyorrhœa alveolaris. The bacteriology of pyorrhœa is very imperfectly known and hence the real cause is unknown. This failure in the bacteriology of pyorrhœa seems to be based on the fact that the cultural methods are insufficient for the isolation and growth of all the numerous varieties of organisms in the alveolar margins of the mouth. Noguchi has isolated in pure cultures from the human mouth *Treponema macrodentium*, *Treponema microdentium* and *Treponema mucosum*, and Ellermann (1904), Weaver and Tunnicliffe (1905), Mühlens (1906), have

obtained the spirochætes of Vincent's angina and the fusiform bacilli in pure cultures; but so far the real pathological cause of pyorrhœa alveolaris is unknown, and it is still considered by many that it is really a result of the condition known as the uric acid diathesis. By the cultural methods so well begun by Noguchi it will be possible in the future to study the toxins and their effects, and so the real explanation of many obscure pathological conditions may be explained.

Some Remarks on the Pathological Significance of Spirochætæ and Treponemata.

Spirochætes, according to Fantham, give a good example of the gradual adaptation to parasitism. In the early stages of their existence these organisms lived in water. From there they gradually found their way into the alimentary tract of the lower and higher animals, and finally certain varieties became so altered as to be capable of living in the tissues and blood-stream of the higher animals. Whether these parasites are of pathological importance when inhabiting the intestines and oral cavities of human beings apparently enjoying good health is doubtful, but when ulceration or abscesses form in these sites, as seen in pyorrhœa alveolaris, cancrum oris, abscesses of the jaw and Vincent's angina, they undoubtedly seem to seize the opportunity for proliferation and increase to such an enormous extent in numbers that they must produce toxins detrimental to the health of the host.

Do these spirochætes of the mouth and intestine remain in the superficial sites made suitable for them or do they finally find their way into the deeper tissues and ultimately into the blood-stream? That the *Treponema pallidum* is capable of proceeding from a superficial abrasion of the skin to the deeper tissues, and finally to every part of the human body, is well known. With their extremely thin bodies, sharp-pointed extremities, rigid, corkscrew-shaped coils and active spiral movements these are peculiarly adapted for making rapid progress in fairly dense media. It is by applying this knowledge of the active movement of these protozoa through a solid medium that Noguchi (1912) has been able to isolate *Treponema macrodentium*, *Treponema microdentium* and *Treponema mucosum* in pure cultures.

There is some cause for anxiety, therefore, when it is considered that perhaps a very narrow line of defence keeps the apparently harmless treponema of the human mouth and intestines from proceeding into the general system. Moritz found spirochætes from 2 μ to 6 μ in length

and with from three to ten coils in the wall of the gut and also in the bone-marrow in a case of severe anæmia. Thomas and Rolleston (1910) describe a case of fatal aplastic anæmia which followed an infection of the gums with the spirilla and fusiform bacilli of Vincent's angina. Proescher and White (1907) found spirochætes in two cases of Hodgkin's disease where they occurred in large numbers in the affected glands. In these cases we are unable to exclude syphilitic infection, but at the same time it must be admitted that in some cases it may be an entirely different species of spirochæte even although it is extremely similar to *Treponema pallidum* in morphology. As a striking example of the marked similarity of the two different species of treponemata as viewed under the microscope we give two drawings (figs. 65 and 66, Plate II) to illustrate the resemblance of *Treponema microdentium* in a culture to *Treponema pallidum* as it occurred in a case of congenital specific liver.

Baermann found *Spirochæta refringens* in the enlarged glands of a monkey infected with syphilis, and this demonstrates the possibility of this organism becoming a blood spirochæte. Scherber states that *Spirochæta balanitidis* can make its way into the blood-stream. If medical men in this country would begin a systematic examination of the mouths of their patients, we have not the slightest doubt that, like ourselves, they would be astonished to find millions of spirochætes in small scrapings from the alveolar margin. These in many cases are too numerous, even if they are considered only saprophytes, to be associated with good health. They must be elaborating toxins of a peculiar character and they must also assist in the general destruction of the tissues in the alveolar margin. Pathologists never seriously consider that in pyorrhœa it may be the spirochætes which are the chief cause of this widespread disease. Noguchi (1912) has recently shown that cultures of *Treponema microdentium* and *Treponema mucosum* cause inflammation and induration when injected into the tissues of animals, and if organisms now gained access to this devitalized tissue an extremely chronic condition such as pyorrhœa is set up in which millions of spirochætes live and flourish in symbiosis with numerous cocci and bacilli. The vaccine treatment of pyorrhœa alveolaris is in most cases unsatisfactory and we venture to suggest that a mixed vaccine of bacteria and spirochætes might yield better results.

The spirochæte associated with Vincent's angina seems now almost definitely to be of pathological importance.

The Pathological Significance of Spirochætes in the Human Fæces.

