Rappelons maintenant que $g(P_0) = 0$ donc $\log\frac{k}{d} \leqq 0$. Il s'en suit $d \geqq k$, donc les domaines contiennent tous le cercle C_k de rayon $k > 0$ et de centre P_0. Par comparaison des valeurs-frontière et en appliquant le principe de maximum des fonctions harmoniques [1]), on voit que chaque $g_n(P_0, P)$ (P intérieur à C_k) vaut au plus la valeur en P de la fonction g correspondant à C_k, c.a.d. vaut au plus la constante $\log\frac{1}{k}$. On est alors arrivé à une contradiction: le membre à gauche de (19) tendant vers infini si $n \to \infty$, ne peut rester $< C$. Il s'en suit même une borne inférieure pour $\varphi(C)$.

En effet, il résulte de (19):

$$\log\frac{1}{r} - \log\frac{1}{k} < C$$

donc

$$r > k\, e^{-C}$$

Il s'en suit

$$\varphi(C) \geqq k\, e^{-C} \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (20)$$

ou avec (18)

$$\varphi(C) \geqq \tfrac{1}{2}\, e^{-\frac{\pi}{2} - C}$$

Par une considération des domaines $G(P, P_0) > C$, on arrive à l'existence d'une fonction $0 < \psi(C) < \infty$. En effet, en supposant que $\psi(C) = \infty$, on peut trouver une suite de domaines $\{\Omega_n\}$ et une suite de points $\{P_n\}$, où P_n est dans Ω_n, telle que $P_n \to P_\infty$ et

$$\log\frac{1}{r_{P_0 P_n}} - g_n(P_0, P_n) > C.$$

Remarquons maintenant que $g_n(P_0, P_n) = g_n(P_n, P_0)$ de sorte qu'on peut prendre le pôle dans P_n. On voit alors que le membre à gauche de cette inégalité doit tendre vers zéro, pourvu que g_n ne tend pas vers une fonction identiquement égal à $-\infty$. Or ceci n'est pas le cas, les frontières Σ_n ayant tous des points intérieur au cercle de rayon 1 et de centre P_0, puisqu'on a supposé $g_n(P_0) = 0$. On est donc arrivé à une contradiction.

Le passage au cas où $g(P_0) \neq 0$ est maintenant simple. Appliquons pour cela la transformation (17). On a

$$\bar{g}(P_0) = g(P_0) + \log\frac{1}{p}.$$

En supposant $\bar{g}(P_0) = 0$ et $g(P_0)$ égale à une valeur donnée, il faut prendre $p = e^{g(P_0)}$. La courbe $\bar{G} = C$ est transformée en $G = C$. La première courbe se trouve entre les cercles $(\varphi(C))$ et $(\psi(C))$, donc la courbe $G = C$ entre les cercles de rayon

$$\varphi(C)\, e^{-g(P_0)} \quad \text{et} \quad \psi(C)\, e^{-g(P_0)}.$$

Des tentatives pour déterminer les valeurs exactes de φ et de ψ, comme données dans l'introduction, par les méthodes précédentes n'avaient pas encore de résultat.

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[1]) Remarquons que $g(P, P_0) < \log\frac{1}{r_{P P_0}}$.

Biochemistry. — *Tissues of prismatic cells containing Biocolloids.* IV. *Morphological changes of the complex coacervate gelatine + gum arabic in consequence of a pH change of the medium flowing along the membrane.* By H. G. BUNGENBERG DE JONG and B. KOK. (Communicated by Prof. H. R. KRUYT.)

(Communicated at the meeting of November 29, 1941.)

1. *Introduction.*

In this and in the next communication we shall discuss the effect of the pH, of some neutral salts and non-electrolytes on a complex coacervate formed in the prismatic cells of a celloidin membrane. Some morphological changes were observed which — in view of what we know of the effect of the variables mentioned on the water percentage of the complex coacervate — are unexpected. These changes are *vacuolization processes* (i.e. de-mixing of new equilibrium liquid from the coacervate) *in spite of the fact that the water percentage of the coacervate increases.*

2. *Methods.*

The methods employed are in principle like those described previously [1]). As in communications I and III a solution of 6 g. gum arabic + 5 g. gelatine + 200 g. water was enclosed in the celloidin membrane. The cuvette used is a modification of that of fig. 2 in the first communication. Instead of one tube there are two, which, by means of two thin flexible rubber tubes are connected with two glass reservoirs with taps. The complex coacervation is brought about by causing 0.01 N acetic acid to flow along the membrane.

