Biochemistry. — *Behaviour of microscopic bodies consisting of biocolloid systems and suspended in an aqueous medium.* IV. *Vacuolation phenomena of complex coacervate drops at a constant temperature. Formation of foam structures and of thin-walled drops with a large central vacuole.* By H. G. BUNGENBERG DE JONG and O. BANK. (Communicated by Prof. H. R. KRUYT.)

(Communicated at the meeting of February 25, 1939.)

Introduction.

After mixing a positively charged gelatin sol with a negatively charged gum arabic sol, provided the mixing-ratio and pH are not too unfavourable, we find complex coacervation [1]).

To every mixing-ratio belongs a certain pH at which the coacervation is optimal and at the same time reversal of charge takes place. At a lower pH the coacervate drops have a positive charge, at a higher pH a negative one. The morphology of the vacuolation phenomena described below is different according as we start from a negatively or a positively charged complex coacervate. Data concerning the systems used for this investigation as regards the pH of optimal coacervation and reversal of charge will therefore be given first: We made use of isoelectric gelatin [2]) and of Na-arabinate [3]), viz. a radical solution was prepared from 12 gr. of Na-arabinate + 10 gr. of isoelectric gelatin + 380 cc of distilled water. At 36° each time 5 cc of this mixture was pipetted into a series of tubes of the shape as produced in fig. 1 and then x cc of HCl 0.1 N + (7,5 — x) cc

Fig. 1.

of distilled water. After mixing thoroughly, the tubes were hung in a thermostat of 36° and after ± 10 minutes a cohesive coacervate layer was obtained by centrifugation during a short time with a hand-centrifuge. After another 10 minutes in the thermostat, the centrifugation was repeated and the coacervate volumes were read (table, column 2). Only the mixtures

[1]) For elaborate researches on complex coacervation see: H. G. BUNGENBERG DE JONG and W. A. L. DEKKER, Koll. Beihefte, **43**, 143 (1935); **43**, 213 (1936).

[2]) For starting-product and preparation of the isoelectric gelatin see in: H. G. BUNGENBERG DE JONG and W. A. L. DEKKER, Koll. Beihefte, **43**, 213 (1936), see p. 256.

[3]) For the preparation we refer to: H. G. BUNGENBERG DE JONG and P. H. TEUNISSEN, Koll. Beihefte, **47**, 254 (1938).

TABELLE I.

Added cc. HCl 0.1 N (= x)	Coacervate volume (cc.)	pH	U Electrophoretic velocity (arbitrary units)
0.45	0		
0.55	0.39		
0.65	0.96		
0.75	1.22	3 58	— 317
0.90	1.22	3.37	— 122
1.00	1.25	3.18	— 1
1.15	1.19	2.98	+ 113
1.25	1.10	2.89	+ 127
1.40	0.73	2.75	+ 133
1.60	0		

with x = 0,55 and 0,65 resp. were centrifuged once more after a long time. The read coacervate volumes, recorded in column 2, of these two x values are somewhat too small, the upper layers still being slightly turbid. Of identically composed mixtures, however, with a final volume of 50 cc

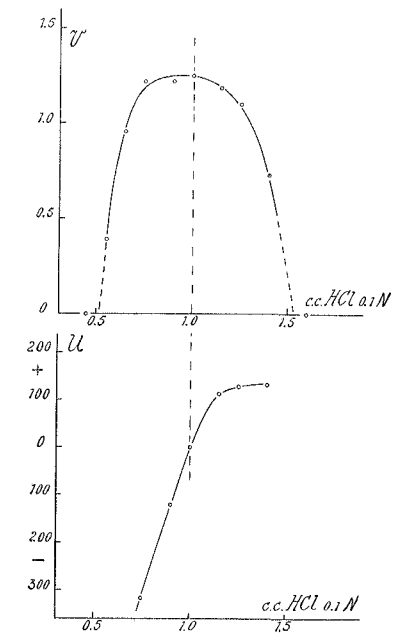

Fig. 2. Connection between optimal coaservation and reversal of charge.
Ordinates: V = volume of the coacervate.
U = electrophoretic velocity in arbitrary units.

instead of 12,5 cc, were yet determined the pH of the systems and the electrophoretic velocity (in arbitrary units), both at 36° [1]).

