Biochemistry. — *Tissues of prismatic cells containing Biocolloids.* I. By H. G. BUNGENBERG DE JONG, B. KOK and D. R. KREGER. (Communicated by Prof. J. VAN DER HOEVE.)

(Communicated at the meeting of March 30, 1940.)

1. *Introduction.*

In a previous communication [1]) the method was described for the inclusion of biocolloidsols in collodion membranes, together with some examples of coacervation of the biocolloids present in the microscopic cavities of the membrane. The method described has a number of drawbacks, however.

a. The membranes are fairly thick, with the result that the cavities in them lie at different levels. The microscopic view is thereby troubled, since these cavities are often seen lying above each other.

b. The preparation requires a fairly long drying-period; if dried too quickly the membrane will not be sufficiently coagulated and the walls of the cavities crack when immersed in water.

c. Before the membrane has at length become sufficiently firm, the watery inclusions sink, the larger ones more especially, owing to their higher specific gravity. If the object-glass is lying with the membrane turned upwards, this is liable to lead to perforation of the membrane on the side touching the surface of the glass. A better way is to dry it with the membrane turned downwards. This perforation is not so liable to take place where air and membrane meet. The membrane separating is so thin, however, that it yet often cracks in contact with the water.

d. A further consequence of the lengthy drying period is the loss of water by the embedded hydrosols. When the dried membrane comes into contact with water, a certain over-pressure is developed in the cavities, which likewise conduces to the large cavities that should be kept intact for the microscopical examination, being injured and the colloids washed away.

Recently a method was found out which is a great improvement upon the method earlier described. It consists herein that the emulsion is not poured out upon an object-glass, but is allowed to spread out upon the surface air/water. The above-mentioned drawbacks are hereby wholly, or for the greater part, eliminated:

a. the preparation takes but a few seconds,

b. the membranes are thin, and the inclusions nearly all lie in the same plane,

c. although some of the larger inclusions may also be injured, the greater number will be found to have the membranes intact.

For other particulars regarding these, see sub 3.

[1]) H. G. BUNGENBERG DE JONG and O. BANK, Proc. Kon. Ned. Akad. v. Wetensch., Amsterdam, **42**, 83 (1939).

2. *Preparation of the tissues.*

In principle the preparation of the emulsion to be poured on the water surface has not changed in the course of time, except that we have substituted for the ordinary collodion celloidin (SCHERING–KAHLBAUM, für allgemeine Zwecke). Half a tablet of celloidin, cut into small dice in a stopper-bottle, is drenched with 400 cc amylalcohol + 400 cc ether. The celloidin swells and, with occasional shaking, gradually dissolves (in 24 hours). The emulsionizing fluid thus obtained has been used by us for over a year, and will probably keep good still longer.

To prepare the emulsion, 2 cc of the hydrosol to be embedded is put into a measuring-cylinder of 10 cc (preferably with a ground-glass stopper) to which 4 cc of the emulsionizing fluid is added. The emulsification is brought about by turning the measuring glass upside down several times. Violent shaking must be avoided, as otherwise the inclusions will turn out very small.

Then one drop of the emulsion (or several drops quickly one after the other) is allowed to fall from a slight distance on the centre of a Petri dish (diameter 15 cm) filled with distilled water, on the bottom of which an object-glass [1]) has previously been laid. The drop of emulsion will quickly spread out to a thin membrane, in which the inclusions cluster together in small groups. When the membrane has been prepared it begins to shrink in extent. After a few seconds, when this shrinkage is clearly in progress, the object-glass is lifted out of the Petri dish, first tilted sideways, then, still in a slanting position, straight upwards.

The membrane in this way is obtained spread out evenly on the object-glass. It is allowed to drip off for a few seconds longer, and then the object-glass is laid with the membrane side downwards on one of those special cuvettes, to be described later, as are used in microscopic examinations.