When the coacervate has become parietal and practically free from vacuoles (occasionally except for a few large ones), we change on to the second reservoir, which contains a different solution of acetic acid or a solution of a neutral salt, respectively a non-electrolyte in 0.01 N acetic acid. The ensuing morphological effects *"inflow effects"* are observed for some time until the picture practically ceases to change, after which we return to the first reservoir (0.01 N acetic acid) and the morphological effects caused *"outflow effects"* are again observed for some time. After this the membrane was always removed and replaced by a fresh one. It is true that the same membrane may be used a few times in succession, but owing probably to the imperfect impermeability of the celloidin membrane the character of the inflow and the outflow effects gradually changes when the cycle is repeated several times. In order therefore to obtain comparable results a membrane is used only once for an inflow and outflow cycle.

In order to replace the original medium as quickly as possible by another one, the clearance was reduced to a minimum by cementing a glass cube in the centre of the cuvette (between membrane and glass cube an opening of ca. 1 mm is left for the flowing medium).

Moreover, in order to prevent complications in consequence of gelation of the complex coacervate, care should be taken that the temperature in the cuvette is above ca. 33° (preferably between 35° and 40°).

We desist from a detailed description of the experiment apparatus, only noting that *a.* the cuvette is sunk in a copper box through which hot water is led, *b.* that a similar heating box is mounted round the objective of the microscope, *c.* the medium liquid enters the cuvette at a temperature of ca. 40° and *d.* the medium liquid leaving the cuvette drips on to a thermometer, so that it can always be ascertained whether the temperature is still at least 35°.

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[1]) See Proc. Kon. Ned. Akad. v. Wetensch., Amsterdam, XLIII, 512, 732 (1940).

3. *Charge condition of the complex coacervate enclosed in the cells when 0.01* N *acetic acid is led past them.*

In the explanation of the in- and outflow effects on variation of the pH, which will be dealt with in 8., the charge condition of the complex coacervate in the original medium is of great significance. The colloid mixture enclosed in the membrane (6 g. gum arabic + 5 g. gelatine + 200 g. H_2O) has been chosen in such a way that the complex coacervate is practically uncharged when 0.01 N acetic acid flows past (pH 3.35). This is apparent from the electrophoretic direction *a* of the coacervate drops in the celloidin cells a short time after they are formed and before they coalesce to a parietal coacervate, *b* the little vacuoles still enclosed in the parietal coacervate (observable for a short time only, disturbances occurring later on in consequence of polarization of the celloidin walls). On coacervation with acetic acid solutions of different concentration this electrophoretic direction is indicative of a strongly negative charge with pH 3.85, 3.76, 3.65, 3.55, of a weakly negative charge with pH 3.45, a weakly positive charge with pH 3.26 and a strongly positive one with pH 3.05, 2.96, 2.8 and 2.65. With pH 3.35 the direction of the motion of the coacervate drops was uncertain, mostly, however, pointing to a weakly negative charge, like the behaviour of the vacuoles. These qualitative observations therefore indicate that the reverse of charge takes place between pH 3.35 and 3.26, and much nearer to the first value than to the second.

Below are given electrophoresis measurements made at 38° in a microscopic cuvette of mixtures consisting of 100 cc acetic acid of varied concentration + 1 cc of the stock solution (6 g. gum arabic + 5 g. gelatine + 200 g. H_2O), which lead to the same conclusion. Moreover the survey gives the results of another, similar series of measurements made in the constant presence of 10 m aeq. p. L., KCl (significant for the in- and outflow effects with KCl containing acetic acid, which will be discussed in the next communication).

