LXXXII. THE MECHANISM OF PRECIPITATION OF COLLOIDAL GUM BENZOIN BY CEREBROSPINAL FLUID.

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IN 1920 the use of a colloidal suspension of gum benzoïn was introduced by Guillain, Laroche and Lechelle [1920] in order to detect certain pathological variations in the cerebrospinal fluid. The test is carried out by preparing in test-tubes 1 cc. of each of a series of dilutions of cerebrospinal fluid, $\frac{3}{4}$, $\frac{1}{2}$, $\frac{1}{8}$, $\frac{1}{16}$, ... $\frac{1}{65,536}$, sixteen dilutions being used. To each tube is then added 1 cc. of a colloidal suspension of gum benzoïn. In our experiments we have, in order to obtain uniform suspensions, slightly modified the original instructions given by the authors of the test, thereby obtaining a very uniform 'benzoïn' sol, but one apparently rather less sensitive than the original. The sol used is fully described in the preceding paper.

Typical results obtained from fluids which are stated to give characteristic precipitations are as follows:

C.S.F. from case of General Paralysis of the Insane	344	443	444	310	000	0
" " Tabes Dorsalis	003	410	241	000	000	0
" " Meningitis (streptococcal)	000	001	444	443	100	0
" " "	122	200	444	430	000	0
" normal*	Between 000	000	242	000	000	0
	and 000	000	000	000	000	0

The system of indicating results is that described in the preceding paper.

* Fluids from absolutely normal individuals are naturally rarely obtained, and so 'normal' denotes fluid from patients where there is every reason to believe there is no essential change in the nature of the fluid, *e.g.* measles, dementia, epilepsy.

The main point to be noticed is that the precipitation occurs in two zones. There is a normal precipitation in the seventh or eighth tube, which in pathological fluids may or may not be increased. It is particularly increased in meningitis, and usually to a less extent in general paralysis; in fact, an extension of the zone to the right often accompanies an increase of protein in the fluid, as shown by the butyric acid test [Noguchi and Moore, 1909]. The first six tubes form another zone of precipitation. Here the precipitate is particularly marked in general paralysis, and it is also notably present in the case of tabetic fluids. That is to say, in syphilis of the central nervous system well-marked precipitation occurs in these tubes. There often is also slight precipitation in cases of meningitis, but in the normal fluid there is no precipitation at all. This zone, then, appears to be affected independently of the other.

Guillain, Laroche and Lechelle showed that neither the individual proteins (obtained by fractional saturation with ammonium sulphate) nor the dialysable constituents of a fluid could reproduce the curve obtained with the original fluid. A mixture of these could, however, do so. Dible [1922] suggests that the various curves are due to variation in cerebrospinal fluids in their globulin, albumin and salt content. In view of the fact that no account was taken by these workers of the actual p_H in the various tubes it is doubtful how these results should be interpreted. It may be noted that previous workers [Cruickshank, 1920; Lange, 1912] have shown that the portion of a cerebrospinal fluid which is most effective in precipitating a gold sol is that which is precipitated by half saturation with ammonium sulphate.

In the previous paper [1923] we have shown that the precipitation of 'benzoin' by gelatin and by oxyhaemoglobin depends very greatly on the hydrogen ion concentration of the medium. At a p_H on the alkaline side of the isoelectric point, no precipitation of the sol by gelatin takes place at any concentration of gelatin. At a p_H of about 4.7, precipitation takes place when 1 cc. of the sol is added to 1 cc. of gelatin diluted 1 in 640,000. As the p_H decreases, and the solution becomes more acid, the amount of gelatin required decreases, until at a p_H of 2.8 the hydrogen ions alone are sufficient to precipitate in the absence of gelatin, and precipitation occurs until a concentration of gelatin of at least 1/640,000 is present. Similar results have been obtained using haemoglobin. In view of the great dependence of the behaviour of proteins towards colloidal 'benzoin' on the reaction, a systematic investigation of the normal and paretic types of curves in this respect appeared to be called for. Accordingly, we have investigated the influence of reaction in the cases of serum globulin, serum albumin, and of whole serum, as well as of cerebrospinal fluids of the paretic and normal types on their behaviour as precipitants of colloidal 'benzoin.' The results explain the constancy of the zone of precipitation in the seventh and eighth tubes, and its variation in degree and not in position, and also demonstrate, we think, that the precipitation in the first five tubes (called by the authors of the test the syphilitic zone), is due, not to ordinary serum globulin or albumin, but to some extra material, which if it is protein in nature possesses a high isoelectric point, p_H between 7 and 8, and that in these cases, the actual serum globulin and albumin may be, at most, very slightly increased.

