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Growth, Phagocytosis, and Other Phenomena in Tissue Cultures of Foetal and Adult Lung

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VII. *Growth, Phagocytosis, and other Phenomena in Tissue Cultures of Fœtal and Adult Lung.*

(A Report to the Medical Research Council.)

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[Plates 16, 17.]

Contents.

(1) Introduction.

This research was undertaken with the following aims in view :—(1) The investigation of the growth phenomena of fœtal and adult lung *in vitro* ; (2) The study of the phagocytic reaction (if any) of such cultures towards sterile carmine and coal particles. As far as I know, these are the first observations as yet made on tissue cultures of lung.*

The material assistance I owe to the Medical Research Council, and I am glad to have this opportunity of expressing my gratitude.

* Since the above was written, Dr. Warren H. Lewis informs me, by letter, that he has been studying tissue cultures of lung.

To my chief, Sir CHARLES SHERRINGTON, I wish to convey my most grateful thanks for valuable suggestions and illuminating discussions on the genesis of this paper. Prof. CHRISTIAN CHAMPY was kind enough to show me his tissue culture technique. I am glad to acknowledge my indebtedness to his very stimulating personality. For assistance in the surgical procedure I am deeply indebted to Messrs. E. G. COX—whose skilful anæsthetizing and knowledge of surgical technique have been invaluable—T. J. SURMAN, who has frequently administered the anæsthetic, and to F. HAYNES for assistance throughout the experiments.

The making of serial sections and assistance in the observations on the living cultures have been Mr. HAYNES' contributions to this work. And it is with pleasure that I now thank him for a continual painstaking and intelligent co-operation.

The credit of producing satisfactory micro-photographs of difficult material is due to Mr. W. CHESTERMAN.

No attempt is here made to review the previous work in Tissue Culture. The literature is considerable, and it has already been reviewed by CARREL (6), LEVI (24, 27), LEWIS (28*c*) and myself (CARLETON (3, 4)).* Consequently only such references are made in this paper as have a direct bearing.

At the outset I would make clear one point. The histological study of tissue growth *in vitro* logically resolves itself into (1) the phenomena of the invasion of the medium by cells derived from the original fragment of tissue or "implant," and (2) the changes within the implant itself. The changes within the original fragment are often a very necessary part of the whole study, though many authors have neglected this point.

CHAMPY, 10 years ago (7, 8, 9 and 10), showed the interest of studying the implant by the sectional method on tissue cultures. Apart from EBELING and FISCHER, few workers have taken the trouble to do this, and fewer still—if any—have sectioned the different stages of their material as a routine procedure. In the present paper the observations have been made by both the hanging-drop technique and the tube method, to which latter only is the sectional method satisfactorily applicable.

A word regarding the tube method of cultivation. CARREL (6), as judged by his criticism of CHAMPY's results that "the tissues were not in a condition of cultivation, but merely of survival," would appear to imply that the tube technique which CHAMPY employed allows of survival but not of growth.

Both tube and hanging-drop methods have been used throughout these experiments, though one must note that the former technique does not give the abundant outgrowth into the medium furnished by the hanging-drop method. But there is no "survival," by which CARREL presumably means the persistence of tissues *in vitro* unchanged. Further, since CARREL does not section the implant as a routine procedure, it is difficult to see how he can tell whether survival or other phenomena are present within it.

* See also LUBARSCH and WOLFF: "Der heutige Stand der Gewebzüchtung, im besonderen in ihrer Bedeutung für die Pathologie;" 'Jahreskurse f. ärzt. Fortbildung,' 1925, January; and ERDMAN: Züchtung von Säugetieregewebe *in vitro*;" 'Anat. Anzeiger,' 1924, vol. 58, p. 247.

The cultivation of lung by the tube method has shown me with regard to the implant itself :—

(i) That cell division occurs even in adult alveolar epithelium, from which it is absent *in vivo*.

(ii) That growth, partly by mitosis, partly by sliding movements of cells, occurs around the body of the implant.

(iii) That many of the pulmonary elements are actively phagocytic.

Cells which exhibit the vital criteria of reproduction, movement and phagocytosis can hardly be regarded as being in a state of mere " survival."

(2) Technique.

(i) *Material.*

All the material for these experiments was derived from the cat or the rabbit. Cultures in autogenic plasma in the case of adult tissues, and maternal plasma in the case of fœtal tissues, were the rule.

(ii) *Methods of Cultivation.*

Three types of culture were used in these experiments.

(*a*) *The Cover-slip slide Method.*—This in no wise differs from the well-known " hanging-drop " technique, currently used in tissue culture.

(*b*) *The Cover-slip cell Method.*—A ground glass ring of about 5 mm. depth is cemented to a cover-slip with Canada balsam dissolved in absolute alcohol. When dry it may be sterilized in the hot air sterilizer without damage. Plasma and implant are added to this cell, which is placed on a mica sheet resting on a large paraffined watch-glass attached to the bottom of a deep Petri dish. To avoid desiccation of the cultures sterile Ringer-Locke solution is kept around the watch glass, which, being paraffined, does not cause wetting of the cultures by " creeping." To examine such a culture with the microscope the cover-slip cell is inverted over a sterile slide. Some four to six cover-slip cells can be lodged in one Petri dish.

(*c*) *The Tube Method of* Champy.—Small flat-bottomed glass tubes, 8 mm. by 10 mm., are nearly filled with plasma. The implants are added, as the plasma is clotting, so as to lie at, or immediately beneath, the surface of the plasma.

The tubes are kept in a small capsule cover, previously paraffined, which is attached to one segment of a Petri dish. A shallow beaker is kept inverted over the " battery " of tubes (usually 6 to 12 in number), and the junction between its mouth and the Petri dish is sealed with a layer of sterile Ringer-Locke solution. The latter, it is true, does not remain sterile, but infection of the cultures does not occur, since the paraffined capsule cover on which they are placed resists any attempt of the encircling fluid to mount its sides.

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(iii) *Relative Merits of the Above Methods.*

(*a*) The cover-slip slide technique is best suited for observations on the living cultures on the warm stage. Very useful " whole preparations " can also be made after suitable fixation and staining. *Disadvantages*: The inability to keep cultures for long (*i.e.*, more than a maximum of five days) without sub-culturing, owing to liquefaction of the small volume of plasma and its contamination with katabolic and degenerative products. Also the difficulty of detaching and orientating the cultures for purposes of sectioning.

(*b*) The cover-slip cell technique enables a deeper layer of plasma to be used than with the " hanging drop " method. Hence, the cultures can be kept longer. They can also be conveniently examined with the microscope while alive. *Disadvantage*: Greater liability to infection during observation, etc., than with methods (*a*) or (*c*); growth into medium less marked than in (*a*).

(*c*) The tube technique, on account of the large volume of plasma, enables the cultures to be kept longer without sub-culturing than with methods (ii) and (iii). Liquefaction of the medium also occurs much later. Finally, a point of great value, the implant can be successfully dissected out in a little block of plasma after fixation. By piercing the plasma block with a very fine hedgehog quill, the implant can be manipulated without damage during the processes of dehydration, clearing and embedding. By means of the same quill it is orientated at the time of casting, so that it can be sectioned in a known and definite plane, usually at right angles to the surface of the plasma. After orientation in the paraffin wax during the casting process the quill is removed.

The method of tube culture has the disadvantage that anything approaching critical microscopic study of the living culture is impossible, while invasion of the medium is usually scanty. In this research the cover-slip slide and tube methods were found to be complementary, the former being chiefly used for observations on the zone of invasion (in both living and stained specimens), while the latter was especially valuable for study of the implant itself in section.

(iv) *Irrigation of the Cultures.*

Cover-slip cell and tube cultures were washed with Ringer-Locke solution to remove katabolic products. The technique is simple: to each tube (or cell) sterile Ringer-Locke solution of 66 per cent. of normal strength is added. After about five minutes it is removed. Such cultures were washed every second day until they were fixed. Although the same bulbed pipette may be employed to add the Ringer-Locke solution to successive batteries of tubes (or cells), it is advisable to suck up the fluid with a fresh pipette for every third culture, in order to diminish the chances of bearing an infection from one culture to the other. It is important, when washing cultures according to the above technique, which I owe to Champy, to use dilute (66 per cent.) Ringer-Locke solution since the medium absorbs a little of this fluid each time the culture is washed. Consequently, if normal Ringer-Locke solution were employed, the medium would become hypertonic after a few washings.

For fixing the cultures dilute Bouin's fluid (one part of fixative to two of distilled water) was generally employed. For "whole mounts" Carnoy's fluid, or 4 per cent. Formol in normal saline gave good results—especially when followed by Heidenhain's Iron Hæmatoxylin.

(v) *Operative Procedure.*

This part of the technique resolves itself into two periods which may be summarized as follows :—

(*a*) *Removal of the Blood and Tissues for Cultivation.*—In the fully etherized animal a cannula is inserted into the carotid according to the technique described in SHERRINGTON'S Mammalian Physiology (38). Blood was collected in paraffin-coated centrifuge tubes packed around with ice in the centrifuge cups. The red blood corpuscles and leucocytes were separated off by five to ten minutes centrifugalization, while pieces of the tissues to be cultivated were dissected out and placed in capsules containing a little Ringer-Locke solution. Standard aseptic procedure was observed.

It was not found necessary to sterilize the carotid cannulæ in paraffin, as clotting could be prevented without it. But it is important to centrifugalize the blood as soon as it is obtained.

(*b*) *Preparation of the Cultures.*—The fragments of tissue to be cultivated are cut into thin lamellæ, which are rinsed in Ringer-Locke solution to remove red blood corpuscles. As already noted by DREW (15) these, when present in any quantity, inhibit growth.

A fragment of tissue is then held in a fine forceps, while a thin fragment is cut off with a fine scissors, and immediately taken up and transferred to the plasma. I have found fine scissors as easy to employ and far more economical than cataract knives for cutting up organs for cultivation.

(vi) *Statistical.*

The observations recorded in this paper are based upon 340 cultures of fœtal and adult lung distributed as follows :—

Cover slip slide cultures	98
Tube cultures	203
Cover slip cell cultures	39

The total percentage of cultures infected works out at 2·6—a small number considering that no sterile chamber was used for making them.

It is of interest to note that only two of 106 cultures of adult lung were infected. This I attribute largely to the fact that the implants were always taken from just beneath the pleura, and hence contained only the smaller bronchi.

The large number of cultures is justified by two considerations. Firstly, the fact that the phenomena of invasion of the medium are best studied by the cover-slip slide method, while the changes within the implant can only be elucidated by making sections of it after tube cultivation made it necessary to duplicate each series or stage in tube and

cover-slip slide cultures respectively. Secondly, individual variations in cultures are common, whence the need for integration of a number of results in order to arrive at satisfactory conclusions.

(3) The Growth-Changes Outside the Implant.

Under this heading are grouped the invasion of the culture medium by cells derived from the implant, their multiplication, and the different ways in which they group themselves. The study of the changes inside the implant itself is dealt with in Section 5.

A. Fœtal Lung.

Fragments in maternal blood plasma formed the material for these observations. The cultures were of the cover-slip slide or cover-slip cell variety. Sometimes the implants were fixed and stained in bulk, and sometimes they were sectioned.

A culture of fœtal lung immediately after implantation shows the fragment of tissue lying in the plasma in which a fine fibrin meshwork rapidly makes its appearance. Around the implant are usually red blood corpuscles and leucocytes in scanty numbers. The edges of the implant are either bounded by the endothelium of the visceral pleura, or else are of less regular outline where sheared by the scissors. In the latter case no definite layer of limiting cells can be discerned for the first 24 to 48 hours after implantation.

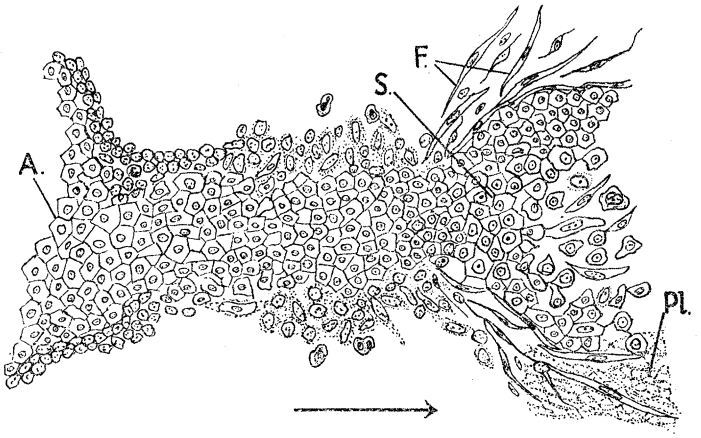
(i) *The latent period.*—The only phenomenon which is noticeable during this phase is the migration of a certain number of leucocytes from the implant to the medium. Frequently at this, or a later time, they migrate not only away from the implant, but also, and especially, upwards, till they come into contact with the cover slip.

