Biochemistry. — *On flocculation and reversal of charge of negative biocolloids through alkaloid salts and basic stains.* III. By H. G. BUNGENBERG DE JONG and C. V. D. MEER. (Communicated by Prof. H. R. KRUYT.)

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1. *Introduction.*

In previous communications we discussed the reversal of charge of a number of biocolloids through four alkaloid salts [1]).

The order of decreasing affinity (increasing reversal of charge concentration) in which these four organic cations arrange themselves:

chinine > strychnine > novocaine > guanidine

appeared to be independent of the composition of the ionogenic group of the biocolloids (phosphate-, sulphate-, carboxylcolloids).

In this investigation we have occupied ourselves with flocculations of biocolloids whether or not occurring with alkaloids and we shall show in how far statistics concerning flocculation can give us a preliminary impression of the order of decreasing affinity for a much greater number of organic cations. The order obtained will be checked in a few instances by electrophoretic measurements. It will be further seen that such statistics also allow of conclusions as regards different tendencies of the biocolloids to flocculation in dependence of the composition of the ionogenic group of the biocolloid.

2. *The autocomplex nature of the flocculation negative biocolloid + alkaloid cation.*

In the first named publication we discussed the fact that the flocculation of negative biocolloids with alkaloid cations is of an autocomplex nature. Analogously with the autocomplex flocculation with anorganic cations [2]) it is to be expected that the change that a given alkaloid causes flocculation will increase: *A.* as the reversal of charge takes place at smaller concentration, *B.* as the colloid possesses a greater charge density.

This is as a matter of fact, indicated by the behaviour of the biocolloids studied in the

TABLE 1. Flocculability of negative biocolloids with 4 alkaloid salts.

Alkaloid salt	Na-Carra-ghene	K-Chon-droitine sulphate	Na-Yeast nucleinate	Soybean phosphatide alcohol insoluble	Na-Pectinate	Na-Arabinate	Soybean phosphatide alcohol soluble	Egg. lecithin
Chinine Cl	+	+	+	+	—	—	—	—
Strychnine Cl	+	+	+	—	—	—	—	—
Novocaine Cl	+	—	—	—	—	—	—	—
Guanidine Cl	—	—	—	—	—	—	—	—
$\frac{10^3}{\text{R.H.N.}}$ of the Colloid anion	4.65	3.92	3.62	1.28	0.98	0.95	0.25	0.05

previous communication towards the four alkaloids used there: see table I, in which + means that the sol will flocculate, — that the soll will remain clear.

[1]) H. G. BUNGENBERG DE JONG and J. G. WAKKIE, I Biochem. Z. **297**, 70 (1938); II Biochem. Z. **297**, 221 (1938).

[2]) H. G. BUNGENBERG DE JONG and P. H. THEUNISSEN, I Kolloid Beihefte **47**, 254 (1938), II **48**, 33 (1938).

In the table the alkaloid chlorides are placed below each other in the order of increasing reversal of charge concentration (decreasing affinity) the biocolloids in the order of the charge density decreasing from left to right. So the regularity mentioned under A and B finds expression in Table I in the fact that the combinations in which there is flocculation are found in a triangular field of the table.

3. *Statistics concerning the flocculation of a greater number of biocolloids through a greater number of alkaloid chlorides.*

When we have at our disposal more extensive statistics concerning the flocculation of a greater number of biocolloids through a greater number of alkaloid chlorides, we can collect the results in a table analogous to Table I. We must then see to it that the alkaloids are placed below each other in such a way that the number of times that flocculation occurs in a horizontal row decreases from top to bottom (in Table I chinine-chloride 4 ×; strychnine chloride 3 ×; novocaine-chloride 1 ×; guanidine chloride 0 ×).

In this way we must then find the series of decreasing affinity of the alkaloid cations for the negative biocolloids.

Table II gives the results of such an investigation of the flocculation power. In two points it differs from Table I: A.) we have not exclusively arranged the biocolloids according to charge density decreasing from left to right, but we have combined this arrangement with the one according to the composition of the ionogenic groups. B.) We have used a greater number of qualifications than only flocculation power (+) or absence of flocculation power (—).