The results have been collected [2]) in table I.

From fig. 2 may be seen that the optimal coacervation takes places at the reversal of charge.

2. *Formation of coacervate drops with numerous smaller enclosed vacuoles, with foam structure and one large central vacuole resp.*

After the coacervate layers had been completely centrifuged, the centrifugal tubes (see fig. 1) were placed in water of room-temperature, as the result of which the coacervates gelatinize and at the same time a loose flocculated mass separates from the upper layer. This flocculated mass is of its nature likewise a gelatinized complex coacervate [3]).

After half an hour the turbid upper liquid was poured from the now opaque [4]) gelatinized coacervate and the tubes rinsed with distilled water, after which to each tube 12.5 cc of distilled water is added. Subsequently the tubes were placed in the thermostat at 36°. The gelatinized layer of coacervate now becomes again liquid and transparent. After a sufficiently long period (5 to 10 min.) the content of the tubes is carefully shaken. Powerful shaking should be avoided, in order to prevent the molten coacervate from dividing into too small droplets. Now a little of the obtained turbid system is brought on an object-glass and the tube is again placed in the thermostat of 36°. Microscopical examination immediately after placing on the object-glass reveals in all coacervates the presence of vacuolation phenomena. Morphologically, however, we can clearly distinguish two types of these phenomena. According to the table II below, the type of vacuolation is closely connected with the condition of the charge of the original coacervate.

If the coacervate originally was uncharged or had a positive charge, several small vacuoles, lying relatively far apart, occur in the coacervate.

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1) It is absolutely necessary to perform the pH and electrophoretic determinations at the same temperature. On cooling of the systems to room-temperature, pH shiftings as well as changes in the electrophoretic velocity occur, accompanying the gelatination of the coacervate drops.

2) A specially constructed cuvette was used for the microscopical measurement of the electrophoretic velocity, enabling us to measure at a higher temperature.

3) By heating these floccules are again completely dissolved in the upper layer. When they are centrifuged and then heated in a little of the upper layer, typical coacervate drops are formed, which after cooling behave in the same was as the original complex coacervate drops. The amount of separated floccules is a minimum at the reversal of charge. At the same time there is a minimum of arabinate + gelatin present in the upper layer; i.e. the solubility of the coacervate at the reversal of charge is a minimum. By cooling this solubility evidently decreases.

4) Opaque, owing to the separation of numerous very small vacuoles. This vacuolation, accompanying the gelatination of the complex coacervates, does not form the subject of this communication.

TABLE II.

cc HCl 0.1 N	Charge	Morphol. appearance of the coacerv. dr. after mixing of molten coacervate with distilled water, after the given number of minutes		
		½	5	10 min.
0.55	— — — —	ring str.	ring str.	ring str.
0.65	— — —	foam str. a. ring str.	„	„
0.75	— —	„ „ „	„	„
0.90	—	„ „ „	foam str. a. ring	foam str. a. ring
1.00	0	vac. str.	vac. str.	vac.
1.15	+	„ „	„ „	„
1.25	+ +	„ „	„ „	„
1.40	+ + +	„ „	„ „	„

Sometimes these vacuoles may yet become fairly large, but they do not flatten each other and consequently retain their globular shape (see fig. 3).

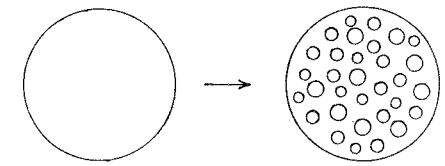


Fig. 3. Vacuolation of the positive coacervate drops.

After five or ten minutes in the thermostat, apart from small alterations in the size of the vacuoles, no change occurs in the morphological appearance of the vacuolated coacervate drops. After a sufficiently long period in the thermostat, the vacuoles disappear (in all probability by passing out) and consequently again exclusively homogeneous coacervate drops are found.