EXPERIMENTAL.

As small quantities of serum albumin and serum globulin apparently exist normally in the cerebrospinal fluid, these two proteins were first of all investigated. The mode of experiment was the same as described in the preceding paper. Fresh serum was fractionated by precipitation, first with half-saturated ammonium sulphate and then with saturated ammonium sulphate. The protein fractions obtained were washed well with ammonium sulphate solution of the requisite strength, and then transferred to a collodion sac, in which they were dialysed for 24-36 hours against tap-water. The albumin

solution was made up to the volume of the original serum and it was then ready for use. The globulin fraction was separated from the dialysed solution. It was filtered off and dissolved in sufficient sodium chloride solution (0.8 %) to make up the original bulk. If necessary it was filtered from some insoluble residue. Dilutions of these protein solutions were prepared, and also solutions of sodium hydroxide and of hydrochloric acid of various strengths. To a mixture of $\frac{1}{2}$ cc. of a globulin solution and $\frac{1}{2}$ cc. of acid or alkali was added 1 cc. of 'benzoin' sol. After shaking thoroughly, and standing overnight, the degree of precipitation was judged. The results are given in Table I, in which the dilutions given are those of the globulin or alkali after mixing, and before adding the 'benzoin.'

Table I.

Effect of p_H on precipitation by Globulin.

(The degree of precipitation is indicated by the same conventional numbers as in the preceding paper.)

		Concentration of globulin solution									
		1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120
Concentration of sodium hydroxide	N/800	0	0	0	0	0	0	0	0	0	0
	N/1600	4 (p_H 5.3)	4	4	4	4	3	0	0	0	0
	N/3200	0	0	0	0	0	4	1	0	0	0
	N/6400	0	0	0	0	0	4	4	0	0	0
	No alkali	0	0	0	0	0	2	4	2	0	0

It will be seen that with serum globulin a zone of precipitation occurs, which, just as in the case of gelatin and of haemoglobin, alters its position on addition of alkali. In the case of globulin the last row in which precipitation occurs has the p_H 5.3. A similar experiment using serum albumin shows no precipitation in solutions of p_H greater than 4.7. That is to say, these proteins do not cause precipitation in solutions the p_H of which is on the alkaline side of the isoelectric point, *i.e.* in solutions in which they exist as negative ions.

Precipitation of 'benzoin' by Serum.

Experiments were carried out to determine the effect of various dilutions of serum on 'benzoin.' The results are given in Table II.

Table II.

Precipitation by Serum.

The p_H given is that of the supernatant fluid. The charges on the colloidal particles are denoted by (+) or (-).

Concentration of serum	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
	0	0	0	0	0(-)	4 (p_H 5.4)	4 (p_H 4.7)	0(+)	0
Concentration of serum	1/1024	1/2048	1/4096	1/8192	1/16384	1/32768	1/65536	1/131072	
	0	0	0(+)	4 (p_H 4.2)	4 (p_H 4.2)	2	0(-)	0(-)	

It will be seen that there are two zones of precipitation—the first is at a concentration of serum 1/8192–1/16,384, or, assuming that the total protein content of serum is 7.5 %, about a concentration of protein of 1/130,000–

1/260,000. The charge on the 'benzoin' particles, as determined by the ultra-microscopic method described in the previous paper, is negative when the concentration of protein in solution is less than that corresponding to precipitation, and positive when it is greater. This zone then apparently corresponds to the normal zone of precipitation obtained with gelatin at concentration of about 1/600,000. The second zone of precipitation occurs at much higher concentrations of serum, 1/32–1/64. The charge on the particles is positive in tube seven and negative in tube four. The p_H of the supernatant fluid in tubes five and six is 5.4 and 4.7 respectively. It is obvious that here we are encountering, at the isoelectric point of the protein, the zone of precipitation which tends to broaden and to extend into regions of high concentration. In tubes one to four the p_H is above 5.4, and so no precipitation occurs and the particles are negatively charged. As soon, however, as the p_H falls below 5.4 precipitation occurs, as some of the protein molecules (globulin) now act as bases and are positively charged and tend to precipitate the 'benzoin.' When too much positively charged protein is present, protection occurs, because too much protein is absorbed, and so in tube seven there is no precipitation and the particles are positively charged. The commencement of the zone of precipitation naturally coincides with the isoelectric point of serum globulin (p_H 5.4) as this is higher than that of serum albumin (p_H 4.7). It would appear that, as the zone of precipitation extends from p_H 5.4 to 4.7, the presence of two proteins in concentrations of the same order of magnitude tends to produce a broad zone extending between the isoelectric points of the two proteins. This is probably to be explained on the consideration that, at a p_H between the two isoelectric points, much negatively, as well as some positively charged protein will be present. In that case there will be much less tendency for the particles of 'benzoin' to acquire a positive charge, although neutralisation may quite readily occur.

