

Biochemistry. — Behaviour of microscopic bodies consisting of biocolloid systems and suspended in an aqueous medium. VIII. Formation and properties of hollow spheres from coacervate drops containing nucleic acid. By H. G. BUNGENBERG DE JONG and C. V. D. MEER. (Communicated by Prof. H. R. KRUYT.)

(Communicated at the meeting of April 25, 1942.)

1. Introduction.

In Communication VII¹⁾ of this series we said that CaCl_2 and $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ cause the formation of hollow spheres from the $G+N+a$ coacervate of the composite coacervate drops which on pH reduction are formed in sol mixtures $G:N:A=3:1:1$ (G = gelatine, N = Na-Nucleinate, A = gum arabic). In § 3 there follow further observations concerning the formation and properties of these objects, preceded in § 2 by some other ways in which hollow spheres can be formed from the $G+N+a$ coacervate. As for colloid preparations used and general methods we refer to Communication VII.

For an explanation of the mechanism of the formation of hollow spheres by salts from the $G+N+a$ coacervate it is important that we already find an analogous formation of hollow spheres in the $G+N$ coacervate. In what follows we shall discuss this more in detail (§ 4). It was desirable to make a preliminary investigation of the effect of salts on the coacervate volume of the $G+N$ coacervate, the results of which we add briefly in § 5.

2. Some other ways in which hollow spheres may be obtained from the $G+N+a$ coacervate.

A. Effect of pH reduction and pH increase on complex coacervate drops.

When according to the directions of § 3 in Communication VII in the auxiliary apparatus described there, we have prepared the composite coacervate drops and reduce the pH by the gradual addition of HCl (e.g. at intervals of 15 minutes each time 5 cc HCl 0.01 N), we note some phenomena which are not very striking: the $G+A+n$ wall of the composite coacervate drops decreases in volume, to disappear finally, while perhaps the vacuolization of the enclosed $G+N+a$ drop increases a little. So we see that pH reduction has an effect analogous to that of KCl in Communication VII.

When we first prepare the composite coacervate drops and then increase the pH on the other hand, by gradually adding Na-acetate (e.g. every five minutes 1 cc Na-acetate 0.1 N) the volume of the $G+A+n$ wall is also first seen to decrease. After it has disappeared entirely the remaining coacervate drop (originally the enclosed $G+A+n$ coacervate of the composite drops) strongly vacuolizes on continued pH increase and after a stage of froth structure a typical hollow sphere is formed with a rather thin wall (Fig. 1 a—f). When the system is cooled to room temperature while under the microscope we add some granules of saccharosis, we observe invagination (Fig. 1 f—g). This disappears on dilution with water, which shows that the invagination was brought about by osmotic dehydration, the changes described in Fig. 1 c—f are analogous to the changes previously described in the complex coacervate gelatine-gum arabic. The formation of hollow spheres described here we shall not discuss further, in this place only mentioning

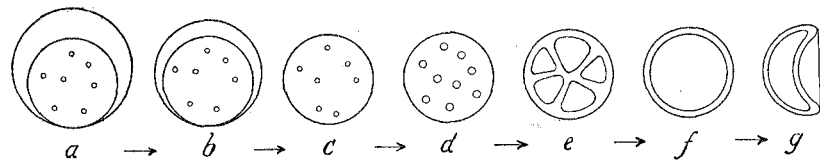


Fig. 1.

¹⁾ H. G. BUNGENBERG DE JONG, Proc. Ned. Akad. v. Wetensch., Amsterdam, 45, 393 (1942).

the fact, that drops of the complex coacervate gelatine-nucleic acid also change into hollow spheres, by increasing the pH which are also invaginated after gelatination, with cane sugar.