Acetic acid conc. in N.	pH	U (arbitrary units)	
		Without salt	With KCl 10 m aeq.
0.030	3.11	+ 333	+ 171
0.025	3.15	+ 275	+ 138
0.020	3.20	+ 194	+ 92
0.015	3.26		
0.008	3.40	— 138	— 122
0.006	3.45	— 238	— 148
0.005	3.50	— 325	— 215
Reversal of charge (graphically interpolated)		pH 3.32	pH 3.28

So we see that the 0.01 N acetic acid used (pH 3.35) in the colloid proportion given must indeed cause a practically uncharged coacervate in the celloidin cells.

The question may be asked why in the above electrophoretic measurements the gelatine-gum arabic mixture was diluted 100 ×. The reply is this: If we should measure colloid systems of considerably greater concentrations, complications would arise which are absent in the celloidin membrane in the coacervated system and which are also practically avoided when the colloid mixture is greatly diluted. These complications are the result of the salt formed in the complex coacervation from the counter ions of the two colloids. In this case the salt formed is Ca acetate, which may interfere in two ways: *a.* by shifting the point of reversal of charge, *b.* by a pH change, the acetate together with the acetic acid forming a buffer system. On complex coacervation in the cells of the membrane the salt formed from the counter ions of the two colloids is washed away and so these complications do not arise. On complex coacervation in vitro, however, the salt formed remains in the system and these complications can only be neglected when the colloid system — and with it the salt concentration — is made very small.

4. *Expectations concerning the nature of in- and outflow effects on increase or decrease of the* pH, *based on the data of 3.*

We know from previous investigations that there is an intimate correlation between condition of charge, water percentage of the coacervate and colloid percentage of the equilibrium liquid, namely so, that with the uncharged complex coacervate the colloid percentage of the equilibrium liquid is minimal and — apart from certain complications — the water percentage of a complex coacervate is also minimal. Since we have seen in 3. that the coacervate formed with 0.01 N acetic acid (pH 3.35) is practically uncharged, we may expect that the increase as well as the decrease of the pH will increase both the colloid percentage of the equilibrium liquid and the water percentage of the coacervate.

Hence no morphological effects may be expected from inflow. The coacervate must remain free from vacuoles, similarly no new coacervate drops may form in the vacuole. On outflow — return to the original pH — on the other hand, the parietal coacervate must vacuolize and new coacervate drops must form in the central vacuole. We shall see in 5. that actually there are important deviations from these expectations.

5. *Inflow and outflow effects on variation of the* pH.

a. Owing to the variation of the acetic acid concentration.

When the original 0.01 N acetic acid is replaced by 1/30 resp. 1/300 N acetic acid, there are practically no changes [1]) in the original picture. So there are no inflow effects of a morphological nature. On subsequent replacement by 0.01 N acetic acid outflow effects are likewise absent.

When the original 0.01 N acetic acid is replaced by 1/10 N acetic acid vacuolization occurs in the parietal coacervate. When after inflow of sufficient duration we return to 0.01 N the vacuolization in the coacervate persists, at most decreases slightly, new coacervate drops forming in the large central vacuole. After sufficient time these drops coalesce with the parietal coacervate, this becoming free from vacuoles.

When the original 0.01 N acetic acid is replaced by 1/10 N acetic acid vacuolization also occurs in the parietal coacervate. On change to 0.01 N acetic acid the same happens in principle as described above with 0.1 N acetic acid, the number of coacervate drops forming in the central vacuole only being smaller. Occasionally we observed that besides the vacuoles originally present, new small vacuoles were formed in the parietal coacervate.

Figure 1 is a schematic summary of the above observation.

b. Owing to variation of the HCl *concentration.*

The question may be asked whether the changes described under *a.* are specific effects of acetic acid or if they are the consequences of pH variation. If the latter is the case it must also be possible to obtain them with isohydric HCl solutions. If for the calculation of the pH of the acetic acid solutions employed we assume pK = 4.7 for acetic acid, it follows that of 0.1 N = pH 2.85; 0.033 N = pH 3.09; 0.01 N = pH 3.35; 0.0033 N = pH 3.59; 0.001 N = pH 3.85.

