Biochemistry. — *Tissues of prismatic celloidin cells containing biocolloids.* VIII. *Gelation of the parietal gelatine-gum arabic complex-coacervate and behaviour of the objects obtained with regard to neutral red at various pH's.* By H. G. BUNGENBERG DE JONG and R. C. BAKHUIZEN VAN DEN BRINK.

(Communicated at the meeting of February 22, 1947.)

1. *Introduction.*

In previous communications [1]) we described the complex-coacervation of a mixture of gelatine and gum arabic sols, present in the cells of an artificial tissue, and studied the influence of pH, neutral salts and non-electrolytes. During these investigations the temperature (33—40°) was always kept above the temperature of gelation of the coacervate (± 28°). In this communication we describe the gelation of the coacervate present in the cells and some properties of the objects obtained, especially shifting of colloid from the gelated coacervate-layer to the central vacuole.

2. *Preparation of the membranes.*

We made use of new experience [2]) with regard to a method of spreading that could be better reproduced. As an emulsionizing fluid we used a solution of 20 gr celloidin-gel in a mixture of 100 cc ether + 50 cc amyl-alcohol + 50 cc benzene. In a graduated stoppered measure of 10 cc, fastened to a revolving disk with 22 rotations a minute, 1 cc of a solution of 3 gr gum arabic + 3 gr gelatine + 100 cc distilled water is emulsionized for 5 minutes in 4 cc of the emulsionizing fluid indicated above. Of the emulsion obtained 0,33 cc is spread on a basin of a circular shape, 30 cm in diameter, filled with fresh tap-water (distance of the lower end of the pipette to the surface of the water 3 cm). Under these circumstances a membrane formed, with an average diameter of about 14 cm, in which the separate round cell-groups are still connected by bundles of little folds, but in which as a rule only two such bundles of little folds spring from each cell-group.

A short time after the preparation, part of the membrane is scooped up with an object-glass, the superfluous appending membrane is removed and the object-glass covered with membrane put as a cover on a cuvette as described in previous communications, which is filled with hot (35°) 2 m aeq. acetate-buffer of pH 3.7 (2 m aeq. Na-acetate + 20 millimol acetic acid p. L.)

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[1]) H. G. BUNGENBERG DE JONG and cooperators, Proc. Kon. Ned. Akad. v. Wetensch., Amsterdam, **43**, 512 (1940), **45**, 51, 67 (1942).

[2]) In a later communication in these Proceedings we hope to give further details about this experience.

After this buffer-mixture has passed through the cuvette for 2 minutes, we switch over to a 20 m aeq. acetate-buffer of the same pH (20 m aeq. Na acetate + 200 millimol acetic acid p. L.). After 5 minutes the morphologically ideal final stage, the parietal coacervate being free from vacuoles (fig. 1) has been reached. The picture remains unchanged even if this 20 m aeq. buffer (35°) has passed for some hours.

3. *Undesired lesions of the tissue-cells.*

It was found that the procedure indicated: 2 min with 2 m aeq. buffer followed by 5 min 20 m aeq. buffer, led much more quickly to a parietal coacervate free from vacuoles than if one works exclusively with 20 m aeq. buffer.

It may occur that in spite of this a few cells in a cell-group do have a vacuolised parietal coacervate, while all the cells surrounding them look normal. Such cells in which vacuolisation continues a long time or even increases are damaged and often microscopically one may observe a perforation of the cell-wall between the cell-cavity and the cuvette-liquid (fig. 1*a*). It was also observed that such lesions occur more frequently if

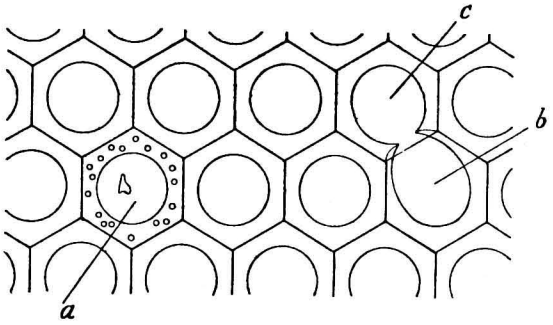

Fig. 1.

an emulsion kept one or more days at room-temperature (± 17°), is used for the preparation of a membrane. Apparently this is correlated with the gelation of the emulsionized drops. Such an un suitable emulsion is much improved by warming to 35° and cooling to room-temperature. In general we recommend using freshly prepared emulsion, as we got the impression that a different kind of lesion may also occur much more frequently when spreading such emulsions that are 1 or more days old. We mean lesions of the side-walls between the cells themselves. Fig. 1 gives the morphological picture of such a lesion of the side-wall between the cells *b* and *c*. The two central vacuoles communicate here, because of a perforation-hole in the side-wall that bulges toward *c*.