It has been shown in this paper that spirochætes occur in the fæces of apparently normal human beings in this country, and this is of great interest as they have been noted in large numbers in the fæces of a case of dysentery by Le Dantec, and in the stools of cholera patients by Kowalski. We found spirochætes not only in a certain number of healthy human beings but also in 50 per cent. of cases affected with chronic rheumatoid arthritis. We also found them in large numbers in the fæces of a case of severe anæmia. Again, it is impossible to state that these are of pathogenic importance, but we have noted that undoubtedly they increase in numbers in certain conditions where the vitality of an animal has been lowered by some intercurrent disease such as trypanosomiasis, severe tuberculosis, or diarrhœa. Balfour noted them in the intestines of animals infected with trypanosomiasis in the Sudan, and Sambon found them in the gastric ulcers of a fox. It must be kept in mind that they occur in apparently normal human beings in more or less perfect health, but again we do not think these ought to be considered as of no pathological importance. It is known that cancer of the mouth is often closely associated with syphilis, and the possibility of spirochætes in the intestine aiding in any way the beginning of that common disease, the carcinoma of the rectum, ought not to be lost sight of. It is interesting to note that in two strong and healthy men, who were feeling just a little out of sorts, very numerous spirochætes of both the varieties described by Werner were found. These were so numerous as to cause the patients to feel a little alarmed, but after the administration of calomel they disappeared in a few days. Metchnikoff suggested years ago that life might be prolonged by proper disinfection of the alimentary tract and proper regulation of the action of the bowels, but little seems to be known as to the exact organism which is most harmful in the production of toxæmia. The work of Sir Arbuthnot Lane in removing by operation portions of the gut and the wonderful effect produced by this procedure in certain joint troubles shows the great importance of a thorough pathological research in this matter.

For years medical men have sought the cause of rheumatoid arthritis, pernicious anæmia, lymphadenoma, and other diseases associated with bad teeth and intestinal trouble without much success, and we venture to suggest that more attention be devoted to the study of the common spirochæte.

SUMMARY.

(1) Several types of spirochæte are associated with *Pyorrhœa alveolaris*, but those which predominate seem to belong to the types *Spirochæta buccalis* and *Spirochæta vincenti*. Vincent's angina is always associated with fusiform bacilli, and severe pyorrhœa may also be associated with these bacilli.

(2) *Spirochæta buccalis* in many respects resembles *Spirochæta refringens* and *Spirochæta balanitidis* in morphological characters. *Spirochæta buccalis* and *Spirochæta vincenti* have been so confused and so imperfectly described that it is quite impossible to determine, from the descriptions of various observers, whether we are dealing with two different species or the same species. From our own observations, however, we recognize two types, one of which we have described as *Spirochæta buccalis* and the other as *Spirochæta vincenti*. Both these may be found associated with Vincent's angina, and also with pyorrhœa.

(3) The type of spirochæte in the mouth described by us as *Spirochæta buccalis* was seen in some specimens to have a series of dark-stained areas suggesting the formation of granules or coccoid bodies. The fixation was by heat and the stain used was gentian violet, so we cannot exclude possible shrinkage of the protoplasm by this method giving rise to such an appearance. We also used wet fixation.

(4) The type of spirochæte described by us as *Spirochæta vincenti* is common in pyorrhœa alveolaris, Vincent's angina, and other suppurative lesions of the mouth. It is a thinner spirochæte than *Spirochæta buccalis;* it is not ribbon-shaped when viewed under dark-ground illumination, and the body is extremely flexible, the coils not being fixed; they show in stained specimens long thin spirochætes with irregular waves (fig. 2, Plate I). This, in our opinion, is quite a distinct type from *Spirochæta buccalis* as far as we can make out from morphology alone.

(5) We are of the opinion that in the conditions known as Vincent's angina, in which spirochætes are found associated with fusiform bacilli, various types of spirochætes occur, and it is this which has caused so much confusion in the descriptions given by various observers of *Spirochæta vincenti*.

(6) The two species of spirochæte found by Werner in the fæces

of an apparently normal human being have been found by us in the fæces of healthy men in this country. In two cases these occurred in very large numbers.

(7) Spirochætes corresponding to the species *Spirochæta eurygyrata* were found in the fæces of 50 per cent. of chronic rheumatoid arthritis cases examined, but always in small numbers, and were sometimes difficult to find.

(8) Spirochætes in very large numbers corresponding to the species *Spirochæta stenogyrata* were found by us in the stools of an obscure case of profound anæmia.

(9) A treponema, probably a new species, has been found in a specimen of human fæces kept in a bottle with some water for several months. This is a much finer organism than we have seen previously described. It may measure 20 μ in length or more, and has fifteen to twenty or more minute fixed coils. Another spirochæte was found in the same specimen which was short and thick.

(10) An undescribed spirochæte has been found in the fæces of healthy and diseased guinea-pigs. This is a short organism with pointed ends, measuring in length from 3·75 μ to about 7 μ as a maximum. The waves are about three in number. When stained with gentian violet a series of dark areas are seen on the body. Another species was also found on one occasion in the fæces of a guinea-pig with diarrhœa (*see* figs. 47 to 52, Plate II).

(11) Spirochætes found by the authors in rats, dogs and fowls have also been described and figured.

(12) It is strongly suggested that more attention be paid by pathologists to the common spirochætes of the mouth, and also the intestines, which are usually considered harmless saprophytes, for there is a considerable amount of evidence that possibly these harmless parasites may proceed under certain conditions to the deeper tissues and even into the blood-stream, and so cause profound and obscure illnesses.