Isohydric HCl solutions were prepared and with them the experiments described in *a.* were repeated. The method of working is as follows. A membrane — each time new-prepared — is washed first with 0.01 N acetic acid (pH = 3.35), until the final condition is reached, then we change to an isohydric HCl solution (0.45 millimol HCl/L) and thus the acetic acid is washed out, no changes occurring in the morphological picture. Then

[1]) With 1/300 N acetic acid some very small vacuoles only form in the large cells of the coacervate, which persist on washing with 1/100 N acetic acid.

we change to a HCl solution of a different pH and after sufficient duration we return to the HCl solution of pH 3.35. The following results were obtained:

pH 2.85 { inflow: strong vacuolization in the parietal coacervate. outflow: the vacuolization decreases slightly, new coacervation drops form in the central vacuole.

pH 3.11 { inflow: no changes. outflow: no changes.

pH 3.61 { inflow: as in the case with acetic acid, practically no changes. outflow: no changes.

pH 3.85 { inflow: slow vacuolization of the parietal coacervate. outflow: vacuolization persists, few small, new coacervate drops form in the central vacuole.

The results are very much like those obtained with acetic acid, so that we are warranted in ascribing the morphological changes to the variation of the pH of the medium led past the membrane.

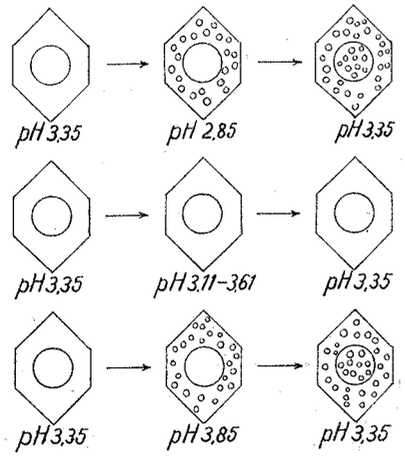

Fig. 1. In- and outflow effects with a medium liquid of lower or higher pH.

6. *Preliminary discussion. The experiment is not in accordance with the expectations.*

When we compare the in- and outflow effects observed with the expectations pronounced in 4., we see that the two do not tally. The absence of any morphological effect on slight pH variations is not so significant, as this may be in consequence of the fact that the displacements of components — which are now also slighter — may take place sufficiently rapidly by diffusion, without causing any new morphological phenomena. So we have to observe the pH changes (of ½ pH unit) which do occasion in- and outflow effects.

The only effect expected which does take place at the expected moment is the formation of new coacervate drops in the central vacuole on outflow. It is true that after outflow the parietal coacervation shows vacuolization, so apparently in accordance with our expectation, but vacuolization had already occurred during inflow, which had not been foreseen.

We must therefore try to find a reason why on increase or decrease of the pH vacuolization of the coacervate occurs, although the water percentage increases at the same time.

7. *New data concerning the influence of the* pH *on complex coacervates.*

The non-accordance stated in 6. made us presume that the knowledge we possess concerning the changes in composition of complex coacervates as f (pH) — which we employed in stating our expectations in 4. —, is incomplete.

The complex coacervation of gelatine and gum arabic has formerly been studied extensively [1]). Then a.o. we analyzed the coacervates and equilibrium liquids which are formed on modification of the mixing proportions of isohydric gelatine and gum arabic sols. The results, however, cannot be directly applied in an explanation of the in- and outflow effects, because here we work with a constant mixing proportion (the mixture of sols enclosed in the cells of the celloidin membrane) and the pH is varied.

Indirectly, however, the results mentioned may be used, owing to the fact that we examined mixing series with five different pH values, which are comparable in every respect. If we base the analysis for one constant mixing proportion on these mixing series, we obtain data which relate to a variation of the pH at constant mixing proportion.

The only mixing proportion occurring in all five mixing series is that of 50 % gum arabic, or rather, in mixing proportions with a very slight variance (between 49.9 and 50.6 % A), but very near 50 % gum arabic.

When these are selected, therefore, we obtain the most complete data, which moreover we have corrected graphically for exactly 50 % gum arabic.

These corrected data are given in the survey and set out graphically in fig. 2.