Remarks.

a. The emulsion is relatively stable. Although the emulsified drops sink, yet for the greater part they do not run into each other. Probably there will be a film on the boundary surface of the emulsified drops. This has no doubt to do with the fact that membranes obtained with freshly prepared emulsion are often less good than when the emulsion has stood for a short time closed. Apparently it does not take long before the film round the emulsified droplets is sufficiently firm. The keeping properties for yielding good membranes last at least one day, often longer. The emulsion may then be shaken up, and, if the result is not good, a few drops of ether may be added to it. In the case of emulsions containing gelatine, it may be advisable to warm up older emulsions slightly (by holding the tube for 10 seconds in water at 60°).

b. In dropping in the emulsion an extremely thin (monomolecular?) layer spreads rapidly out as far as the sides of the Petri dish, which sometimes hinders a proper

[1]) Instead of the usual object-glasses we use somewhat wider ones (76 × 32 mm). Hereby it is possible to make use of cuvettes (§ 5) which allow a sufficiently broad strip (15 mm) of membrane for the microscopic examination, and yet have a broad ebonite frame which confines it better.

spreading of the emulsion itself. This can be prevented by running the finger quickly round the rim of the dish directly after the drop of emulsion has fallen on the surface of the water. Good films can also be obtained without this manipulation, however, if the emulsionizing tube be held horizontally for a few seconds close above the surface of the water before letting the drop fall. The ether-vapour over the water-surface will have a good effect here. Or a few drops of ether may be dropped upon the water, and the emulsion poured on when the rippling of the surface has ceased.

c. It will not be necessary to renew the water in the dish before preparing a new tissue membrane. Water which has been used several times for this purpose yields even better membranes than freshly distilled water, as is comprehensible from the effect of the ether upon the spreading. There are, however, some impurities which act unfavourably upon the quality of the membrane, although they do not interfere much with the actual spreading. If, for instance, a little grease be added to the emulsion, fatty acid, paraffine oil (and many other organic compounds), the membrane will lose its cohesive properties more or less after the coagulation.

For such deleterious effects, however, noticeable quantities would have to be used, and they need not be feared from the slight greasy impurities found on ordinary clean laboratory glasses or on the surface of the water/air from the dipped in finger.

3. *The distribution of the inclusions in the celloidin membrane.*

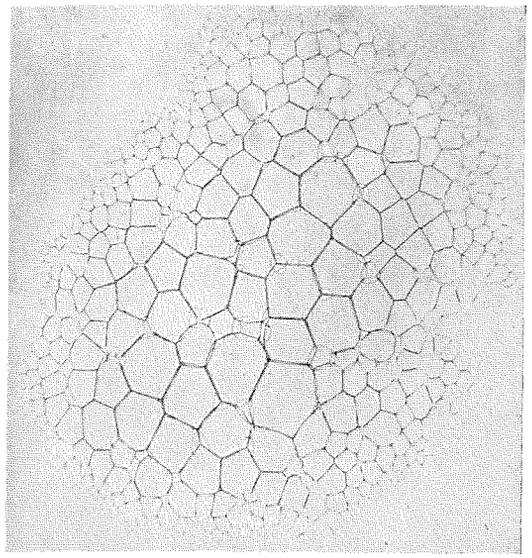
As already mentioned in the introduction, the inclusions in the extremely thin tissue are found as a rule in one and the same plane.

As regards the distribution of the inclusions in the tissue it is possible to modify it oneself. It appears that the volume ratio of the original emulsion determines the structure of the membrane.

If in preparing the original emulsion, with the same volume of biocolloidsol much more celloidin solution be taken than given above (sub 2), then the inclusion will lie mainly solitary. Through the microscope they will then appear round, and considering the thinness of the tissue their shape is almost an oblate spheroid.

The proportions of hydrosol and celloidin solution given above, however, result in membranes whereby the inclusions will lie close together in little clusters (e.g. of 20 or more). (See microphoto *A*). The shape of the inclusion is now that of a flat prism, and the majority are 5 or 6-sided prismata. (See microphoto *B*). These clusters are formed during the spreading, but the process is so rapid that the movement of the inclusions in the still fluid membrane towards each other is not perceptible. With respect to the distribution of the individual cells in the cell-groups, we may remark that the largest cells are found in the middle; round about these are the smaller ones, while the very smallest are found at the outside edge. Likewise the cells decrease in height from the centre to the periphery (see also microphoto *B*), so that in principle a cell group in section will have the aspect as shown in Figure 1.