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The Royal Society of Medicine.

Marcus Beck Laboratory Reports.—No. 4.

Controlled Growth *en masse* (Somatic Growth) of Embryonic Chick Tissue *in vitro*.

By David Thomson, M.B., Ch.B.Edin., D.P.H.Cantab.[1]

Introduction.

In a previous paper (Thomson, 1914)[2] it was shown that small portions of live tissue (chick and human) when placed in a medium composed of blood plasma of a fowl one part, plus extract of chick embryo one part, and incubated at 37·5° C., produced a new growth, which radiated out from original tissue on all sides in an uncontrolled fashion. A portion of heart, liver, or spleen, &c., did not grow into any definite structure or shape like a heart, liver, or spleen. The cells simply multiplied without any tendency to produce a definite grouping, or in other words, there was no somatic control. In the same paper, however, I showed that when definite uninjured masses of a chick embryo were placed in the medium, then these masses increased in size while retaining their original shape; in other words, they showed a controlled increase in size or growth. A total chick embryo twenty-four hours old increased in size as a whole without producing any radiating uncontrolled growth of cells around it. The tail of a young chick embryo and also a toe showed the same increase under control. In the two latter cases

[1] Grocers' Research Scholar. From the Marcus Beck Laboratory, Royal Society of Medicine, London.

[2] *Proc. Roy. Soc. Med.*, 1914, vii (Marcus Beck Lab. Rep. No. 2), p. 34.

uncontrolled growth occurred only at the cut or injured margin of the tail and toe.

It was suggested to me that the increase in size obtained might only be apparent, due to the flattening out of the mass of tissue, but I have since satisfied myself that this is not the case, but that the increase in size is due to a true and definite growth.

(I) Growth under Control (Somatic Growth) in vitro of an Embryonic Chick Toe.

This phenomenon is illustrated by photomicrographs in the accompanying plate. The toe was cut from a nine-day-old chick embryo, placed in the culture medium and incubated at 37·5° C. Fig. 1 shows the size of the toe when newly placed in the medium (40 diameters). The remaining photographs show exactly the same magnification; fig. 2 shows an increase in size after twenty hours' incubation, and fig. 3 shows a further increase after forty-eight hours' incubation. Note also that there is an uncontrolled growth of cells from the cut or injured end. On the third day the uncontrolled growth was cut away and the toe washed for thirty seconds in Ringer's solution and then transferred to fresh medium. On the fifth day this was repeated, but after this no further increase in size was obtained. Fig. 4 represents the size of the toe on the fifth day of incubation. I am quite satisfied that this increase in size is a real growth under control and that it is not due to any flattening out of the original tissue. The culture preparation is of the nature of a hanging drop, the medium being of a solid gelatinous consistence. It is inconceivable that in these circumstances the toe could flatten out to attain an increase in size as depicted. Figs. 5 and 6 show the increase in size of the tip of the tongue cut from a fifteen-day-old chick embryo, after seven days' incubation in the same medium. Again note the uncontrolled growth from the cut margin. It would appear that all circumscribed masses of tissue will increase in size *in vitro* in a similar manner. A young embryonic feather of a twelve-day-old chick embryo increased in size *in vitro* by more than 50 per cent. in four days, and the lens of the eye of a five-day-old chick embryo showed a similar increase in area *in vitro* in four days. In both these cases the definite shape was retained and no uncontrolled growth occurred from the definite margins of these structures.

(II) Conclusions.

It would appear to me that uncontrolled growth can only take place from injured surfaces where there is no basement membrane. The uninjured intact surfaces form a definite membrane, from which a new uncontrolled growth cannot radiate. It is a characteristic of malignant tumours that they do not possess a basement membrane, and it is probable that this is the deciding cause of their malignancy, since the growth is not limited in a definite confined structure, but is able to radiate in any direction in an uncontrolled fashion. Carrel has shown that this form of uncontrolled growth in chick embryo tissue can be continued indefinitely (for over two years) provided the tissue be cut into smaller portions and planted in fresh medium. I have no doubt that the toe in my experiments ceased to grow further on obtaining a certain size due to the difficulty of obtaining nutriment from the surrounding medium. When it became too large, the nutriment was unable to penetrate by osmosis into the central portion, since there was no circulation. A further increase *in vitro* would probably be difficult, unless by some means an artificial circulation could be set up so as to enable the nutrient medium to get at the central portions of the tissue.

EXPLANATION OF PLATE.

Photomicrographs 1 to 6 are all of identical magnification, × 40 diameters. All were taken from the live growing tissue.

FIG. 1.—Toe of a nine-day-old chick embryo newly placed in a culture medium composed of blood plasma of a hen one part plus extract of chick embryo one part.

FIG. 2.—The same after twenty hours' incubation at 37·5° C.

FIG. 3.—The same after forty-eight hours' incubation.

