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CONTRIBUTION TO THE STUDY OF EPITHELIAL MOVEMENT. THE CORNEAL EPITHELIUM OF THE FROG IN TISSUE CULTURE

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NINE FIGURES

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The mechanism of the movement of epithelium, embryonic as well as adult, has been discussed by various observers, but still further investigation is needed by the use of the tissue-culture method, where direct observation is possible.

The experimental work reported in the present paper is concerned with a study of the movement of corneal epithelium, and especially with its behavior with reference to certain mechanical supports.

Several papers have already appeared which deal with the movement of epithelium of various kinds in vitro (Ruth, '11, Champy, '14, Loeb, '02, Osowski, '14, and others). The observation of frog skin in vitro has been described by Holmes ('14) and Uhlenhuth ('14) recently in detail. Previous to this, Harrison ('10) Carrel and Burrows ('11), Lambert and Hanes ('13), and others mentioned it briefly in their papers on tissue culture. Among the works which deal with the culture of the corneal epithelium, that of Oppel ('12), who made use of the cornea of certain warm-blooded animals in his investigations, demands special attention. Harde ('16) made brief mention of an active lateral spreading of the corneal epithelium in the culture of vaccinia with corneal tissue. However, the materials which were used by Oppel and Harde are evidently not very suitable for direct observation. For this purpose cornea of more simple structure is desirable. It must be added that a number of investigations on the problem of the wound healing of the cornea have been made, such as those of Peters ('85), Salzer ('11), Löwenstein ('13), and others, which must, of course, be taken into consideration.

I. BEHAVIOR OF EPITHELIUM CULTIVATED IN VITRO

The cornea of the adult frog (especially R. pipiens) was used. After thoroughly washing its whole surface with sterilized Ringer's solution by means of a pipette, the entire cornea was cut out with a razor and put into Ringer's solution (or serum), after which it was divided into small pieces with very sharp scissors so that the fragments showed sharp edges. Pieces cut radially were preferred.

The cultures were all made by the hanging-drop method (Harrison, '10), the technique of which need not be detailed here. The piece of cornea was taken from the Ringer's solution and dropped on the surface of the cover-glass; excess solution was removed and a drop of plasma (or serum) run over the fragment; autoplasma was used in most of the cultures. The cover-glass was then inverted upon a thin glass ring and sealed on with vaselin (Harrison, '14).

Though all precautions were taken to keep the cultures free from bacteria, it was sometimes necessary to throw away a whole series as a result of infection. This was due obviously to the difficulty of perfect sterilization of the tissue.

In the aggregate, more than 1800 cultures were made, and in nearly all of the experiments two kinds of culture medium (plasma and serum) were used. The following descriptions are the results of the study of about 1500 cultures which were free from faulty technique.

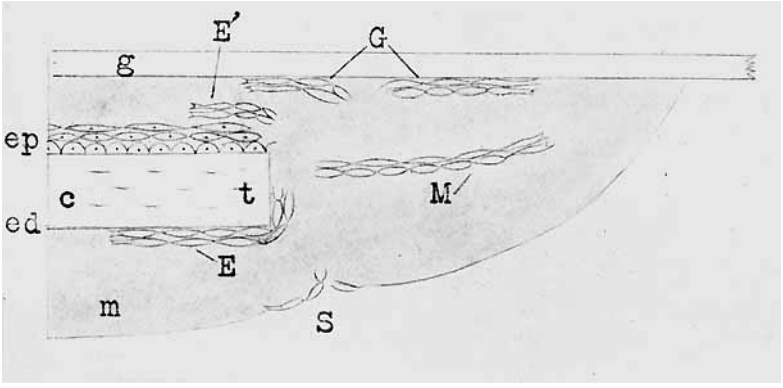

Fig. 1. Diagram showing various types of movement of corneal epithelium cultivated in plasma. *g*, cover-glass; *c*, tissue of cornea cultivated; *ep*, corneal epithelium; *ed*, endothelial surface; *m*, culture medium; *t*, cut end of the piece of cornea. Cell movement into the plasma (*M*), along the endothelial surface (*E*), on the epithelial surface (*E'*), along the cover-glass (*G*), and along the lower surface of plasma (*S*).