The film is here supposed to be on the underside of the object glass. The thickness of the membranes and the height of the cells has been considerably exaggerated in the drawing. If an object-glass with a membrane be examined at the place where the membrane is bend round the edge of the slide, it will be seen that the membrane over each cell is more or



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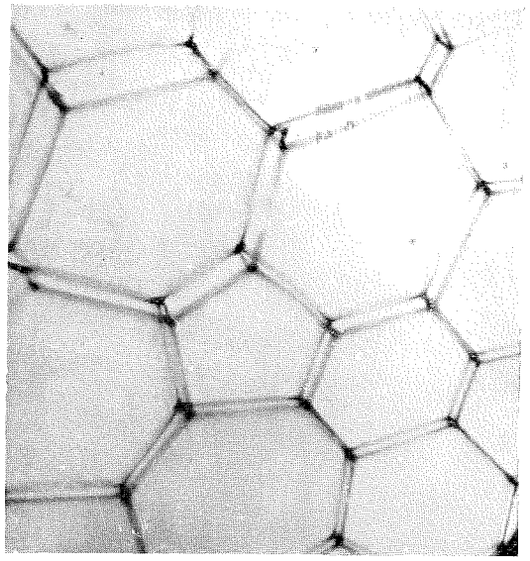

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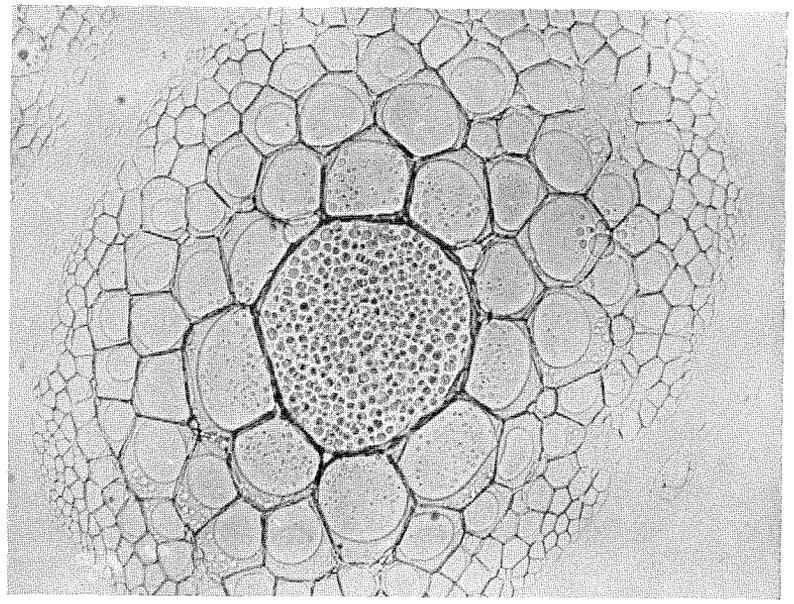

C 134 ×

less convex. This convexity, also to be seen in Fig. 1, is not always equally clear, however.

By carefully focussing the microscope a rather faint circle inside the cell, the larger ones at least, can be observed. This is probably situated in the above mentioned curved membranes of the cell. Respecting the significance and the reason of this particular, we are uncertain. So much we know, however, it is not a circular perforation of the cell membrane, but a thin spot.

Slight disturbances in the structure of a cell-group (as shown in figure 1) are not infrequent. Thus, small cells can be seen pressed in between larger

Fig. 1.

ones, and these are often pushed then to one side of the tissue, whereby the beginning of a three-dimensional tissue-structure is seen (See microphoto *A*).

If a larger plate be used to spread the membrane, and more drops of the emulsion be added on the surface of the water, larger films will be obtained, but the arrangement of the cell-groups and the distribution of the cells in the group is but little different. If the area of the water surface is held constant, but more emulsion be added on the water surface, the cell-groups will be larger.

If less than the above quantity of emulsifying medium be used, the membrane loses in cohesion.