Ad A. The reason why in the table we have divided the biocolloids into 3 groups will be discussed in § 5.

Ad. B. Although it seems an easy matter to determine through qualitative experiments whether a biocolloid will flocculate with an alkaloid chloride, it is not simple, because besides the very evident cases of flocculation and perfect clearness, all transitional stages possible were observed (slight turbidity, weak and very weak opalescence).

The following signs are used in the table:

++	evident flocculation or marked turbidity
+	slight turbidity [1])
?	slight opalescence
??	very slight opalescence
—	perfect clearness of the sol.

On arranging the alkaloids in the order of Table II we have taken this into account. Therefore we gave marks to each of these notations and these were added in each horizontal row. We counted ++ as 4; + (+) = 3; + = 2; ? = 1 and ?? and — = 0. The "flocculation power" (fl.p.) thus obtained for each alkaloid is given directly after the name in Table II. From top to bottom the alkaloids in this table are arranged according to decreasing flocculation power. As a few combinations, namely those marked with an asterisk in the table, have not been tested, the order may have slightly changed. As we shall see, the absence of some data does not affect our final conclusion.

Here follow some details about the method applied in determining the qualifications mentioned (++, +, ?, ??). In so far as the sols used in the experiments are formed by simple solution in cold, resp. boiling (agar) water, they were prepared from the purified colloid preparations described previously [2]).

We used 1 % sols of Na-yeast nucleinate, Na-arabinate and Na-pectinate; 0.5)% sols of Na-agar and chondroitine sulphate: A 0.25 % sol of Na-pectate; A 0.2 % sol of Na-semen-line-mucilage and A 0.1 % sol of Na-carraghene. The phosphatide sols used

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[1]) In a few cases we have used +(+) as a qualification, signifying a case between ++ and +.

[2]) H. G. BUNGENBERG DE JONG and P. H. THEUNISSEN, Koll. Beihefte, loc. cit. I.

TABLE II.

Alkaloid-chloride	Fl. P.	Phosphate colloids: Na-Yeast nucleinate	Soybean phosphatide alcohol non-soluble	Soybean phosphatide alcohol soluble	Egg-lecithin	Fl. P.	Sulphate colloids: Na-Carraghene	K-Chondroitine sulphate	Na-agar	Fl. P.	Carboxyl colloids: Na-Pectate	Na-Semen Lini mucilage	Na-pectinate	Na-Arabinate	Fl.P.
Aeth.hydrocupr.	26	++	++	−	?	9	+(+)	++	+	9	++	+	+	−	8
Papaverine	26	++	++	−	−	8	++	++	+	10	+	++	+	−	8
Chinine	23	++	++	−	?	9	++	++	+	10	++	−	−	−	4
Narcotine	23	++	?	−	+	7	++	++	−	8	++	+	+	−	8
Apomorphine	22	++	++	−	−	8	+	++	−	6	++	++	−	−	8
Hydrastine	18	++	*	?	?	6	++	++	*	8	++	−	−	−	4
Thebaine	20	++	++	−	−	8	++	++	−	8	+	+	−	−	4
Brucine	18	++	*	−	−	4	++	++	−	8	++	+	−	−	6
Pantocaine	16	++	*	−	−	4	+	++	*	6	++	+	−	−	6
Emetine	14	++	++	−	−	8	??	++	−	4	+	−	−	−	2
Strychnine	14	++	??	−	−	4	++	++	−	8	+	−	−	−	2
Tropacocaine	10	++	+	??	−	6	++	−	−	4	−	−	−	−	0
Heroine	8	++	*	−	−	4	+	+	−	4	−	−	−	−	0
Aethylmorphine	7	+(+)	−	−	−	3	++	−	−	4	−	−	−	−	0
$N(CH_3)_4$	6	++	*	+	−	6	−	−	*	0	−	−	−	−	0
Tutocaine	6	+	??	−	−	2	++	−	−	4	−	−	−	−	0
Morphine	6	++	−	−	−	4	+	−	−	2	−	−	−	−	0
Eucaine	5	+	−	−	−	2	(+(+)	−	−	3	−	−	−	−	0
Cocaine	4	??	−	−	−	0	++	−	−	4	−	−	−	−	0
Cotarnine	4	+	−	−	−	2	+	−	*	2	−	−	−	−	0
Alypine	4	−	??	−	−	0	++	−	−	4	−	−	−	−	0
Novocaine	4	−	−	−	−	0	++	−	−	4	−	−	−	−	0
Stovaine	3	?	??	−	−	1	+	−	−	2	−	−	−	−	0
Codeine	2	??	−	−	−	0	+	−	−	2	−	−	−	−	0
Scopolamine (Br)	1	−	−	−	−	0	?	−	−	1	−	−	−	−	0
Homotropine (Br)	1	−	−	−	−	0	?	−	−	1	−	−	−	−	0
Ephedrine	0	−	*	−	−	0	−	−	−	0	−	−	−	−	0
Hydrastinine	0	??	*	−	−	0	−	−	*	0	−	−	−	−	0
Guanidine	0	−	−	−	−	0	−	−	*	0	−	−	−	−	0
Pilocarpine	0	−	−	−	−	0	−	−	−	0	−	−	−	−	0
Acetylcholine	0	−	−	−	−	0	−	−	−	0	−	−	−	−	0
$\frac{10^3}{\text{R. H. N.}}$ of the colloid anion		3.62	1.28	0.25	0.05		4.65	3.92	0.45		5.46	1.84	0.98	0.95	
Flocculability of biocolloids with alkaloid salts		70	27	3	5		74	46	6		36	18	6	0	