1. Description of the types of epithelial movement in plasma (fig. 1)

The cornea of the frog was very suitable for this purpose. For the observance of the intimate cell structure and a closer study of the mechanism of cell movement, high powers could be used. It was, of course, necessary to supplement the study of the living tissue by fixed and stained preparations of whole cultures and by serial sections. The observation of the living cultures was limited to the first week.

a. Movement in the medium. When a culture of cornea, freshly prepared in vitro, was examined under the microscope, the edges of the piece were seen to be sharply defined. One or two hours later the epithelium on the cut ends gave the impression of becoming a little translucent and swollen, a narrow clear rim appearing along the edges. Here and there, around the edges of the fragment, isolated round epithelial cells were to be seen, singly or in groups, having been detached by mechanical injury during the operation.

There was a short latent period before any movement was noticed, the first cellular activity appearing between the third and tenth hours. Accordingly, examination after twelve to twenty-four hours showed an active outgrowth of epithelial cells presenting amoeboid processes. As a rule, the corneal epithelium showed characteristic sheet-like extension during the period of active movement; the advancing edge was always furnished with an amoeboid border of hyaline ectoplasma, as has been described by Harrison ('10), Carrel and Burrows ('11), Lambert and Hanes ('13) and Holmes ('14). Some of the epithelium exhibited marked motility, recalling the movements of amoeba, so that an exact camera-lucida drawing could not be made; pseudopodia were formed, and through their activity the cells changed shape or moved from place to place. Some of the cells which showed filiform processes were over 0.2 mm. in length.

The strong tendency of the cells to lateral spreading brought about the formation of a continuous membrane, extending nearly horizontally, usually slanting upward a little toward the end. The spreading membrane also changed its direction of movement, showing contraction under various circumstances. Growth in strands was also observed. The growing epithelium, usually two cells in thickness, sometimes covered an area a little larger than the original corneal piece, whereby the cells became flattened and the intercellular spaces grew wider.

There was apparently little growth after the third (sometimes the fourth) day. The tissue itself then gave the impression of being less compact and more translucent than formerly. The epithelial cells showed a tendency to round off. The rupture

of strands or sheets of cells into isolated masses was of frequent occurrence. The number of round cells increased rapidly from day to day, and generally fat droplets grew in number and size with the age of the culture, until the cells were often literally packed with them. No marked increase of mitotic figures was seen.

The activity of cell movement into the plasma depends on the consistency of the latter. Around the explanted tissue, liquefaction and retraction of the plasma were often observed which caused changes of the arrangement of cells.

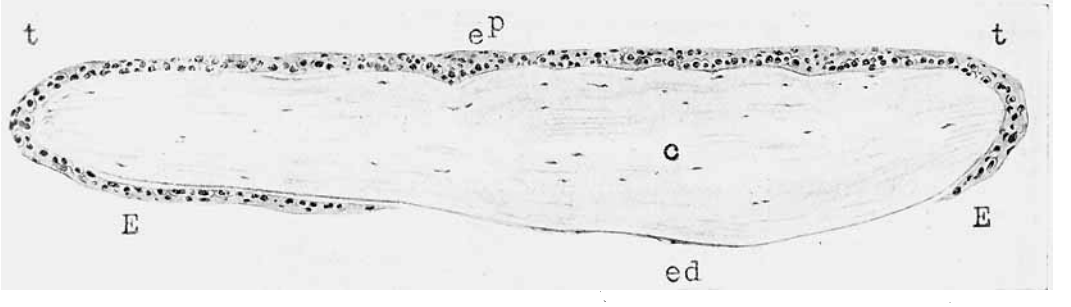

Fig. 2 Vertical section of corneal tissue cultivated in plasma. Experiment XXXVIII, 5, showing the epithelial movement (*E*) along the endothelial surface (*ed*). Age of the culture, four days. Drawn from one of the serial sections. *t*, cut ends; *ep*, epithelial, *ed*, endothelial surface; *c*, part of connective tissue of cornea. × 98.

b. Movement on tissue. The next important type of the epithelial movement is that on the corneal tissue. By virtue of this, the growing epithelium spreads over the edges of the fragment and along the endothelial surface. Figure 2 shows an example of this very clearly. A movement of this type occurred in some cases on all edges of a fragment and in others only on a part of it, combined with other types. It was also observed that part of the cut end of the epithelium might remain almost inactive, while in the other part marked activity occurred.

c. Other types of movement. Whenever the growing epithelial cells came into contact with the cover-glass, they moved actively over it. The character of cell movement, however, was essen-

tially the same as on the endothelial surface. The advancing edge of the growth was composed of a very delicate sheet of protoplasm, showing amoeboid movement, with numerous branching hyaline pseudopodia. When the delicate membrane spread out to an extreme degree, it resulted in the breaking up of the sheet into isolated masses.