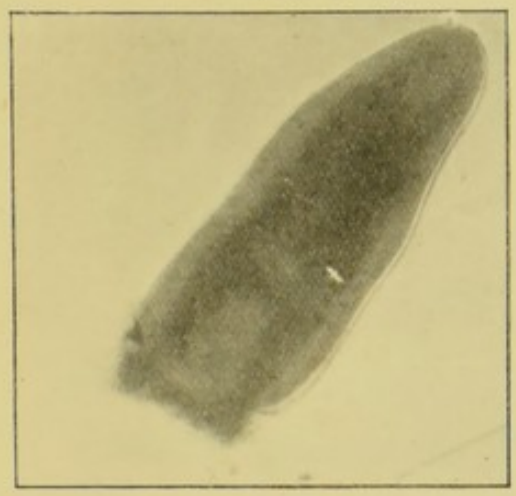
FIG. 4.—The same after five days' incubation (third passage into fresh medium).

FIG. 5.—Tip of tongue of fifteen-day-old chick embryo, newly placed in the culture medium.

FIG. 6.—The same after seven days' incubation at 37·5° C.

Plate.

1



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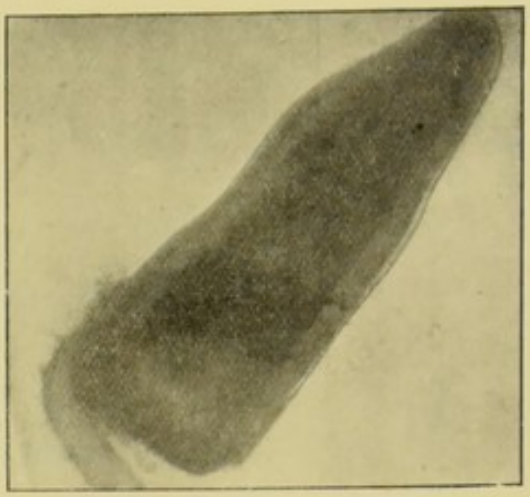

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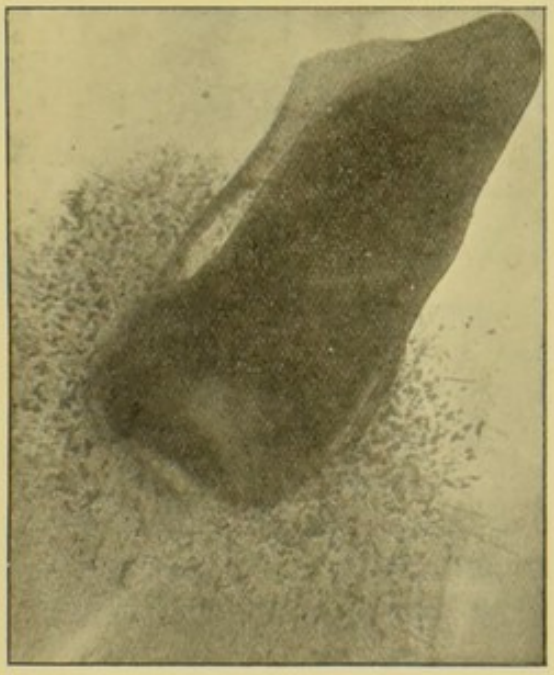

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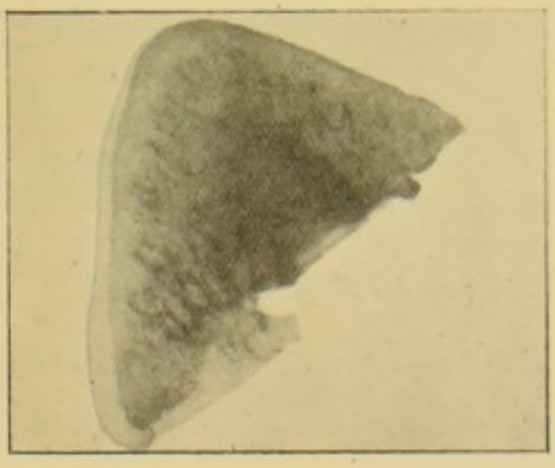

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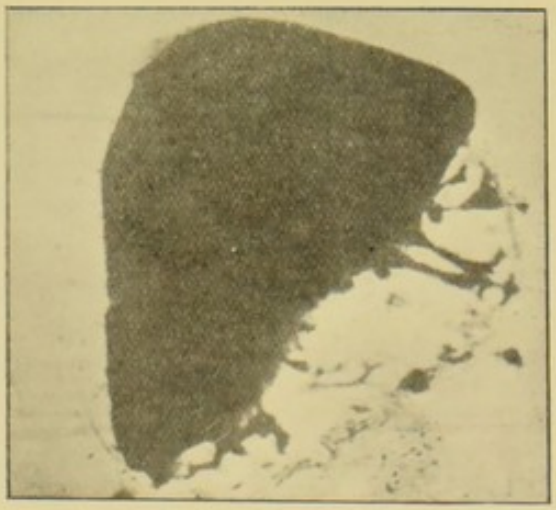


(D. Thomson, photo).

The Royal Society of Medicine.

Marcus Beck Laboratory Reports.—No. 5.

Some Observations on the Development of Red Blood Cells as seen during the Growth of Embryonic Chick Tissue *in vitro*.

By David Thomson, M.B., Ch.B.Edin., D.P.H.Cantab.[1]

(I) Introduction.

