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Biochemistry. — *Behaviour of microscopic bodies consisting of biocolloid systems and suspended in an aqueous medium.* III. *Coacervation phenomena in droplets of biocolloid sols enclosed in a collodion film. Accumulation of basic dyes.* By H. G. BUNGENBERG DE JONG and O. BANK. (Communicated by Prof. J. VAN DER HOEVE.)

(Communicated at the meeting of December 17, 1938.)

1. *Preparation of the collodion films mentioned in the title.*

In the following a simple preparation of the films mentioned in the title is described in broad outlines, while other methods of preparation and questions concerning details will later be discussed more elaborately elsewhere.

The principle of the preparation is this that in a collodion solution, which cannot be mixed with water, the aqueous solution of biocolloids is emulsified, this emulsion in a thin layer is spread on an object glass and, after gelatination of the collodion, the organic solvents are washed out with water. In order to fix the collodion film to the object glass, the latter after heating is rubbed with a piece of bee-wax on one end and in the middle. After cooling the emulsion, which will be indicated later on, is poured over the surface, the object glass is for a while held in vertical position to enable the excess to trickle down, then turned with the wet side downwards and left to dry in the air fastened in horizontal position in a stand-clamp during a sufficiently long time (e.g. 20—30 min.).

The emulsion to be poured on consists of one volume of aqueous solution of one or more biocolloids in 6 volumes of the emulsifying medium. This emulsifying medium is prepared by adding 45 cc of isoamyl alcohol and 45 cc of ether to 1 vol. 10 % collodion solution (für technische u. fotografische Zwecke RIEDEL–DE HAËN).

After sufficient drying the object glass is placed in distilled water in order to remove the amyl alcohol and any salts which may be present. If the collodion membrane has not become sufficiently strong, it is possible that the thin walls of many, if not of all cavities in the membrane burst and the contents are washed away. Even in the best preparations there will always be cavities in which none of the coacervation phenomena which will be described afterwards take place, since they have been emptied by a lesion. The methods for the correction of these difficulties we shall revert later.

The microscopical image of the collodion film, prepared and washed after the method which has been described, shows that the cavities are

usually surrounded by a condensed zone of the collodion, which gives the impression of a "membrane".

2. *Complex coacervation of a sol mixture of gelatin and gum arabic enclosed in the cavities.*

a. Choice of the biocolloid combination.

It was our object to study the morphological phenomena occurring in case of coacervation of biocolloids present in the cavities. In the first place was selected the so-called complex coacervation, i.e. the mutual flocculation of two oppositely charged biocolloids, the flocculated substance having the nature of a liquid ("coacervate"). Here the conditions which make coacervation possible are the most simple from a biological point of view.

It is only necessary that the P_H reaches a value where the one colloid component (e.g. clupeine, gelatin, serum albumin, egg albumin) has a sufficiently positive charge, the other colloid component (e.g. gum arabic, nucleinate, chondroitin sulphate) still a sufficiently negative charge and besides the two colloid components must be present in a fairly favourable mixing-ratio.

For our purposes egg albumin and serum albumin cannot be used, since denaturation sets in upon contact with the emulsifying medium containing amylalcohol-ether. In the following we restrict ourselves to the combination gelatin-gum arabic, in which case complex coacervation is very easily realized. However, for the microscopical examination it has the drawback that it requires a temperature which is at least 30° C. or higher. It is true, at room-temperature, shortly after cooling of the gelatin sol, still liquid coacervates are formed, but these gelatinize soon, accompanied by simultaneous vacuolization phenomena. In order to be able to microscopize at room-temperature, a preliminary treatment of the gelatin is necessary.

b. Preparing of gelatin preparations with a reduced gelatinizing capacity.

It is possible by means of a suitable preliminary treatment of the gelatin with acids or bases at higher temperatures to weaken the gelatinizing capacity to a sufficient extent, so that preparations are obtained which at room-temperature may be used for our purpose. A too long or too powerful treatment with the mentioned substances should be avoided, since the gelatin ultimately is so strongly affected that complex coacervation with gum arabic is no longer possible.