B. Hollow sphere formation in consequence of dilution with much dist. water.

Here follow some observations made several times, and very interesting from a morphological point of view, presenting many problems the solutions of which we have not yet attempted. When in the usual way (10 cc buffer + 10 cc dist. water + 5 cc stock sol $G:A:N=3:1:1$) we have prepared the system of coexisting coacervate drops, adding a great quantity (e.g. 50 cc or more) dist. water, we sometimes see that the $G+N+a$ coacervate is gradually lifted from the edge of the composite drop by a cavity, although locally the contact persists (Fig. 2 a—b). In other cases we get the impression that the

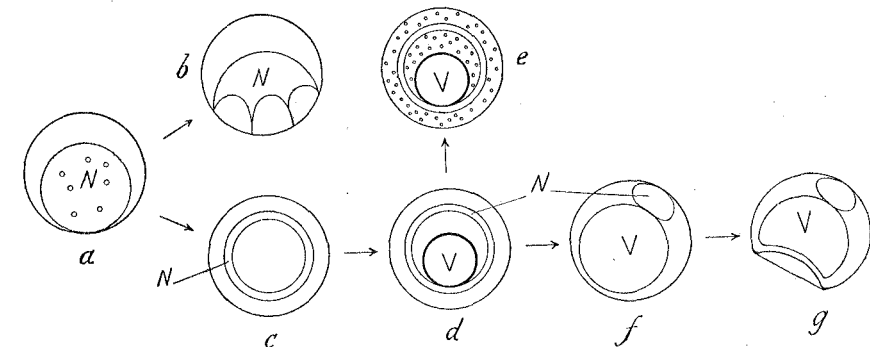


Fig. 2.

enclosed $G+N+a$ coacervate has lost contact with the outside of the drop enclosed, gradually passing as an independent body into a hollow sphere with a rather thick wall (Fig. 2 a—c).

Here we have frequently observed that within the cavity in the $G+N+a$ coacervate there is formed another cavity much more sharply circumscribed. By focussing the microscope at different depths we can observe that this second cavity is optically not so dense as the one in the $G+N+a$ coacervate, so that here there is a *vacuole* (Fig. 2d V). The rest of the cavity in the $G+N+a$ coacervate must then be filled with newly formed $G+A+n$ coacervate. This is also indicated by the same behaviour of this cavity and the enveloping $G+A+n$ coacervate on pH reduction, when granulation arises, see Fig. 2e (formation of small, new $G+N+a$ coacervate drops).

Finally we note that the morphologic condition of d does not represent an equilibrium, as when a preparation is long left to itself in the auxiliary apparatus at 40° the condition of Fig. 2f is gradually reached, the entire contents of the hollow $G+N+a$ drop being broken through, the two $G+A+n$ coacervates having united, whereas the vacuole is still enclosed in the $G+A+n$ coacervate. On cooling and after the addition of cane sugar the $G+A+n$ wall invaginates (Fig. 2g), becoming round again when dist. water is added.

Here we make brief mention of the remarkable morphologic structure of the bodies of Fig. 2f, which has many points in common with some naked plant cells (occurring for instance in the pulp of ripe berries). The morphologic constellation present, is otherwise the same as occurs in the prismatic cells of celloidin membranes¹⁾, which have much in common with the morphologic structure of normal protoplasts enclosed in cells. The only difference is that here we find this constellation without an enclosing wall substance.

¹⁾ H. G. BUNGENBERG DE JONG, Proc. Ned. Akad. v. Wetensch., Amsterdam, 45, 76 (1942).

3. Further details concerning the formation of hollow spheres from the G + N + a coacervate by the addition of salts.

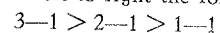
A. Effect of an excess of salts 2—1 and 3—1 on the hollow spheres thus formed.

In Communication VII we described how CaCl_2 and $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ cause the formation of hollow spheres from the G + N + a coacervate. With slightly greater concentrations there is a new effect, namely that the originally beautifully homogeneous pictures become less fine, sometimes even granular (markedly with $\text{Co}(\text{NH}_3)_6\text{Cl}_3$). We get the impression that the G + N + a coacervate assumes a more membrane-like character. It has often a corrugated surface, becomes blurred and granular. But also in the equilibrium liquid a finely granular precipitation is formed. The cause of this morphologic degeneration is to be found in the formation of the autocomplex system: bi- resp. trivalent cation + nucleinate colloid anion.

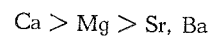
Hence nucleinate sols show a similar granular flocculation with bi- and trivalent cations, but not with salts of type 1—1, 1—2 or 1—3.