A considerable amount of research has been done on the development of red cells in many kinds of animals by several observers, *vide* Keibel and Mall (1912) and Schäfer (1910). The entire process has, however, not yet been fully elucidated, and there is a considerable amount of controversy on the subject. It would appear to me that observations on the growth *in vitro* of blood-forming tissues must be a very important method of investigating this subject, since the process can be watched continuously under the microscope *in vivo*.

(II) Technique.

A simplification of Carrel's technique for the cultivation of tissues *in vitro* has already been fully described (Thomson, 1914). It is unnecessary, therefore, to describe it again in detail. Small portions of blood-forming tissues of the embryonic chick (spleen and bone-marrow) were grown in a medium composed of the blood plasma of a hen one part, plus extract of embryonic chick one part. The tissue was placed in a drop of this medium on a sterile cover-slip, which was then inverted over a hollowed-out thick glass slide and hermetically sealed

[1] Grocers' Research Scholar. From the Marcus Beck Laboratory, Royal Society of Medicine, London.

EXPLANATION OF PLATE I.

PEN AND INK DRAWINGS FROM LIVE CULTURES.

Magnification about 800 diameters.

FIG. 1A.—Amœboid cell containing highly refractile granules and two pink vacuoles (*a*). Drawn from a live culture of spleen tissue *in vitro*, after forty-eight hours' incubation at 37·5° C. (Spleen tissue obtained from a twelve-days-old chick embryo.)

FIG. 2A.—The same cell two hours later, showing three larger pink vacuoles.

FIG. 3A.—The same, one hour later than 2A, showing the discharge of three red cells.

FIGS. 1B to 3B, drawn from the same culture preparation, show the production of six red cells from one amœboid cell. Only two hours elapsed between 1B and 3B.

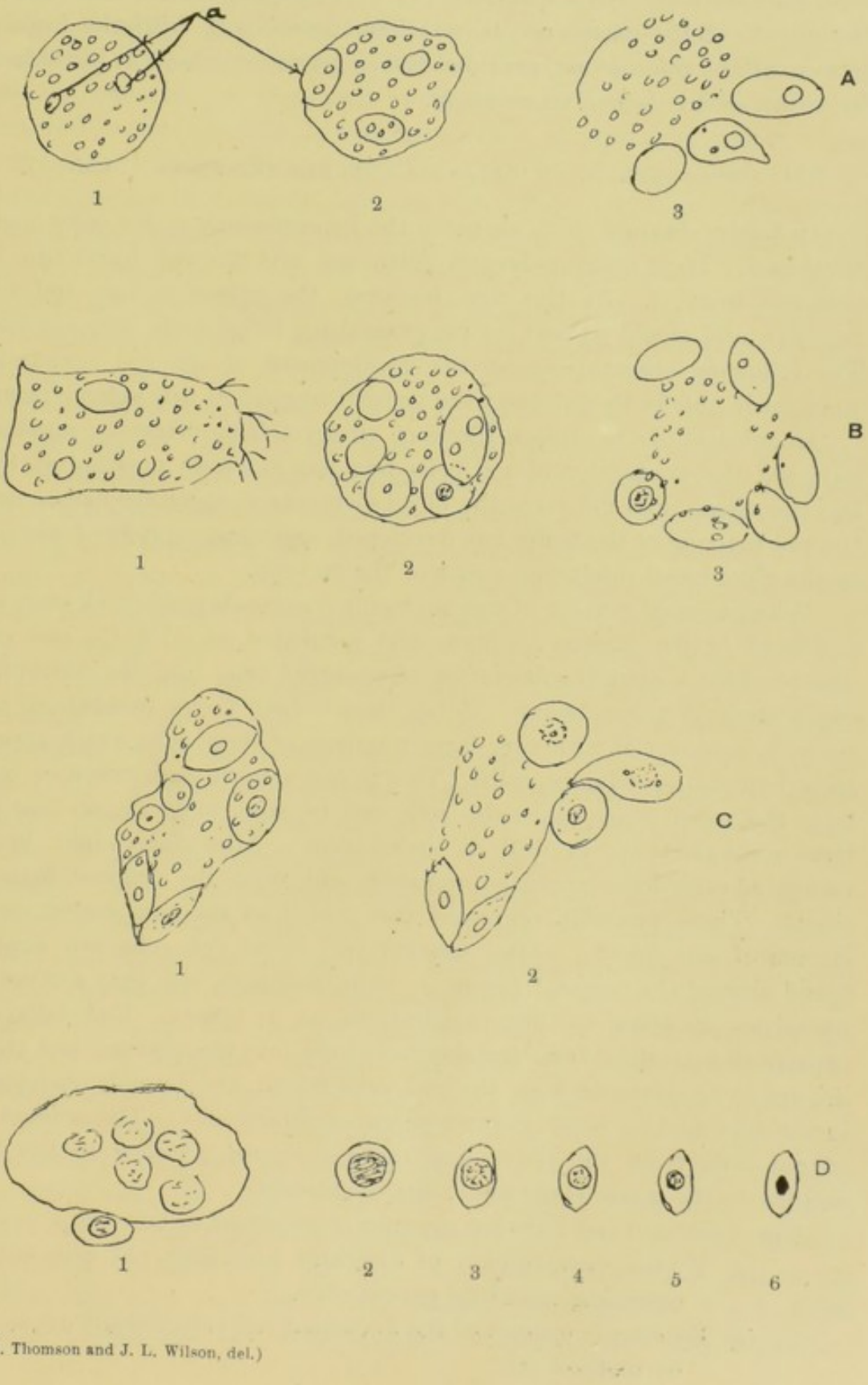
FIGS. 1C and 2C show the same process. The time which elapsed between 1C and 2C was only fifteen minutes.