were the same as in communications I (soybean phosphatide alcohol soluble) and II (soybean phosphatide alcohol non-soluble and egg-lecithin).

In judging the flocculation power we used three methods:

A. A drop of sol and a drop of cold-saturated solution of the alkaloid chloride were placed side by side on an objectglass and a covering glass was carefully placed on it. The contact zone of the two drops was then examined microscopically. When floccules, fibrils or coacervate drops are observed we mark this biocolloid-alkaloid chloride combination $++$.

For with a positive result of the microscopic examination the experiments described sub B and C are also markedly positive. In the table the qualification $+(+)$ occurs three times, namely where with A a slightly positive result was obtained while by B and C the result was markedly positive.

In some cases one can also place crystals of the alkaloid-chlorides in the undiluted sol and in their vicinity look for the occurrence of floccules, fibrils or coacervate drops.

B. Sol and saturated alkaloid chloride solution are mixed in test tubes in different proportions and the occurrence of flocculation, turbidity resp. opalescence is noted with transmitted light. When there was slight but evident turbidity, as with method C, while a negative result was obtained by method A, we marked the combination $+$. When with method B there is a slight or very slight opalescence we marked the combination ? resp. ??.

C. We proceed as in B with this difference that crystals of the alkaloid chloride are added to the undiluted sol. Usually C gives the same result as B, so that method C need only be applied when with B the result was ? resp. ??.

Finally we remark that the experiments with agar were made at a higher temperature (over 40°), in order to prevent disturbances in consequence of gelatination. In the experiment by A we used an electrically heated microscope table, by B and C a thermostat of 50°.

4. *Electrophoretic measurements for control of the alkaloid order of Table II.*

Table II is drawn up in analogy with Table I. The flocculation power as regards the 11 biocolloids examined decreases from top to bottom in the table. So we may expect that the order of the alkaloids thus found will also be the one in which the reversal of charge concentration of the average biocolloid will increase. For the sake of control we determined with a number of alkaloids the reversal of charge concentrations for a sol of alcohol-soluble soybean phosphatide (Table III), while we also measured the reversal of charge concentrations for SiO_2 powder (Table IV). In these tables are also given the values taken from Table II for the flocculation power as regards all 11 biocolloids and as regards the group of the biocolloids belonging to the object examined electrophoretically (phosphate colloids in the case of Table III) or which from an electrochemical point of view may be considered related (sulphate colloids in the case of Table IV) [1].