In a small percentage of the cultures the spreading of cells over the outer epithelial surface of the cornea (fig. 1, *E'*) as well as along the lower surface of the culture medium (fig. 1, S) was noted.

The elements of the connective tissue showed neither active growth nor movement. The study of the lymphocytes found in the corneal tissue needs special investigation.

2. Movement of the epithelium cultivated in serum

Generally, the cornea cultivated in serum showed some details of cell movement, different from those noted in plasma cultures. No amoeboid migration of the cells into the medium took place. During the first and second hours, the cells of the edges became clear and round, sometimes even swollen in appearance; they soon started to move.

The most striking and constant phenomenon noted in the use of serum was the spreading out of epithelium over the tissue itself, especially on the endothelial surface. The epithelial rim extended usually parallel with the cut edges, showing amoeboid movement on the advancing border. The rapidity of spreading out of cells was sometimes quite remarkable. In a small percentage of the cultures, movement of cells was also observed on the original outer epithelial surface of the cornea.

If single cells or a part of the rim were in contact with the coverglass, they clung to it and spread over the surface of the glass with marked activity. The boundaries of individual cells were often hard to distinguish under the microscope. When such preparations are properly impregnated with silver nitrate (Lewis and Lewis, '12) the intercellular spaces can be clearly demonstrated (fig. 3). When the delicate membrane had extended to the

utmost degree, the cells along the periphery became loose and isolated.

The appended tables give some examples of the frequency of various types of epithelial movement in plasma (table 1) and in serum (table 2), respectively.

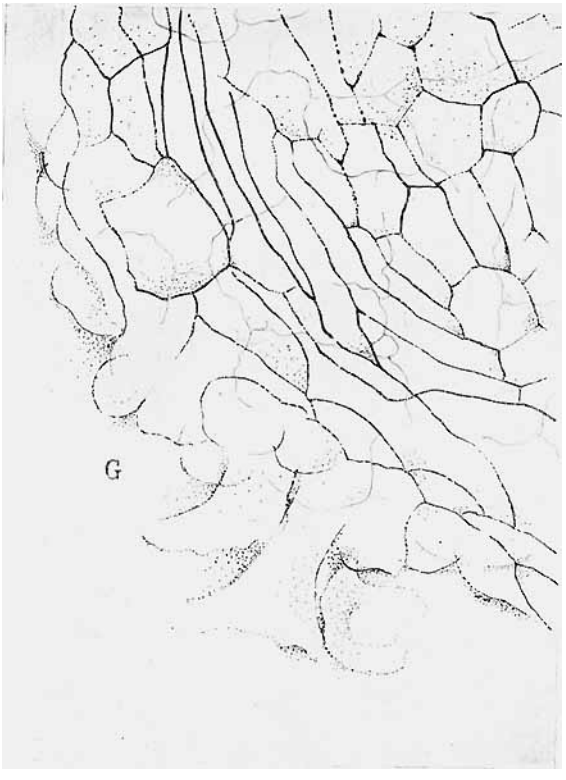

Fig. 3 Experiment XLIX, 2. Silver impregnation, demonstrating the boundaries of individual cells. Drawn from a part of actively moving border closely attached to the lower surface of cover-glass. Cultivated in serum; three days' growth. *G*, advancing border. × 450.

3. Velocity of epithelial movement

The velocity of cell movement in vitro exhibits a considerable variation under different circumstances, and, as stated before,

TABLE 1

Cultures in plasma

SERIES NO.	NUMBER OF PREPARATIONS	CELL MOVEMENT					NO MOVEMENT
		Into medium	On endothelial surface	On epithelial surface	On cover glass	On surface film	
50 a	18	14	14	1	1		
50 b	14	5	13				1
46	10	3	10		4		
45	6	5	5	2	2	1	1
43	20	13	11		2	1 (?)	
52	6	5	4	1	3		
57	10	9	3	2	4		
61	10	8	6	2	2	1	
62	2	2	2	2	2		
63	34	24	33				
102	33	18	31				
106	15	15	14				
104	15	1	15				
107	11	6	11				
110	8	6	8				
111	9	4	9				
212	18	11	17				
Total.....	239	149	206	10	20	3	2

combinations of several types of movement are often to be seen in a single preparation.