It would seem that then a volume ratio is reached readily resulting in an emulsion of the reverse type (oil in water). With the proportions as given above this is far from likely to occur. However in a tissue there is a cell-group here and there in which a tiny round, spotted brownish ball is seen, which has originated because here a droplet of the emulsion medium happened to get completely embedded in an emulsified drop of the aqueous system.

4. *Permeability of the celloidin walls for biocolloids.*

It is possible in the above-described manner to embed all kinds of typical hydrophile biocolloids. But not all of them yield membranes serviceable for the purposes which we have in view. A requirement is that, in contact with water, the colloids will not gradually diffuse outwards. The method is fairly successful with gelatine, gum Arabic, and mixtures of the two. On the other hand, Na-yeast-nucleinate, in a less degree Na-thymus-mucleinate and K-Chondroitinsulphate, appear to wash readily out of the cells into the surrounding aqueous milieu. Up till now we have not managed, by adding any substance to the emulsion medium, to render the walls of the cells less permeable. Lecithin, cholesterol, fats, fatty acids, camphor, etc., merely led to the membranes losing their cohesive properties after being prepared.

5. *Cuvettes.*

a. Simple cuvette.

For studying the influence of the medium upon the biocolloids embedded in the cells, a simple cuvette may be employed (fig. 2), which consists of a rectangular ebonite frame a few mm in height, cemented on to a glass sheet. Thin glass tubes through the short sides of the ebonite frame are

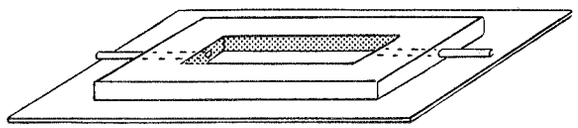

Fig. 2.

connected via flexible thin rubber tubes (diameter 3 mm) with a reservoir for the fluid on a higher level and a glass tube on a lower level through which the fluid could drop away freely. The cuvette is used in the following way: The upper edge of the ebonite frame is dried with a cloth, and distilled water out of the reservoir is allowed to flow slowly into the cuvette till it is dropping regularly out through the outlet. Then the outlet is closed by a clip, and water is allowed to flow from the reservoir till the cuvette is quite full, and the fluid shows a good head, but does not spread over the ebonite. Then the supply is also cut off with a clip, a membrane prepared, and the object-glass laid on the top of the water with the membrane downwards, whereby a little of the redundant water escapes between the object-glass and the ebonite frame, and the object-glass closes upon the cuvette like a lid. Now the outlet is opened in consequence of which the object-glass is sucked firmly down on to the ebonite frame. The outlet tube must be only so much lower that there is sufficient suction, but no air sucked in through the capillary slit between the ebonite and object-glass. When the supply is then opened, water or any fluid as desired can be allowed to flow past the tissue. Care must only be taken that this flow is not too rapid, as otherwise there is risk of the fluid leaking out through the capillary slit between object-glass and ebonite frame.

It is an advantage to take the glass foundation plate somewhat larger, and to cement round it a second rectangular ebonite frame, so that a space of about 1 cm is left between it and the cuvette proper. In this space the water can collect that is forced out when the object-glass is laid on it, as otherwise the object-table of the microscope is liable to be spilt upon.

When gelatine, or a mixture of gelatine and gum Arabic, is embedded, it is necessary, in studying the coacervation phenomena to work at a higher temperature (e.g. 35°—40°). In such a case the cuvette may be placed on an object-table that can be heated, and the fluid to be flowed continually past the membrane should also be heated beforehand.

b. Cuvette for the study of the influence of the electric field.

The cuvette for this purpose (fig. 3) likewise consists of a rectangular ebonite frame which in this case, however, is thicker (e.g. 1 cm). This is again cemented on to a glass foundation-plate, while, moreover, two blocks of plate-glass (dotted in fig. 3) are cemented into the cuvette, so that a space of only 1 mm is left beneath the object-glass with the tissue.