The latent period of fœtal lung, in these experiments, was found to vary from 21 to 96 hours. Usually, however, the first signs of invasion of the medium by cells other than leucocytes appear at the end of the first, or during the second, day. The average time for this worked out, for some 20 cover-slip slide cultures, at 43 hours.

Plate 16, fig. 1, shows the condition of things in a 5-day specimen of fœtal lung of the cat (Culture No. 521). Considerable growth around the implant has occurred, and, on closer examination, this sheet of newly formed tissue, some two to four cells in depth, can be resolved into cells of epithelial and cells of connective tissue origin.

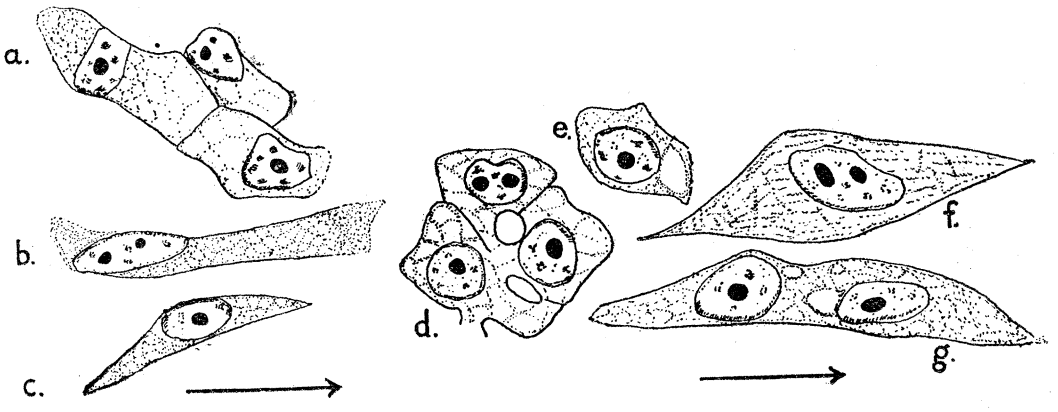
(ii) *Growth-formations of epithelial origin.*—In the implant, the developing alveoli, lined by polygonal cells, can be seen clearly demarcated from the surrounding mesenchyme (see Plate 16, fig. 1, and text-fig. 1). If one traces the cells of the cut end of such an alveolus as shown in text-fig. 1 they can be seen to form a sheet-like extension, which ultimately expands fan-wise to form a mass of increasingly discrete (*i.e.*, separated) cells. The limits of this mass are bounded on each side by fibroblasts. Fibre-formation (whether of the collagen or elastic type) does not occur. As the alveolar epithelial cells reach the

boundary of the invading zone, and come into contact with the medium, they gradually lose their polygonal shape and become fusiform; text-figs. 1 and 2, the latter high power, illustrate this change. The shape of these cells, then, may be largely ascribed to local conditions. In the alveolus itself they are polygonal because of mutual pressure. In the sheet-like extension from the cut alveolus similar pressure conditions exist and



TEXT-FIG. 1.

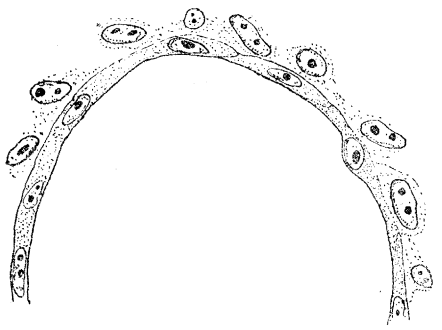
consequently the shape remains the same. But, as the sheet widens out, and is only encircled by sparsely-distributed fibroblasts, the cells pass through intermediate stages (in which their shape varies from the spherical to the roughly rectangular) to a fusiform condition when, released from all considerations of mutual cell-pressure, they encounter and invade the resisting fibrin meshwork of the culture medium. This polymorphism



TEXT-FIG. 2.

of the epithelial elements is also evidenced by the shapes they assume when they surround a vacuole in the medium. Such vacuoles are the result of the fibrinolytic powers of tissues *in vitro*. As pointed out by CHAMPY (12), the capacity to dissolve fibrin is especially marked in cultures of epithelium. Vacuoles appear around which the epithelial elements

group themselves as a lining membrane (see text-fig. 3). These cells are flattened and vary from the spindle shaped to the cubical. Their outlines are often indistinct, and it is possible that sometimes the constitution of the cellular investment of plasmatic vacuoles is syncytial rather than cellular.



TEXT-FIG. 3.

It should be mentioned that the cell membrane is apparently incomplete in some of the cells of the epithelial sheets derived from the cut ends of the alveoli (see text-fig. 3).

(iii) *Growth-formations derived from Connective Tissue.*—Often the bulk of the newly formed cells cannot be traced to the alveolar constituents but to the abundant mesenchyme which, in the later stages of fœtal development, becomes relatively less abundant as it suffers increasing subdivision by the growing alveoli. About half term, in both cat and rabbit embryos, the mesenchyme is composed of triangular cells between which lie fine collagen fibres. In such cultures an abundant proliferation often occurs from the inter-alveolar connective tissue. The cells derived from it are embryonic in character, and are never associated with fibrils (either collagen or elastic) in their process of invasion of the medium. The derivation of these cells from the mesenchyme of the implant is proved not only by their shape and mode of growth (which identifies them with the fibroblast) but also by the direct connection of thick strands of these cells with the mesenchyme of the implant. Reference to text-fig. 6 illustrates this point, which has frequently been confirmed in whole mounts and in sections of other cultures. As to whether the network of fibroblasts is syncytial or not, it is hard to say. But the capabilities of individual growth, the sliding movements of such elements over one another, and the transformations of shape which they undergo *in vitro*, are suggestive, as pointed out by LEWIS (28), of cellular individuality. Furthermore, it by no means follows that a delicate surface membrane, if present, can be microscopically resolved. The evidence derived from tissue cultures of lung is, on the whole, in favour of LEWIS's contention that the mesenchyme cells are not syncytial.*

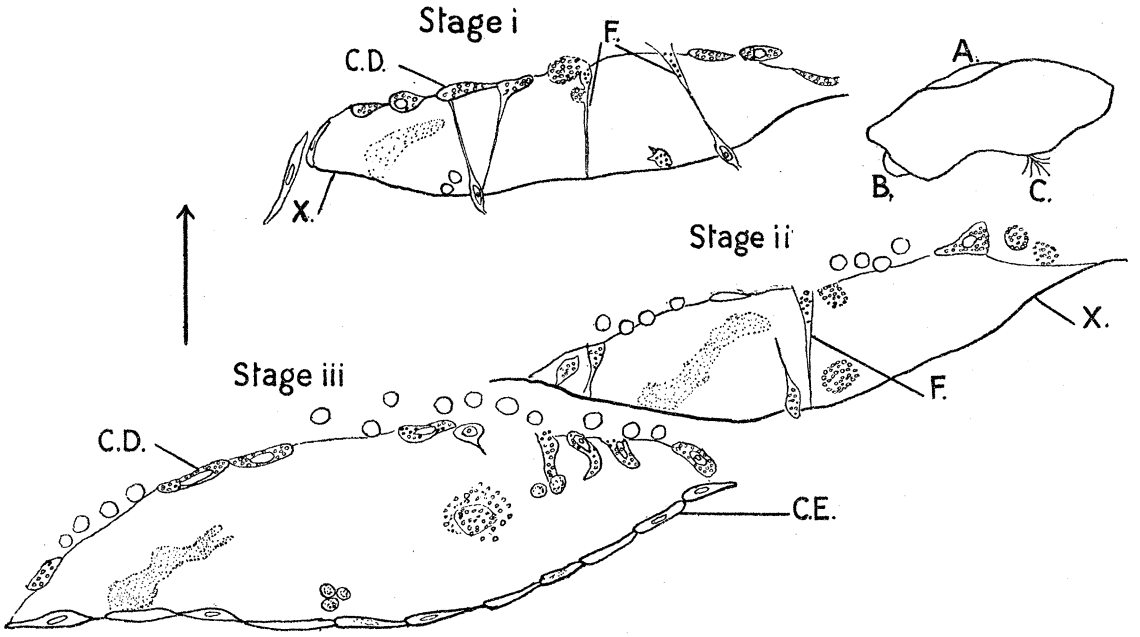
(iv) "*Membrane-formation.*"—Under this heading are grouped curious growth changes which, so far as I know, have not been described before. The onset of membrane formation is rapid, and is frequently followed by regression of the structure evolved in the preceding hours.

Membrane formation is very common in cover-slip slide cultures of fœtal lung. It seems to be most frequent in specimens taken from embryos in the later stages of fœtal life. Chronological description of the growth changes exhibited in two cultures (continuously observed with the warm stage) will serve as an illustration.

* LEVI (25), in the course of careful observations on fibroblasts *in vitro*, claims that continuity of cytoplasm does occasionally exist, as evidenced by the passage of mitochondria from one cell to another.

NOTE.—The cicatricial epithelium, involved in this type of growth, is fully dealt with in the next section of this paper (p. 377 *et seq.*). It will suffice to say here that a sheet of cells, derived from the cut ends of the bronchi, grows over the surface of the implant and covers the naked tissues.

Culture 695.—From a full-term cat embryo. (Text-fig. 4.) At 4 p.m. (28 hours after incubation) a slight outgrowth of fibroblasts of the type already described was noted on one side of the implant. By 11 p.m., in addition to this outgrowth, the cicatricial epithelium had separated from part of the main body of the implant at A in the inset figure, thereby forming a cleft-like space bounded on one side by the cells detached from the cicatricial epithelium, and on the other by the implant (*see* stage i).

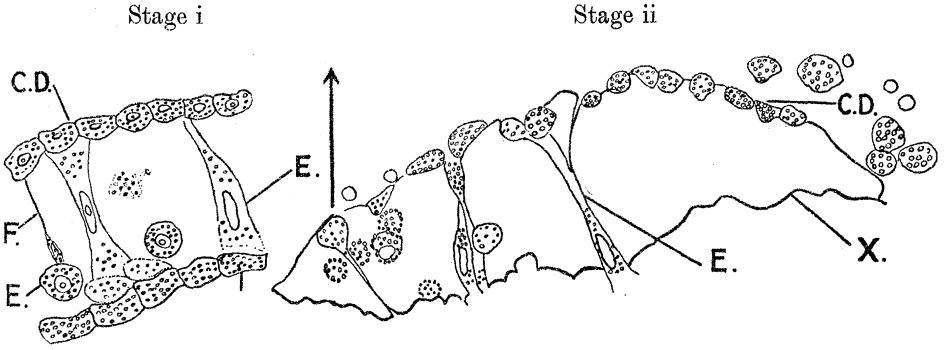


TEXT-FIG. 4.

The space contained cells, the majority of which were undoubtedly fibroblasts, as evidenced by their shape and mode of growth. These cells had originated from the implant, and both their origin on its surface and their insertion on the cells lining the cleft could be clearly seen. By midnight (see stage ii) some of the fibroblasts had undergone retraction towards the cells lining the cleft—henceforth known as the "vacuole membrane," while new fibroblasts had to some extent replaced the old ones. Certain other elements had migrated into the implant vacuole. They were rounded, had a single spherical nucleus and many granular mitochondria. Some of them were leucocytes, others, possibly, migrated alveolar elements. By 4 a.m. (see stage iii) the cleft had increased in width, the delicate bridges of fibroblasts had broken off and undergone more or less retraction towards the vacuole membrane. The cicatricial epithelium of the implant had reformed, being visible as a layer of flattened cells.

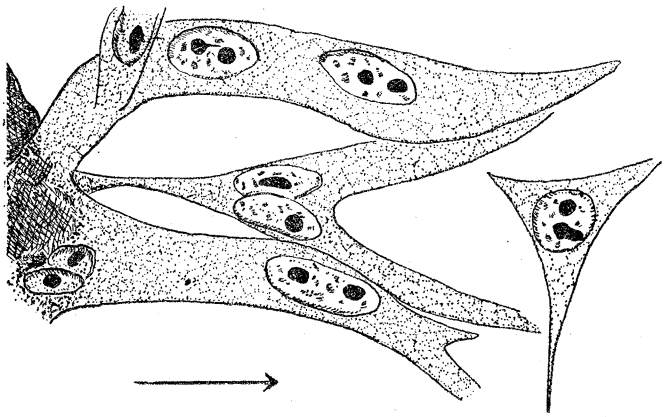
Between this observation and the last the cicatricial epithelium had split off at another point (*see* inset figure B) on the periphery of the implant, and growth of fibroblasts across this vacuole was actively progressing by 4 a.m. At 6 a.m. the culture was fixed and stained and the later findings were confirmed. In addition, it was noticed that the cells of the second vacuole formation could, in contrast with the first, be divided into two types—the mesenchymal and the epithelial. The forms were as noted in the description of the growth-changes in vacuole A; the latter appeared as large flat cells in process of detachment from the cicatricial epithelium. On the top of the culture, immediately beneath the cover glass, was the usual cluster of leucocytes (chiefly polymorphs and lymphocytes).

