Chemistry. — *The Coacervation of Gelatin and Chondroitin Sulphuric Acid.* By H. R. KRUYT and A. H. A. DE WILLIGEN.

(Communicated at the meeting of November 28, 1931.)

In an address which one of us delivered before the Colloid section of the Ned. Chem. Ver. in December 1930[1], he pointed out that the behaviour of the gelatin sol seems to indicate that this sol is complex, and coacervation phenomena appear near the isoelectric point. This view is brought in connection with the relation of gelatin and collagen. As one of the arguments supporting these views, the fact is mentioned, found in the earlier literature, that gelatin under certain conditions can be precipitated with chondroitin sulphuric acid. L. W. JANSSEN had directed our attention to a sentence in the Lehrbuch der physiologischen Chemie by HAMMARSTEN, in which (edition of 1907 p. 432) we read:

„In Lösungen von Leim oder Eiweiss rufen angesäuerte Lösungen der chondroitinschwefelsauren Salzen Niederschläge hervor."

This statement rests on a communication by MÖRNER[2], which runs as follows:

„Ein mechanisches Gemenge von Chondroitsäure und Glutin in welchem das letztere in ziemlich grossem Ueberschuss vorhanden sein muss, bietet Reagentien gegenüber ein interessantes Verhalten dar; es gibt eine solche Lösung mit *Essigsäure* eine im Ueberschuss unlösliche, aus feinen, microscopisch kleinen Kügelchen verschiedener Grösse bestehende, flockige Fällung; mit *Salzsäure, Schwefelsäure, Salpetersäure* eine in überschüssiger Säure leicht lösliche Fällung von derselben physikalischen Beschaffenheit: Kochsalz oder Ferrocyankalium verhindert das Auftreten dieser Fällungen und löst sie, wenn entstanden, wieder auf."

We have undertaken a closer investigation of this comparatively vague statement, in order to study the phenomenon in the light of the knowledge of complex-coacervation acquired since then[3].

Preparation of chondroitin sulphuric acid.

We have prepared chondroitin sulphuric acid starting from a description by P. A. LEVENE[4].

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[1] Cf. H. R. KRUYT, Chem. Weekbl. **28**, 148 (1931).

[2] C. TH. MÖRNER, Skand. Arch. Physiologie **1**, 228 (1889).

[3] Cf. H. G. BUNGENBERG DE JONG and H. R. KRUYT, These Proceedings **32**, 849 (1929) and further the transactions of the former with his pupils in Bioch. Z. **212**, 318 (1929); **221**, 182, 392 and 403 (1930); **232**. 338; **234**, 367; **235**, 185 (1931).

[4] P. A. LEVENE, Monographs Rockefeller Inst. med. Research Nr. 18 (1922).

Cartilage of the nose of cows and of nose, shoulder-blade and ribs of calves was preserved in 30 % alcohol for at least three days, after which all the adherent parts could be easily removed. 5 kg. of the cut up pieces were washed in water, grinded in a mincing machine, and treated for two days with 2 litres of 2 % potassium hydroxide. The liquid was poured off; the residue was again treated with 5 l. of potassium hydroxide for 12 hours and washed twice with 3 l. of water.

All the liquid was filtered through cloth, and after addition of barium carbonate, it was evaporated in flat dishes on waterbath, till the volume was reduced to 5 litres. Then a thick cake was deposited, the viscid liquid standing over it was filtered through cloth, once more evaporated with barium carbonate to a volume of 2 litres, centrifuged and poured out in 16 l. of glacial acetic acid. The deposit was washed with alcohol and ether, and dried in vacuum over potassium hydroxide and sulphuric acid.

The raw product was dissolved in water, precipitated with basic lead acetate, and the precipitate was washed till the *Ba*-reaction had disappeared. For this purpose it was dissolved in diluted acetic acid with 10 % sodium acetate, and the lead was precipitated with hydrogen sulphide. A salt solution of this concentration is necessary in order to get the lead sulphide in a somewhat filtrable form (at a lower concentration there is formed a very stable lead-sulphide sol).

The yellow-brown solution, which is free from lead and barium, was dialysed in an electrodialyser between parchment membranes.