In both tables the alkaloids have been arranged according to increasing reversal of charge concentrations, i.e. according to decreasing affinity of the alkaloid cation for the negatively charged ionized group. So we must expect this also to be the order in which they are found in Table II. In how far this is true may at once be seen from the figures in Tables III and IV of the flocculation power, which must decrease from top to bottom. We see then that on the whole this expectation has become true. The slight deviations do not disappear when we view the flocculation power as regards the group of bio-

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1) The reversal of charge "spectre" of SiO_2 shows the characteristics of the sulphate colloids. The analogous spectre of TiO_2 shows similarity with that of the carboxyl-colloids) (see H. G. BUNGENBERG DE JONG and P. H. THEUNISSEN, loc. cit. II, p. 88—90). We also tried to measure the reversal of charge of TiO_2 with alkaloid salts, but the results were very irregular. (Rapid change of the electrophoretic velocity in course of time), so that we had to abandon this plan.

colloids which one would in the first place consider in this respect, and not as regards all 11 biocolloids.

But naturally there are so many possible sources of errors, e.g. in consequence of the scale of appreciation used, that in spite of the deviations we may be satisfied. Hence we

TABLE III.

Reversal of charge with soybean phosphatide soluble in alcohol with different alkaloid salts.

Alkaloid-chloride	log C (C=reversal of charge conc.)	Flocculation power	
		all colloids	phosphate colloids
Aethylhydro-cupreine	0.05—3	26	9
Apomorphine	0.21—3	25	8
Chinine	0.39—3 0.22—3 [1]	23	9
Papeverine	0.73—3	26	8
Thebaine	0.96—3	20	8
Narcotine	0.07—2	22	7
Strychnine	0.24—2 0.22—2 [1]	14	4
Brucine	0.46—2	18	4
Heroine	0.63—2	8	4
Aethyl-morphine	0.87—2	7	3
Morphine	0.02—1	6	4
Codeine	0.18—1	2	0
Novocaine [1]	0.36—1	4	0
Guanidine [1]	0.74—1	0	0

[1] Value taken from Communication I.

TABLE IV.

Reversal of charge of SiO_2 particles with different alkaloid salts.

Alkaloid-chloride	log C (C=reversal of charge conc.)	Flocculation power	
		all colloids	sulphate coll.
Apomorphine	0.03—3	25	8
Papaverine	0.41—3	26	10
Aethylhydro-cupreine	0.72—3	26	9
Chinine	0.76—3	23	10
Thebaine	0.87—3	20	8
Brucine	0.89—3	18	8
Narcotine	0.04—2	22	8
Strychnine	0.31—2	14	8
Heroine	0.44—2	8	4
Cotarnine	0.54—2? [1]	4	2
Tropacocaine	0.73—2? [2]	10	4
Aethyl-morphine	0.84—2	7	4
Morphine	0.91—2	6	2
Novocaine	0.89—2 [3]	4	4
Codeine	0.94—2	2	2
Scopolamine (Br)	0.15—1	1	1
Pilocarpine	0.26—1	0	0
Guanidine	0.01 [3]	0	0

[1] The electrophoresis velocity here changed fairly rapidly in course of time, hence the value log C = 0.54 — 2 is very uncertain.

[2] Owing to changes of the electrophoresis velocity in course of time, the value log C = 0.73 — 2 is uncertain.

[3] Value taken from Communication II.

conclude that the order of decreasing affinity following from the flocculation statistics is on the whole confirmed by electrophoretic measurements.

5. *Connection between flocculation power of the biocolloids, their charge density and the composition of their ionogenic groups.*

Table I clearly shows the connection between flocculability and charge density of the biocolloid. As the density is greater there is flocculation with alkaloid cations which are further to the right in the affinity order of the alkaloids chinine > strychnine > novocaine > guanidine.

With the analogous autocomplex flocculation with anorganic cations it seems that with less extensive statistic material the charge density of the biocolloids is the predominant factor. But with more extensive statistic material it appears that the composition of the ionogenic groups of the biocolloid is also an important factor [1]).