Culture No. 691.—Implant *b*. From a full-term cat embryo. (Text-fig. 5.) At 5 p.m. on April 26th, 1924, a few radiating fibroblasts appeared, 27 hours after incubation. Extension of growth continued until 11.45 p.m., when a cleft-like space



TEXT-FIG. 5.

began to appear on each side of the implant. This occurred by detachment of the cicatricial epithelium. By 1.30 a.m. (see stage i) cells had grown out from the



TEXT-FIG. 6.

implant towards the vacuole membrane. Some extremely slender ones were fibroblasts, others, flattened and larger, were of epithelial origin and, in view of the

observations already recorded on the fixed 4-day specimen No. 519 already described, I regard them as being derived from the alveolar lining. A few large rounded cells lay in the vacuole cavity. By 5 a.m. (stage ii) the bridges had increased in number. Owing to the increase in size of the vacuole and the consequent tension upon the cells bridging it, both epithelial elements and fibroblasts are very slender, and it is occasionally difficult to say which is which. Some of the bridges have broken their connections, leaving rounded elements against either the implant or the vacuole membrane. Sometimes such cells try to regain their connection with the implant by growing from the vacuole membrane to the implant. " Reversed growth " of this type was noted in this and often in other cultures. Concomitant with the breaking down of the cell bridges in the vacuole under consideration, another one began to form in a similar manner. The latter observations were confirmed after fixing (and staining) it at 6 a.m., 54 hours after implantation of the fragment into the plasma.

Observation of cultures kept for longer periods up to 5 days shows that these vacuoles, originally derived from the cicatricial epithelium, increase enormously in size, as shown in Plate 16, fig. 2. Usually there are one or two, the smaller ones, if formed, becoming confluent with the larger spaces. Until degeneration sets in they continue to be lined by cells derived from the cicatricial epithelium of the implant. Scanty mitoses are present in the vacuole membrane, which may be one to several cells in thickness. Much of the growth in such formations is due to the cells sliding over one another, or else increasing in length. Sliding movement of this type is very common in tissue cultures, and has been noted by many observers: Oppel (31, 32, 33), Lewis (28), Champy (8), Ross Harrison (22).

As the result of extensive membrane formation all round the implant, such marked fibrinolysis may occur that the implant lies centrally in the vacuole. Plate 16, fig. 4, illustrates this type of growth Fibroblasts (darkly stained) and epithelial cells (dot-like at the low magnification) may be seen in the implant vacuole. At the periphery of the latter, sheet-like invasion of the plasma by fibroblasts, and especially epithelial cells, can be noted.

In section the invading cells usually appear as a thin sheet at the surface of the medium. Plate 16, fig. 5, depicts the epithelial outgrowth from the implant in a tube culture. Unlimited extension of the word " survival " is needed to apply it to such a phenomenon.

(v) *Unusual Types of Growth.*—Under this heading may be grouped (*a*) the tendency for cells, after destruction of the bridges, to grow back from the cicatricial epithelium towards the implant. Such attempts occur only in the later stages of vacuole formation and are frequently successful. (*b*) Growth from the implant, into the implant vacuole, of blunt lobed processes, bounded by the regenerated cicatricial epithelium of the implant. Plate 16, figs. 2 and 3 illustrate these blunt projections from the implant. Examination with the high power in living and stained preparations shows the thin investing layer of flattened cells, already mentioned, and inside the processes bounded

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by them (*a*) fine branching cells and (*b*) more stumpy, triangular or rounded elements. The former I regard as fibroblasts, the latter as epithelial elements.

The implant cavity, even at its first appearance as a narrow cleft between the cicatricial epithelium and the main body of the implant, always contains a clear, colourless fluid. That the contents are fluid is evidenced by the Brownian motion of small debris, &c., suspended in it.

(vi) *The Growth Rate of Fibroblasts* in the implant vacuoles was noted in some instances. The most active growth rate, as measured by increase in length of two fibroblasts, was in one case 322μ in 4 hours, and, in the other, 221μ in 1 hour 10 minutes. This implies a rate of growth of 80·5μ and 189·5μ per hour respectively. The growing end of the fibroblast is sometimes pointed, sometimes fan-shaped—very like the fan-shaped processes first noted in the outgrowing axones of cultivated nerve cells by Ross Harrison (22). Curious changes in shape may also occur by the retraction of the growing end and the transformation of the fibroblast into a small, amœboid cell. The *in vitro* changes in connective tissue cells, having been very carefully studied by Lewis (28A, 28B) and De Garis (14A), are not further dealt with here.

(vii) *The Distinction between the Mesenchymatous and the Epithelial Elements* in vitro.—Apart from the obvious means of distinction by tracking groups of questionable cells back to their point of origin in the implant (the method is applicable to both "whole mounts" and sections), the next best criterion is the size of the cells. For the epithelial elements when they stretch out and become fusiform, lag behind the cells derived from the mesenchyme, which are nearly always longer and usually many times larger than the modified epithelial cells. Even taking the fibroblasts at the periphery of the implant, where they tend to be shortest, they are considerably larger, and usually stouter, than the epithelial cells. Comparison of text-figs. 2 and 6 illustrates this point. Furthermore, when present in any numbers, the apparent anastomosis causes the mesenchymatous formation to appear as a network, in contrast with the majority of the epithelial cells.

(viii) *Cell Division in the Invading Zone.*—The chief type is by ordinary mitosis. Mitotic figures are rare in the mesenchyme, frequent in the developing bronchi and alveoli, in the lungs of half-term cat and rabbit embryos. In the cells in the zone of invasion of an actively growing cover-slip slide culture mitoses are most frequent in the fibroblastic portions. The epithelial sheets and other epithelial formations contain fewer dividing cells, even when well developed. Further, much of the growth of epithelial formations is due to extension by sliding and amœboid movements rather than to actual cell division. Abnormal mitoses are extremely rare in the cells outside the implant, nor have I ever observed a case of undoubted amitosis. The details of mitosis in the living cell are not dealt with in this paper, since they have already been admirably described by Strangeways (40) in cultures of cells of the chick.

B. Adult Lung.

The following remarks apply to cultures of adult cat and rabbit lung. Invasion of the medium is slight, and is only by cells derived from the fibrous tissue of the implants Many of the cultures do not proliferate into the plasma at all, while those which do always show a far longer latent period than that of fœtal lung. Fibroblastic invasion of the medium has been noted from 45 to 96 hours after implantation. Independent of, or preceding, this there usually occurs a migration of leucocytes into the medium. In addition, rather similar cells, but of larger dimensions, sometimes migrate from the implant. These, for reasons pointed out in the next section (" Changes in the Implant," p. 379), are probably modified alveolar epithelial elements. Vacuole formation is rare, doubtless because of the absence of any concerted epithelial invasion of the plasma and the consequent fibrinolysis which is the prime cause of such formations.

Briefly summarized, the plasma-invading powers of adult lung are very slight and are restricted to outgrowth of the connective tissue elements. The *in vitro* changes, as shown in the next section, are restricted almost entirely to the implant.

(4) The Changes within the Implant.

Of the 200 tube cultures of fœtal and adult lung, the greater proportion were serially sectioned. So also were some of the cultures of the cover-slip slide and cover-slip cell varieties. Processes such as the overgrowth of one type of tissue, the appearance of large numbers of mitoses in the alveolar epithelium of adult lung, the detachment of alveolar epithelial cells, the phagocytic response of the lung parenchyma when the surfaces of the implant are dusted with sterile particles, and the mode of formation of the cicatricial epithelium are phenomena which are best studied by the sectional method, and sometimes can only be so studied.

Cultures were fixed for sectioning at periods varying from 6 hours to 10 days after incubation. It was not attempted to prolong cultivation beyond the latter period, since by then the implant was either degenerating, or had become transformed into connective tissue cells and fibres. Subculturing was deliberately avoided, owing to the liability of disturbing the relationships of growing tissues.

Control fragments of lung were nearly always examined.

A. Fœtal Lung.—The material for these experiments was furnished by cat and rabbit embryos dating from half term to practically full term. The *in vitro* behaviour of half or full-term embryos is essentially the same. Consequently the following observations have not been specially grouped according to the age of the embryos.

(i) *The Cicatricial Epithelium.*—This term, introduced by Champy, applies to a phenomenon first noted by Oppel (31, 32, 33). Champy (8, 9, 10) noted that in sections of cultures of testis, kidney and thyroid the epithelial elements grow around the cut edges of the implant to form the " épithélium de cicatrisation." The cells of the cicatricial (and of other) epithelia *in vitro* often show " dedifferentiation," a term first

applied by PÉREZ (34A) to this process in insect larvæ, and subsequently extended to tissue cultures by CHAMPY.

The mode of formation of the cicatricial epithelium being the same in both fœtal and adult lung, the micro-photographs illustrating it are taken from specimens of one type only (the adult).

About 24 hours after implantation a layer of cells begins to invest the cut edges of the implant. In serial sections its derivation can nearly always be traced to the epithelium of a bronchus or bronchiole. In the earlier stages of its formation a bronchus —as seen in a vertical longitudinal section—appears as a tubular structure with a flange-like portion abutting on the surface of the implant. The tubular segment represents the original segment of the bronchus, the flange the layer of cells now growing centrifugally from the lumen over the surface of the implant. Further examination reveals a varying number of mitoses in the flange-like portion, which is covered over by a layer of epithelial cells continuous with, and derived from, the pre-existing bronchial epithelium. The cilia, when present (as in the larger bronchi in the later period of fœtal life), disappear *in vitro*. Consequently they cannot be found either in the bronchus itself or in the layer of cells derived from it. Frequently, for reasons unknown to me, a striated border is found at the unattached poles of the cells of the cicatricial epithelium. They are very polymorphic and, generally speaking, the further they grow from the parental epithelium, the less like it do they become. Owing to the fact that, quite apart from mitotic division, much of the growth of the cicatricial epithelium is due to that curious sliding motion of cells which is so frequent an occurrence in these cultures, the contour of a rapidly-growing cicatricial epithelium is irregular, owing to differences in the number of cell-layers in it, and also to the very variable shape of its constituent cells, as they slide over one another. This type of movement *en masse* is very like that which occurs during regeneration of epithelia *in vivo*, as shown by RANVIER (37), GIROUD (18), and others.

By the third or, at the most, by the fourth day the cicatricial epithelium is complete and has invested all the naked surfaces of the culture. Its formation would appear to be the rule in all cultures which show growth.

Its mode of origin is illustrated in Plate 16, fig. 6, which depicts in section of a four-day tube culture of adult lung. The connective tissue of the bronchus partakes but feebly in the outgrowth of the cicatricial epithelium from the bronchial lumen.

The fully-formed cicatricial epithelium sometimes ceases to grow once it has completely invested the culture. In such cases its outline accurately follows that of the implant. But often its growth continues and, sheet-like extension being no longer possible, it forms spheres or blunt finger-like processes which retain continuity with the epithelial sheet. Such processes either project into the plasma or grow in the opposite direction a little way into the implant (see Plate 16, fig. 6). They are identical with the "boyaux épithéliaux" noted by CHAMPY in the cicatricial epithelia of many other organs *in vitro*.

Some of the implants, having been cut off vertically from the surface of the lung, are bounded on one side by pleural endothelium. Such cultures can be recognized in section by the layer of (usually) flattened endothelial elements lying on a layer of collagen connective tissue fibres. The *in vitro* activity of the pleura is scanty, and is restricted to an increase in thickness due to cell division.

(ii) *The Alveolar Epithelium.*—On implantation in the plasma, fœtal lung undergoes a slight collapse. The elastic fibres of the alveolar walls, bronchi, etc., hence appear thicker and more tortuous than in the control fragments. After about 24 hours of incubation the alveolar epithelial cells begin to swell. As a result of this, the alveolar cavities begin to be obliterated. This blocking out of the air cavities is in no wise due to the slight collapse of the alveolar walls, since it occurs about 24 hours later and is obviously caused by the swelling of the epithelial cells. Next, but not in all of the cultures, mitoses appear in the swollen alveolar epithelium some 24 to 48 hours after incubation. About a quarter of the tube cultures of fœtal lung show this phenomenon. It is noteworthy that the number of mitoses in the implants of fœtal lung is not related to the number in the control fragments. Thus, cell-division does not occur in the alveolar epithelium of full-term cat embryos, though it may appear in tissue cultures. Inversely, although the developing alveoli in specimens of lung of half-term cat embryos show active cell division, this is sometimes altogether absent *in vitro*, sometimes more active and sometimes less.