Apparently the lesion has been caused by an over-pressure in cell *b* relatively with regard to the pressure in *c*. Such side-wall lesions may sometimes become very frequent and whole series of cells in the cell-group communicate with one another in the same way as *b* and *c*.

4. *Gelation of the parietal coacervate.*

With reduction to room-temperature, while the 20 m aeq. buffer continues to pass along the membrane, vacuolisation occurs in the parietal coacervate. It depends further on the rate of reduction in temperature whether many small (quick reduction in temperature) resp. relatively few larger vacuoles (slow reduction in temperature) are ultimately present in the gelated parietal coacervate. Vacuolisation is a process that also occurs in a coacervate layer in a sedimentation tube, beginning to appear at about 28° [3]).

While the original central vacuole was round, fig. 2*a*, it has now become more or less angular in shape, fig. 2*b*, and the gelated coacervate-layer is less thick than the fluid one.

With warming up the original situation ($b \rightarrow a$) is re-established.

5. *Morphological changes of the gelated complex-coacervate-layer, when the complex-relations are removed, shifting of colloid to the vacuole.*

When now, starting from the condition *b* of fig. 2, KCl is added to the buffer (pH 3.7) at room-temperature, to an amount of 200 m aeq. p. L.,

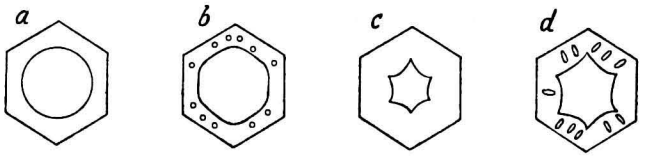

Fig. 2.

the parietal coacervate-layer swells, the central vacuole becomes irregular in shape, while the optic contrast between vacuole and gelated coacervate-layer diminishes strongly. The boundary of the central vacuole is not distinctly visible and the vacuoles in the pariotal coacervate layer become also invisible (fig. $b \rightarrow c$).

When the KCl concentration is sufficiently reduced, the vacuoles become visible again, however. The KCl action is based on a removal of the electrostatic interaction of gelatine (positive) and gum arabic (negative). We know from other experiments that under these circumstances gum arabic goes out of the gelated coacervate and so in our case the central vacuole must begin to contain gum arabic.

This cannot be shown, however, with a basic dye, as the accumulation is prevented by the high KCl concentration.

The complex-relations can also be removed by increasing the pH above the I.E.P. of the gelatine used (I.E.P. $\pm$ 5). If, starting from the condition

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[3]) H. G. BUNGENBERG DE JONG, E. G. HOSKAM and L. H. V. D. BRANDHOF-SCHAEGEN, Proc. Kon. Ned. Akad. v. Wetensch., Amsterdam, **44**, 1104 (1941).

b at room-temperature a very much diluted borax-solution ($\frac{1}{400}$ mol. p. L.) is led along the membrane, the gelated coacervate-layer also swells, the central vacuole being strongly curved in and the vacuoles of gelation stretched out in the shape of a pear or elliptically (fig. 2*d*).

After this, when rinsing with a diluted solution of a basic dye, it can be proved that the gum arabic has gone over into the central vacuole, because this vacuole colours more strongly than the gelated coacervate-layer.

The results depend on the dye used and on the pH. With methylene blue the accumulation can be realised even at pH 9, but the coacervate-layer also still colours weakly here. A better contrast can be obtained with neutral red. For this reason we have examined this more in detail.

6. *Accumulation of neutral red in the central vacuole.*

In the case of neutral red the choice of the pH is limited on account of the easy flocculation at higher pH's. Borax exclusively (pH 9.24) is therefore completely unsuitable and even in borax-KH_2PO_4 buffers of pH 7.5 flocculation still occurs. pH 7.0 can just be used but pH 6.0 is more favourable in every respect.

In 5) colouring of the vacuoles was attained in two stages: a treatment beforehand with a buffer, followed by a treatment with the diluted dye-solution. It appears that it is also possible to obtain the same result by leading only a buffered neutral red solution along the membrane. It takes a rather long time then, however, as apparently at the lower pH's (7 resp. 6) the expulsion of the gum arabic to the central vacuole occurs more slowly than at pH 9.24.