During the period of activity, a change in the type or direction of movement was often observed, and such a change in one part was apt to modify the movement of neighboring cells. Single cells or small groups of cells, as a rule, moved more freely than those in the spreading membrane. The consistency of the plasma, the liquefaction and the contraction of the fibrin influence both the rapidity and the type of movement into the plasma. The temperature has both direct and indirect influences on the movement. There is also individual variation which cannot be attributed to any of the above factors. For these reasons, the velocity of the epithelial movement varies from hour to hour, and it is very difficult to analyze the factors influencing it.

Usually, the most vigorous activity occurred during the first twenty-four hours. For instance, the epithelium on a piece of

TABLE 2

Cultures in serum

SERIES NO.	NUMBER OF PREPARATIONS	CELL MOVEMENT					NO MOVEMENT
		Into medium	On endothelial surface	On epithelial surface	On cover glass	On surface film	
56	28		28				
94	28		28		5	1	
58	7		6				1
60	8		7				1
139	7		7				
138	7		6				1
140	5		5				
135	8		8				
165	14		14				
166	12		12				
167	15		15				
208	23		23	1			
219	18		18				
221	20		20				
232	7		7				
233	9		9				
234	9		9				
Total.....	225		212	1	5	1	3

cornea cut radially into eight parts, spread out into the plasma within twenty-four hours in an extension which was even larger than their original area, though not in the same degree of rapidity at all cut ends. Similarly, in successful preparations, it was seen that the whole endothelial surface was covered within twenty-four hours by the epithelium spreading over the entire circumference of the piece. When the moving borders met each other, movement ceased.

Some measurements are given in table 3.

TABLE 3

	PREPARATION NO.	MOVEMENT ON ENDOTHELIAL SURFACE			MOVEMENT INTO PLASMA		
		After 5 hrs.	24 hrs.	48 hrs.	5 hrs.	24 hrs.	48 hrs.
Experiment 210, plasma culture, at 21°C.	1	0.14	0.4	0.5	0.06	0.3	0.5
	2	0.14	0.7	1.0	0.1	0.3	1.8
	3	0.1	0.8	0.8	0.11	1.1	1.6
	4	0.14	0.65	0.7	?	1.0	0.9
	5	0.14	1.0	1.0	0.08	0.8	1.0
	6	0.1	0.3	0.9			
	7	0.1	0.6	0.9			
	8	0.1	0.6	0.8			
Average movement in millimeters		0.12	0.63	0.83	0.087	0.7	1.16
					20 hrs.	40 hrs.	60 hrs.
Experiment 211A, plasma culture, at 20°C.	1				0.5	2.2	2.0
	2				0.8	4.0	5.4
	3				1.0	2.5	2.8
Average movement in millimeters					0.77	2.8	3.4
		20 hrs.	40 hrs.		20 hrs.		
Experiment 211B, plasma culture, at 20°C.	1	0.7	1.2				
	2	0.6	0.7				
	3	1.0	1.0				
	4	0.6	0.7				
	5	0.4	0.5		0.2		
	6	0.6	0.6				
Average movement in millimeters		0.65	0.78				
		20 hrs.					
Experiment 208, serum culture, at 20°C.*	1	0.3					
	2	0.4					
	3	0.8					
	4	0.8					
	5	0.6					
	6	0.7					
Average movement in millimeters		0.6					

*Pieces which measured: 1.2 x 0.6 mm.; 1.8 x 0.5 mm., 2.0 x 0.6 mm., 2.4 x 1.1 mm., and 0.8 x 0.8 mm., were entirely covered up with moving epithelium in seventeen hours.

II. ON THE REACTION OF EPITHELIAL CELLS TO CERTAIN SOLID SUPPORTS

1. Movement on flat surfaces

a. On glass and celloidin. We shall first consider movement on the glass cover-slip.[1] There is difficulty by means of usual culture method in bringing the epithelial rim into contact with the cover-glass from the beginning. Attempts to do this by reducing the plasma failed, as the epithelium did not show activity unless the medium was used in sufficient quantities.