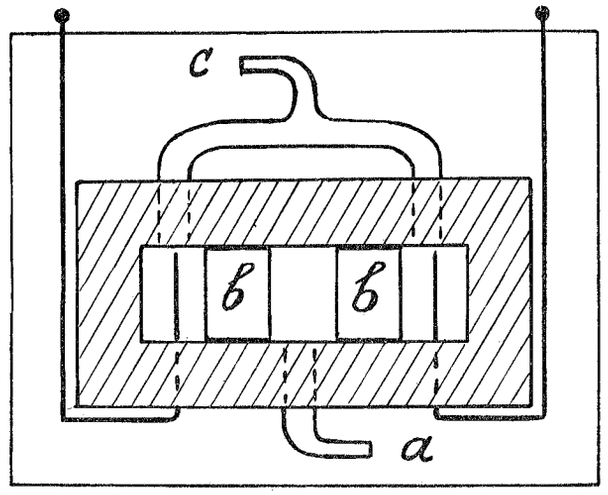

Fig. 3.

This cuvette is employed under a constant flow of the medium, which serves to wash away the electrolyse products on the electrodes. The fluid enters at *a* into the through-like space between the glass-blocks (*b*), flows then over the glass-blocks to the two trough-like spaces in which are the two electrodes (Pt, Ag or even Cu wires), and from thence leaves the cuvette at *c*. The opening *c* is purposely made in the middle of the connecting pipe, so that the resistance of the fluid may be equally great each way along right and left. The glass-blocks (*b*) assist in increasing considerably the speed of the fluid flowing past the membrane, without affecting the economy in the consumption of the fluid. Moreover, this prevents convexion currents in the cuvette, whereby electrolyse products would get to the cell-groups. The proper functioning of the flushing system can be controlled with a quartz suspension, and the off-flow of the fluid can be regulated by placing in the supply-pipe a capillary of such

dimensions that, with the intensity of field used, the rate of the ions with the highest velocity in the electric field (H ions) is exceeded several times.

With the cuvette which we employed, namely glass-blocks 1 cm broad and 1 cm long, with a space of 1 mm between glass-block and tissue, we used only 5 cc flushing fluid per minute, and calculated for a potential difference of 60 volts (i.e. about 30 volts per cm over the glass-blocks [1]) a safety margin about fivefold.

Just as in the case of the former cuvette, it is advisable here, with a view to avoid spilling upon the object-table of the microscope, to fix a second rectangular ebonite frame on to the glass-plate at some distance round the cuvette proper. This is not shown in figure 3.

6. *Complex coacervation of gum Arabic with gelatine in the cells of the tissue.*

The apparatus described is particularly suited to study the coacervation of biocolloids and the influence of a different milieu (pH, salts, etc.), or of an electric field applied to coacervaties already produced respectively. Here, and in the following paragraphs, we shall confine ourselves to describing the formation of a coacervate and the final position of the coacervate in the cell compartment.

If we embed a mixture of gum Arabic and gelatine (6 gr gum Arabic + 5 gr gelatine dissolved in 200 gr water) in the membrane, and allow to flow over the membrane first warm (40°) distilled water, and then for a short time warm (40°) 0.01 N acetic acid, complex coacervation will occur. The way in which this takes place (Cf. fig. 4*A*) is that in each cell very tiny coacervate droplets are separated which perform a distinct Brownian movement. These droplets gradually coalesce to larger ones and these sag in the cell, and as soon as they come in contact with the wall, they lose their outline: the coacervate moistens the wall and gradually the final state can be observed to occur, whereby the coacervate, that still has a few vacuoles, is entirely parietal. This final stadium is more readily obtained in smaller cells than in the larger ones. Compare microphotography C, where in the large middle cell the coacervation is in an incipient stadium (many small coacervate droplets). In the smaller cells lying round about there are still some separate coacervate droplets present, but a considerable number has already moved off to the wall, and forms a parietal coacervate coating. In the cells lying still further outwards the coacervate has already become completely parietal.