It is of interest to find that a somewhat similar phenomenon may be produced if gelatin be diluted 1 in 1000 in Ringer's solution and then diluted with an equal volume of distilled water in a series of successive tubes and 'benzoin' be added. The results of such an experiment will be found in Table III.

Table III.

Precipitation by Gelatin in Ringer's Solution.

Gelatin (1 part in 1000 parts of Ringer-Tyrode solution) was prepared, and this was diluted with distilled water to the dilutions given, an equal volume of 'benzoin' sol was added to each dilution and the results read after standing overnight.

Concentration of gelatin $\times 1000$	.1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
	0	0	0	0(-)	0(-)	1	0(+)	2	4	4	3½

Here two zones of precipitation appear, the first corresponding with a change in the charge on the particles from negative in high concentration to positive in low, the second with a change from positive in high concentration to negative in low. It is to be noted that Ringer's solution alone has no precipitating

effect on 'benzoin,' notwithstanding its comparatively high concentration of salt. This is because its p_H is 7.6.

Precipitation of 'benzoin' by Normal Cerebrospinal Fluid.

A normal cerebrospinal fluid was examined in regard to precipitation of 'benzoin' and the charges on the particles in the unprecipitated zones. The curve obtained was as follows:

000, 000, 320, 000, 000, 0.

Precipitation of about the usual amount was seen in the seventh and eighth tubes. On investigating the reaction in tube 7 this was found to be equivalent to p_H 4.5. It was, however, interesting to find that the charges on the particles of 'benzoin' were negative on either side of the zone of precipitation. The explanation is evident from the following experiment which shows the effect of the addition of acid or alkali to another normal fluid.

Table IV.

Effect of p_H on precipitation by normal C.S.F.

$\frac{1}{2}$ cc. of diluted C.S.F. and of acid or alkali added to each tube and then 1 cc. of 'benzoin' solution. Readings after standing overnight.

		Concentration of Cerebrospinal Fluid (Normal)										
		1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048
Concen- tration of hydro- chloric acid	Distilled water	0	0	0	0	0	1	0	0	0	0	—
	N/6400	0	0	0	0	0	0	0	0	0	—	—
	N/1600	0	0	0	0	0	0	0	0	0	—	—
	N/800	0	0	0	0	0	0	0	0	0	—	—
	N/400	0	0	0	0	0	0	0	0	0	—	—
Concen- tration of sodium hydroxide	Distilled water	0	0	0	0	0	3 (p_H 4.4)	1	0	0 (-)	0	0
	N/6400	0	0	0	0	0	4	4 (p_H 3.4)	1	0	0	0
	N/1600	0	0	0	0	4 (p_H 4.6)	4	4 (p_H 3.4)	4	4 (p_H 3.0)	3	2
	N/800	0	0	0 (-)	4 (p_H 4.7)	4 (p_H 3.4)	4 (p_H 3.3)	4	4 (p_H 3.0)	3	3	0
	N/400	0	0 (-)	4 (p_H 4.7)	0 (+)	0	4 (p_H 3)	4 (p_H 3)	4 (p_H 2.8)	4	4	4

Fig. 1 shows the resulting zone of precipitation if the concentration of fluid be plotted against the p_H of the tubes showing precipitation.

Here it will be seen that the added acid or alkali had a marked effect on the position of the zone of precipitation. This zone varies in a similar fashion to a zone of precipitation obtained with a protein. It does not extend to tubes which have a p_H greater than 4.7. This corresponds to the isoelectric point of serum albumin.

It will be evident from the table and figure that in a normal C.S.F. test, the zone of precipitation is not really crossed at all. It is entered and left again, as shown by the dotted line in the figure, which indicates approximately the p_H of the liquid in the various tubes after the 'benzoin' has been added, but where neither acid nor alkali is added. The result is that the 'benzoin' particles on both sides of the zone of precipitation are negatively charged since the positive region is never entered.