In the later experiments, the piece of cornea (cut off tangentially to eyeball) was placed on the cover-slip, mainly with the inner surface down, and subjected to slight pressure by means of thin silver wires or glasses and kept in that position for a certain period after mounting with serum, so that the cut ends came into contact with the glass. When the silver wires were properly placed and controlled under the microscope, it was possible in almost every instance to bring certain parts of the moving epithelium into contact with the cover. Of course, where even a minimal space existed between the tissue and cover-glass surface, the epithelium crept on the underlying tissue.

Cover-slips coated with celloidin were also used. Thus the movement of the epithelium on celloidin and glass surfaces, respectively, could be compared. A comparison could also be made in the same preparation, by employing a cover-slip, only one half of which was coated with celloidin. Such preparations must be handled carefully, otherwise a change of cell form readily occurs.

No characteristic difference whatever in the mode of movement on the two different surfaces could be noticed. If the epithelium grew out vigorously, closely attached to the cover-slip, each cell became flattened into an exceedingly thin layer. In some instances it was seen that the advancing border became very

[1] It should be stated here that the cover-glasses used in these experiments were throughly cleaned and washed in the vapor of distilled water, as is usually done to remove any trace of alkali, in order to exclude any chemical influence from that source. (Ostwald-Ruther, Physiko-chemische Untersuchungen.)

irregular. This was most marked when active cells on the border, showing unusual protoplasmic activities, tore themselves loose from their connection. Similar conditions, however, were often to be noticed in the actively mobile epithelial layer growing out into firmly clotted plasma. In the epithelial movement on the endothelial surface, such a condition but rarely occurred; as a rule, the cells moved more uniformly, showing a smooth advancing border.

In other instances small pieces of cover-glass or celloidin membrane were placed on the endothelial surface and pressed against it, so that a certain portion or the whole of this surface, which is a favorable support for moving cells, was covered. In these cases the epithelium was also seen to move over the celloidin or glass, if they were suitably mounted and the cells were not injured. Where the cell movement on such artificial supports failed, examination proved that the placing of the membrane was not suitable; thus, by control of pressure, it was possible to direct the moving cells to these artificial surfaces, though this was not always an easy matter. At any rate, it is an established fact that the epithelium is able to creep on objects of such nature that chemical influences are excluded.

b. On dead cornea. If in a part of an explanted piece of cornea an epithelial defect existed, it was seen that the epithelial cells were able to cover the spot, spreading out from all edges, practically with the same rapidity as on the endothelial surface.

The same was true of a spot which had been killed by touching it with a heated needle. The cells injured by the latter procedure became round and detached, and the growing epithelium crept beneath them along the surface of the killed tissue.

Preparations were made of large strips of corneal tissue (about 2 x 4 mm.), across the center of which a sharply defined epithelial defect was produced, after which one half of the remaining corneal tissue was killed by means of a heated needle, making the tissue surface look opaque and wrinkled. In such preparations it was seen that the epithelial movement over the wound occurred uniformly, covering both the live and the dead tissue with the same rapidity.

Analogous experiments were made with pieces of cornea in which an epithelial defect existed at one extreme end and in which the tissue at the other end had been killed; similarly, the cell movement on a burnt wound was compared with a control preparation, in which the underlying tissue was left intact with simply an epithelial defect.

The use of tissue, vitally stained by neutral red[2] or nile blue, facilitated the observation of epithelial movement on such wound surfaces.

Analogous observations were made by using the surface of the cartilaginous layer of the sclerotic coat of the frog's eye. This tissue could readily be isolated from the other layers after boiling, and it was then thoroughly washed before using. It was translucent, showing characteristic convexity, which admirably fitted on the inner surface of the similarly curved cornea; even without any pressing, the two tissues would lie so closely together in the culture medium (serum) that the epithelium from the cut ends of cornea crept over the cartilage. It proved helpful, however, if the pieces were slightly pressed together.

Out of twenty-six experiments, movement of the epithelium over the cartilaginous plate took place in twenty-one; in three cases no movement was observed owing to injury of cells. An example of this is illustrated in figures. 4 and 5.

2. Movement of fiber-like supports

Next, the response of the epithelium to the various fiber-like supports were tested, such as spider web, silk fiber, glass wool, and asbestos.

a. Spider web as support. Experiments were made similar to those of Harrison ('14), using spider webs. In each preparation a sufficient quantity of serum was used as culture medium.

Figure 6 represents one of the preparations of the series; many cultures were found where the cells clung to the fibers. Out of thirty cultures twenty-four were positive, four doubtful, two infected.