7. *The three ways in which the final stadium may be attained, whereby the coacervate is parietal.*

Coacervation does not invariably take place in the manner described

[1]) The field collects practically wholly in the narrow spaces above the glass-blocks. Thus, for studying the behaviour of the biocolloid-system embedded in the tissue (e.g. coacervates), the membrane is examined above the glass-blocks.

above. As a matter of fact three ways are known to us by which the final stadium may be reached (Cf. fig. 4). These are:

a. The coacervate separates as small drops which increase in number and size (partly by coalescing as described above).

b. In the cell compartment tiny vacuoles occur, which increase in number and size, and which finally coalesce with each other to one large central vacuole, where the coacervate, which still holds smaller vacuoles, is also parietal in the final stadium (Fig. 4*B*).

c. In the cell compartment fibres and strands appear which meet in the middle at one or more points. The liberated mass contracts at these points

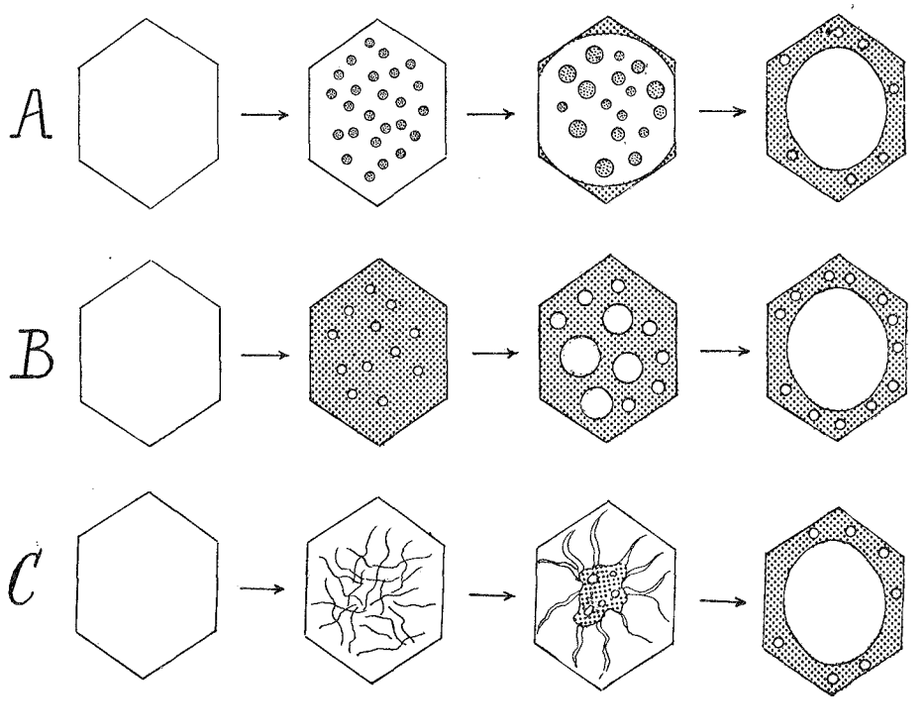

Fig. 4.

to a more or less rounded off and vacuolated clump. This gradually becomes fluid, after which it loses the above mentioned aspect and takes on that of a parietal coacervate (fig. 4*C*).

The way described sub *b* is followed when, proceeding from the same membranes containing gum Arabic + gelatine, we take for a flushing liquid medium first warm (40°) 0.01 N, acetic acid + 40 m. aeq. NaCl, when no changes take place yet, and then 0.01 N acetic acid + 20 m. aeq. NaCl, when the parietal coacervate is brought about via vacuolisation. The explanation is as follows:

The watercontent of complex coacervates is augmented by neutral salts, and the coacervation is suppressed in the case of sufficiently high concentrations. For our combination this already takes place with 40 m. aeq.

NaCl. When we reduce the NaCl, concentration to 20 m. aeq. coacervation is possible. We thus pass a certain salt concentration, whereby a coacervate of high water content i.e. an extremely voluminous, coacervate occurs which fills the whole cell compartment.

With a further reduction of the salt concentration this coacervate becomes poor in water, and the expelled water is liberated in the form of vacuoles. Since, from the nature of the case, the parietal coacervate is not produced by the spreading of coacervate droplets upon the cell-wall, but by the coalescing of the vacuoles, this parietal coacervate still contains, as a rule, a considerable quantity of smaller vacuoles.