The reason for the constancy of position of the zones of precipitation obtained with normal fluids is evident. In the tubes in the first column, the amount of C.S.F. present is high and so there is considerable buffering of the

solution. The result is that after addition of the 'benzoin' the p_H remains high and it is not until the seventh or eighth tube that the acidity of the 'benzoin' is sufficient to lower the p_H to about 4.7. At this point the protein will have

Fig. 1. Precipitation of 'benzoin' by normal cerebrospinal fluid as influenced by the p_H . The dotted curve represents approximately the actual p_H of the tubes to which no acid or alkali is added. As will be seen, the region of complete precipitation is not entered, the maximum precipitation being of the order 1/2. In other cases the line may enter and leave but does not cross the zone of complete precipitation. (+) and (-) refer to the charge on the 'benzoin' particles in the different regions.

lost its negative charge and so will begin to act as a precipitant of the 'benzoin.' Naturally, owing to slight variations in amount of buffering of the solution (CO_2 content, etc.), and possibly in the amount of protein the exact p_H in the seventh and eighth tubes will vary slightly; hence the slight variations in the amount of precipitation produced at this point even with normal cerebrospinal fluids. As we have remarked above, with some there is no precipitation at all. It is clear that this will occur where the protein content is low, or the degree of buffering is high. On the other hand, it is clear that in fluids, *e.g.* from a case of meningitis, where the protein content is much in excess, the zone will be extended into regions of higher dilution.

Precipitation of 'Benzoin' by a Cerebrospinal Fluid from a case of General Paralysis.

In the following experiment to determine the effect of acid and alkali on the zone of precipitation, a typical fluid from a case of general paralysis giving the following curve was used:

444, 443, 443, 100, 000, 0.

The essential point of difference between this and the normal curve is that complete precipitation has occurred in the first five tubes.

The details of the experiment recorded are the same as in the case of the normal fluid.

The result is shown in Table V.

Table V.

Effect of p_H on precipitation by Paretic C.S.F.

		Concentration of Cerebrospinal Fluid (G.P.I.).										
		1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
Concentration of sodium hydroxide	N/100	—	0	0	0	0	0	0	0	0	0	0
	N/200	—	0	0	0	0	0	0	0	0	0	0
	N/400	3 (p_H 7.8)	0	0	0	0	0	0	0	0	0	0
	N/800	4 (p_H 7.6)	0	0	0	0	0	0	0	0	0	0
	N/1600	4 (p_H 7.8)	4 (p_H 7.5)	3	0	0	0	0	0	0	0	0
	N/12800	4 (p_H 7.6)	4 (p_H 7.2)	4 (p_H 6.8)	0 (—)	4 (p_H 5.1)	2	0	0	0	0	0
	Distilled water	4 (p_H 7.3)	4 (p_H 7)	4 (p_H 6.8)	3	4 (p_H 5)	3 (p_H 4.4)	0	0	0	0	0
Concentration of hydrochloric acid	N/6400	—	4 (p_H 7.2)	4 (p_H 7)	4 (p_H 5)	4 (p_H 4.6)	4 (p_H 4.1)	3	0	0	0	0
	N/1600	—	4 (p_H 7)	4 (p_H 6.6)	0 (+)	0	4 (p_H 3.8)	4 (p_H 3.6)	3 (p_H 3.4)	0	0	0
	N/1200	4 (p_H 7.4)	4 (p_H 6.9)	4 (p_H 5.4)	—	—	—	—	—	—	—	—
	N/800	4	4 (p_H 6.9)	0	—	—	—	—	—	—	—	—
	N/600	4	4 (p_H 6.2)	0	—	—	—	—	—	—	—	—
	N/400	4 (p_H 7)	4 (p_H 5.5)	0	0	0	0 (+)	4 (p_H 3)	4	4	4	4
	N/300	4 (p_H 7)	0	0	—	—	—	—	—	—	—	—
	N/200	4 (p_H 5.9)	0	0	0	0	0	4 (p_H 2.6)	4	4	4	4
	N/100	0	0	0	0	0	0	4 (p_H 2.4)	4	4	4	4

Fig. 2 shows the zone of precipitation if the p_H be plotted against the concentration of fluid in tubes showing precipitation.