If the dialysis is continued for a long time, a preparation is obtained, which, evaporated by means of a hot current of air, is deposited as transparent brown leaflets possessing an ash-content of only 4.0 % of the dry substance. An *N*-content was found of 2.35 resp. 2.38 %, an *S*-content of 6.20 resp. 6.26 %. A solution prepared from this appears, however, soon to present sulphate reaction, to possess a low viscosity (¼ % sol had a relative viscosity of about 1.05) and a diminished power of precipitating gelatin. Therefore the following investigations (unless explicitly stated otherwise) have been made with a less far dialysed sol which was used as it was obtained, i.e. it was not first evaporated, nor were the sols prepared by dissolving the dried substance. The analysis of this sol yielded the following results: in 5 cc. 42.4 mg. of dry substance; of this 6.0 mg. of ashes; *N*-content of the ash-free dry substance 3.09 resp. 3.13 %; *S*-content 6.54 resp. 6.42 %.

Measurements of the Viscosity of the Chondroitin Sulphuric Acid Sol.

First of all we desired to know the characteristic properties of the viscosity of the chondroitin sulphuric acid sol; for this purpose the far dialysed sol was used.

POISEUILLE's law is followed; the same sol was measured in the same viscosimeter at three different pressures, in which the outflow times for water were resp. 243.3, 314.8 and 434.7 sec.; the corresponding relative viscosities for the sol were 1.089, 1.089 and 1.087 [1]).

Then the dependence of the viscosity with regard to the *pH* was examined. Table I gives the result.

TABLE I.

pH.	Relative viscosity
2.3	1.189
2.6	1.203
3.0	1.208
3.8	1.220
4.4	1.223
6.4	1 224
8.0	1.112

Table IV (0.0—5.0) gives the same series of observations for the less far dialysed preparation, which is richer in ashes. It appears from both series that no minimum value is passed, as we always find with albumens, i.e. with ampholytes. Chondroitin sulphuric acid is a negatively charged colloid, which accordingly does not change the sign of its charge between the *pH* values 2 to 8.

Coacervation Phenomena in Mixtures of Gelatin and Chondroitin Sulphuric Acid Sols.

a. *Exterior.*

When mixtures are made in different proportions, starting from a 0.25 % gelatin sol and a 0.35 % chondroitin sulphuric acid sol, which had previously been brought to an equal *pH*, the typical complex-coacervation appears; turbidity takes place, which is maximum at a definite proportion of the mixture. This maximum lies at a different proportion of the mixed components, when the *pH* is modified. In the sols of the just-mentioned strengths the maxima lay as follows [2]):

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[1]) In anticipation of experiments to be mentioned presently it may be stated here that also coacervating sols follow POISEUILLE's law: a mixture 4.0—1.0, which will be described more fully later and which is very turbid at a *pH* 3.22, gave the relative viscosities 1.033, 1.033 and 1.032 for this *pH* in the same viscosimeter under different pressures, i. e. differences which lie on the verge of accuracy.

[2]) Observed visually. Photometric investigations are in progress.

TABLE II.

pH.	Proportion of the volume of the sols	Proportion of the weight gelatin : chondroitin	
		In weight	In percentages
2.5	4 cc. gel. + 1 cc ch.	0.20 : 0.07	74 : 26
3.75	4.5 ,, ,, + 0.5 ,, ,,	0.225 : 0.055	87 : 15
4.5	4.9 ,, ,, + 0.1 ,, ,,	0.25 : 0.007	97 : 3

The character of the turbidity differs depending on the temperature. At room temperature the precipitate is floculent, under the microscope it appears to consist of long rows of very small spherical particles. After mixture at 40° the spheres are larger, clearly liquid, and present less tendency to join to flakes. If at 80° somewhat more concentrated solutions e.g. 2 or 3 % are brought together, a separation into two layers appears. On cooling the macro-coacervate gelatinates, becomes again liquid at 32—33°, and turbid when heated again. With a 10 % sodiumchloride solution it becomes first strongly opalising, and then dissolves.

All these results are in perfect harmony with the results of earlier investigation on complex-coacervation. The viscosity of the mixtures could now lead to a definitive conclusion.

b. Measurements of the Viscosity.