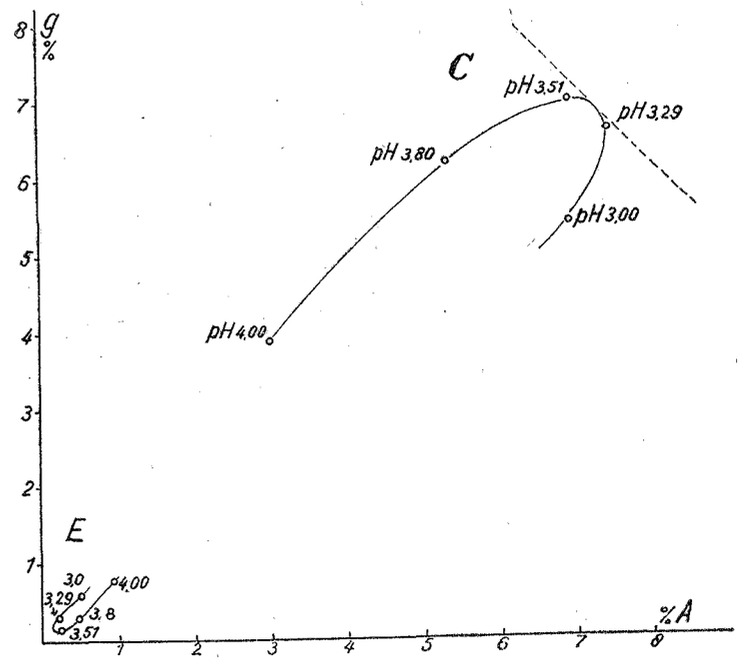

Fig. 2. Change in the composition of coacervate (C) and equilibrium liquid (E), with pH, at constant colloid proportion in the total system.

The following five points become apparent, of which one and two are in accordance with the premises employed in the deduction of the expectations in 4.:

[1]) H. G. BUNGENBERG DE JONG and W. A. DEKKER, Koll. Beih. **43**, 213 (1936). The analysis results mentioned further in this text are mentioned in that article on p.p. 222 and 223.

1. At a certain pH — here ca 3.4 — the dryweight of the coacervate (i.e. A + G, that is the gelatine + gum arabic percentage), attains a maximum, i.e. the water percentage of the coacervate reaches a minimum (in Fig. 1 at the spot where the dotted line drawn at an angle of 45° touches the upper curve).

2. At practically the same pH the colloid percentage of the equilibrium liquid is also minimal (in Fig. 1 obtainable analogously by drawing a tangent to the lower curve at an angle of 45°).

3. The relative proportion of the two colloids in the coacervate is changed at a variation of the pH and the coacervate becomes relatively richer in gum arabic when the pH decreases.

4. The relative proportion of the two colloids in the equilibrium liquid is changed in a pH section round the pH mentioned in 1. and 2. at first in a reversed sense.

5. The proportion of the two colloids in the coacervate at the pH mentioned in 1. and 2. is equal to that in the equilibrium liquid.

pH	% Coacervate		Equilibr. liquid		% (A + G		% A / % G	
	% gelatine	% gum ar.	% gelatine	% gum ar.	Coacervate	Equilibrium liquid	Coacervate	Equilibrium liquid
3.00	5.44	6.92	0.59	0.495	12.36	1.085	1.27	0.84
3.29	6.68	7.39	0.277	0.183	14.07	0.46	1.11	0.66
3.51	7.06	6.92	0.175	0.195	13.98	0.37	0.98	1.11
3.80	6.25	5.27	0.324	0.446	11.52	0.77	0.84	1.38
4.00	3.9	3.0	0.80	0.90	6.9	1.70	0.77	1.12

Fig. 3. *A.* Change of the colloid percentage of the coacervate (C), resp. of the equilibrium liquid (E) with the pH.
B. Change of the colloid proportion in the coacervate (C), resp. in the equilibrium liquid (E) with the pH.

These five points become more apparent in the separate graphs of fig. 3 (and in the schemes of figure 4), in which curves C apply to the coacervate and E to the equilibrium liquid.