B. Specific salt effects on the formation of hollow spheres.

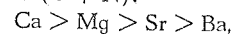
In Communication VII we have seen that with regard to CaCl_2 , $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ acts equally strongly already at smaller concentrations, while KCl does not cause the formation of hollow spheres (at least as long as there are composite coacervate drops). So to the activity of the salts decreasing from left to right the following valence rule applies:



It has appeared that hollow spheres may also be formed owing to KCl, K_2SO_4 and $\text{K}_3\text{CH}(\text{SO}_3)_3$, although this only happens with concentrations at which the surrounding G + A + n coacervate has already been neutralized. The salts mentioned then arrange themselves in the valence rule of the anions $1-3 > 1-2 > 1-1$. We also investigated by the same method if there are any differences between MgCl_2 , CaCl_2 , SrCl_2 and BaCl_2 . Here we found that the last two have the weakest effect, but we could not obtain any certainty as to the order of Sr and Ba. The order of the four cations was found to be as follows (according to their activity decreasing from left to right concerning the formation of hollow spheres)



The order of the ions agrees with the order found by O. BANK and E. G. HOSKAM¹⁾ in their investigations of the specific effect of ions on the neutralization of the complex coacervate gelatine — nucleic acid (G + N).



While as regards the general valence effect it was also found²⁾ that $3-1 > 2-1 > 1-1$ and $1-3 > 1-2 > 1-1$. When to this we add the observation published in another Communication, that KCl, added to a coacervated G + N system causes the formation of hollow spheres³⁾ then it is very likely that

a. The problem of the formation of hollow spheres from the G + N + a coacervate by the addition of salts is fundamentally the same as in the G + N coacervate.

b. The formation of hollow spheres in the G + N + a coacervate and in the G + N coacervate is intimately connected with the processes attending the neutralization of the complex coacervate by added salts.

It is for these reasons that we have further studied the G + N coacervate and in the following section (§ 4) we shall first see if what has been said in a. applies, while in § 5 we shall further discuss the supposition in b.

¹⁾ O. BANK and E. G. HOSKAM, *Protoplasma* 34, 188 (1940).

²⁾ O. BANK and E. G. HOSKAM, loc. cit. (Valence of cations), H. G. BUNGENBERG DE JONG and ONG SIAN GWAN, *Biochem. Z.* 221 (1930).

³⁾ H. G. BUNGENBERG DE JONG, O. BANK and E. G. HOSKAM, *Protoplasma* 34, 30 (1940) p. 41.

4. Formation of hollow spheres from the G + N coacervate by the addition of salts.

A. Method.

With the same pH value the water percentage of gelatine-nucleic acid coacervates is considerably below that of gelatine-gum arabic coacervates (the consequence of greater density of charge of the nucleinate colloid anion). The result is that when one wants to prepare G + N coacervates which are sufficiently liquid and suitable for morphological investigation, the pH must be chosen considerably higher. Whereas for the G + A coacervates pH 3.7 is a suitable pH, a considerably higher pH is preferable with G + N coacervates. Moreover, in the complex combination G + N there is an additional complication on acidification in the formation of nucleic acid. As we shall see in § 5 these disturbances occur already below pH 3.8. In what follows we worked with pH 4.4, when we could easily obtain coacervate drops which were beautifully homogeneous and of sufficient size for morphological investigation.

We always started from a stock sol consisting of 9 gram gelatine F00 extra + 3 gr. Na nucleinate + 108 cc H_2O . After the mixture has been left one night in the refrigerator it is dissolved by heating and used at once in experiment (for preparations and the method see Communication VII).

In the auxiliary apparatus we place at 50°:

18.2 cc dist. water + 1.8 cc $\text{HCl} 0.1 \text{N}$ and then 5 cc of the above stock sol (these quantities are in agreement with optimal coacervation see § 5). After 5 min. the coacervate drops are sufficiently large and homogeneous, after which the study of the effect of added substances can set in. When 10 cc dist. water is added, no morphologic changes take place.