The changes displayed by the originally negative coacervate drops are of quite a different nature. Immediately after the shaking of the molten coacervate layer with the distilled water, miscroscopical examination reveals by the side of other forms discussed below coacervate drops with a foam structure. The often fairly large vacuoles flatten each other and are only separated by thin coacervate lamellae. The foam structure develops here peripherally, which may be excellently observed in large coacervate drops. In order to be able to observe the various stages of foam formation in one and the same preparation side by side, we should in particular examine the coacervates which originally have a weak negative charge, e.g. the coacervate formed with 0.9 cc of HCl 0.1 N. Evidently the source of

energy playing a part in the formation of the voluminous vacuoles is fairly soon exhausted here and we observe all the different stages side by side immediately or after 5 or 10 min. In some usually very large coacervate drops we notice then that the foam formation starts in the peripheral layers of the coacervate drop (see fig. $4a \rightarrow b \rightarrow c$), in other smaller ones the transition into a foam mass has already been completed (see microphotograph A).

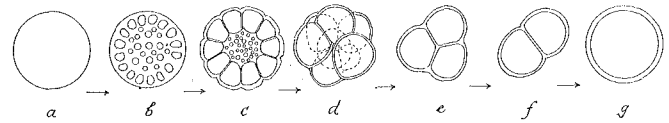


Fig. 4. Formation of the liquid hollow spheres from negative coacervate drops.
a. Initial stage: homogeneous coaiervate drops.
b and c. Peripheral formation of the foam vacuoles. In the centre a granulation may be observed, probably produced by numerous small vacuoles.
d. The whole coacervate drop has changed into a complex of foam vacuoles.
e and f. By bursting of the foam lamellae, forms occur with a small number of foam vacuoles.
g. Final stage: liquid hollow spheres.
The various forms a — g have been drawn in this diagram arbitrarily all of about the same size.

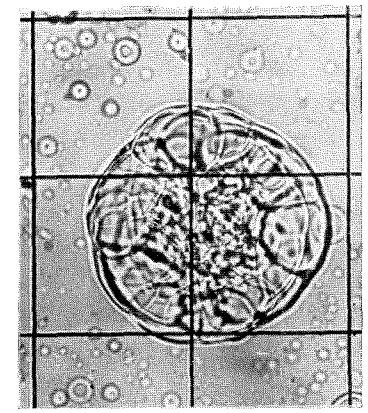
The number of individual vacuoles, separated by coacervate lamellae, may vary greatly. Frequently also forms are observed with a small number, e.g. 4, 3 or 2, while constantly a large number of coacervate drops is found with only a single very large vacuole (see microphotograph B).

Upon examination of the preparation under the microscope, no or hardly any modifications of the various forms are observed. However, this is the result of the gelatination of the coacervate lamellae by cooling. At 36°, however, alterations do occur (provided the original coacervate had a sufficient negative charge). After 5 to 10 min. in the thermostat there is usually very little left of the polymorphy of the coacervate drops with foam structure, which is observed immediately after shaking. Now almost exclusively [1]) coacervate drops are found with a very large central vacuole (see Table II: "ring structure", according to the form of the optic section, which is observed microscopically). Evidently these liquid hollow spheres (see microphotograph C) originate from the above-mentioned coacervate drops with foam structure by successive bursting of the coacervate lamellae in between the vacuoles (see fig. $4d \rightarrow e \rightarrow f \rightarrow g$).

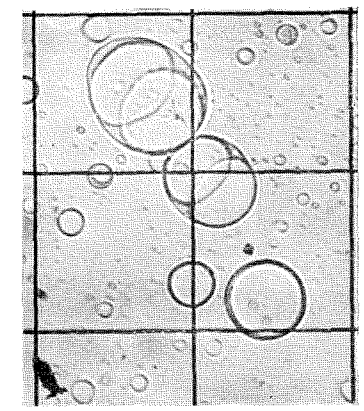
3. *Liquid hollow spheres.*

The hollow spheres, formed after a short while from the coacervate drops with foam structure, are in several respects remarkable. In the first place, the relative stability of this structural form. At 36° they remain to exist for a fairly long time (e.g. more than 20 min.), in spite of the fact