Yet another curious process has to be noted. A number of the alveolar epithelial cells, at times varying from 24 hours to 4 days after incubation, detach themselves from the alveolar walls. This process is very common in cultures of fœtal and adult lung. The stages in the detachment of these cells, and their behaviour towards sterile coal and carmine particles are so similar in both fœtal (from half term onwards) and adult lung that they are described in the sections dealing with adult lung (p. 381) and phagocytosis *in vitro* (p. 383).

The next stage in the history of fœtal lung *in vitro* is mainly one of degeneration. Proliferative and desquamative phenomena cease about the fourth day and the alveolar elements—both free and attached—degenerate. Their nuclei become shrunken and pycnotic, and appear as homogeneous black spots. Their cytoplasm is finely granular after fixation, the cell-outlines disappear. Finally, a condition is reached in which only the more resistant chromatin granules can be detected. By the seventh day of cultivation, degeneration has destroyed the alveolar epithelium, and only the connective tissue cells and collagen fibres are left within the implant, except for a few scattered alveolar elements.

(iii) *The Connective Tissue.*—Concurrent with the degeneration of the alveolar epithelium is the hypertrophy of the connective tissue, which may be reduced to a collagenous overgrowth and transformation of the implant, since the elastic fibres play no part in the matter. By the third or fourth day the collagen meshwork is seen to be thicker than in control specimens. The "fibrosis" of the implant is sometimes focal

in origin, in that a radiating invasion of the implant occurs either from the peri-bronchial fibrous tissue, or from the adventitia of a blood vessel. Often the process is diffuse and occurs in the collagen meshwork throughout the implant. The alveolar epithelial cells are caught up in this steadily increasing network of connective tissue; they become isolated from each other, and those which do not already bear the stigmata of degeneration rapidly assume them as they become progressively strangled by the growing meshes which encircle them. An " old " culture, *i.e.*, one of 7 to 12 days, shows thick collagen bands and fibres, with fusiform connective tissue nuclei interspersed between them. Scattered and surrounded by the collagenous elements, alveolar and bronchial epithelial cells can still be detected, as shown in Plate 16, fig. 7.

(iv) *Various Subsidiary Phenomena.*—The larger blood vessels are generally quite passive, except for the proliferation of collagen fibres from the peri-vascular connective tissue sheath already mentioned. In the later stages of cultivation (*e.g.*, from 7 days onwards) degeneration of the larger blood vessels may supervene. Proliferation from, or swelling of, the endothelial cells is extremely rare.

As noted by CHAMPY (8), the central portion of the implant undergoes, sooner or later, an aseptic necrosis. This must be primarily ascribed to lack of oxygen.

Invasion of the plasma in tube cultures is usually scanty as compared with the cover-slip slide variety. When it occurs it may be epithelial in origin—in which case the invading sheet of cells is usually derived from the cicatricial epithelium, as shown in Plate 16, fig. 5—or of a connective tissue nature, especially in old cultures in which the epithelial elements have degenerated. In the former case the cells are polymorphic; those farthest away from the implant are often flattened and endothelial-like; those nearer, irregular in shape and disposition; often the long axes of these cells are perpendicular to the surface of the plasma, a phenomenon which must be ascribed to the greater cell-pressure around the implant.

B. Adult Lung.—The cultures on which these observations are based were derived from adult etherized cats and rabbits. The minimum duration of culture was 5 hours, the maximum 8 days. Control fragments of lung show capillary congestion and some desquamation of the alveolar epithelium, in addition to the collapse which always occurs in adult lung unless special precautions are taken to prevent it. The epithelial catarrh is ascribable to the irritant effect of the ether.

(i) *The Cicatricial Epithelium.*—The account already given (pp. 377 and 378) of the formation of the cicatricial epithelium in fœtal lung is almost entirely applicable to its behaviour in cultures of adult lung. Such a culture is depicted in Plate 16, fig. 6.

Owing to the fact that all the bronchi of adult lung (excepting, of course, the terminal bronchioles) are ciliated, whereas in fœtal lung only the largest bronchi are ciliated, the degree of " dedifferentiation " of the bronchial and cicatricial epithelia in adult lung is greater than in fœtal lung. The ciliated columnar epithelial cell is obviously more highly specialized, in structure and in function, than its non-ciliated rather cubical

precursor. Hence its degree of dedifferentiation is greater in its transformation into the polymorphic cell of the cicatricial epithelium.

(ii) *The Alveolar Epithelium.*—Cultures examined 5 hours after implantation show no change beyond collapse of the alveoli, which is naturally far more marked than in the case of fœtal lung. The next phenomenon consists in the swelling up, and subsequent detachment of, large numbers of alveolar epithelial cells.

Sometimes synchronous with this is the appearance of mitoses in the swollen alveolar epithelium, but a point which must be stressed is that the swelling and detachment of the alveolar epithelial cells is frequently independent of any kind of cell division. Nearly all cultures show this desquamation of alveolar cells, while only a smaller proportion show any evidence of mitoses in these elements. Furthermore, only very rarely have I noticed mitosis in alveolar epithelial cells which were being shed, the process being apparently restricted to attached alveolar epithelium. And, lastly, cultures in which the process of detachment of alveolar elements is general and extremely active often show no mitoses at all. Therefore, the phenomena of detachment of the alveolar epithelial cells, and their cell division, seem to be synchronous rather than causally related, and it is preferable to deal with each under a separate heading.

(*a*) *The Detachment of the Alveolar Epithelial Cells.*—This process is so general in cultures of from 24 hours onwards that its different stages may easily be reconstructed by the study of sections through the implant. A general view of the implant in this condition is shown in Plate 16, fig. 8. The following account is applicable in all essentials to both fœtal and adult lung, and has been repeatedly verified in different cultures. The cells whose history we are now concerned with are not, of course, the large anucleate respiratory squames (present only in adult lung), but the smaller cubical cells. The fate of the squames would appear to be a rapid disintegration either *in situ* or after desquamation.

Plate 17, figs. 12 to 16 illustrate the stages of detachment in a 24-hour culture of adult cat lung. The smaller alveolar cells, after fixation, have a finely granular cytoplasm. In the course of their evolution towards detachment they swell so that the free surface of the cell juts into the alveolar cavity. At the same time, the cytoplasm becomes vacuolated, and, in the next stage, the cell tends to become spherical, with the result that proportionately less and less of its surface membrane is in contact with the alveolar wall. The nucleus, in contrast with the increasingly vacuolated cytoplasm, does not become any larger except for a brief initial increase. Finally, the cell undergoes detachment from the alveolar wall. When free in the alveolus it becomes more or less spherical. By this time the cytoplasm is highly vacuolated, but the nucleus shows no sign of degeneration, and such cells may live *in vitro* for at least as long as 8 days.

In the course of their detachment the alveolar epithelial cells lose their previously fairly definite polyhedral or cubical shape and become rounded. Concomitant with

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this loss of structural specialization is the appearance of functions not usually associated with specialized cells, *i.e.*, motility and phagocytic power.

The dedifferentiation of the alveolar epithelium is thus functional as well as structural.

Sometimes alveolar collapse is so marked in implants of adult lung, and the swelling of the alveolar epithelium so general and synchronous, that comparatively few of the cells can undergo detachment, owing to obliteration of the alveolar cavities. Such a culture, in section, shows closely packed strands of rounded, oval or cubical cells of large size and a not very deeply staining cytoplasm. Their character, in fact, is definitely embryonic and indifferent—so much so that these cells would afford no clue to the type of epithelium from which they were derived to one who had not followed the history of their derivation. Adult lung, *in vitro*, rapidly dedifferentiates, and, in so doing, it frequently passes through a stage in which its elements are strikingly "fœtal" in appearance, owing to the small alveolar cavities and the thickened epithelial cells lining them. This seems to be the pulmonary counterpart of reversed development described by CHAMPY (8) in cultures of adult kidney. Degeneration, usually heralded by pycnosis and karyorhexis, is often present, especially in the centre of cultures of adult lung, owing largely, I imagine, to oxygen want. The process of dedifferentiation, however, is quite distinct from it, in time of onset, in the stages through which it passes, and in appearance.

(*b*) *Cell Division in the Alveolar Epithelium.*—Many cultures of adult lung show this curious phenomenon which is never present in normal (*i.e.*, control) adult lung. Mitoses often appear as early as 24 hours after incubation and persist up to the fourth day. Cell division in the alveolar epithelium, although common, is by no means as general as dedifferentiation and detachment of the alveolar cells. Thus, mitoses were noted in 12 cultures of adult lung out of a total of 45 from 24 hours of incubation onwards.

The vast majority of mitotic figures are normal in appearance. Irregular distribution of the chromosomes was only once noted.

(iii) *The Connective Tissue.*—As with fœtal lung, a progressive overgrowth of the collagen fibres is the rule from the third or fourth day of cultivation onwards. This "fibrosis" of the implant of adult lung is essentially the same as in the fœtal organ, which has already been described.

(iv) *Subsidiary Phenomena.*—The larger blood vessels are usually passive in so far as the tunicæ intima and media are concerned. The collagen fibres of the adventitia sometimes act as a focus from which, in the later stages of cultivation, invading strands arise. Dedifferentiation of the muscle fibres of the type described by CHAMPY (7) and LEVI (26) was once noted.

The capillaries collapse, while no proliferation of the capillary endothelium has been noted.

(5) Phagocytosis in Tissue Cultures of Lung.

For the addition of sterile particles of coal or carmine to the cultures it suffices lightly to brush the surface of the plasma, or, better, the implant itself in either cover-slip slide or tube cultures.

Carmine is very energetically taken up by tissue cultures of fœtal and adult lung. The phagocytic response invoked by coal is less brisk, though well marked; the majority of the coal particles, even after prolonged grinding in an agate mortar are larger than the carmine grains, consequently the carmine stands a better chance of being taken up by cells, so that it is unwarranted to suppose, merely on the evidence of these experiments, that carmine stimulates phagocytosis more readily than coal.

The response of the implant itself is so similar in the cases of fœtal and adult lung that they are described together. In the case of adult lung the invasion of the medium is too slight for any useful observations on phagocytosis by its constituent cells to be made.

A. Phagocytosis in the Zone of Invasion (Fœtal Lung only).

When carmine is added to a culture at the time of inoculation it may be observed within cells some 24 hours afterwards. Intra-cellular carmine granules are found in the following elements:—

(*a*) In white blood corpuscles.

(*b*) In the cells lining the implant cavity. These, as have been shown, are outgrowths from the bronchi. Consequently phagocytosis of carmine granules by dedifferentiated epithelial cells of bronchial origin has to be admitted. The epithelial elements which bridge the implant cavity also frequently contain intra-cellular carmine.

(*c*) Possibly in the fibroblasts. This phenomenon is certainly rare owing to the far greater avidity of white blood corpuscles and epithelial elements for the carmine granules. Further, differentiation between the fibroblast and the isolated epithelial cell is sometimes difficult; but distinct possibility of phagocytosis by fibroblasts in lung cultures has to be considered. I hope to develop this question in a subsequent paper.*

The addition of coal particles calls forth all the above types of phagocytic response, but on a smaller scale.

B. Phagocytosis within the Implant.

The different stages in the phagocytosis of coal and carmine particles can be more accurately studied within the implant than in the zone of invasion for two reasons:—

(i) Serial sections can be made.

(ii) The (usually) bad optical conditions due to observation through a non-homogeneous

* M. R. Lewis has noted the ingestion of *Bacillus radicola* by embryonic connective tissue cells *in vitro*. (M. R. Lewis: 'Johns Hopkins Hosp. Bull.,' vol. 34, p. 223 (1923).)

medium are obviated, since by cutting fairly thin (8μ) sections, the fibrin mesh work can be reduced almost to one plane.

The practical advantages of (i) and (ii) mean that the criteria for the occurrence of phagocytosis, and the facilities for observing the derivation of the cells responsible, are considerably increased.

(1) *The Criteria of Phagocytosis.*—Foreign particles such as coal, or carmine, or bacteria in infected cultures, may sometimes be seen plastered on to the surface membrane of the cell. Such instances, especially when the suspected cells are *not* sectioned, and the particles (or bacteria) are very small, often look extremely like genuine ingestion.

The criteria adopted in these experiments for ascertaining if phagocytosis had truly occurred were as follows :—

(*a*) To note whether the cell membrane could be seen *outside* the particles at the borders of the cell.

(*b*) To note whether, at a high magnification (*e.g.*, one of 1,300 to 2,780 diameters), some of the particles could be brought by careful focussing into the same focal plane as the nucleolus of the cell under observation.

(2) *Phagocytosis and the Alveolar Epithelium.*—The examination of sections of implants, the surface of which has been lightly touched with coal or carmine, shows that the majority of the cells, which have absorbed the pigment, are the same alveolar elements, the swelling up and detachment of which has been already described. As with the processes of detachment, so with phagocytosis ; there is no essential difference in the behaviour of fœtal or adult lung. Even the facility with which these cells ingest coal and carmine seems to be about the same in fœtal and adult lung.