FIGS. 1D to 6D were drawn from a Giemsa-stained smear of the bone-marrow of a sixteen-day-old chick embryo.

FIG. 1D shows the nucleus split into five, preparatory to the formation of nucleated red cells.

FIGS. 2D to 6D show the young nucleated red cells in a gradual transition from round to oval, and show how the nucleus gradually becomes smaller in size.

PLATE I.



(D. Thomson and J. L. Wilson, del.)

with paraffin wax. In a transparent culture preparation of this nature, the growth of the tissues can be watched continuously with the highest powers of the microscope, provided the preparation is kept at blood-heat by means of an electric warm stage.

(III) Observations on Spleen Cultures (Embryonic Chick).

In twelve-day-old chick embryos the bone-marrow is not completely developed. It is quite pale and colourless and no red blood can be obtained from it. At this age, however, the spleen is very red and vascular, and would appear to be generating blood cells very actively. The following observations on the development of the red corpuscles were made chiefly by watching the growth *in vitro* of this splenic tissue.

In cultures of the spleen tissue of embryos from fifteen to nineteen days old the production of red cells was less marked; possibly at this age the spleen does not form the blood elements so vigorously since now the red marrow of the bones has developed, and most probably functions as the chief blood-producing tissue of the embryo.

When a small portion of splenic tissue (twelve-day-old chick embryo) is placed in the plasma medium and incubated at 37·5° C., one can observe quite a definite emigration of amœboid cells into the surrounding plasma in three hours. After twenty-four hours incubation the tissue is surrounded with very large numbers of these cells which appear to be leucocyte-producing cells. This is not a mere emigration of cells from the tissue mass, since the cells can be seen to divide, so that we have multiplication as well as emigration. After forty-eight hours elongated cells have appeared radiating out from the original mass of tissue. These probably represent the growth of the connective tissue stroma of the spleen. After seventy-two hours red cells are usually found around the original tissue in large numbers, but they are not so numerous in some culture preparations as in others. Red cells are capable of migrating from the original tissue into the plasma, and these are apt to be confused with the red cells which are actually developed in the surrounding medium from parent amœboid cells. The production of red cells from the latter has, however, been watched in the live culture on the warm stage under the microscope.

The nucleated red cells are apparently produced in two ways:—

(*a*) By the development of daughter nucleated red cells within colourless amœboid parent cells.

(*b*) By simple mitosis of the nucleated red cells already developed by method (*a*).

(*a*) The colourless parent cells destined to produce red cells show active amœboid movement and contain numerous highly refractile granules. In the live condition the nucleus is scarcely visible. The first evidence of the formation of daughter red cells within them (as observed in live cultures) is the appearance of pinkish vacuoles in the protoplasm. These vacuoles increase in size and finally the faint outline of red cells can be discerned forming within the protoplasm of the parent cell. These become more distinct and more pink in colour and eventually they are discharged as definite red cells, or it might be more accurate to say that the colourless amœboid cell eventually breaks up into a number of nucleated red cells. Some of these are apparently capable of producing as many as six of the latter. I have attempted to illustrate this phenomenon as it appeared to occur in the live culture preparations by the pen-and-ink sketches, Plate I. Fig. 1A shows a parent amœboid cell containing highly refractile granules and two small pink vacuoles (*a*). Fig. 2 is the same cell two hours later, showing three large pink vacuoles, and fig. 3 is the same cell one hour later than fig. 2, showing the discharge of three red cells. Figs. 1, 2, and 3B show a similar evolution of six red cells, the time elapsing between 1 and 3 being two hours. Fig. 1C shows the red cells forming within a large parent cell and fig. 2C represents the same cell fifteen minutes later, showing the discharge of three red cells. The red cells developing within the parent cell undergo a gradual change in colour and shape. At first they are round with a large homogeneous nucleus and clear, colourless protoplasm. They gradually become more oval and simultaneously they develop a pinkish colour, while the nucleus becomes smaller and granular. This gradual transition in shape and in the size of the nucleus is depicted in figs. 2 to 6D. In the live culture specimens it is very difficult to see the nuclei of the parent amœboid cells. From stained specimens of these cultures, however, it would appear that the nucleus splits up into several portions, each of which becomes the nucleus of a daughter red cell. In Plate II I have attempted to illustrate by photomicrographs what I have already depicted in the pen-and-ink drawings. Photomicrograph 1, taken from the live growth of embryonic chick spleen *in vitro*, shows a few parent amœboid cells and also some red cells which have been discharged from them. No. 2 shows exactly the same area twenty-one hours later, and the marked increase in the number of red cells is quite evident. The remaining photomicrographs were taken from stained preparations. No. 3 shows a large parent cell (P) with daughter nucleated red cells within it.

EXPLANATION OF PLATE II (PHOTOMICROGRAPHS).