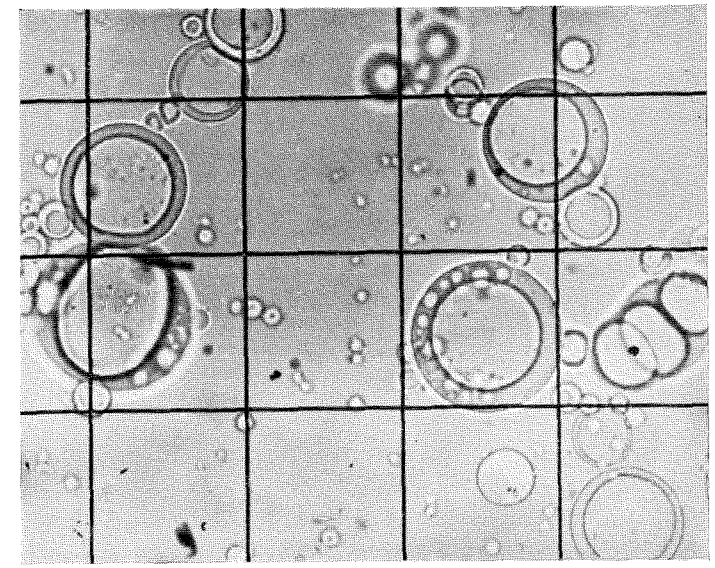
1) By the side of a small fraction of more complex forms, chiefly f of fig. 4.



A (160 ×)



B (160 ×)



C (160 ×)

that the coacervate lamella enclosing the large vacuole is very thin and the internal coacervate lamellae separating the vacuoles in the coacervate drops with foam structure are indeed rapidly destroyed. The outer surface of the hollow sphere may be in a special condition.

In the typically developed hollow spheres (see microphotograph *B*) the volume of the central vacuole is often a multiple of the volume of the globular skin of coacervate enclosing it. Frequently we find that the total diameter and the diameter of the vacuole stand in the relation of 9 : 8, which corresponds with a volume of the vacuole which is 2.4 times larger than that of the coacervate. It is also remarkable that during the beginning of the existence of the hollow spheres the thickness of the wall of the coacervate layer everywhere appears to be equally great. In all probability there is a mechanism which, when the centring of the vacuole threatens to get lost, corrects this again and which is connected with the relative long duration of their existence.

In spite of this, yet the hollow spheres ultimately degenerate, viz. by the decreasing volume of the central vacuole (and perhaps by passing out), so that finally they change into homogeneous coacervate drops [1]). The hollow spheres reproduced in microphoto *C* are already in this stage of decrease of the volume of the central vacuole. In the larger specimens this volume is here averagely somewhat smaller than the volume of the globular skin of coacervate ($\pm$ 0.8). Besides, in some drops we already notice defects with regard to an ideal centring of the vacuole. If now we raise the question for what reason the mentioned decrease in volume of the central vacuole takes place, we should bear in mind that the form of the liquid hollow sphere from the point of view of free boundary surface energy cannot be a stable one. During its formation, by a process which will be discussed below, work has been done against the free boundary surface energy. There is now every reason to assume that this process doing work has not ceased immediately but with a gradually decreasing intensity continues for some time.

Consequently, during the time that the typical hollow spheres exist, we have constantly to deal with two free energies:

A. one trying to press water into the vacuole;
B. the free boundary surface energy, constantly aiming at the smallest possible macro boundary surface of the coacervate and therefore trying to press water from the vacuole.

During the formation of the hollow spheres *A*. prevails entirely, after some time *B*. begins to predominate. In between, consequently, there must

1) These coacervate drops may be distinguished from the original ones, and also from the coacervate drops formed by dist. water from the original positive coacervate, in this way that at 36° after sedimentation in the tube they can not or only with great difficulty melt into a homogeneous coacervate layer. The supposition suggested above, that the outer surface of the hollow spheres is in a special condition (e.g.gel condition), thus gains in probability.

be a time when the hollow sphere does not change in dimensions, the two processes counterbalancing each other. The hollow sphere then seems to be in equilibrium with its surroundings, although in reality it is in a stationary condition.

Observations.

1. If it were possible to cause the free energy mentioned under A. to remain constant from now onwards or to place it on another constant level, the liquid hollow sphere in the first case would remain unaltered in dimensions, in the secound case it would change into one with other but again constant dimensions.

This seems not devoid of interest with regard to biology. It might be possible that not all cell structures have a static character but some also such a dynamic nature. A change in the functional condition of the cell might then be accompanied by in principle reversible changes of these dynamic structures.