[2] S. Matsumoto. Demonstration of epithelial movement by the use of vital staining. This paper will appear in the next number of this journal.

b. Silk fiber as support. Other experiments were made, using silk fibers. In these a number of fibers of raw silk were stretched on the glass ring and cover-glass, imitating the experiment with the spider webs.

Out of twenty-nine cases there were fourteen that gave positive results, one being infected.

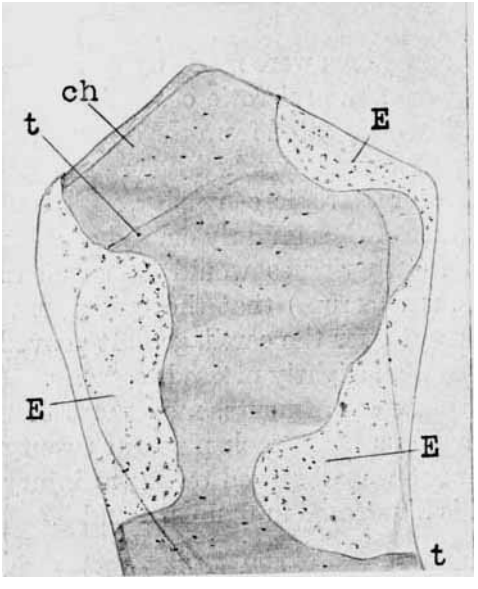

Fig. 4 Experiment 233, 4, showing epithelial movement (*E*) on the boiled cartilaginous plate (*ch*) of sclerotic coat closely placed on the endothelial surface of cornea. Cultivated in serum; forty hours' growth. *t*, cut ends of the piece of cornea are clearly visible through the transparent cartilagineous plate. × 98.

Similar experiments were made by Carrel and Burrows ('11a) with embryonic chick tissue.

c. Glass wool as support. Glass wool (Merck) was thoroughly cleaned and sterilized, then placed in the culture, so that the epithelium might attach itself to the fibers.

Out of twenty-eight cultures sixteen gave positive results. An example is shown in figure 7.

d. Asbestos fiber as support. Asbestos fibers were ignited and treated with pure conc. HCl; the fine cloudy suspension was collected and washed in distilled water to remove completely all traces of HCl, and then sterilized before use.

In the serum culture of the cornea the fibers were mixed densely so that, they appeared like a nest, in which the tissue lay. As the fibers were very fine, they did not hinder microscopical examination, if not too densely placed.

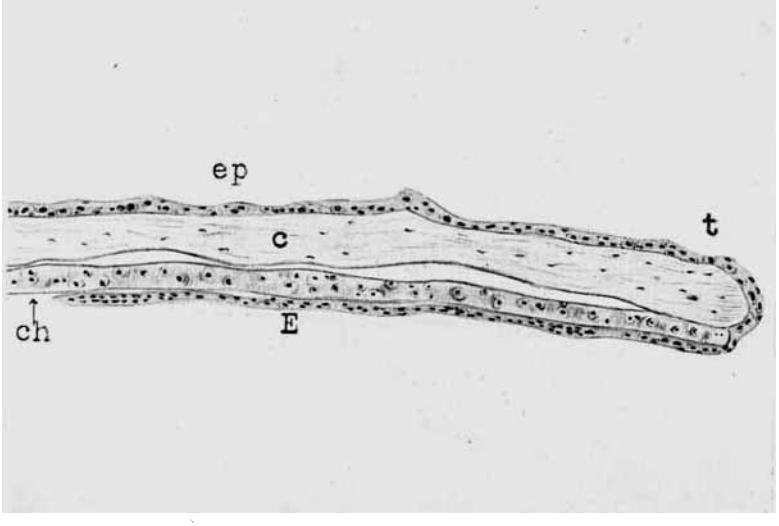

Fig. 5 Experiment 233; 4. Same preparation as shown in fig. 4. Portion of vertical section, drawn from one of the serial sections. *ep*, epithelial surface of cornea; *c*, connective tissue; *ch*, cartilaginous plate, placed on the endothelial surface of cornea; *t*, cut end of cornea. Note the epithelial movement (*E*) on the sclerotic cartilage (*ch*). Forty hours' growth. × 98.

Thirty cultures were made in this group, with positive results in twenty-two. Figure 8 shows an example of this series.

e. Movement on pith, etc. Further studies were made with pith. Thin pieces were used, which were kept in distilled water which was changed every three days for a period of over three months.