It will be seen that up to a p_H of about 5 the curve is very similar to that given by a normal fluid. Notwithstanding the increase in total protein content, as shown by the Noguchi reaction, the zone of precipitation at p_H 4 occurs at a concentration of about 1/64 just as with the normal fluid. It would appear from this that there is no definite increase in the amount of serum globulin or albumin present, because if this were increased one would expect that the zone would be shifted to the right. On the other hand, precipitation is seen to occur in a region between p_H 5 and p_H 7 to 8. This is a zone non-existent with a normal fluid, serum globulin or serum albumin and so presumably the substance which causes this precipitation is not present in these.

If it is a protein, and the marked increase in the Noguchi reaction in most cases of general paralysis might suggest this, it is presumably a protein of isoelectric point p_H 8, since the 'benzoin' is negatively charged and proteins, as far as our experiments go, precipitate it only when they are at, or on the acid side of, their isoelectric point. They fail to do so when they are negatively charged. At p_H 7 the normal proteins (serum albumin and serum globulin) are negatively charged and it is difficult to understand how they could be the active agents.

It may be noted that precipitation occurs over a continuous area. This might at first sight appear rather inexplicable, as instead two zones might rather be expected. However, the explanation is, no doubt, similar to that of the existence of a zone with normal serum extending approximately from

p_H 5.4 to 4.7, that is, between the isoelectric points of serum globulin and of serum albumin. If two proteins are present in comparable concentrations, then at a p_H between the two isoelectric points, the positively charged particles of the protein with the higher isoelectric point will neutralise the negative

Fig. 2. Precipitation of 'benzoin' by cerebrospinal fluid from patient with G.P.I. The dotted curve represents approximately the actual p_H of the tubes to which no acid or alkali is added.

(+) and (-) refer to the change in the 'benzoin' particles.

charge on the colloidal 'benzoin' particles, but it is doubtful whether it will confer a positive change on them, as the other protein in solution is still completely negative, and so will tend to prevent the positive protein being absorbed on the particles.

DISCUSSION.

Goebel [1921] has made the observation that in the case of the mastic reaction, on diluting cerebrospinal fluid from a case of general paralysis with a normal fluid, the curves obtained are still essentially of the paretic type. He further suggests that there are two fundamental types of curve—the meningeal type indicating increased permeability of the vessels and consequent increase in serum protein in the fluid, and the paretic type characteristic of conditions associated with tissue destruction. Lange [1912] would appear

to suggest that in paretic fluids evidence of the presence of nucleoprotein may be obtained. Cruickshank [1920] suggests that in general paralysis there is a change in the ratio of globulin to albumin in the cerebrospinal fluid and as the globulins from a normal fluid do not give the reaction even on concentration, this is associated with an alteration in the electrical charge on the globulins.

Our own results confirm the view that there is a qualitative difference between the paretic cerebrospinal fluid and the normal, as in the table showing precipitation by a paretic fluid the zone due to the normal constituents can be identified and differentiated from the peculiar and characteristic abnormal zone. The essential feature of the latter is that it occurs between p_H 5.5 and 7.5, and it is clear that no interpretation or explanation of a curve can be made without taking into account the reaction of the solution in which precipitation occurs. This, of course, is in accordance with the fact that the physico-chemical behaviour of proteins is a function of the reaction, more particularly of the reaction relative to their isoelectric point.

The general effect of alkalinity on the colloidal reactions of the cerebrospinal fluid has already been noted in the literature. For instance, Bannerman [1919] has shown that the globulin fraction of a paretic fluid can reproduce the paretic curve of gold, if it is made slightly alkaline, and Presser and Weintraub [1922] and Sahlgren [1922] attributed the non-precipitation of mastic by high concentrations of normal cerebrospinal fluid to the protective action of the alkali present. But the precipitation of a colloid is determined by the two factors protein and reaction together, and these cannot be considered independently, for it is the electrical condition of the protein that is of importance.

CONCLUSIONS.

1. The zone of precipitation occurring in normal and pathological cerebrospinal fluids in the seventh and eighth tubes in the colloidal 'benzoin' test is due to precipitation of the negatively charged 'benzoin' particle by the positively charged protein of the fluid.
2. The constancy of its position depends on the fact that the p_H must be below 4.7 before any such precipitation can occur. The variations in the degree of precipitation and in the number of the tubes included in the zone, depends on the relative amount of protein present and the degree of buffering of the fluid.
3. The precipitation occurring in the first five tubes in fluids from cases of general paralysis of the insane is due to the presence of some abnormal constituent, and not to an increased amount of the proteins responsible for the normal precipitation.
4. The general dependence of precipitation by cerebrospinal fluid on the hydrogen ion concentration has been investigated.

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