2. The above-described hollow spheres are not specific to the complex coacervate from positive gelatin + negative arabic acid, but were also obtained in other complex coacervates, as far as they are examined in this respect (positive gelatin + negative nucleic acid; positive egg albumin + negative arabic acid), again by sufficient negativation. We may expect, consequently, that we have to deal here with a common characteristic of the complex coacervates. It is hardly doubtful, therefore, that the morphological forms, which W. BERG[1]) observed microscopically already more than thirty years ago in the reaction of protamine solutions (clupeine sulphate) on dried ammonium nucleinate lamellae and which he called „Hohlkörper", likewise belong to the same category.

The description of his experiments does not enable us to get acquainted with all the factors (e.g. of the pH) playing a part in their formation, but yet we may conclude from it that here also a strong negativation first made the formation possible.

The original separated homogeneous unmixing drops with excess of protamine in the solution (read: positively charged complex coacervate drops) were vacuolated and changed into Hohlkörper, if by the existing convection current in the liquid they were driven very close to the nucleinate lamela (read: by absorption of nucleinate in the coacervate reversal of charge and strong negativation set in). W. BERG also observed that already formed Hohlkörper lose their characteristic form and again change into homogeneous drops, if a protamine solution in excess reacts upon them. It should be remarked here that also with the above-described liquid hollow spheres from gelatin and arabinate a positivation (e.g. by addition of a little HCl) makes them rapidly change into homogeneous coacervate drops.

3. Finally it may be observed that the phenomena described here of abnormal enlargement of the vacuoles are not restricted to the complex colloidal systems in the limited sense of the word (consisting of two oppositely charged biocolloids) but may occur as well in autocomplex systems (where the role of one of the biocolloids is taken over by a similarly charged crystalloid ion). In the first communication of this Series[2]), namely, pulsating vacuoles were described in coacervates formed out of gum arabic + basic dyes. Transitory forms occurred there, which here we called liquid hollow spheres, and again under circumstances causing a very strong negativation, viz. when the originally homogeneous coacervate drops were surrounded by the gum arabic solution.

4. *Provisional theory of the vacuolation phenomena.*

In the experiments described above the molten gelatinized coacervate

[1]) W. BERG, Archiv. f. Mikrosk. Anat. **65**, 298 (1905).

[2]) H. G. BUNGENBERG DE JONG, Proc. Kon. Ned. Akad. v. Wetensch., Amsterdam, **41**, 643 (1938).

layer was shaken with 12.5 cc of distilled water. If a larger volume of distilled water is taken, also originally uncharged or positive coacervates may produce foam- and ring-structured coacervate drops respectively. Upon 50 cc of distilled water being taken, this is for example the case with the coacervates prepared with 1.00, 1.15 and 1.25 cc of HCl 0.1 N. The striking connection between morphological appearance and original condition of the charge, observed after mixing with a little distilled water, is then no longer present. However, electrophoretic measurements show that the coacervate drops, which are now formed, have a strong negative charge.

Coacervate drops with a typical "ring structure" may also be obtained from a positive coacervate by a repeated treatment with 12.5 cc of distilled water. After the first treatment vacuolated coacervate drops are formed from the positive coacervate prepared with 1.25 cc of HCl 0.1 N. If now these are centrifuged, the coacervate layer which now is turbid owing to the enclosed vacuoles is left to gelatinize, the upper layer is poured off and 12.5 cc of distilled water are added, we see that, after melting of the gelatinized coacervate layer and shaking at 36°, typical coacervate drops are formed with a large central vacuole.

This experiment also points in the direction that the fact whether foam- and ring-structured coacervate drops are formed is not so much connected with the original sign of the charge of the coacervate as with the question whether the negativation of the coacervate, produced by a raised pH, goes so far that the coacervate drops ultimately possess a strong negative charge.