It stands to reason that directions of general validity cannot be given, owing to the very different characteristics of the various kinds of gelatin on the market.

The following directions, applicable to "Gelatin for bacteriological purposes" of the Glue and Gelatin Works "Delft" at Delft, may give an impression of the formation of preparations obtained by acid and alkaline reactions respectively.

A. (*obtained by acid reaction*).

20 gr. of gelatin are left in 100 cc of distilled water at room-temperature to swell during a sufficient time, then the gelatin is dissolved at a higher temperature, the solution is boiled in a boiler with a round bottom and back-flow cooler, 5 cc of glacial acetic acid being added. After boiling during 1 hour the substance is cooled, 10.8 gr. of Na-acetate being added, left for some time and then dropped into 500 cc of alcohol. The separated tough gelatin mass is now repeatedly treated with 200 cc of alcohol. The removal of Na-acetate and acetic acid is promoted by picking the gelatin mass after sufficient hardening into smaller bits. Finally it has to be treated several times with acetone and dried in the air. Yield — 14.5 gr.

The obtained preparation has not entirely lost the capacity of gelatination and does not dissolve completely in cold water but needs some heating. The 3 % solution at room-temperature remains liquid for a long time, but yet it gelatinizes in the end.

B. (*obtained by alkaline reaction*).

20 gr. of gelatin are left to swell in 180 cc distilled water during 1 hour, after which time the flask is placed during half an hour in a thermostat of 50°, in order to dissolve the gelatin completely. Now 4 gr. of NaOH dissolved in 6 cc of distilled water are added. Since alkali in this concentration and at this temperature (50°) affects the gelatin much more quickly than acetic acid in the abovementioned method, only a short time of reaction should be allowed. At special intervals 50 cc are pipetted from the flask into small flasks with 3 cc of glacial acetic acid. This amount of glacial acetic acid is chosen in such a way that after neutralization of the NaOH a buffer mixture Na-acetate-acetic acid is formed in the ratio of roughly 1 : 1.

It becomes now apparent that by a treatment of 10 minutes a suitable gelatin solution is obtained, which at room-temperature within 24 hours does not gelatinize and still produces complex coacervation. After a reaction of 20 minutes, however, the gelatin has broken down too far.

As was indicated above under *A*, we can of course separate the gelatin again from this solution; however, this is not quite necessary for the following experiments, since the salts impeding coacervation may later be diffused.

c. Morphology of the complex coacervation of the sol mixture enclosed in the cavities.

A 5 % solution of the gelatin preparation *A* is prepared or the gelatin solution obtained according to *B* is diluted to half its concentration and mixed with an equal volume 5 % solution of gum arabic [1]).

According to the method indicated in 1. now a collodion film is prepared, in which drops of this sol mixture are enclosed. If the object glass is laid in a PETRI dish and this is filled with glacial acetic acid 250 × diluted with distilled water and placed on the object table of the microscope, we can observe that the complex coacervation now taking place is formed in the following way (cf. also fig. 1):

a. Gradually more and more coacervate droplets are formed in the cavities;

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[1]) We used a very good quality in clear pieces (Gomme Senegal, petite boule blanche from ALLAND et ROBERT, Paris) which after being crushed was dissolved in water. It is to be recommended to centrifuge this solution.

β. They increase in number and size to such an extent as to touch each other and fuse;

γ. The events in *b*. lead to a "phase reversal", i.e. the collodion wall is completely moistened by the coacervate and this fills the whole cavity, a large number of small vacuoles being embedded in it;

δ. The vacuoles are gradually fused, soon one large vacuole is formed by the side of not yet fused smaller vacuoles, and finally the image is found of a coacervate moistening the wall with one central vacuole.

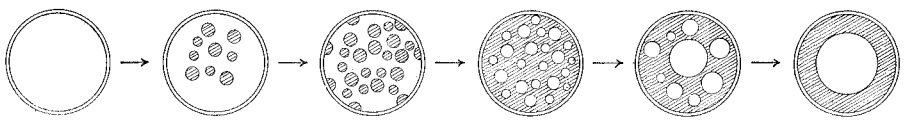

Fig. 1.