The manner described sub *c* is seen when a membrane is cast from an emulsion that has stood a long time cold and this is first flushed with cold 0.01 N acetic acid (when nothing will yet be seen) and then the temperature of the 0.01 N acetic acid be slowly raised to 30—35°. The explanation is that the gelatine in the cold emulsion is present in a gelatinous state. It is true that a complex gel will be produced by allowing cold 0.01 N acetic acid to flow over it, but this, with the microscopic size of our cells, renders the aspect but little different; scarcely anything is seen to take place in the cells.

With slow heating, a gradual transition from the gelatinous state of the gelatine to the sol stadium takes place, whereby the complex gel condenses to a complex coacervate. With the half-melted plastic intermediate stadia there form the coalescing stadia described sub *c*) attached to the wall by strands, while when the transition from gel to sol is complete the normal figure of the parietal coacervate results as the final stadium.

8. *The relative position of coacervate and vacuole.*

In the foregoing it has already been discussed that the final stadium is invariably that whereby in the cell compartment the coacervate is parietal. The remaining cavity, giving rise to the three ways (mentioned sub 7), we have designated as "vacuole".

We are, however, not absolutely certain that this fluid cavity may indeed be termed a vacuole. This would be the case when this fluid hollow is separated on all sides from the cell-wall by coacervate, however thin this coacervate layer might be. If the microphoto C be examined, we get at first sight the impression that the coacervate in the larger cells at least does not entirely surround the liquid cavity. We will now, however, argue that it must not yet be concluded from this that the liquid cavity is not a vacuole in the strictest sense. Proceeding from the assumption that this cavity is indeed surrounded on all sides by coacervate, it then follows from what was discussed in paragraph 3 that, for the smaller cell group illustrated in microphoto C, just a relative position of the vacuole and main mass of the coacervate may be expected in the smaller cells grouped round the larger central cel, as this microphoto clearly shows. We always see there

that the main mass of the coacervate lies turned from the centre of the cell group.

Now, according to 3 the section of a cell group resembles that of a plane-convex lens, in which the large cells lying in the middle are also of the greatest height. This height decreases towards the periphery and, especially when a cell group is fairly small in the case of cells lying round the centrum, the lateral edges turned towards the centre of the group will be longer than the lateral edges turned towards the periphery.

If now, in such a more peripheral cell a certain portion of the volume is taken up by the coacervate, and this completely moistens the cell-wall, the vacuole will seek that position whereby the surface vacuole/coacervate will be as small as possible.

Of the three positions drawn in fig. 5, this surface will decrease in the direction $a \rightarrow b \rightarrow c$. From the point of view that the free surface energy strives to a minimum, we may thus expect just what is seen in the micro-

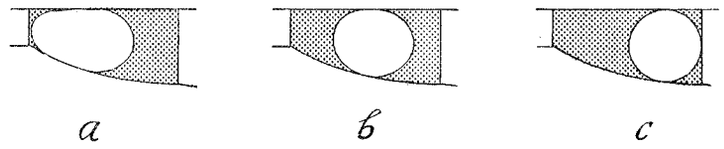

Fig. 5.

photo C viz. in the cells round about the large central cell, the main mass of the coacervate lies turned away from the centrum of the cell-group.

9. *Lesions of the dividing-walls.*

We consider it probable that the coacervate moistens the cell-wall entirely, i.e. that the liquid cavity is a vacuole in the strictest sense.

Nevertheless cells are frequently met with which look as if there is after all a terminal contact/angle coacervate/wall. This aspect is caused, however, by a lesion in the cell-wall there. It can be seen, namely, that in the contiguous cell the coacervate also stands with a terminal angle upon the cell-wall, and just at the same place in the cell-wall. The two cells communicate here, and have only one common vacuole.

In the microphoto C, we see, for instance, in the left lower quandrant three medium-sized cells, lying perpendicularly one above the other, the partition-walls of which have been perforated and which, thus, have one common vacuole.

If coacervation is brought about in the way described in § 6, then we shall also see here and there in the tissue that coacervate droplets flow from the one cell to the contiguous one, proving that the cell-wall here has been injured. If the final stadium of the partietal coacervate be awaited we shall find in these cells the morphological peculiarities which were described above.

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March 1940.