Experiments showed that the epithelium was able to cling to the cell walls of the thin piece of pith. Figure 9 shows clearly

the growing out of epithelial cells on the support. Out of twenty-seven experiments nineteen gave positive results.

On the shell membrane of hen's egg, used as support, after thoroughly washing and boiling, a similar condition was observed.

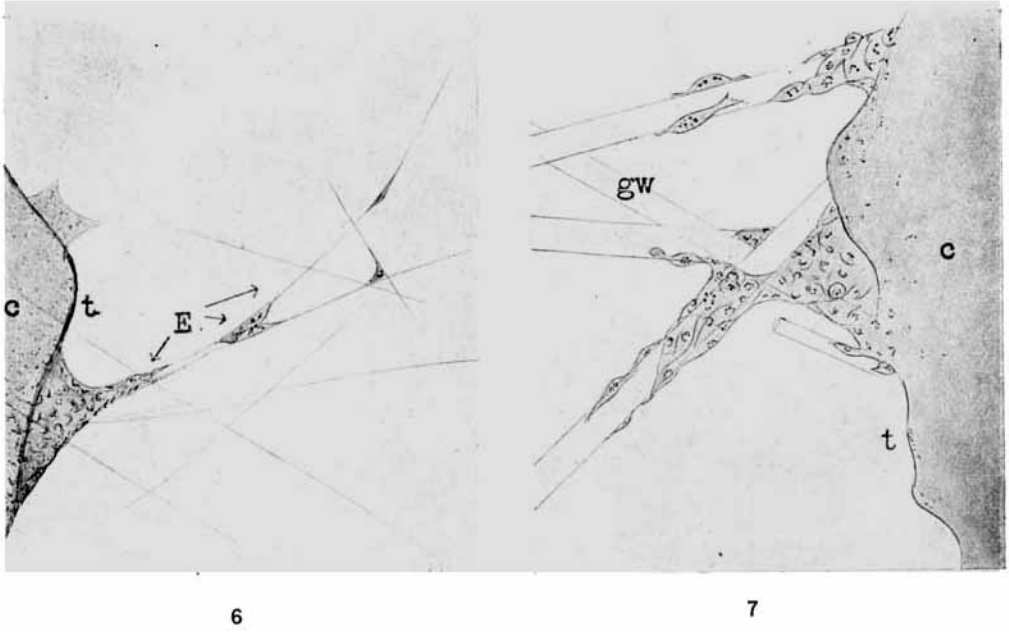

Fig. 6 Experiment 238, 11. Epithelial movement in serum on spider web. In the neighborhood of the explanted tissue (*c*) a good many isolated cells, round in shape, were noticed, which are omitted in this figure; they showed no active movement. *t*, edge of the piece; above, some cells move out in sheets on the cover-glass. Compare Jour. Exp. Zoöl., 17, 521, figs. 4 to 7, 12. × 98.

Fig. 7 Experiment 49, 7. Epithelial movement on glass wool (*gw*); cultivated four days in serum. *t*, edge of tissue. × 98.

III. RÉSUMÉ

This paper deals with the movement of corneal epithelium of the adult frog in vitro. Frog cornea is very suitable for this purpose, as it is so transparent and thin that it permits of direct observation of the cell movements.

The corneal epithelium cultivated in plasma shows various types of movement, according to the nature of the substratum

(fig. 1). The movement is of an amoeboid character. As a rule the cells have a strong tendency to cling to their own kind and thus extend in sheets, although under certain conditions active movement of isolated cells is also to be seen.

In the majority of cultures movement into the medium or along the endothelial surface (or both) takes place according to the consistency of the culture medium. The fact, that the epi-