On the first microphotograph reproduced here (fig. 2) the last stages of this process of fusion are represented. In the small cavities, where the events succeed each other more rapidly than in the large ones, the final stage is often already reached. In the large ones, on the other hand, a central vacuole is present but the coacervate moistening the wall still contains many smaller vacuoles. On the microphotograph also some cavities are to be seen which contain no complex coacervate. Owing to a lesion, they have already been emptied but consequently they show the more clearly the condensed zone of the collodion round the cavities, which condensed zone makes the impression of a membrane.

Further researches concerning the model described here will be published later in Protoplasma.

Observation.

Although the gelatinizing capacity of the previously treated gelatin has strongly decreased, it has not been completely neutralized. In the complex coacervate this characteristic becomes again clearly manifest. Consequently together with the coacervation gelatination sets in again and only the smaller cavities reach the final stage; the larger ones on the other hand often do not. By heating the dilute acetic acid to 40—50°, here also the final stage may be reached. Accelerated fusion of the vacuoles in large cavities, however, is also possible at room-temperature, if to the dilute acetic acid a sufficient quantity of resorcin is added (e.g. in addition to 0.4 % of acetic acid besides 5 % of resorcin). This fusion may also be brought about by locally in the PETRI dish dropping some drops of concentrated resorcin solution on the preparation. Resorcin, namely, in a sufficiently strong concentration neutralizes the gelatination, but there is a possibility that now in the coacervate new small vacuoles are formed, since resorcin by the side of this property also causes dehydration in a certain range of concentrations.

H. G. BUNGENBERG DE JONG AND O. BANK: BEHAVIOUR OF MICROSCOPIC BODIES CONSISTING OF BIOCOLLOID SYSTEMS AND SUSPENDED IN AN AQUEOUS MEDIUM. III. COACERVATION PHENOMENA IN DROPLETS OF BIOCOLLOID SOLS ENCLOSED IN A COLLODION FILM. ACCUMULATION OF BASIC DYES.

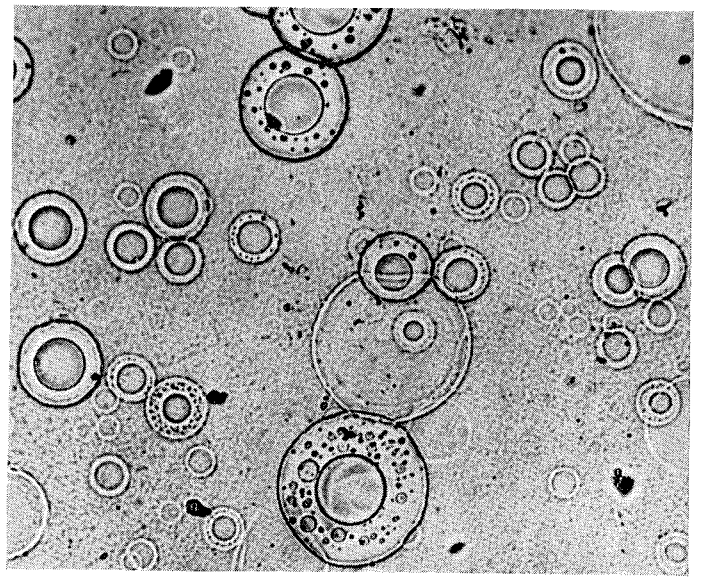

Fig. 2 (180 ×).