So when with an added salt solution, usually a much smaller volume than 10 cc, we do note changes, this is to be ascribed to the salt and not to the water in which it is dissolved. In examining the salts the following method may be followed.

We first prepare a coacervated system, after 5 min. we add a smaller quantity (e.g. 1 resp. $\frac{1}{2}$ cc $\text{CaCl}_2 0.1 \text{N}$) salt solution, observe possible morphologic changes for 5 min. add the same quantity of salt solution, observe for another 5 min. thus continuing the intermittent addition of the salt solution.

A variant of this method is the addition of salt solution every 30 min. instead of every 5 minutes. In what follows the two methods are referred to as the 5 minute and the half hour method.

B. Effect of the cation and anion valence on the change of the G + N coacervate drops into hollow spheres.

By the 5 minute and the half hour method we first investigated the effect of the valence of the cation and the anion. We found that in sufficient concentrations *all the salts examined change the coacervate drops into hollow spheres.*

The changes are similar to those described in Communication VII for the effect of CaCl_2 and $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ on the G + N + a coacervate, namely first increased vacuolization, then coalescence of the vacuoles to few vacuoles, the drop sometimes assuming the character of a froth mass, after which the hollow sphere stage is reached.

The results of the 5 minutes and the half hour method are shown in Table I.

In column 3 are stated the values of the quantities added every 5 resp. 30 minutes of the salt solutions mentioned in column 2 (to $\text{K}_3\text{CH}(\text{SO}_3)_3$ we first added solid salt till 15,20 resp. 22 m.aeq. final concentration and after ca. 10 min. we continued by adding the 40 m.aeq. salt solution).

The final concentrations which are just too low for the maximal effect are found in column 4, they precede those which are sufficient.

By the two methods we find the orders:

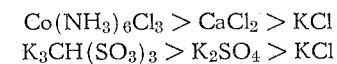


TABLE I.

Addition of salt to 25 cc coacervated G + N system, which are not yet and just (underlined) sufficient to change practically all the coacervate drops into hollow spheres.

	Salt solution	Addition in cc	Final conc. in M.AEQ.pL.
5 min. method	Co(NH ₃) ₆ Cl ₃ 0.1 N	0.5 — 0.5 — 0.1 — <u>0.1</u>	4.2 — 4.6
	CaCl ₂ 0.1 N	1 — 1 — 0.5 — <u>0.5</u> — 0.5 — <u>0.5</u>	12.5 — 13.8
	KCL 0.5 N	0.5 — 0.5 — 0.5 — 0.5 — <u>0.1</u>	37.0 — 38.7
	K ₂ SO ₄ 0.5 N	0.5 — 0.5 — 0.5 — 0.2 — <u>0.1</u>	31.8 — 33.6
	K ₃ CH(SO ₃) ₃ 0.04N	<u>1.0</u>	22.0 — 23.7
22 m.aeq.			
½ hour method	Co(NH ₃) ₆ Cl ₃ 0.1 N	0.7 — 0.1 — 0.1 — <u>0.1</u>	3.5 — 3.8
	CaCl ₂ 0.1 N	2.0 — 0.5 — <u>0.5</u>	9.1 — 10.7
	KCl 0.5 N	1.0 — 0.25 — <u>0.25</u> — <u>0.25</u>	28.3 — 32.7
	K ₂ SO ₄ 0.5 N	1.0 — 0.1 — <u>0.1</u>	21.1 — 22.9
	K ₃ CH(SO ₃) ₃ 15 m.aeq.	0.04N 0.5 — 0.5 — 1.0 — 1.0	
	K ₃ CH(SO ₃) ₃ 20 m.aeq.	0.04N <u>1.0</u>	17.8 — 20.7

that is, the order in which the salts from left to right have a decreasing neutralizing effect on the complex coacervates:

$$3 - 1 > 2 - 1 > 1 - 1$$

$$1 - 3 > 1 - 2 > 1 - 1 \text{ "double valence rule"}$$

C. Specific effect of the cations.

In order to investigate accurately the specific effect of MgCl₂, CaCl₂, SrCl₂ and BaCl₂, we observed the morphologic changes of the following mixtures with each time 4 of these salts during 5 min. and 30 min.:

25 cc coacervated system + 2.5 cc 0.1 N salt solution

25 cc coacervated system + 3 cc 0.1 N salt solution

25 cc coacervated system + 3.5 cc 0.1 N salt solution

and we stated the order:

$$\text{Ca} > \text{Mg} > \text{Sr}, \text{Ba}$$

analogously LiCl, NaCl and KCl were compared with each other, but the differences are so slight, that we can only pronounce the supposition that the order is

$$\text{Li} > \text{Na} > \text{K}$$

D. Other points of agreement between the G + N and G + N + a coacervates.

With CaCl₂ and Co(NH₃)₆Cl₃ degeneration phenomena also occur in the G + N coacervate. In the 5 min. method the first degeneration symptoms coalesce approximately with the optimal formation of hollow spheres. In the 30 min., method the two are distinct, so that in a certain concentration section, sound hollow spheres may be obtained. Again the hollow spheres show lengthy invagination with cane sugar. The invagination is of short duration with salts (e.g. after the additions of some granules of KCl under the microscope). The G + N coacervate also gives hollow spheres on pH increase (NaOH) which also invaginate with cane sugar.

5. On the mechanism of the change of G + N + a resp. G + N coacervate drops into hollow spheres through the addition of salts.

From what was mentioned in § 4 b—d appears the great similarity in the change into hollow spheres of G + N + a coacervate drops and of G + N coacervate drops through

the addition of salts, so that we may safely assume that if we can account for the G + N coacervate, the same will apply to the G + N + a coacervate. In order to make quite sure we also tried if the addition of KCl will cause the formation of hollow spheres from the G + A coacervate. The result is vacuolization, sometimes a large central vacuole is formed, but it disappears spontaneously after 5—10 minutes, leaving only homogeneous coacervate drops. On the other hand, the walls of the hollow spheres which are formed by adding KCl to the G + N coacervate are thinner and the spheres last much longer (e.g. 6 hours at 50°), after which homogeneous coacervate drops are gradually formed again. In these respects, therefore, the hollow spheres from the G + N + a coacervate are very much like those of the G + N coacervate and unlike those of the G + A coacervate.

As regards the mechanism of the formation of hollow spheres from the G + N coacervate, the occurrence of the double valence rule and of the specific cation order:

Ca > Mg > Sr, Ba (and possibly Li > Na > K) point to the intimate connection with events attending the neutralization by salts of complex coacervate G + N. In order further to support this conclusion, we made preliminary measurements at 40° of the effect of salts by the coacervate volume method, which we have often applied in our study of the G + A coacervate¹⁾. These tests are conducted so that they are practically comparable with the method described above: 25 cc final volume, containing 5 cc stock sol (9 G + 3 N + 108 H₂O). The coacervate volumes were noted after centrifuging to constant volume.

Fig. 3 shows the effect of added HCl. It is seen that the coacervate volume reaches

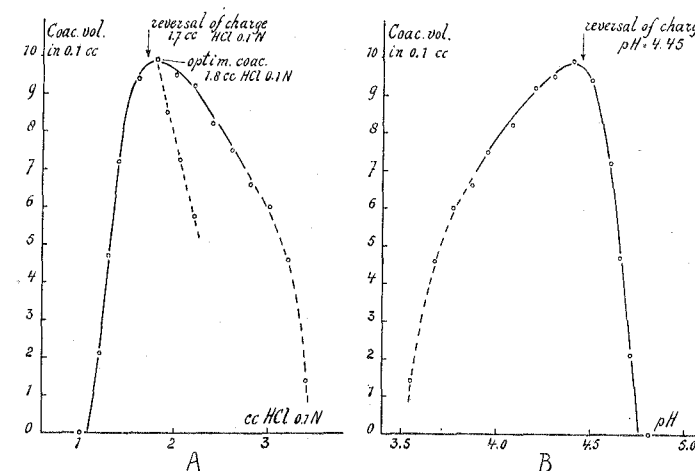


Fig. 3.

a maximum at 1.8 cc HCl 0.1 N (determined graphically by constructing a bisecting line).