Of the alkaloids it can also be said that if with the more extensive statistic material of Table II we had arranged the biocolloids exclusively according to charge density decreasing from left to right, there would be nothing left of the connection between charge density and flocculability, which seems so evident in Table I. For then we should get the following values for the flocculability with charge density decreasing from left to right:

$$36 - 74 - 46 - 80 - 18 - 27 - 6 - 0 - 6 - 3 - 5.$$

The connection between charge density and flocculability, however, is evident in Table II, where we have divided the biocolloids into three groups according to the composition of the ionized groups.

Within each division the connection between charge density and flocculability is very evident.

When we set out graphically the values of the "flocculability" (indicated in Fig. 1 as Fl and mentioned at the bottom of the columns in Table II) as function of the charge density and then connect with each other the points belonging to the phosphate colloids, likewise of the sulphate colloids and again those of the carboxyl colloids, we get Fig. 1, which shows what we have discussed above:

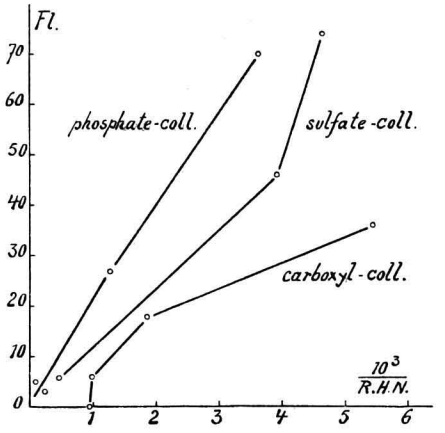

Fig. 1.

A. of each of the three groups of biocolloids it may be said that the flocculability increases with increasing charge density;

B. with equal charge density a phosphate colloid has greater flocculability than a sulphate colloid, and the latter than a carboxylcolloid.

The order: phosphate colloid > sulphate colloid > carboxylcolloid is different from the order found with the inorganic cations. This difference is possibly owing to the fact that with inorganic cations the polarizability of the ionized groups of the biocolloid plays a part, with the great organic cations (see Communication II) there is no question of polarization of the ionized groups of the biocolloid. The order phosphate colloid > carboxylcolloid > sulphate colloid found for the inorganic cations is therefore the one of decreasing polarizability power of the ionized groups. The cause of the order found here with the great organic cations cannot yet be indicated.

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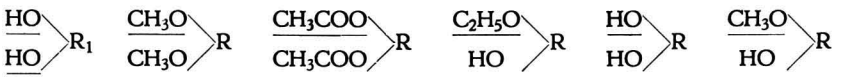
[1]) H. G. BUNGENBERG DE JONG and P. T. THEUNISSEN, loc. cit. I, p. 307.

6. *Connection between structure of the alkaloid cations and place in the affinity series.*

In Tables III and IV we have included a number of nearly related alkaloids, which are important for the problem of the connection between structure of the alkaloid cation and place in the affinity series. It is the following:

apomorphine > thebaine > heroine > aethylmorphine > morphine > codeine



The order of which is the same for reversal of charge of the phosphatide and of SiO_2.

In the schematic structure formulae given we have underlined the phenolhydroxyl of morphine and the CH_3O—, C_2H_5O—, resp. CH_3COO groups each time taking its place, the alcoholic hydroxyl in morphine resp. the groups corresponding with it, on the other hand, have not been underlined.

With apomorphine, where the ring skeleton is slightly different (hence R_1 instead of R) there are two phenolhydroxyles in the molecule.

For the explanation of the order found it seems plausible to start from the assumption that the introduction of a hydroxyl group will decrease the affinity. On substitution of these hydrophile groups by the much less hydrophile CH_3O—, C_2H_5O—resp. CH_3COO groups the affinity must increase again. On the ground of this assumption the order thebaine > heroine > aethylmorphine > morphine can be accounted for, but it is not explained why the reversal of charge of codeine takes place with higher concentrations than of morphine, nor why apomorphine is found entirely on the left side of the affinity series. The facts allow of explanation however, when it is assumed that exclusively *an alcoholic hydroxyl has a weakening effect on the affinity, whereas the effect of a phenol hydroxyl is strengthening.*

We shall further use the results of a previous systematic investigation of L. THEUNISSEN VAN ZIJP [1]) from which it was seen that when a carbon chain is lengthened the affinity increases. This is evident for instance in the series aethylmorphine > codeine.