Plate 17, figs. 15 and 16 show definitely intra-cellular carmine granules in a strand of swollen alveolar cells four days after implantation and dusting with the pigment. The following stages are shown in fig. 15 :—One of the " cubical " epithelial cells of the alveolar wall—" petites cellules épithéliales " of the French authors—is shown at *a* ; *b* is a swollen example of the same ; *c* is a group of such cells which contain carmine granules ; *d* and *e* are still later stages, in which the frequent vacuolation of such cells is marked. All these stages were contiguous in the section depicted in the drawing. Finally, the recently detached epithelial cell, bearing its load of intra-cellular carmine, is shown, at a higher magnification, in fig. 16. Some of the cells depicted (fig. 15, *d* and *e*, and fig. 16) contain unmistakable carbon particles in addition to the carmine granules. Since no coal was added to the cultures under consideration, it is obvious that these carbon particles must have been in the lungs prior to cultivation. The lungs of adult domestic animals in towns (including cats) always contain, as is well known, a good deal of carbon.

When finely ground-up coal is dusted over the implant exactly the same phenomena are observable, but on a smaller scale. When very large coal particles come into contact with the alveolar epithelium they may sometimes be seen encircled by cells

derived from the alveolar epithelium. Since fœtal lung contains no coal, it is certain that all the pigment in it dates from the period of inoculation with coal dust. In the case of adult lung one cannot be certain, although extremely fine intra-cellular particles are in all probability derived from carbon suspended in the inspired air during life It should be noted that only the finest particles attain the alveoli during life, owing to the stoppage of all the larger, and many of the smaller, ones by the nasal and bronchial epithelia. Experiments on dust inhalation in guinea pigs (5) have convinced me that it is only after the most intensive "dusting"—such as never occurs under normal conditions of life—that the *larger* particles attain the alveoli. Further, it is usually easy, even when coal particles are added to cultures of adult lung, to distinguish, even inside the implant, between the particles inhaled during life and those introduced *in vitro*, since it is almost impossible by grinding coal to make the particles as small as the carbon granules shown in Plate 17, figs. 15 and 16, in which the criteria of phagocytosis adopted in this paper are realized.

Another type of isolation of particles added to the culture medium is that in which they become surrounded by cells derived from the cicatricial epithelium, as shown in Plate 16, fig. 9. It is possible that this is the early stage in the formation of a multi-nucleate syncytial mass, or giant cell, as noted around *Lycopodium* spores in tissue cultures of spleen by Lambert (23), and by Bond (1) when toxic substances or bodies are added to white blood corpuscles *in vitro*.

The phagocytic powers of the cicatricial epithelium in cultures of adult and fœtal lung are slight as compared with the remarkable *in vitro* behaviour of the alveolar epithelium towards coal and carmine particles. But phagocytosis by cells of the cicatricial epithelium does occur (see Plate 17, fig. 17).

(3) *Phagocytosis by White Blood Corpuscles.*—The white blood corpuscles occupy an intermediate position between the alveolar elements and the cicatricial epithelium as regards the degree of phagocytosis which they display towards coal and carmine particles. A fair number of coal, and especially carmine, particles are ingested by white cells inside the implant.

(4) *The Transformation of Alveolar Epithelial Cells into Fibroblastic Forms.*—In a series of 10 cultures of fœtal lung, inoculated some with coal and some with carmine immediately after implantation, and fixed on the fourth day of incubation, a curious transformation was noted.

Many of the detached alveolar epithelial cells underwent a transformation into elements which a histologist who was not cognizant with their derivation might well call fibroblasts. Transitional forms are numerous in sections of the implant, and were noted in both coal and carmine series. The outcome of this transformation is a cell which, varying from the branching to the fusiform has the appearance of the connective tissue cells of fœtal lung. That it is not such is strongly suggested by the following considerations:—(i) there are numerous transitions in shape between it and the typical phagocyte derived from the alveolar epithelium, or *alveolar phagocyte*, to extend Charlton

Briscoe's term (14). (ii) Most of these cells, like the swollen alveolar elements, contain intra-cellular carmine or coal particles. (iii) When the number of alveolar epithelial elements—either free or attached—is relatively small, the transitional and "fibroblastic" forms are numerous. (iv) The number of cells which have migrated into the plasma around the implant is totally insufficient to compensate for the missing alveolar cells, even assuming that all these migrated cells are of alveolar origin, which is most unlikely. (v) The alveolar elements cannot reasonably be supposed to have degenerated *in situ* since no cellular detritus is present.

Consequently the conclusion is forced upon one that the swollen and detached alveolar epithelial cells have become transformed into elements which look like fibroblasts. The conversion of phagocytic cells into fibroblasts has been stated to occur by Metchnikoff (30) and Mavrogordato (29), the latter, in the course of experiments on the Pneumoconioses, claiming that he can trace the macrophage "through all the stages to white fibres."

As to whether such fibroblastic forms should be regarded as true fibroblasts is mainly a question of nomenclature. Certainly on an embryological basis the fibroblastic forms derived from the alveolar epithelial cells can hardly be regarded as true fibroblasts in view of the different germ layers concerned in their formation.

(6) Commentary on the Foregoing Observations.

(i) *The Stimulus to Epithelial Hypertrophy.*—A clear cut instance of this is seen in that proliferation of the bronchial epithelium, which results in the bounding of the cut edges of the implant by the cicatricial epithelium. What is the stimulus which causes the bronchial epithelium, devoid, in the adult, of mitoses, to evolve in such a manner? The fact that the cicatricial epithelium is formed as readily around adult as fœtal implants suggests that no mysterious, endogenous factors peculiar to the growing bronchial epithelium of the fœtus can be invoked. The factor producing the hypertrophy of the bronchial epithelium seems to be simply the act of cutting the bronchus. In the effort to restore epithelial continuity the cells grow out from the cut end and invest the implant. As to *why* the epithelium of a cut bronchus in a tissue culture should be suddenly stimulated to grow, is as inexplicable as nerve, or, indeed, epithelial or any other form of regeneration. We are totally ignorant, as recently pointed out by Sherrington (38), of the physico-chemical repercussion on the cells in all such instances. We only know that damaged tissues tend to repair themselves.

(ii) *Detachment of the Alveolar Epithelial Cells.*—Not only the epithelium of the bronchi becomes hyperactive *in vitro*, but that of the alveoli exhibits the phenomena of detachment, movement and cell division. The swelling up and detachment of the alveolar epithelial cells is a process which does not occur to any appreciable extent in the normal —*i.e.*, non-irritated—lung. Regenerative activity on the part of the smaller cubical constituents of the alveolar epithelium is generally recognized. For instance, the relining of the alveoli with epithelium after pneumonia is due to the remaining cubical

elements. Some observers claim that the phagocytosis of foreign bodies is largely the work of detached alveolar elements (13, 14). Others that phagocytosis in the lung is entirely due to leucocytes (30, 41). Recently the view that dust-cells are really transformed endothelial leucocytes has found widespread support, especially in the United States (34, 35, 36).

In any case, the power to grow and to multiply is present in the alveolar epithelium *in vitro*. Here, as in the case of the bronchial epithelium, the stimulus for epithelial extension into the plasma may be the cutting—*i.e.*, the abolition of continuity—of the original epithelial organization.

The reason for the swelling of the alveolar epithelium within the implant, likewise that responsible for the frequent appearance of cell division in adult lung *in vitro*, is hard to conjecture. Possibly oxygen lack may be a factor.

The connective tissue of lung cultures, on the other hand, would seem to be largely inhibited by the presence of the dividing epithelial elements. Once degeneration of the alveolar and bronchial epithelium has begun, the connective tissue hypertrophy becomes increasingly active. The evidence of these experiments supports the idea of mutual inhibition between connective tissue and epithelia.

(iv) *The Phagocytosis of Coal and Carmine by Cells of the Cicatricial Epithelium.*—This is an illustration of how primitive protoplasmic functions, lost by specialized cells, may reappear, should such elements lose their previous specialization. Thus, mitoses are certainly extremely rare (and, in my experience, absent) in the ciliated bronchial epithelium of the adult lung. Similarly, these cells are almost unanimously regarded as being incapable of phagocytosis. Nor do they show sliding movements. But, under conditions of culture, structural specialization (cilia, definite shape, etc.) are lost, while their derivatives, as they grow around the implant, regain the properties of mitotic division, movement of a sliding type, and the power of ingestion of foreign bodies.

(v) *Phagocytosis by Alveolar Epithelial Cells.*—With regard to the possibility of phagocytosis by the alveolar epithelium *in vivo*, the question is still unsettled. That the alveolar epithelium is phagocytic *in vitro* appears to me to be certain from these experiments. But is it capable of ingesting foreign bodies (*e.g.*, dust particles) *in vivo*? The presence, in cultures of adult lung, of both carmine particles—which had been added to the culture—and of minute carbon granules within cells obviously derived from the alveolar epithelium suggests, at any rate, the possibility of phagocytosis *in vivo* by these cells. And this view is strengthened by the fact that free carbon granules could not be found in the control specimens of lung. But one fact stands out: *the alveolar epithelial cells* in vitro *are capable of swelling up and of detachment, and, under the conditions of these experiments, were found to be actively phagocytic for both coal and carmine particles.* The phenomenon often occurs on such a large scale that it is difficult to suggest any other interpretation.

The controversy over the origin of the dust cells is beyond the scope of this paper,

and is also one which has been recently discussed elsewhere (Drinker (17); Carleton (5)). I would point out, however, that phagocytic capacity of the alveolar epithelium certainly exists in some instances, even if it be debatable in others. Guieyesse-Pellissier (19, 20, 21) has shown that olive oil, if introduced into the lung, is rapidly absorbed—and finally digested—by cells derived from the alveolar epithelium. I have repeated his experiments and find that my observations confirm his. The phenomena of swelling up, and detachment of, the alveolar epithelium are so widespread in sections of such lungs, and the phagocytosis is so intense (*see* Plate 16, fig. 10, and Plate 17, figs. 18 and 19), that I do not think that anyone who cares to repeat these experiments can have any doubt as to the alveolar origin of the cells.* Another instance, *in vivo*, of detachment and swelling up of the units of alveolar epithelium "*en masse*" is to be seen in the lungs of cats subjected to "mustard gas"—dichlor-ethyl sulphide—(Carleton (2)). Here the alveolar epithelium desquamates from the alveolar wall to form nucleated mulberry-like spheres, as shown in Plate 16, fig. 11.

It is of importance to note that both in the cases of phagocytosis by alveolar epithelial cells *in vitro*, and in the case of ingestion of oil droplets by the same cells *in vivo*, no appreciable endothelial reaction can be demonstrated. This does not imply, of course, that the very ingenious experiments of Permar (34, 35, 36) on the phagocytosis of carmine suspension in saline by the lung *in vivo* are incorrect. But the introduction of a substance (*e.g.*, carmine) suspended in a fluid medium must necessarily invoke a vascular response. Permar's observations would rather point out the possibility that while one type of foreign body in a fluid medium elicits a phagocytosis by endothelial cells, another stimulates the alveolar epithelium to ingest it, when administered through inhalation.

We know that different types of micro-organisms are ingested by different types of leucocyte, and I think it possible that future researches will show that while one dust, administered under certain conditions, will elicit, say, an endothelial response, another kind of foreign body will be ingested by cells derived from the alveolar epithelium.

(7) Summary.

The results obtained from these experiments on the *in vitro* growth of fœtal and adult lung, and the deductions derived from them, may be summarized as follows:—

(1) The cover-slip slide (hanging drop) technique favours growth from the implant into the medium more than the tube method of cultivation, though the view that only

* The swelling of these alveolar phagocytes is cytoplasmic rather than nuclear, especially once detachment has occurred. Probably—as suggested to me by my friend Mr. J. B. S. Haldane—the extraordinary œdema of these cells must be attributed to the digestion of the oil, the breaking down of which causes an increase in the number of molecules, whence the rise of the intra-cellular osmotic pressure, and the absorption of water through the cell membrane to compensate this. That the alveolar epithelial cells really do digest the oil droplets is shown by the variable degree of staining of these with osmic acid—black to pale grey—and the formation of cytoplasmic vacuoles around the more faintly staining oil droplets.

survival, and not growth, of tissues occurs by the latter method was found to be without foundation in the material studied.

(2) In the invasion of the medium in plasma cultures of fœtal lung (from half to full term), the following types of growth play varying parts :—

(*a*) *Epithelial growth,* in which a sheet-like extension occurs from the epithelium of cut alveoli or bronchi. The cells become increasingly polymorphic as they extend further from the original focus or foci of growth. The fusiform shape of the epithelial cells at the border of the zone of invasion is due largely to the absence of mutual cell-pressure and to the resistance of the fibrin meshwork. Owing to the fibrinolytic power of epithelial tissues *in vitro,* vacuoles are often formed in the medium. Such vacuoles tend to be lined by epithelial elements.