Further researches, which will be published more extensively elsewhere, show that in the explanation of the vacuolation forms described here we have to distinguish:

a. a primary vacuolation, connected with the washing away of the neutral salt formed during the complex coacervation;

b. possible secondary changes of the formed vacuoles, which are connected with a sufficiently strong negative charge of the coacervate drops.

ad a. The primary vacuolation may be understood from the results of previous researches[1]). It appeared from analyses that addition of a neutral salt in a low concentration causes the water content of the complex coacervate to increase. Withdrawal of a neutral salt from a complex coacervate consequently results in water repulsion = vacuolation. Upon shaking of the coacervate with distilled water indeed withdrawal of a neutral salt takes place, viz. in our case of NaCl, which has been formed during complex coacervation and distributed over the two layers. With certain restrictions, discussed more elaboratly elsewhere[2]), complex coacer-

[1]) H. G. BUNGENBERG DE JONG and W. A. L. DEKKER, Kolloid Beihefte **43**, 213 (1936), see p. 255—261.

[2]) H. G. BUNGENBERG DE JONG, Kolloid Z. **80**, 221 (1937).

vation may namely be regarded as a double conversion. At optimal coacervation this may be formulated:

Gelatin chloride + Na-arabinate → gelatin arabinate. n aq. + NaCl.

In our case we can also calculate roughly how strong is the concentration of the NaCl after coacervation [1]). At the reversal of charge, for example, has been added 1 cc of HCl 0.1 N per 12.5 cc final volume, which yields a concentration of 8 m. aeq. per L. The pH is 3.18, i.e. the H conc. is only 0.66 m.aeq. per L. From this it follows that at the double conversion NaCl is formed of a concentration up to ± 7.3 m.aeq. per L.

If we shake the isolated coacervate layer with a dilute NaCl solution (e.g. 10—15 m.aeq.) instead of distilled water, we obtain only homogeneous coacervate drops. This may be easily understood, since the withdrawal of NaCl from the coacervate, resulting in primary vacuolation, now no longer can take place.

ad b. Above we came already to the conclusion that, when upon shaking of the coacervate drops with distilled water the coacervate obtains a sufficiently strong negative charge, the vacuoles formed by primary vacuolation swell strongly, while the coacervate lamellae separating them grow thin (foam structure) and finally burst (ring structure). Apparently a water transport takes place from outside through the coacervate to the vacuoles. Concerning the mechanism of this water transport we can, in expectation of more extensive researches, only express our opinion with some reserve.

First of all we might be inclined to think of an ordinary osmotic water transport through the coacervate to the vacuole containing a little salt (here NaCl). However, the fact that the formation of hollow spheres is not prevented, if we shake the coacervate layer with cane sugar solutions instead of distilled water (e.g. with a solution of 0.2 mol per L. which certainly is strongly hypertonic with regard to the 7.5 m.aeq. NaCl solution), renders this supposition highly improbable. Moreover, it remains unexplained why in positive coacervates no hollow spheres are formed. For this reason we are inclined to ascribe the water transport towards the vacuoles to an abnormal osmosis.

On account of the importance of the sign of the charge of the coacervate, we think in particular of an electro-endosmotic liquid transport. Here a membrane potential between vacuolar liquid and outer liquid would play a part, in such a way that the first is negative with regard to the second. In case of a negative charge of the coacervate an electro-endosmotic transport from outside inwards would then be the result.

This supposition, which however is suggested with due reserve, is in agreement with the different behaviour of the liquid hollow spheres towards addition of non-electrolytes (e.g. cane sugar) and electrolytes (e.g. salts) in low concentrations. If to an existing system with liquid hollow spheres cane sugar and NaCl respectively are added to a

[1]) According to recent researches on the distribution of a neutral salt over a coacervate layer and the equilibrial layer, the concentration in the coacervate is about equally strong as in the equilibrial layer.

final concentration of 10 millimol per L., in the first case practically no alteration takes place; with NaCl on the other hand the volume of the central vacuole decreases rapidly and they change into homogeneous coacervate drops. It might be supposed that in the latter case a new membrane potential has been introduced against the original one which had already strongly decreased in intensity, owing to which, provided the sign of the charge of the coacervate lamella in between remained the same, the vacuolar liquid is now rapidly electro-endosmotically transported outside.

Elaborate researches on the mechanism of the liquid displacements discussed here are still being made and will be published elsewhere.

Significance for biology.