FIG. 1.—Photomicrograph of red-cell-producing tissue growing *in vitro*. (Spleen tissue of twelve days' embryonic chick.) Magnification about 420 diameters.

FIG. 2.—The same twenty-one hours later, showing a marked increase in the number of red cells. N.B.—1 and 2 are photomicrographs of the live tissue. The remaining photographs are taken from stained preparations.

FIG. 3 shows nucleated red cells forming within a large cell (P) from spleen of a twelve days' chick embryo after forty-nine hours' growth *in vitro*. (× 420.)

FIG. 4 shows a mass of red-cell-producing tissue. The cells are united by protoplasmic processes and contain from one to six nuclei. From spleen tissue (of twelve days' chick embryo) growing *in vitro*.

FIG. 5 shows two nucleated red cells forming inside a large round cell (P). (From growth of spleen tissue *in vitro*.) (× 250.)

FIG. 6 shows the formation of four nucleated red cells, also a pair, one of which is already oval but not yet separated. (× 420.)

FIGS. 7 and 8 show the formation of three and four nucleated red cells respectively. (× 420.)

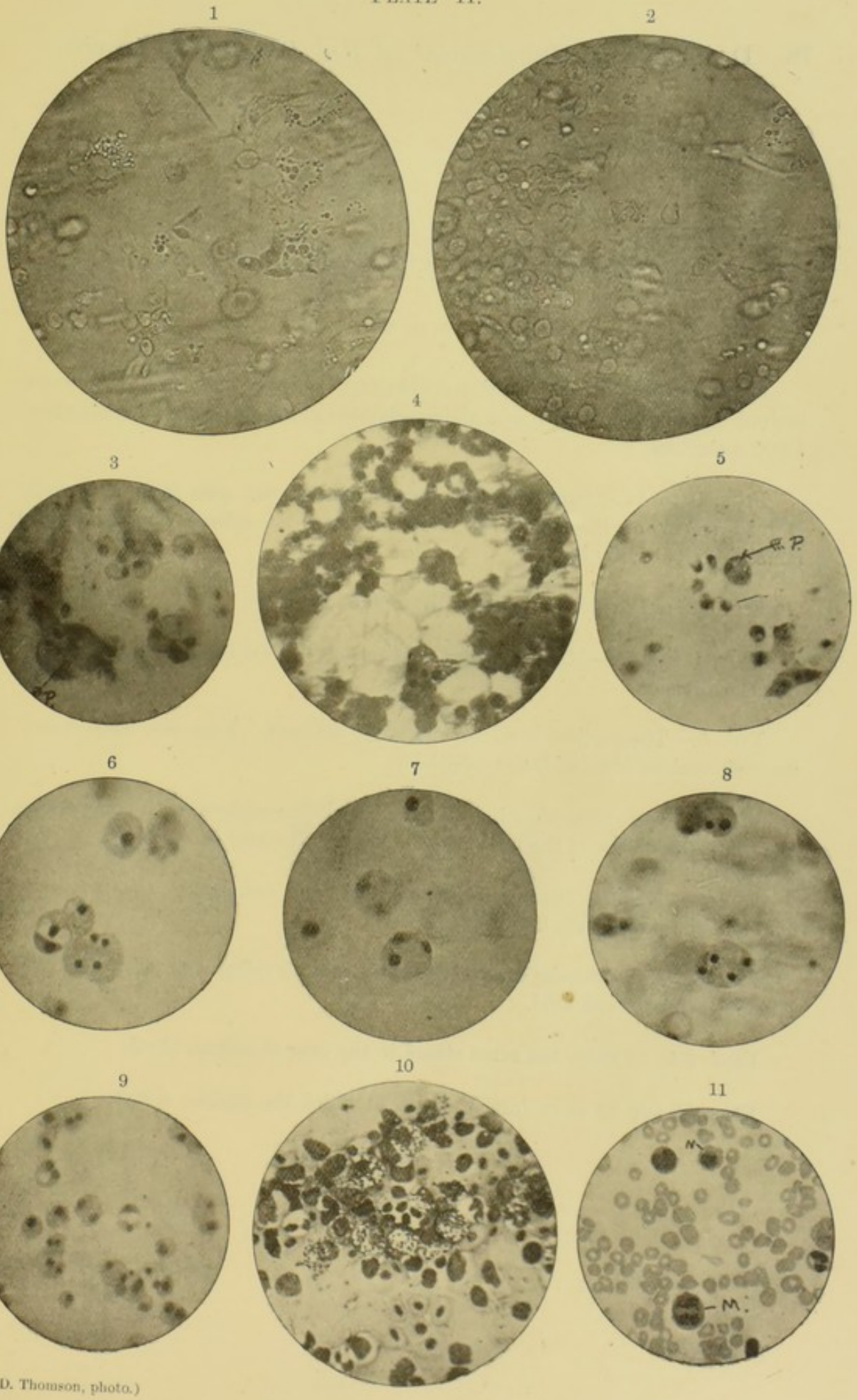
FIG. 9.—Ditto. Shows several pairs. This is possibly simple mitosis of nucleated red cells. (× 250.)