(*b*) *Connective tissue growth,* due to direct extension of the mesenchyme of the implant into the medium. Such growth is of the radiating type.

(*c*) The complex phenomenon of "*membrane-formation.*" In this type of growth part of the cicatricial epithelium becomes detached from the implant. Outgrowth of mesenchymatous and epithelial cells (alveolar or bronchial) takes place in the cleft thus formed. The invasion by these cells of the membrane cavity is usually a transitory phenomenon, the outgrowth of connective tissue elements not being commensurate with the enlargement of the membrane cavity. Consequently the bridges, etc., invading the membrane cavity are very liable to regression.

(3) In the case of adult lung, invasion of the medium is restricted to a scanty outgrowth of fibroblasts.

(4) The chief changes within the implant, in both fœtal and adult lung, resolve themselves into :—

(*a*) The swelling up and detachment of the alveolar epithelial cells.

(*b*) The frequent appearance of mitotic figures in these—even in the case of adult lung, from which they are normally absent.

(*c*) The formation of a cicatricial epithelium. This is derived, in the case of cultures of fœtal and adult lung, from the cut edges of the bronchi. Its dedifferentiation appears to be the rule.

(*d*) The fibroid transformation of the implant, due to hypertrophy of the connective tissue (collagen) fibres which ultimately strangle and cause to degenerate the epithelial elements.

(5) The stimulus causing the bronchial epithelium to form the cicatricial epithelium undoubtedly appears to be the cutting of the bronchi. Rupture of epithelial continuity *in vitro* would thus seem to be as potent a factor in inducing its repair as it is *in vivo.*

(6) The inhibition of connective tissue by epithelium appears to exist in cultures of

lung. It is certain that the "fibrosis" of the implant only commences when the epithelial elements within it begin to degenerate.

(7) Cultures of fœtal and adult lung are actively phagocytic in respect of sterile coal and carmine particles. Both the cells of the zone of invasion and those inside the implant participate in this process. The phagocytic cells comprise :—

(*a*) *Leucocytes*, as has often been observed *in vitro*.

(*b*) *Alveolar phagocytes*, the swollen and detached cells derived from the alveolar epithelium. The ingestion of coal or carmine particles by these "alveolar phagocytes" is very marked inside implants the surface of which was lightly dusted with the pigment.

(*c*) *Dedifferentiated Cells of the Cicatricial Epithelium.*—This is of rarer occurrence than (*a*) or (*b*).

(8) The transformation of coal or carmine laden alveolar phagocytes into branching or fusiform cells simulating fibroblasts (but of a different embryological origin) was noted on a large scale in certain cultures.

(9) The scantiness *in vitro* of any phagocytic response on the part of the endothelium of the blood vessels is noteworthy.

(10) Although observations on the *in vitro* behaviour should only be extended with extreme caution to the organ *in vivo*, these experiments indicate at least the possibility of phagocytosis by the alveolar epithelium of dust particles. It is significant that the stages in the production of alveolar phagocytes *in vitro* has at least one counterpart *in vivo*, namely, the phagocytosis and subsequent digestion of oil by the alveolar epithelium. In this case the cells which absorb the oil *in vivo* are identical in origin and appearance with those which ingest coal and carmine particles *in vitro*.

(11) Slight phagocytic capacity is shown by the cells of the cicatricial epithelium, a point of interest in view of the derivation of such cells from the originally highly specialized bronchial epithelium. Its dedifferentiated products, however, may reacquire the fundamental properties of movement and phagocytosis at the sacrifice of their previous specialization.

(8) Explanation of Plates.

Plate 16 represents untouched microphotographs of both "whole mounts" and sections of plasma cultures of fœtal and adult lung.

The original magnification is indicated for each figure, there being no reduction.

The following abbreviations are used to indicate the type of culture : *C.s.s.* = Cover-slip slide, *C.s.c.* = Cover-slip cell, *T.* = Tube.

Lettering of Plate 16 as follows :—

A.E. = alveolar epithelial cells.
A.P. = alveolar phagocyte.
B.E. = bronchial epithelium.
C.D. = detached cicatricial epithelium investing the implant.

C.E. = cells of cicatricial epithelium investing the implant.
C.P. = collagen plates.
C.T. = connective tissue of bronchus.
E.A. = epithelial extension from alveoli.
F. = fibroblasts.
I. = implant.
I.C. = ingrowths of cicatricial epithelium into implant.
Mi. = mitotic figure.
P. = blunt processes, invested by fibroblasts and containing alveolar epithelial cells, developed from the implant.
Pl. = plasma.
Z.I. = zone of invasion.
X. = see the explanation of the figure.

PLATE 16, FIGS. 1 TO 11.

FIG. 1.—General view of a 5-day c.s.s. culture (No. 521) of fœtal lung (cat). *Technique.*—Carnoy fixation; stained iron hæmatoxylin. × 30. The implant (I) with the bronchi and developing alveoli is darkly stained. The limit of the zone of invasion is indicated at Z.I. Its constitution is largely epithelial, partly mesenchymatous. The alveolar outgrowth E.A. is figured at a higher magnification in text-fig. 2. The vacuoles, formed by the fibrinolytic action of the epithelial cells, are shown.

FIG. 2.—Membrane-formation in its later stages in a c.s.s. culture (No. 692) of fœtal lung (cat) after 54 hours' incubation. × 48. *Technique.*—5 per cent. formol in 0·9 per cent. NaCl; iron hæmatoxylin. The membrane cavity—still bounded by cells representing a detached portion of the cicatricial epithelium—has enormously enlarged. The implant is at the "top" of the photograph. Curious blunt processes are seen at P. Such a culture has grown "within itself," *i.e.*, within the "skin" of cicatricial epithelium investing it which has undergone increasing detachment and extension.

FIG. 3.—The lobed processes ("P" in the preceding figure) seen with a higher magnification (× 126). Each process consists of an investment of mesenchymatous cells, containing elements derived from the alveolar epithelium.

FIG. 4.—Another example of membrane-formation. Fœtal lung (cat); c.s.s. culture (No. 741); three days' incubation. × 30. *Technique.*—5 per cent. formol in 0·9 per cent. NaCl; iron hæmatoxylin. Stretching across the membrane cavity towards its investing layers of cells are fibroblasts—thin and darkly stained—and epithelial cells—dot-like. Invasion of the medium by epithelial elements (X.) has occurred from the periphery of the membrane.

FIG. 5.—Invasion of the medium seen in a section vertical to the surface of the plasma. Culture No. 377. T. Seven days' continuous cultivation of fœtal lung (cat). Daily irrigation with 66 per cent. Ringer-Locke solution. Fixed dilute Bouin; stained iron hæmatoxylin. × 136. Note the extensive sheet-like zone of invasion, composed in this case of cells derived from the cicatricial epithelium.

FIG. 6.—The fully-formed cicatricial epithelium completely investing the implant. The bronchial derivation of the cicatricial cells is very clear. Ingrowths of the cicatricial epithelium into the implant have occurred. Vertical section of the implant in an eight-day T. culture (No. 474) of adult lung (cat). × 146. *Technique.*—Dilute Bouin; iron hæmatoxylin and Ponceau S substitute for Van Gieson.

FIG. 7.—Fibroid transformation of a nine-day T. culture (No. 537) of fœtal lung (cat) in section. × 400. *Technique.*—Dilute Bouin ; iron hæmatoxylin and eosin. C.P. = collagen fibres and plates in which the nuclei of the alveolar cells lie embedded. Mi = mitotic figure.

FIG. 8.—Swelling and detachment of the alveolar epithelial cells in a section of a 24 hours T. culture (No. 630) of adult lung (cat). × 93. *Technique.*—Dilute Bouin ; iron hæmatoxylin and Ponceau S substitute for Van Gieson.

FIG. 9.—A coal particle (X) added to the culture, being surrounded by cells derived from the cicatricial epithelium (C.E.) in a section of a four-day T. culture (No. 652) of fœtal lung (cat). × 400. *Technique.*—Dilute Bouin ; Ehrlich's hæmatoxylin and orange G.

FIG. 10.—The phagocytosis of olive oil, introduced by intratracheal injection into the cat's lung. The cells responsible for the phagocytosis, and subsequent digestion, of the oil are derived from the alveolar epithelium. × 950. *Technique.*—Fixation in Flemming's fluid ; section stained in iron hæmatoxylin and lichtgrün. These cells and the elements which undergo detachment from the alveolar wall *in vitro* are the same.

FIG. 11.—Section of a cat's lung exposed to mustard gas and killed four days after exposure. The detachment of the alveolar epithelial cells is here complete ; each alveolus contains a mulberry-like mass of such elements. × 650. Another example of œdema and detachment of the alveolar epithelium *in vivo*. X = capillaries acutely congested and containing many white blood corpuscles. *Technique.*—Bouin's fluid ; iron hæmatoxylin and eosin.

PLATE 17, FIGS. 12 TO 24.

High-power camera lucida drawings of sections.

FIG. 12.—× 2780. Stages in the detachment of alveolar epithelial cells in the implant of a 24 hours' culture of adult lung (cat). *Technique.*—Dilute Bouin and iron hæmatoxylin. *a*, *b* and *c* represent consecutive stages in the œdema and detachment of the alveolar epithelial cell *in vitro*.

FIG. 13.—× 2780. The same culture. Another œdematous alveolar epithelial cell. Note the vacuolated cytoplasm—typical in such cells. The increase in volume of the cell is largely cytoplasmic, the nucleus remaining the same in size after a brief initial increase.

FIG. 14.—× 2780. The same culture. A detached alveolar cell. Both it and its predecessors in figs. 12 and 13 contain minute carbon particles. Since no dust was added to the culture the carbon must have been in the lung (if not actually in the cells) prior to cultivation.

FIG. 15.—× 1300. Illustrating the phagocytosis of carmine granules by the alveolar epithelium *in vitro*. A four days' culture of adult lung (cat) inoculated with carmine at the time of implantation. *Technique.*—Dilute Bouin ; Ehrlich's hæmatoxylin and orange G. *a* to *e* show the different stages in the swelling up and ingestion of carmine by cells derived from the cubical cells of the alveolar epithelium. Such a cell is depicted at *a*. The same cells which have ingested the carmine contain carbon granules, which can only represent carbon inhaled *in vivo*.

FIG. 16.—× 2780. The same culture. A just-detached alveolar phagocyte containing coal and carmine granules.

FIG. 17.—× 2780. Intra-cellular carmine granules in a cell of the cicatricial epithelium of a four-day culture of fœtal lung (cat). *Technique.*—Dilute Bouin ; Ehrlich's hæmatoxylin and orange G. The existence of this phenomenon, though rare, shows that, *in vitro*, phagocytosis may occur by dedifferentiated cells derived from the previously highly specialized bronchial epithelium.

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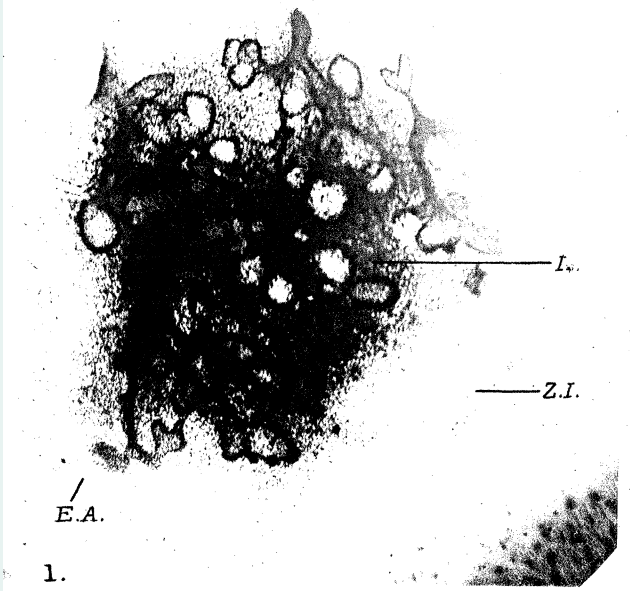

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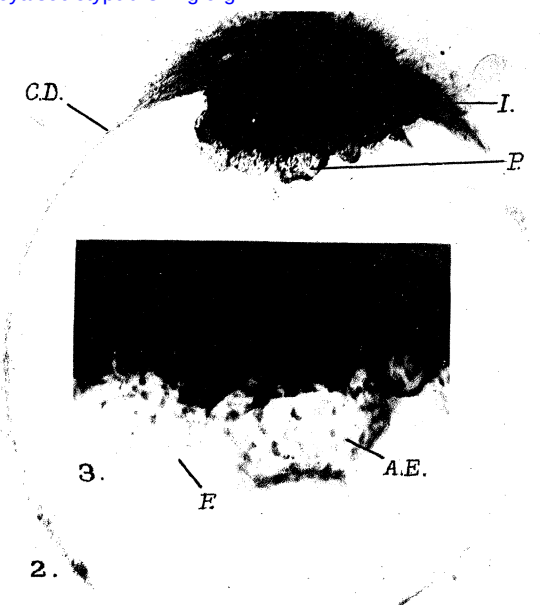