In Fig. 3b the coacervate volumes are set out against the pH values measured at 40°; it appears that the peak of the curve lies at pH 4.40. The optimal coacervation, according to electrophoresis measurements lies at 40° very near the reversal of charge, which we found to be with 1.7 cc HCl = pH 4.45²⁾.

The part of the curves of Fig. 3, where the equilibrium liquids as well as the coacervates were entirely clear has been drawn in full, only towards a greater quantity of HCl (resp. towards a lower pH) the equilibrium liquid (formation of nucleic acid, see § 4a) begins

¹⁾ e.g. Proc. Kon. Ned. Akad. v. Wetensch., Amsterdam, 42, 247 (1939), 45, 3 (1942).

²⁾ Here we wish to express our thanks to Dr. H. L. BOOY for his assistance in measuring the electrophoresis velocity.

to be opalescent (pH 3.87—3.67). The coacervate is then fairly transparent still, but by still lower pH values, it becomes more and more opalescent and less transparent, like the equilibrium liquid.

Fig. 4a and 4b show the effect of neutral salts at optimal coacervation (i.e. at 1.8 cc HCl constant)¹⁾. Here we see the order of the salts (double valence rule) characteristic

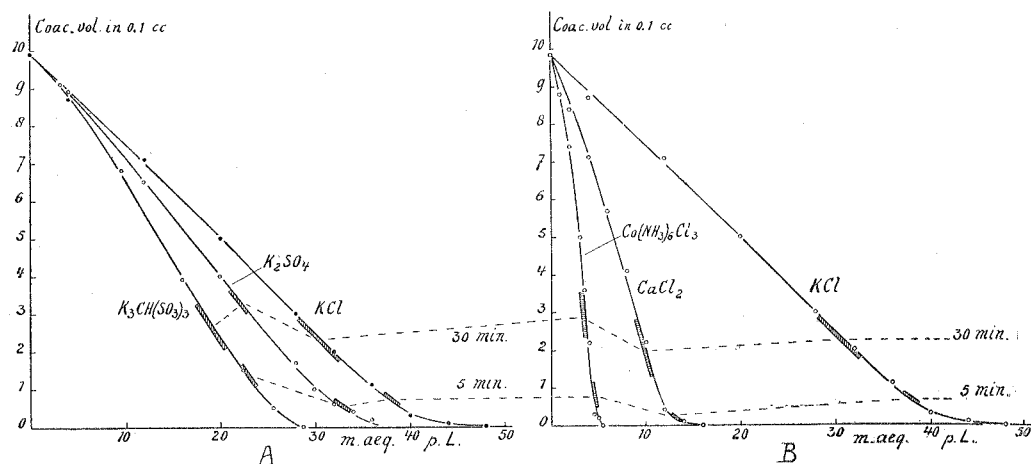


Fig. 4.

of the neutralization of complex coacervates. In Fig. 4 we have clearly marked the concentration sections extending between the two each time named concentrations in Table 1.

Indeed we see that the concentrations from which the hollow spheres are characteristically formed lie at the bottom of the coacervate volume curves (by the 5 min. method at roughly 0.1 and by the half hour method at roughly 0.25 of the original coacervate volume).

So hollow spheres do, indeed, develop as a co-effect of the neutralization of the coacervate by salts, rapidly (in 5 min.) when 0.9, slowly (in 30 min.) when 0.75 of the original coacervate volume will disappear through neutralization by salts.

The following observations are of importance for the problem of the mechanism of the formation of hollow spheres:

A. Cane sugar does not produce hollow spheres at ca. 100 m. mol final concentration. The presence of cane sugar in this concentration does not prevent the formation of hollow spheres by salts (e.g. KCl,) they also persist when afterwards cane sugar is added.

B. When through a salt a system of hollow spheres has first been formed, the extra addition of 10 cc dist. water does not cause the spheres to disappear, but the walls become a little thicker. When, however, 10 cc 10% amyllum solubile (MERCK) is added to a system of hollow spheres formed by a salt, the hollow sphere changes in 10—15 min. into a homogeneous coacervate drop, which condition is still present after 2 hours, 10 cc 10% dextrine has the same effect. When to the original coacervate system 10 cc 10% amyllum solubile is added first, it is no longer possible to obtain hollow spheres by the addition of KCl.