Use was made of a liquid consisting of 10 cc (borax + KH_2PO_4) buffer [4]) + 5 cc neutral red 0.1 % + 85 cc distilled water.

Starting from condition *b* (fig. 2), within half an hour strong accumulation in the vacuoles is obtained at pH 7.0 and strong accumulation followed by coacervation at pH 6.0. From now on we shall indicate the first type by *diffuse accumulation*, the second by *grain-accumulation*. We found further that with these $\frac{1}{200}$ % neutral red solutions at pH 6 diffuse accumulation occurs instead of grain-accumulation, when the passing liquid also contains 50 m aeq. p. L. KCl. When switching over to the same liquid without KCl, the diffuse accumulation changes into grain-accumulation. When the liquid containing KCl is carried over, grain-accumulation is again replaced by diffuse accumulation.

The reversible change of grain-accumulation into diffuse accumulation is not only possible by increasing the salt-concentration, there being constant dye-concentration, but can also be realised by exclusively changing the dye-concentration.

If at pH 6 with $\frac{1}{200}$ % neutral red grain-accumulation (fig. 3*b*) has been obtained, it changes into diffuse accumulation when $\frac{1}{4000}$ % neutral red

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[4]) By mixing 0.05 molar borax with 0.1 molar KH_2PO_4.

(pH 6) passes. Increasing the neutral red concentration to $\frac{1}{200}$ % then again gives grain-accumulation (fig. 3$a \rightarrow b$).

7. *Colouring of the gelated coacervate-layer with neutral red.*

If one starts from the grain-accumulation with $\frac{1}{400}$ % neutral red at pH 6 and if the liquid that passes through is replaced by a $\frac{1}{200}$ % neutral red solution of pH 5, the darkly coloured grains disappear in the vacuole and the vacuole liquid loses its colour, while on the other hand the gelated coacervate layer itself is coloured (fig. 3$b \rightarrow c$).

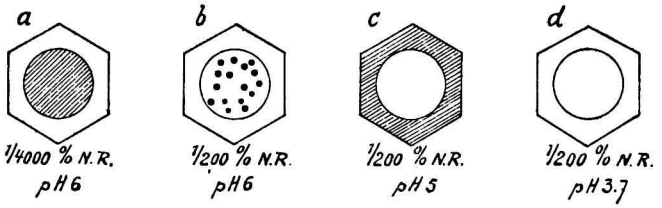

Fig. 3.

So at this pH, which about agrees with the I.E.P. of the gelatine used, practically all the gum arabic already goes again from the vacuole into the parietal gelated coacervate layer, but as it is here only very imperfectly compensated by the positive partial charges of the gelatine, the possibility of colouring with neutral red remains.

At pH 3.7, where complete compensation does take place, neutral red does not colour the gelated coacervate layer at all (fig. 3*d*).

That this non-colouring is indeed due to the fact that gum arabic combines with gelatine and not to a possibly unfavourable pH is clear, because with membranes which only include gum arabic, at pH 3.7 strong accumulation and coacervation do take place. It is only at a lower pH value (2.3), where the ionisation of the COOH groups of the gum arabic is practically suppressed, that no accumulation occurs any longer.

Essentially the same phenomena as with neutral red were also observed with methylene blue.

Summary.

1. Undesired lesion-symptoms of the cells filled with gelatine and gum arabic, and their prevention, are described.

2. The morphological changes which accompany the gelation of the parietal complex coacervate and the influence on this gelated coacervate of a neutral salt addition or a pH increase are described.

3. At pH's higher than the I.E.P. of the gelatine, gum arabic passes into the central vacuole, as a consequence of which accumulation, sometimes followed by coacervation, with a basic dye (neutral red $_4\frac{1}{400} - \frac{1}{200}$ %) may occur.

4. The reversible change (neutral red, pH 6) between diffuse and

grain-accumulation as a consequence of a change of the salt resp. of the dye-concentration is described.

5. The change of the colouring of the vacuole (neutral red pH 6) into the colouring of the gelated coacervate layer (neutral red pH 5) points to an absorption of gum arabic by the gelatine, the gum arabic being compensated very insufficiently, so that it can still combine with neutral red.

6. At pH 3.7 the gelated coacervate layer is no longer coloured by neutral red, though gum arabic, if only present in the cell compartiments, may still accumulate neutral red at this pH. So because of the combination with the positive gelatine the power to combine with neutral red is suppressed.

7. Gum arabic, if only present in the cell compartiments, no longer accumulates neutral red at pH 2.3. This can be explained by suppression of the ionisation of the COOH groups of the gum arabic.