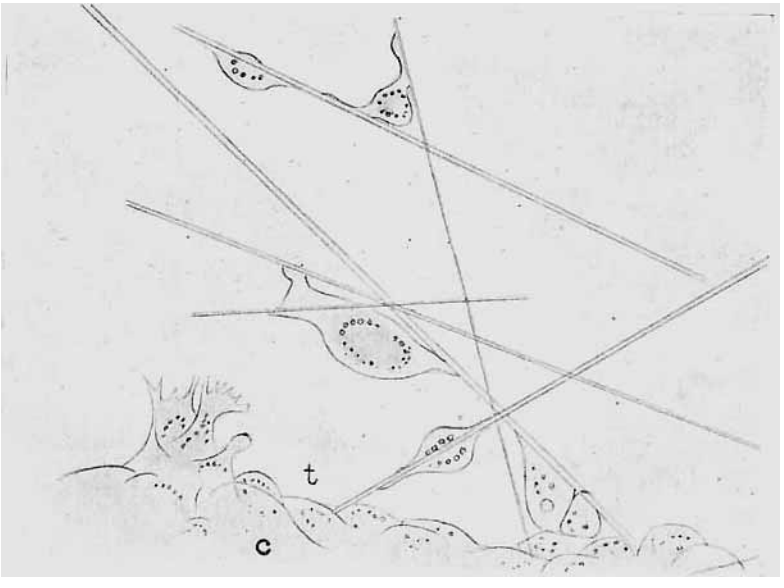

Fig. 8 Experiment 238, 1. Epithelial movement on fibers of asbestos; cultivated forty hours in serum. At the extreme left cells are to be seen moving along cover-slip. Note the adaptation of single cells to asbestos fibers. × 450.

thelium moves along the epithelial or endothelial surface is very important from various points of view; such a movement may easily be overlooked in the culture of non-transparent tissue, such as skin.

In the preparations in which serum is used, no migration of the epithelium into the medium takes place. There is mainly a movement of cells over the tissue, especially on the endothelial surface.

Naturally, the question arises, whether the epithelial movement on the tissue, which is so frequently to be seen in the fluid medium, is chemotactic or thigmotactic in nature, It might even be that the type of movement is due both to mechanical and to chemical influences acting simultaneously. However, the epithelium is able to move with practically the same velocity both on a substratum where the covering epithelium has been simply

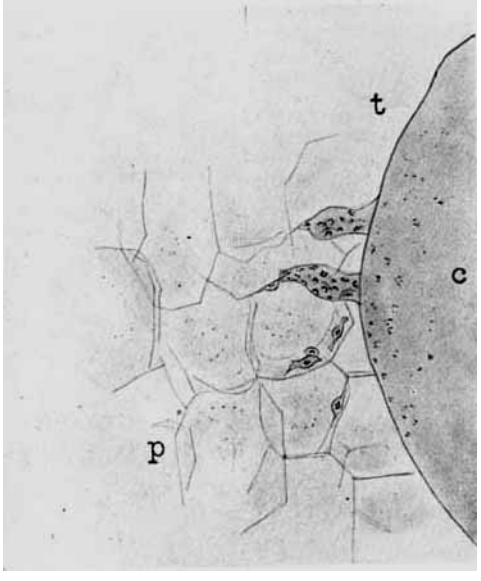

Fig. 9. Experiment 239. Corneal piece cultivated in serum with pith; three days after explanation. Note the epithelium clinging to the piece of pith (*p*) moving out of the cut end (*t*) of cornea (*c*). × 98.

scraped off and on one where the underlying tissue has been killed by heating. The movement in the latter cases cannot, therefore, be dependent upon chemotactic influences from the living tissue. The same is true of the movement taking place on the surface of the cartilaginous plate of the sclerotic coat, previously killed by boiling.

Furthermore, the epithelium is able to move on the surface of glass, on a celloidin film, and also on such fiber-like supports

as spider web, glass wool, asbestos, etc., when it is brought into contact with them.

The cell movement on the glass and celloidin film is very vigorous, sometimes more rapid than on the endothelial surface. In the movement on such supports chemotactic influences are to be considered as excluded.

It has been repeatedly observed by various writers that a suitable support for the growing cells is an important requisite; Harrison ('14) demonstrated lately the importance of such factors for the movement of embryonic cells very clearly. The facts above described confirm this view, that stereotropism plays an important rôle in cell movement.

The behavior of corneal epithelium in vitro serves to throw some light on the mechanism of epithelial growth in vivo.

The experiments show clearly that the epithelium is able to extend from the cut end quite rapidly in sheets into the medium (plasma), or on the tissue (plasma and serum), and can cover a large area, whereas mitotic cell divisions are not necessary at all.

That Oppel ('12) as well as Osowski ('14) did not observe amoeboid activity of the epithelium was perhaps due to the difficulty of direct observation. Special, careful observation on the movement of epithelium of warm-blooded animals, however, is necessary.

In conclusion, I wish to express my indebtedness to Prof. R. G. Harrison for his direction and valuable advice.

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