¹⁾ Measurements to the left of the peak (at 1.3 cc HCl 0.1 N) and to the right of the peak (at 2.6 cc HCl 0.1 N) show a course of the curves analogous to that we lately described for the G + A coacervate (H. G. BUNGENBERG DE JONG and C. V. D. MEER, Proc. Ned. Akad. v. Wetensch., Amsterdam, 42, 490 (1942) i.e. in the first case (negative coacervate) in small salt concentrations there is the order of curves (from top to bottom) 3—1 ... 2—1... 1—1...1—2...1—3 in the second case (positive coacervate) the order is reversed. In the higher salt concentrations the curves in both cases approach the absciss axis in the order of the double valence rule.

So we state a difference between a molecularly dissolved non-electrolyte (cane sugar) and a colloiddally dissolved non-electrolyte¹⁾ (amyllum, dextrine).

The former penetrates into the coacervate, the latter probably does not (at least we know this for complex coacervate G + A). Although after gelatination the wall of the hollow spheres is not easily penetrated by saccharosis, this is apparently not the case at a working temperature of 50°, according to A. But according to B, we must assume impenetrability for amyllum solubile, even at 50°.

So we arrive at the conclusion that according to B. we can prevent the formation of hollow spheres, resp. that we can change hollow spheres that have formed already into homogeneous coacervate drops, by giving a certain colloid-osmotic pressure to the surrounding equilibrium liquid. Therefore it seems probable to us that the hollow spheres are also formed in consequence of colloid-osmotic overpressure of the contents of little vacuoles, formed primarily as a result of added salts. The question remains why with the G + N coacervate and to a less extent with the G + A coacervate, there is temporarily a colloid osmotic overpressure of the contents of the vacuoles with regard to the medium surrounding the coacervate drops. We tentatively offer the following explanation, based on the rapid reaction to changes of the medium of the G + A coacervate and on the slow reaction of the G + N coacervate.

This can indeed in principle account for the above: the contents of the vacuoles will have assumed the higher colloid percentage of the equilibrium liquid belonging to the salt concentration long before this is the case with the great volume of the medium surrounding the coacervate drops. In this period there is colloid-osmotic overpressure of the vacuole contents, so that they want to take up water. When this happens, however the colloid osmotic pressure of the vacuole contents is not diminished because the water taken up, at once passes again into equilibrium liquid by taking up colloids from the coacervate surrounding the vacuole.

The driving force for the inflow of water into the vacuoles, owing to which they become ever larger and coalesce so that finally hollow spheres are formed, persists until at last the surrounding medium liquid has also assumed the composition of the equilibrium liquid. Owing to the rapid reaction of the G + A coacervate to changes of the medium, the colloid osmotic non-equilibrium between primary vacuoles and surrounding medium liquid is of short duration, so that the formation of hollow spheres takes place during a short time only.

Summary.

1. Further details are given concerning the formation and properties of hollow spheres from the G + N + a complex coacervate of composite coacervate drops.
2. Added salts also change drops of the G + N complex coacervate into hollow spheres and here again we find the influence of ion valence (double valence rule) and the specific ion order: Ca > Mg > Sr, Ba.
3. Measurements of the effect of salts on the volume of the G + N coacervate show that with concentrations in which typical hollow spheres are formed the coacervate is neutralized for the greater part.
4. A provisional explanation is given of the mechanism of the formation of hollow spheres by added salts, according to which a temporarily existing colloid-osmotic non-equilibrium between medium and contents of the primary vacuoles is taken as starting point. This explanation also accounts for the fact that the formation of hollow spheres by adding salts to the G + A complex coacervate is not nearly so striking.

Leiden, Laboratory for Medical Chemistry.

¹⁾ Amyllum solubile MERCK, although it has a weak capillar electric negative charge, has a very high reciprocal hexol number and can therefore be considered practically a non-electrolyte. As a matter of fact it does not form a complex coacervate with positive gelatine.