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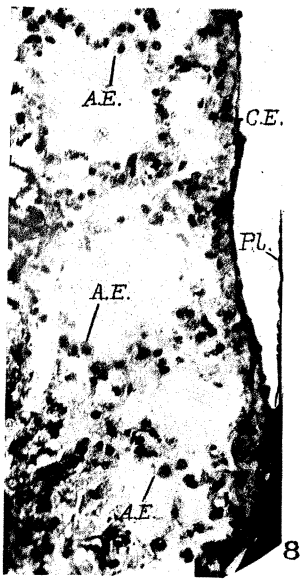

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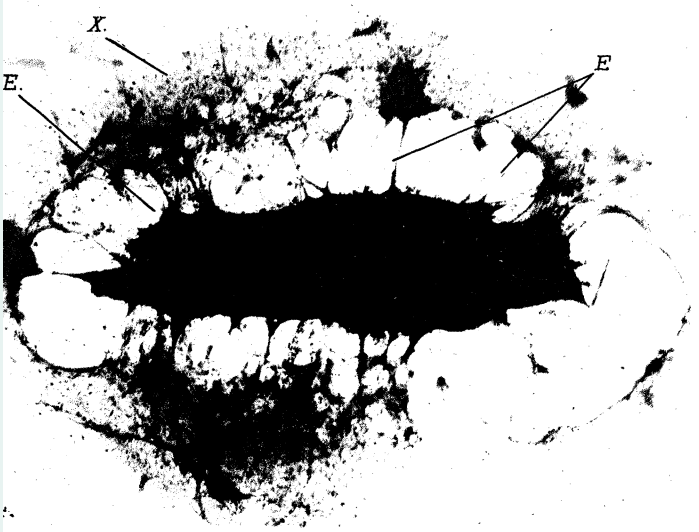

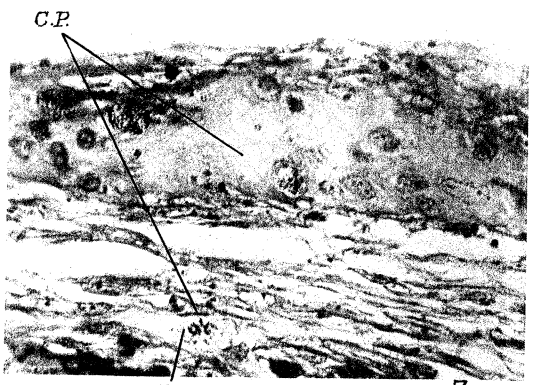

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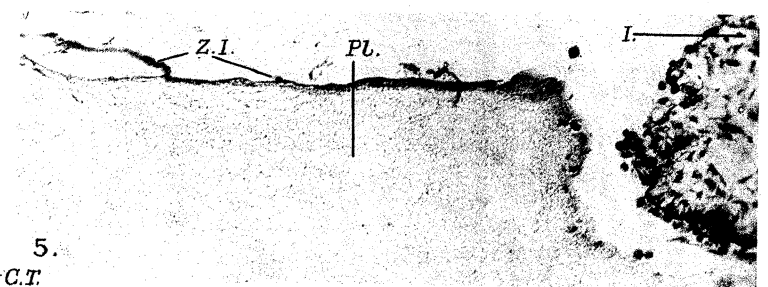

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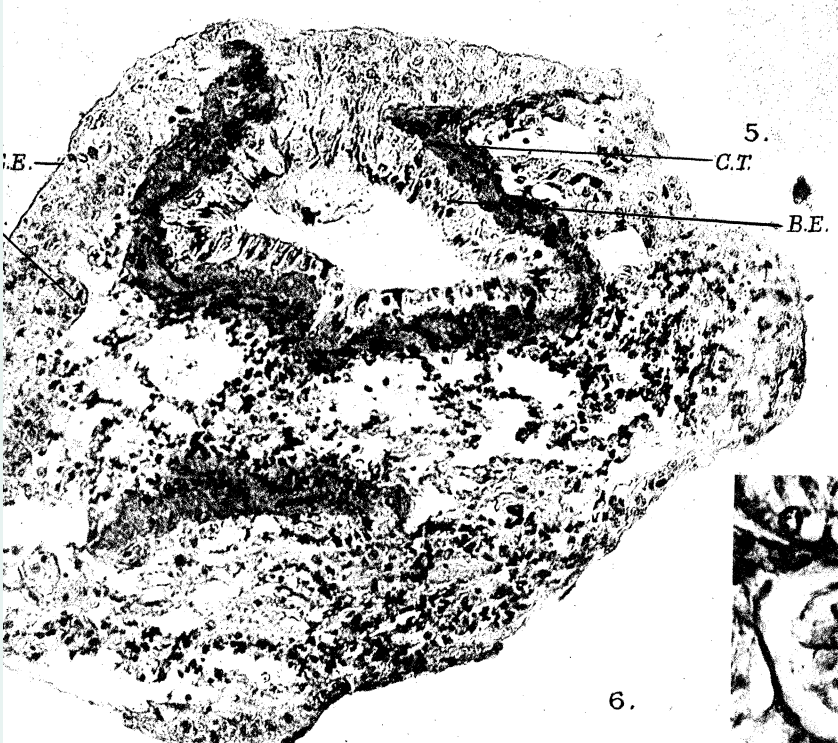

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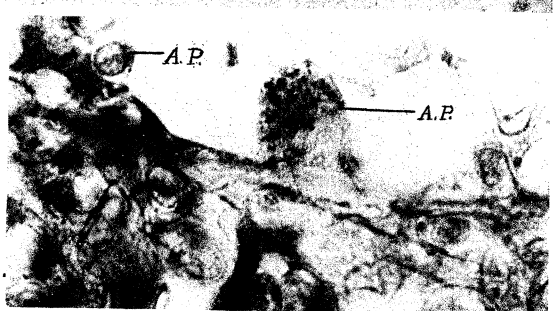

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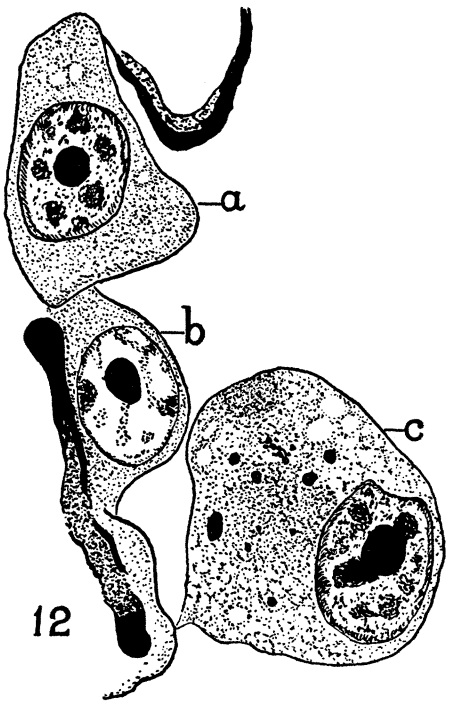

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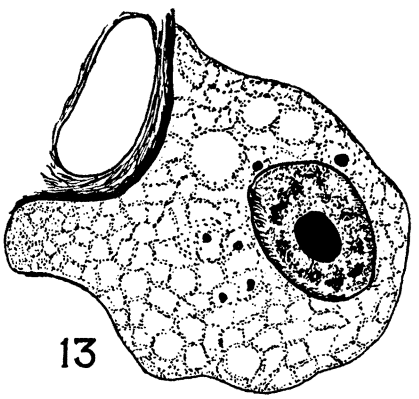
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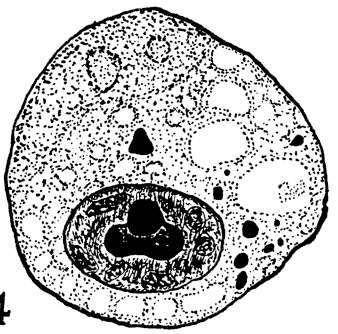
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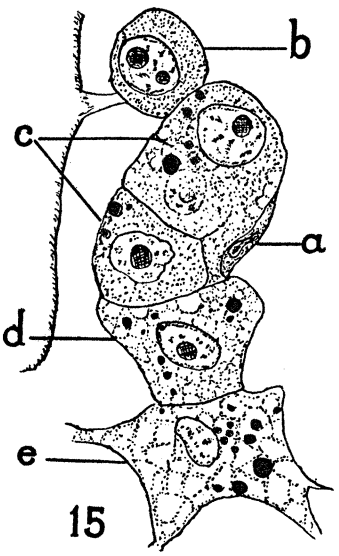

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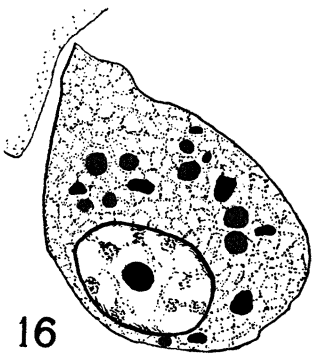
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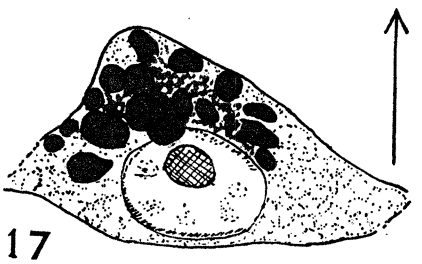
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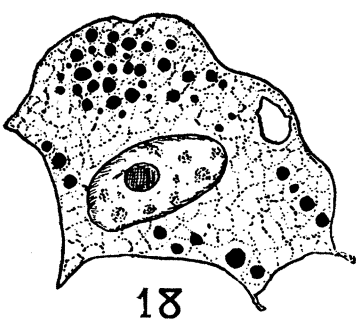
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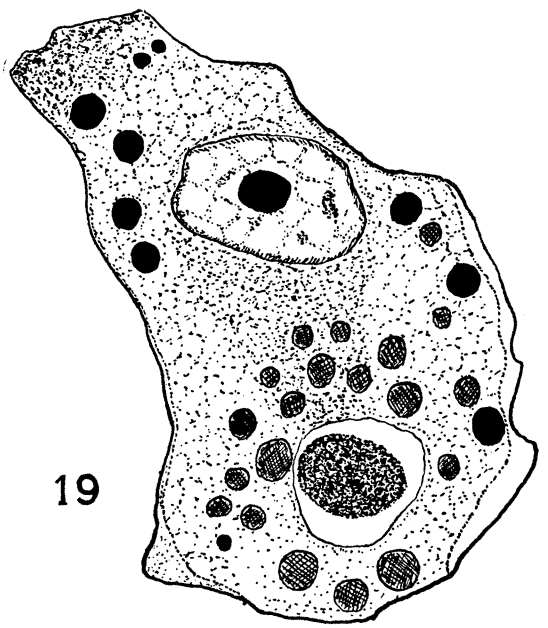
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FIGS. 18 and 19.—×2700 (approx.). Detached cells, derived from the alveolar epithelium of the cat's lung after an introtracheal injection of olive oil. Animal killed 24 hours after injection. *Technique.*—Fixation in Flemming's fluid. Stained lightly in iron hæmatoxylin, counterstained in lichtgrün.

FIG. 18 is such a cell shortly after phagocytosis of the oil.

FIG. 19 represents a cell in which phagocytosis is being followed by digestion of some of the oil, now present as greyish droplets. The enormous increase in cytoplasmic, but not nuclear, volume is remarkable.