It might be considered whether the vacuolation phenomena discussed above, which are accompanied by abnormal enlargement and fusion of the vacuoles under formation of liquid hollow spheres, in addition to other (e.g. normal osmotic) processes may be of importance in the formation of the central vacuole of the typical cell of the higher plants. However this question may be answered, it seems to us that the described vacuolation mechanism probably is of importance in the formation of pathological structures in the cytoplasm, particularly in the so-called "schaumige Degeneration". The supposition that the cytoplasm is a negatively charged complex system is in excellent agreement with the fact that it is in alkaline media that this schaumige Degeneration is produced [1]). Alkalization (raising of pH) namely results in a strong negativation of a complex system, which, as we saw in 2. and 4., is exactly what is needed for the formation of foam structure and ring structure. The phenomena discussed here yield models not only for the schaumige Degeneration of not or hardly vacuolated, e.g. animal cells and their possible reversibility, but also for the analogous phenomena in the typical cell of the higher plants, where foam formation occurs in the very thin layer of cytoplasm.

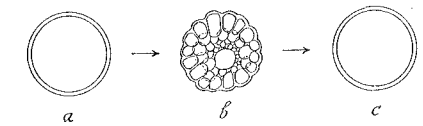


Fig. 5. Influence of a raised pH on liquid hollow spheres.
a. original condition.
b. foam formation in the globular skin of coacervate, the original vacuole growing smaller and often incognizable.
c. Final stage, formed after some time spontaneously from b.

If namely in an already formed system of liquid hollow spheres a further negativation is brought about by careful addition of NaOH, once more foam formation occurs in the globular skin of coacervate (fig. 5, $a \rightarrow b$). The original central vacuole may then shrink considerably and the microscopical image may even be dominated entirely by the new, strongly enlarged vacuoles, formed in the skin of coacervate.

[1]) E. KÜSTER, Pathologie der Pflanzenzelle Teil I, Protoplasma Monographien, Dritter Band, Gebr. Borntraeger, Berlin 1929. See p. 143.

After this system has been left for some time at 36°, the foam structure disappears spontaneously and the typical ring structure is restored (fig. 5, $b \rightarrow c$).

It would lead us too far to enter into details concerning the mechanism of the discussed changes, in particular of the shrinking of the original central vacuole. This will be reserved for a later more extensive publication in Protoplasma.

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Biochemistry. — *Behaviour of microscopic bodies consisting of biocolloid systems and suspended in an aqueous medium. V. Gelatinized hollow spheres. Temporary invagination to gastrula-like bodies by mechanical or osmotic removal of water from the central cavity.* By H. G. BUNGENBERG DE JONG and O. BANK. (Communicated by Prof. H. R. KRUYT.)

(Communicated at the meeting of February 25, 1939.)

1. In the preceding communication complex coacervate drops were described with a very large central vacuole (liquid hollow spheres, Hohlkörper). These morphological forms have only a limited stability. It is true that the hollow spheres at 36° remain to exist in the surrounding liquid during at least 20 min., but a real equilibrium is out of the question. The volume of the central vacuole is subject to constant changes. Immediately after the formation of the liquid hollow spheres the volume of the vacuole expands, first rapidly, afterwards more slowly, until after a short stationary condition the volume of the vacuole begins to decrease, first slowly, later more rapidly.

Since during these changes the character of hollow sphere is for the present maintained, they must be accompanied by a constant rearrangement of the liquid particles with regard to each other in the skin of coacervate.

In case of a decrease in volume of the vacuole, for example, the thickness of the skin becomes greater and its two globular surfaces smaller (fig. 1A).

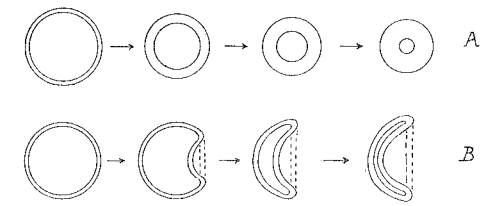

Fig. 1. Removal of liquid from the vacuole in a liquid (A) and a gelatinized (B) hollow sphere. It is supposed that the volume of the coacervate and of the gel during this withdrawal of liquid remains constant.

In this communication we shall discuss a few characteristics of the gel bodies, obtained by cooling of the liquid hollow spheres, in particular the changes they display upon removal of liquid from the central cavity.

Owing to the change of the coacervate into a gel, in these gel bodies rearrangements of the particles with regard to each other, as were possible during the liquid state in the coacervate skin, are not or only to a very