These have been carried out in analogy with those on the system gelatin-gum arabic 1). It appeared, however, to be exceedingly difficult to perform a series of measurements always at constant *pH*: the *pH* of the mixtures was not always that of the initial sols, and small *pH* differences, especially on the side of the gelatin, affect the viscosity greatly. Therefore mixtures of the components were made, the *pH* was changed by addition of acid or alkali to the gelatin solution, and the viscosity and the actual *pH* of the contents of the viscosimeter were measured. The latter was done both potentiometrically with the chinhydron electrode, and colorimetrically with bromine cresol green; these methods yielded satisfactory concordant results. The *pH*-values in the subjoined table marked with ± have been determined *only* colorimetrically.

Starting from the solutions of the above-mentioned strength (0.25 % gelatin and 0.35 % chondroitin sulphuric acid), the following mixtures were made (Table III).

In table IV the viscosity values at 40° are recorded for these sols at the different *pH* values indicated there. Corrections have been applied for time reactions. The sols are indicated according to the last column of table III.

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1) H. G. BUNGENBERG DE JONG and W. A. J. DEKKER, Bioch. Z. **212**, 318 (1929).

TABLE III.

Volumes		Percentages of weight of the sol		Percentages of weight of the dry substance		Notation
Gelatin	Chondroitin	Gelatin	Chondroitin	Gelatin	Chondroitin	
5.0 cc	0 cc	0.25	—	100	0	5.0—0
4.9 „	0.1 „	0.245	0.007	97	3	4.9—0.1
4.5 „	0.5 „	0.225	0.035	87	13	4.5—0.5
4.0 „	1.0 „	0.20	0.07	74	26	4.0—1.0
3.0 „	2.0 „	0.15	0.14	51	49	3.0—2.0
2.0 „	3.0 „	0.10	0.21	32	68	2.0—3.0
1„0 „	4.0 „	0.05	0.28	15	85	1.0—4.0
0	5.0 „	—	0.35	0	100	0—5.0

TABLE IV.

Mixture	pH	Relative Viscosity
5.0—0	4.72	1.080
	4.40	1.094
	4.24	1.160
	3.40	1.444
	2.33	1.366
idem (duplo)	4.72	1.079
	4.47	1.089
	4.14	1.174
	3.88	1.262
	± 3.4	1.459
4.9—0.1	2.26	1.3
	3.78	1.136
	3.95	1.039
	4.27	1.046
	4.69	1.043

TABLE IV (*Continued*).

Mixture	pH	Relative viscosity
4.5—0.5	2.25	1.138
	3.29	1.011
	4.04	1.008
	4.34	1.019
	4.85	1.068
	(± 3.85	1.003)
4.0—1.0	2.23	1.018
	3.02	1.017
	± 3.3	1.019
	3.85	1.025
	4.47	1.040
	5.05	1.103
3.0—2.0	2.39	1.026
	3.33	1.057
	3.98	1.064
	4.43	1.085
	5.08	1.105
2.0—3.0	2.39	1.070
	2.81	1.093
	3.01	1.100
	3.75	1.113
	4.14	1.119
	4.66	1.146
*) 1.0—4.0	(2.91	1.147)
	(4.27	1.150)

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*) Accurate measurement was rendered difficult by the readiness of these sols to show flocculation.

TABLE IV *(Continued)*.

Mixture	pH	Relative Viscosity
0.0—5.0	2.30	1.193
	3.17	1.201
	3.62	1.199
	4.17	1.187
	4.56	1.171

The results of table IV are graphically represented in the figures 1 and 2. By interpolation in these figures it can be ascertained what the viscosity