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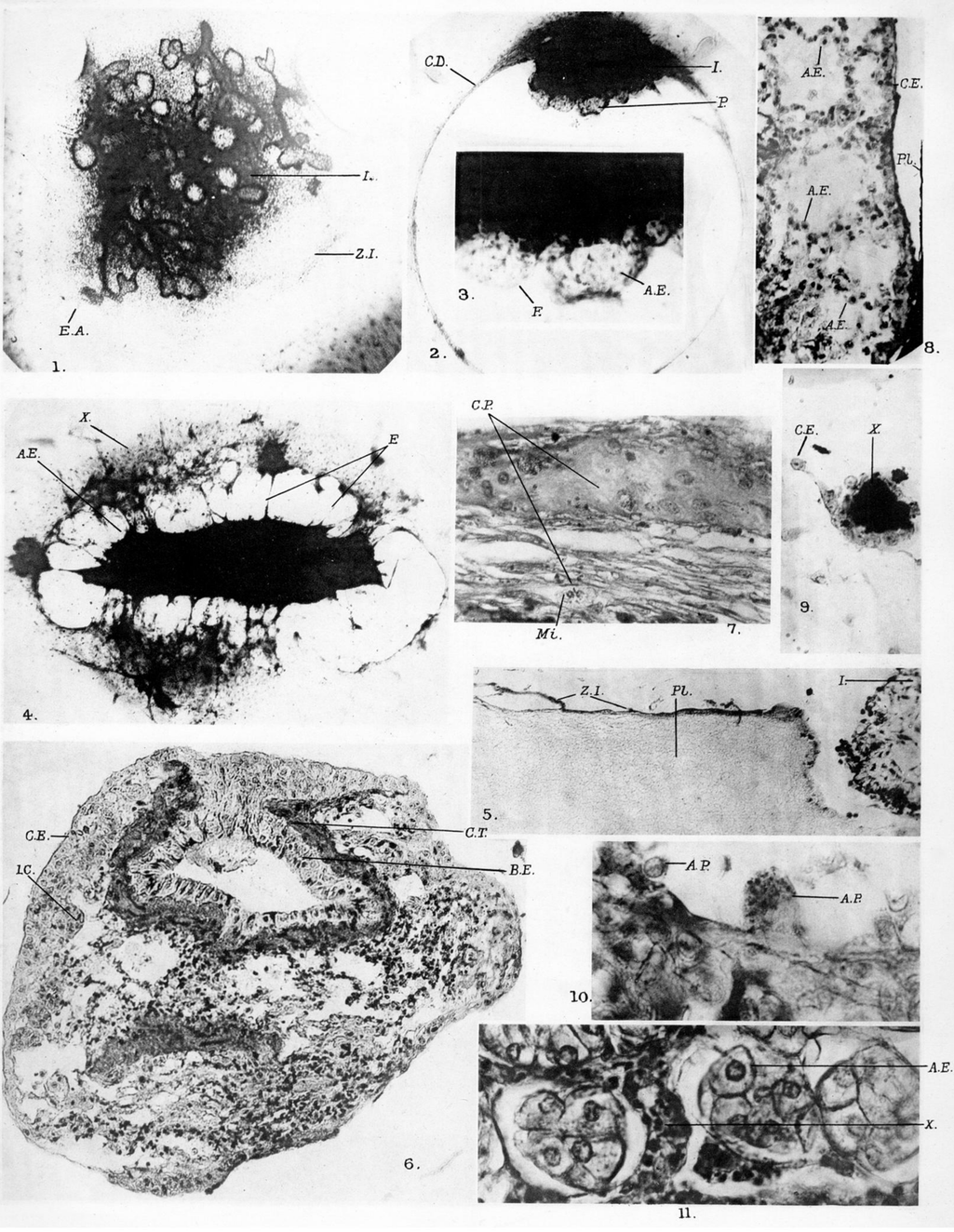

PLATE 16, FIGS. 1 TO 11.

FIG. 1.—General view of a 5-day c.s.s. culture (No. 521) of fœtal lung (cat). *Technique.*—Carnoy fixation; stained iron hæmatoxylin. × 30. The implant (I) with the bronchi and developing alveoli is darkly stained. The limit of the zone of invasion is indicated at Z.I. Its constitution is largely epithelial, partly mesenchymatous. The alveolar outgrowth E.A. is figured at a higher magnification in text-fig. 2. The vacuoles, formed by the fibrinolytic action of the epithelial cells, are shown.

FIG. 2.—Membrane-formation in its later stages in a c.s.s. culture (No. 692) of fœtal lung (cat) after 54 hours' incubation. × 48. *Technique.*—5 per cent. formol in 0·9 per cent. NaCl; iron hæmatoxylin. The membrane cavity—still bounded by cells representing a detached portion of the cicatricial epithelium—has enormously enlarged. The implant is at the "top" of the photograph. Curious blunt processes are seen at P. Such a culture has grown "within itself," *i.e.*, within the "skin" of cicatricial epithelium investing it which has undergone increasing detachment and extension.

FIG. 3.—The lobed processes ("P" in the preceding figure) seen with a higher magnification (× 126). Each process consists of an investment of mesenchymatous cells, containing elements derived from the alveolar epithelium.

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FIG. 5.—Invasion of the medium seen in a section vertical to the surface of the plasma. Culture No. 377. T. Seven days' continuous cultivation of fœtal lung (cat). Daily irrigation with 66 per cent. Ringer-Locke solution. Fixed dilute Bouin; stained iron hæmatoxylin. × 136. Note the extensive sheet-like zone of invasion, composed in this case of cells derived from the cicatricial epithelium.

FIG. 6.—The fully-formed cicatricial epithelium completely investing the implant. The bronchial derivation of the cicatricial cells is very clear. Ingrowths of the cicatricial epithelium into the implant have occurred. Vertical section of the implant in an eight-day T. culture (No. 474) of adult lung (cat). × 146. *Technique.*—Dilute Bouin; iron hæmatoxylin and Ponceau S substitute for Van Gieson.

FIG. 7.—Fibroid transformation of a nine-day T. culture (No. 537) of fœtal lung (cat) in section. × 400. *Technique.*—Dilute Bouin; iron hæmatoxylin and eosin. C.P. = collagen fibres and plates in which the nuclei of the alveolar cells lie embedded. Mi = mitotic figure.

FIG. 8.—Swelling and detachment of the alveolar epithelial cells in a section of a 24 hours T. culture (No. 630) of adult lung (cat). × 93. *Technique.*—Dilute Bouin; iron hæmatoxylin and Ponceau S substitute for Van Gieson.

FIG. 9.—A coal particle (X) added to the culture, being surrounded by cells derived from the cicatricial epithelium (C.E.) in a section of a four-day T. culture (No. 652) of fœtal lung (cat). × 400. *Technique.*—Dilute Bouin; Ehrlich's hæmatoxylin and orange G.

FIG. 10.—The phagocytosis of olive oil, introduced by intratracheal injection into the cat's lung. The cells responsible for the phagocytosis, and subsequent digestion, of the oil are derived from the alveolar epithelium. × 950. *Technique.*—Fixation in Flemming's fluid; section stained in iron hæmatoxylin and lichtgrün. These cells and the elements which undergo detachment from the alveolar wall *in vitro* are the same.

FIG. 11.—Section of a cat's lung exposed to mustard gas and killed four days after exposure. The detachment of the alveolar epithelial cells is here complete; each alveolus contains a mulberry-like mass of such elements. × 650. Another example of œdema and detachment of the alveolar epithelium *in vivo*. X = capillaries acutely congested and containing many white blood corpuscles. *Technique.*—Bouin's fluid; iron hæmatoxylin and eosin.

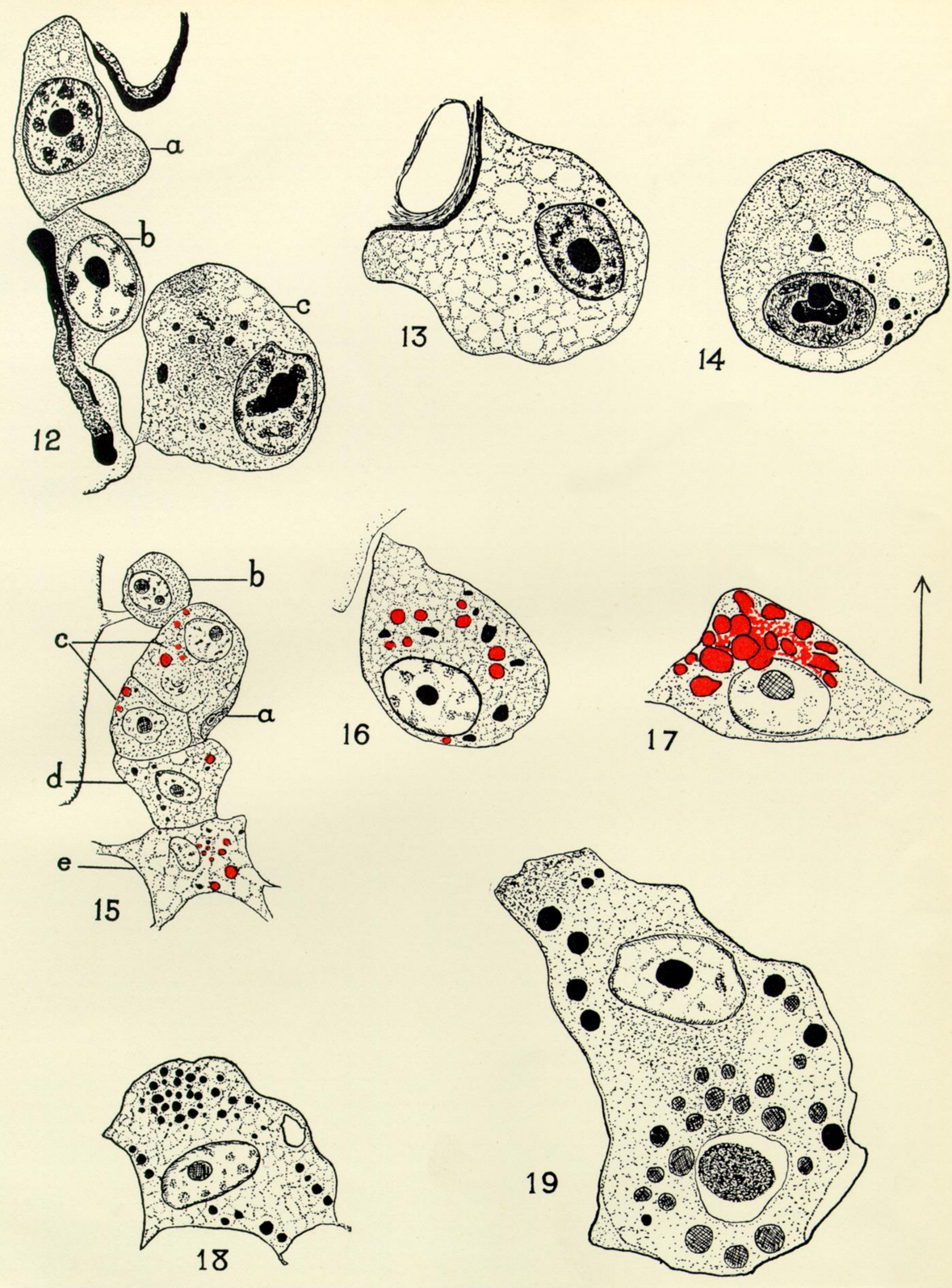

PLATE 17, FIGS. 12 TO 24.

High-power camera lucida drawings of sections.

FIG. 12.—× 2780. Stages in the detachment of alveolar epithelial cells in the implant of a 24 hours' culture of adult lung (cat). *Technique.*—Dilute Bouin and iron hæmatoxylin. *a*, *b* and *c* represent consecutive stages in the œdema and detachment of the alveolar epithelial cell *in vitro*.

FIG. 13.—× 2780. The same culture. Another œdematous alveolar epithelial cell. Note the vacuolated cytoplasm—typical in such cells. The increase in volume of the cell is largely cytoplasmic, the nucleus remaining the same in size after a brief initial increase.

FIG. 14.—× 2780. The same culture. A detached alveolar cell. Both it and its predecessors in figs. 12 and 13 contain minute carbon particles. Since no dust was added to the culture the carbon must have been in the lung (if not actually in the cells) prior to cultivation.

FIG. 15.—× 1300. Illustrating the phagocytosis of carmine granules by the alveolar epithelium *in vitro*. A four days' culture of adult lung (cat) inoculated with carmine at the time of implantation. *Technique.*—Dilute Bouin; Ehrlich's hæmatoxylin and orange G. *a* to *e* show the different stages in the swelling up and ingestion of carmine by cells derived from the cubical cells of the alveolar epithelium. Such a cell is depicted at *a*. The same cells which have ingested the carmine contain carbon granules, which can only represent carbon inhaled *in vivo*.

FIG. 16.—× 2780. The same culture. A just-detached alveolar phagocyte containing coal and carmine granules.

FIG. 17.—× 2780. Intra-cellular carmine granules in a cell of the cicatricial epithelium of a four-day culture of fœtal lung (cat). *Technique.*—Dilute Bouin; Ehrlich's hæmatoxylin and orange G. The existence of this phenomenon, though rare, shows that, *in vitro*, phagocytosis may occur by dedifferentiated cells derived from the previously highly specialized bronchial epithelium.

FIGS. 18 and 19.—×2700 (approx.). Detached cells, derived from the alveolar epithelium of the cat's lung after an introtracheal injection of olive oil. Animal killed 24 hours after injection. *Technique.*—Fixation in Flemming's fluid. Stained lightly in iron hæmatoxylin, counterstained in lichtgrün.

FIG. 18 is such a cell shortly after phagocytosis of the oil.

FIG. 19 represents a cell in which phagocytosis is being followed by digestion of some of the oil, now present as greyish droplets. The enormous increase in cytoplasmic, but not nuclear, volume is remarkable.

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