FIGS. 7 to 9 were taken from the growth *in vitro* of tissues (undetermined) from a three-day-old chick embryo.

FIG. 10.—Spleen smear of a sixteen-day-old chick embryo. Note multinucleated mass in centre. (× 420.)

FIG. 11.—Smear from spleen puncture, from a human case of splenic anæmia. Note nucleated red cell (N), also dividing red cell (M). (× 420.)

PLATE II.



(D. Thomson, photo.)

EXPLANATION OF COLOURED PLATE III.

FIGS. 1 to 8.—Stages in the development of red cells, from a Giemsa-stained smear of the spleen of a sixteen-day-old chick embryo. Magnification, 1,000 diameters.

FIGS. 9 to 14.—Stages in the development of red cells from a Giemsa-stained smear of the spleen of a human case of splenic anæmia (spleen puncture). (× 1,000.)

FIGS. 1 and 2.—Cells destined to produce red cells.

FIGS. 3 and 4.—Mitotic figures preparatory to the formation of red cells (nuclear division).

FIG. 5.—The earliest form of nucleated red cell. Note the large homogeneous nucleus and the blue protoplasm.

FIG. 6.—Next stage showing a smaller clock-like nucleus, with commencing change in the colour of the protoplasm. (Cell still round in shape.)

FIG. 7.—Nucleus becoming smaller and more compact. Cell becoming oval and the protoplasm becoming red.

FIG. 8.—Fully developed nucleated red cell. Protoplasm red. Cell oval. Nucleus compact and oval.

FIGS. 9 to 12 show the same stages in the case of human blood.

FIGS. 13 and 14 show further stages in which the nucleus is lost.

PLATE III.

1 2 3 4

5 6 7 8

9 10 11 12

13 14

(D. Thomson, pinx.)

It was impossible, unfortunately, to get the whole of this cell completely in focus. No. 5 shows a similar parent cell (lower magnification) with two daughter red cells within it. No. 4 shows a mass of parent amœboid cells, each containing from one to six nuclei. The cells are connected with each other by protoplasmic processes. This photograph resembles markedly fig. 30, p. 30, in Schäfer's "Essentials of Histology" (1910), in which is shown the development of blood corpuscles in the vascular area of the guinea-pig. Photomicrographs 6 to 9 (growth *in vitro* of blood-forming tissues of an embryonic chick three days old) show the formation of nucleated red cells in pairs, threes and fours; some of these form at the margin in the shape of a nucleated crescent. No. 10 is taken from a Giemsa-stained smear of the spleen of a sixteen-day-old chick embryo, and shows in the centre a cell mass containing six nuclei. This I believe is a parent colourless cell about to form six daughter nucleated red cells. No. 11, from a Giemsa-stained smear (spleen puncture) from a case of human splenic anæmia, shows a nucleated red cell (N), and also one in the process of mitosis (M). It is interesting to note that the human nucleated red cell is about exactly the same size as the nucleated red cells of the chick as shown in photomicrograph 10 taken at an identical magnification, and that the non-nucleated human red cells are much smaller than the nucleated forms.

(*b*) *The Multiplication of Nucleated Red Cells by Simple Division.*—It is well known that nucleated red cells can multiply by simple mitosis. This process has been fully observed in the blood of newts and a cinematographic film of the phenomenon has even been taken by Dr. Comandon (Pathé Frères). I am in doubt as to whether the photomicrographs 6 to 9, Plate II, represent simply the division of already existing nucleated red cells of the chick or whether they show the production of daughter red cells from the original amœboid parent cells. If they represent the former then the photographs would appear to indicate that a nucleated red cell is capable of dividing into two, three and four red cells.

(IV) Observations on Bone-marrow Cultures (Embryonic Chick).

In these the development of red cells was similar to that already described under the spleen cultures. Foot (1913) made observations on the cultivation *in vitro* of bone-marrow of chicks, more especially with regard to the development of the leucocytes. He does not appear to

have observed the development of red cells, and he even states that the red cells in the tissue die off in the culture preparation. I am unable to agree with his observations on this point.

(V) Further Observations.

I should have stated that the young daughter red cells produced from the parent amœboid cells vary in size. Some are at least twice as large as others. As a rule the daughter red cells each contain a vacuole in the early stage. This vacuole is situated on one side of the nucleus, and in unstained specimens appears like a highly refractile granule (*vide* photomicrograph 1, Plate II). In the coloured plate, figs. 1 to 8 are painted from a Giemsa-stained smear of the spleen of a sixteen-day-old chick embryo. Figs. 1 to 4 show what I believe to be the parent amœboid cells, and in the latter two the nucleus is undergoing division. Fig. 5 is a young daughter red cell, and figs. 6, 7, and 8 show the gradual transition to the fully developed red cell. Figs. 9 to 14 are painted from a Giemsa-stained smear of a spleen puncture from a case of human splenic anæmia. Here it would appear that similar transitions are occurring to those seen in the bone-marrow smear of the embryonic chick. Fig. 10, however, is probably simply a divided red cell and not a parent amœboid cell. In the human case the same transition in colour is also noticeable.

Before concluding I wish to acknowledge the help given me by Dr. J. Leitch Wilson, with regard to the continuous microscopic observations made on the live spleen cultures.

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