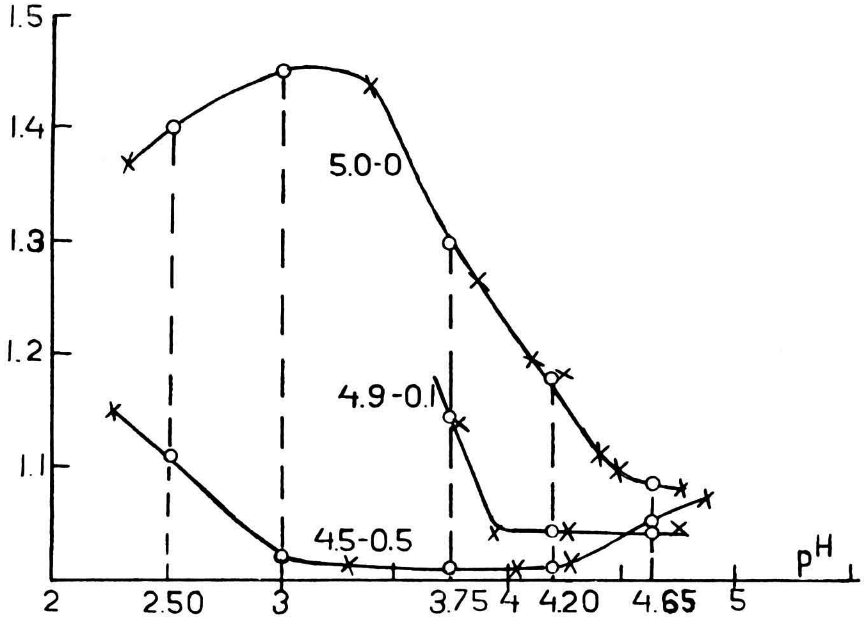

Fig. 1

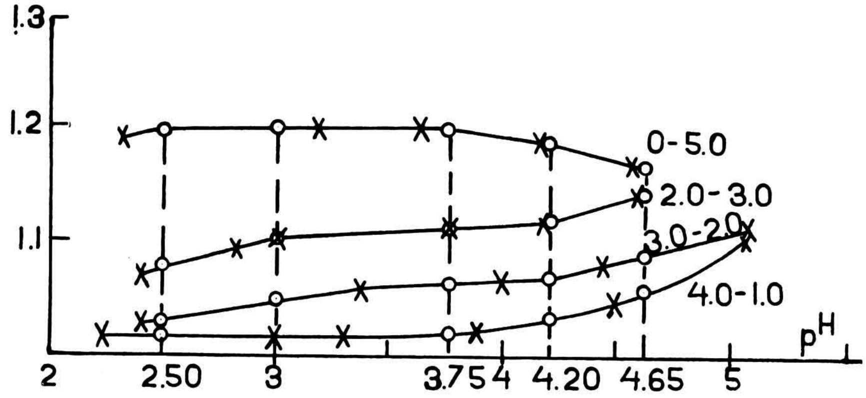

Fig. 2

is in all these mixtures at the *pH*'s 2.5, 3.0, 3.75, 4.20 and 4.65; these values of the viscosity are given in the third column of table V and indicated by O in fig. 1 and 2. In the fourth column the values of the viscosity are found calculated on the supposition that the viscosity could be calculated additively from that of the components. In the fifth column it is found what percentage the value of the third column is of that of the fourth, when both have first been diminished by unity.

The values of column 5 are represented as ordinates in the figures 3—7; the volume-mixing proportion of the sols is represented as abscissae.