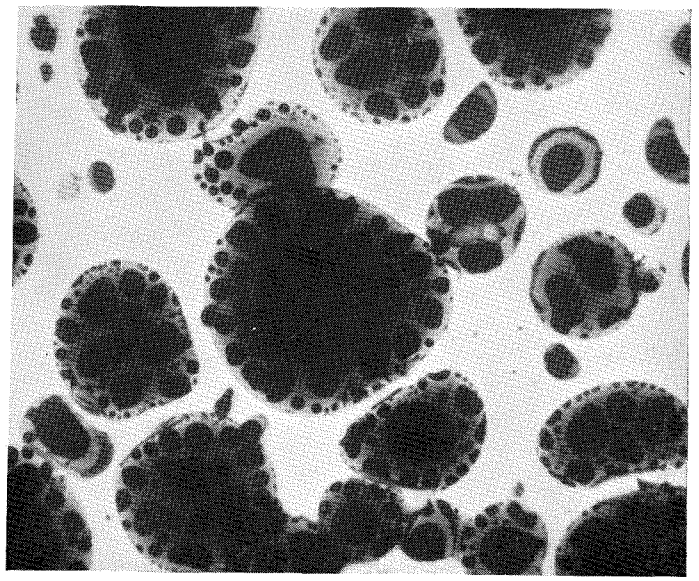

Fig. 3 (200 ×).

d. Significance for biology: Morphological model for parietal cytoplasma with central vacuole.

The adult cell of the higher plants is usually characterized by the possession of one large central vacuole. In young cells this is not yet present, but is gradually formed by fusion of a large number of smaller vacuoles, owing to which phenomenon ultimately the cytoplasma (except plasma strands) comes to be situated entirely against the wall.

Modern cytologists think that the cytoplasma is usually a liquid and that this neither on the outer surface nor on the interface with the vacuole possesses a microscopically visible membrane. In 1932 one of us suggested the possibility that the cytoplasma (and possibly other cell components) might have the nature of a coacervate or at any rate that upon formation of morphological structures coacervation phenomena might be temporarily placed in between [1]). What has been said under *c.* shows that the typical morphological constellation: wall — cytoplasma — vacuole, as well as the formation of the central vacuole by fusion of many smaller ones, may be imitated with models in which coacervates take part.

3. *Accumulation of a basic dye in the cavities containing gum arabic solution and coacervation of the gum arabic in case of sufficient accumulation.*

a. Accumulation of basic dyes.

The following refers to analogously prepared collodion films, with this difference that instead of the sol mixture exclusively a 5 % solution of gum arabic was emulsified in the amylalcoholic-etherial collodion solution.

If a membrane prepared in this way is laid in a dilute solution of basic dyes, e.g. neutral red, methylene blue, brilliant cresyl blue, toluidine blue, fuchsin, etc., accumulation of the dye takes place in the enclosed gum arabic solution.

In principle DONNAN's equilibrium may account for this accumulation. The arabinate ion cannot diffuse, but the cations belonging to it (mainly Ca-ions) will exchange with the dye cations till the ratio of the concentrations (activities) of the anorganic and dye cations in the gum arabic solution is equally large as in the outer liquid. If now the concentration of the original dye solution is low as compared to the concentration of the gum arabic, then after the equilibrium being reached, the dye concentration in the gum arabic solution will be considerably higher than in the outer liquid.

b. Coacervation of the gum arabic.

When as the result of the exchange mentioned in *a.* the substitution of the original anorganic cations, accompanying the arabinate ion, by dye cations exceeds a certain value, coacervation of the gum arabic takes place.

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[1]) H. G. BUNGENBERG DE JONG, Protoplasma, 15, 1932.

In the cavities small deeply stained coacervate drops are separated, which may fuse and are also deposited on the wall. We refer to the microphotograph (fig. 3) where this coacervation has been formed after the preparation being placed in a c. 0.02 % solution of toluidine blue.

c. Significance for biology: Vital staining of vacuoles, unmixing of the vacuolar liquid.

The above-described accumulation of basic dyes and possible following phenomena of unmixing may perhaps be regarded as models for the vital staining of vacuoles in plant cells and for the unmixing phenomena which may accompany them. It has to be assumed then that diffusible cations from the vacuoles exchange with dye cations. However, there is this difference that this model shows the described phenomena with all typical basic dyes, whereas in plant cells only a group, the so-called vacuolar dyes, is able to bring this about. This may be due to differences between the dyes mutually as regards the penetrability of the protoplasm: The vacuolar dyes can pass this as a whole, the others cannot.

Further particulars concerning accumulation of basic dyes and the coacervation following a very strong accumulation, as well as a discussion as to how far the mechanism of vital staining of the vacuole might be explained in this way, will be published later in Protoplasma.

Laboratory for Medical Chemistry at Leiden.

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