On methylating morphine to codeine the affinity decreases, which we explain as follows: the introduction of CH_3 has two effects: increase of the affinity owing to the introduction of the CH_3 group itself and decrease of the affinity owing to the elimination of the phenolhydroxyl, the latter effect preponderating.

In morphine, codeine and aethylmorphine there is still the greatly weakening alcoholic hydroxylgroup. Elimination of the latter by methylation, resp. acetylation causes a strong increase of the affinity.

All this explains to us the order:

thebaine resp. heroine > aethylmorphine > morphine > codeine,

but we cannot answer the question why methylation has a greater effect than acetylation [2]).

Finally the distinction we have made between alcoholic and phenolhydroxyls also accounts for the position on the extreme left in the affinity series of apomorphine. For both hydroxyl groups are here phenolhydroxyls.

7. *Significance of charge density and chemical composition of the ionized groups of negative biocolloids for their flocculation with stain cations.*

With the same 11 biocolloids we made an analogous investigation of the flocculation power with 14 basic stains (preparations of Messrs. Dr. G. GRÜBLER). The stains examined (with the number of their flocculation power in brackets) were: nile blue (40), toluidinblue (40), trypaflavin (40), indulin scharlach rot (36), neutral red (36), methy-

[1]) L. THEUNISSEN VAN ZIJP, Dissertation, Leiden 1939.

[2]) Possibly the acetylgroup is more hydrophile than the methoxylgroup.

lene blue (35), crystal violet (33), pyronine (30), auramin (28), chrysoidine (28), fuchsine (28), methylgreen (20), brilliant green (20), malachite green (20).

The same regularities discussed in detail in Table II are also expressed in this statistic material.

We shall first consider the values for the flocculability of the biocolloids:

Phosphate colloids: yeast nucleinate (56) soybean phosphatide, alcohol nonsoluble (56) soybean phosphatide alcohol soluble (44) egglecithin (9).

Sulphate colloids: Na-carraghene (56), K chondtroitin sulphate (56) Na-agar (4).

Carboxyl colloids: Na-pectate (54), Na-semen lini mucilage (42), Na-arabinate (34), Na-pectinate (23).

It is true that here too we see the connection — so clear in Table II — between decreasing flocculability and decreasing charge density, but this can no longer find expression between the two phosphate colloids mentioned first and between the two sulphate colloids mentioned first which all four are marked 56. This is owing to the much greater affinity of the stain cations than the alkaloid cations for the biocolloids. As these four biocolloids flocculate strongly with all the stains examined (=4), they obtain the maximal value of 56 ($= 14 \times 4$).

When the values of the flocculability are set out against the charge density of the biocolloids in the same way in Fig. 1, we again obtain the order: phosphate colloid > sulphate colloid > corboxyl colloid.

Summary.

1. The flocculation (coacervation resp.) of negatively charged biocolloids with alkaloid cations, resp. stain cations is of an autocomplex nature. It is to be expected that from more extensive statistic material concerning the flocculability of a number of biocolloids by a number of alkaloids, resp. stains, conclusions can be drawn in a general way as to the affinity order of these cations.

2. The statistic material necessary is given (flocculability of 11 biocolloids with 31 alkaloids and 14 basic stains).

3. The resulting affinity order is checked by some electrophoretic measurements and is on the whole found correct.

4. The tendency to flocculation of the biocolloids with alkaloids, resp. stains increases with increasing charge density of the colloid anion, it is moreover dependent on the chemical composition of the ionized groups of the biocolloid:

phosphate group > sulphate group > carboxyl group.

5. It would seem that from the affinity order:

apomorphine > thebaine > heroine > aethylmorphine > morphine > codeine

it follows that the affinity of the alkaloid cation for the biocolloid anion is diminished by an alcoholic hydroxyl group, but is increased by a phenolhydroxyl.

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