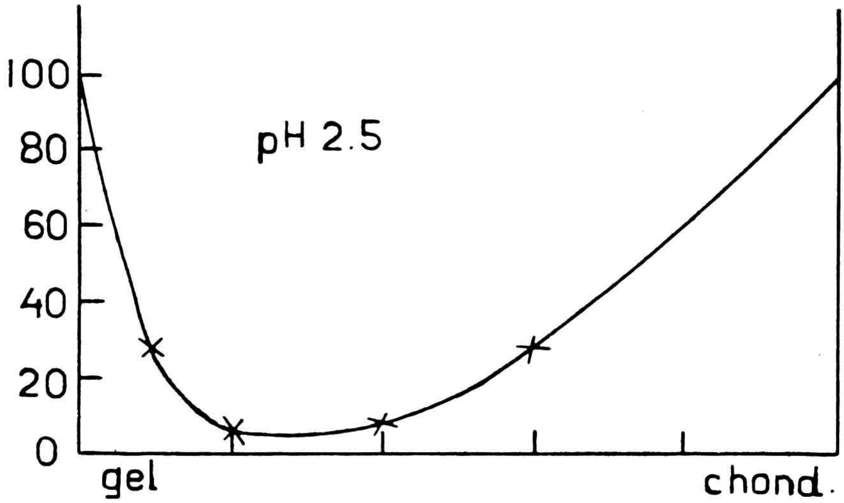

Fig. 3

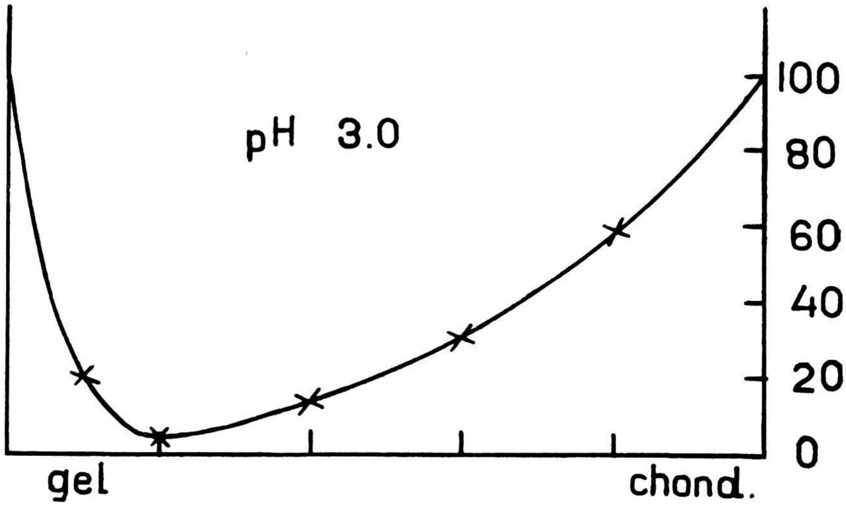

Fig. 4

These results are in perfect harmony with those found in the system

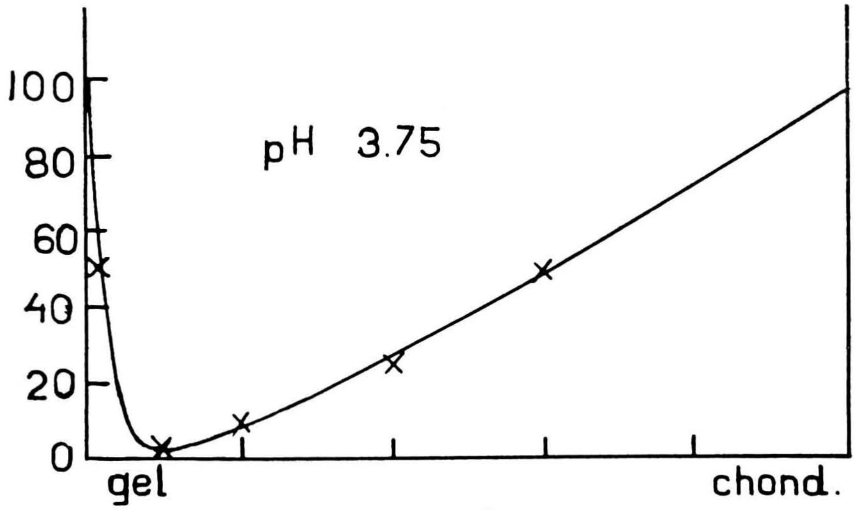

Fig. 5

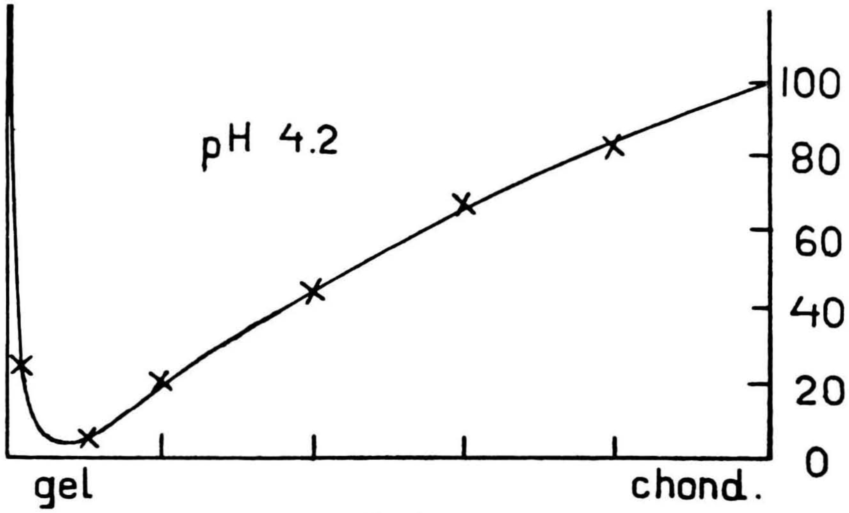

Fig. 6

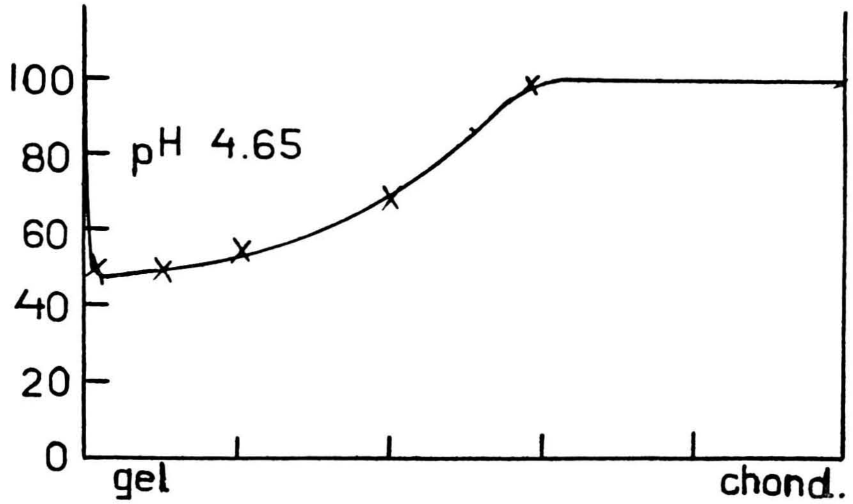

Fig. 7

TABLE V.

pH.	Mixture	Viscosity found	Viscosity additively calculated	Viscosity found as $^0/_0$ of the additively calcuted viscosity
2.5	5.0—0	1.40	1.40	100
	4.5—0.5	1.11	1.38	29
	4.0—1.0	1.017	1.36	5
	3.0—2.0	1.03	1.32	9
	2.0—3.0	1.08	1.28	28
	0—5.0	1.20	1.20	100
3.0	5.0—0	1.45	1.45	100
	4.5—0.5	1.04	1.42	10
	4.0—1.0	1.017	1.40	4
	3.0—2.0	1.05	1.35	14
	2.0—3.0	1.10	1.30	33
	(1.0—4.0	1.15	1.25	60)
	0—5.0	1.20	1.20	100
3.75	5.0—0	1.29	1.29	100
	4.9—0.1	1.14	1.29	50
	4.5—0.5	1.01	1.26	4
	4.0—1.0	1.02	1.26	8
	3.0—2.0	1.06	1.25	24
	2.0—3.0	1.11	1.23	48
	0.—5.0	1.20	1.20	109
4.2	5.0—0	1.16	1.16	100
	4.9—0.1	1.04	1.16	25
	4.5—0.5	1.01	1.16	6
	4.0—1.0	1.035	1.17	21
	3.0—2.0	1.07	1.17	42
	2.0—3.0	1.12	1.18	67
	(1.0—4.0	1.15	1.18	83)
	0—5.0	1.19	1.19	100
4.65	5.0—0	1.09	1.09	100
	4.9—0.1	1.043	1.09	48
	4.5—0.5	1.05	1.10	50
	4.0—1.0	1.06	1.11	55
	3.0—2.0	1.09	1.12	75
	2.0—3.0	1.14	1.14	100
	0—5.0	1.17	1.17	100

gelatin-gum arabic. The strong minima that indicate the deviations of the additive course (for additive behaviour would give a horizontal line in fig. 3—7), characterise the dehydration which causes the coacervation. The minima accordingly coincide with the maxima of turbidity, mentioned in table II.

The investigations are being continued.

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