8. *Explanation of the inflow and outflow effects.*

When in a given mixing proportion of the two colloids in the total system the pH has been selected so that the coacervation is optimal we are at the points given in fig. 4, namely at the maximum of curve C and the minimum of curve E in fig. 4a and at the point of intersection of curves C and E in fig. 4b.

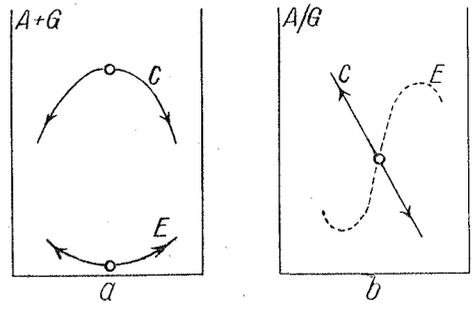

Fig. 4.

According to what has been said in 3. the complex coacervate enclosed in the celloidin cells is in this condition at pH 3.35 (optimal coacervation takes place practically at the point of reverse of charge). For the explanation of the in- and outflow effects we may start immediately from these schemes. Arrows indicate the direction in which during the inflow with a liquid of different pH the working point shifts along curves C and E of fig. 4A and along curve C of fig. 4B. As for the interpretation of the in- and outflow effects the consideration of the change of the A/G proportion in the equilibrium liquid does not open new aspects, curve E in fig. 4B is dotted and no arrows are inserted. On outflow the shifted working point moves in opposite direction along the curves to its original place.

We will now see what morphological changes may be the consequence of a shifting of the working point along curves C and E of fig. 3A and along curve C of fig. 4B and it will become clear that a summation of the changes considered separately before is in accordance with the in- and outflow effects observed.

So the three shiftings show:

I. Increase of the water percentage in the coacervate.
II. Increase of the colloid percentage in the equilibrium liquid.
III. Modification of the colloid percentage in the coacervate.

The results of I. and II. need not be discussed at length, they have already been treated in 4. and together they show: absence of inflow effects and on outflow they show vacuolization of the parietal coacervate and formation of new coacervate drops in the central vacuole.

The surprising result that already on inflow vacuolization of the parietal coacervate occurs, although the coacervate becomes richer in water may now be understood by taking into account the change of the colloid proportion in the coacervate (III). On increase of the pH the gum arabic percentage for instance decreases relatively as regards the gelatine.

This change under simultaneous change of the composition of the equilibrium liquid must take place throughout the coacervate.

With the slight diffusion velocity of the colloids this can only occur without any

morphological changes with a thin lamella of coacervate lying directly against the central vacuole. The rest of the coacervate, however, must then vacuolize i.e. the change in the colloid proportion takes place here under the formation in the parietal coacervate of new vacuoles, containing equilibrium liquid.

When, therefore, we observe the colloid proportion in the coacervate it becomes clear that the inflow effect consists in vacuolization, in spite of the fact that the coacervate becomes richer in water at the same time.

We wonder what the effect will be on outflow. On return to the original pH the proportion of the two colloids will return to the original one. Here too we must consider whether the colloid displacement between coacervate and equilibrium liquid may be effected sufficiently rapidly by diffusion only.

As for this, however, we are in a more favourable position than with inflow, because now the parietal coacervate layer is full of vacuoles (containing equilibrium liquid). When for a moment we leave out of consideration the fact that on return to the original pH the coacervate becomes poorer in water, so when we exclusively observe the change of the colloid proportions, two possibilities are seen to exist:

a. the colloid displacement by diffusion is sufficiently rapid, in which case there will be no new vacuolization and so the picture caused on inflow will be retained,

b. the colloid displacement by diffusion is not rapid enough, in which case a new generation of vacuoles must be formed in addition to those formed on inflow.

A summation of the three effects discussed I, II and III may lead to the expectation that the total effect on inflow will be vacuolization in the parietal coacervate and on outflow that the coacervate remains vacuolized (new vacuoles may even form), new coacervate drops forming in the central vacuole.

These expectations are in accordance with the in- and outflow effects observed.

SUMMARY.

1. We studied the morphological changes in consequence of pH variations of an uncharged complex coacervate enclosed in the cells of a celloidin membrane.

2. Vacuolization of the parietal coacervate takes place both on sufficient pH increase and decrease.

3. On return to the original pH the coacervate at first remains vacuolized, while new coacervate drops are formed in the central vacuole.

4. Considering that the uncharged complex coacervate becomes richer in water on increase as well as on decrease of the pH, 2. is unexpected.

5. On pH change the colloid proportion in the coacervate is also modified, as is seen from new data concerning the effect of the pH on the composition of a coacervate and equilibrium liquid at constant proportion of the colloids in the total system.

6. The morphological changes mentioned in 2 and 3 may be understood from the summation of three effects resulting from pH decrease or increase:

a. the increase of the water percentage of the coacervate,

b. the increase of the colloid percentage in the equilibrium liquid,

c. the modification of the colloid proportion in the coacervate.

Biochemistry. — *Effect of neutral salts on the composition of complex coacervate (gelatine + gum arabic) and equilibrium liquid at constant* pH *and constant mixing proportion of the two colloids in the total system.* By H. G. BUNGENBERG DE JONG and E. G. HOSKAM. (Communicated by Prof. H. R. KRUYT.)

(Communicated at the meeting of November 29, 1941.)

1. *First method of investigation.*

Although the question put in the title can only be answered directly by analysis, the experiments rescribed in the following pages enable us to answer the question indirectly. We followed two methods, which led to the same conclusion.

The first method is the simplest experimentally, as we avoid the preparation of isohydric gelatine and gum arabic sols, necessary in the second method.

It makes use of the fact that when in a number of mixing proportions of gelatine and gum arabic which are constant within each series, the coacervate volume is determined as a function of the added quantity of HCl, the coacervate volume curve is generally asymmetrical, becoming symmetrical with a certain mixing proportion.

Starting from purified gelatine [1]) and gum arabic [2]) we made 2½ % (air dry) mixed stock solutions, such that the proportion of gelatine and gum arabic changes mutually. This mixing proportion we express in what follows in % *A* (= gum arabic) of the colloid mixture.

In sedimentation tubes provided at their lower ends with narrower cylindrical tubes divided into 0.1 cc, is pipetted 10 cc stock solution and then a cc HCl 0.1 N + (2.5 — a) cc H_2O. After mixing the tubes are placed in the thermostat at 40° and after one night the coacervate volume is noted.

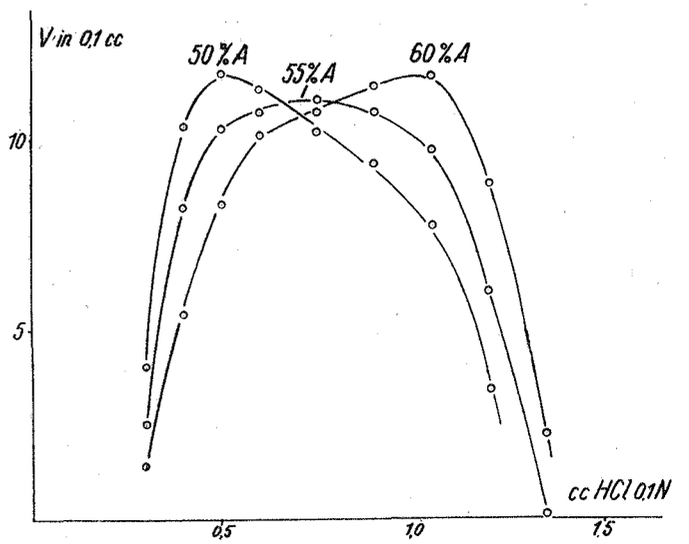

Fig. 1. Shape of the coacervate volume curves in some mixing proportions of the two colloids in the total system.

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1) F00 extra of the "Lijm- en Gelatinefabriek 'Delft' " at Delft, purified by a method described previously (Kolloid Beihefte **43**, 256, 1936), a modification of LOEB's method.

2) Gomme Sénégal petite boule blanche I, of Allan et Robert, Paris.