XXIX. THE OPACITY OF SERUM DILUTED WITH DISTILLED WATER, PHYSIOLOGICALLY NORMAL SALINE, AND RINGER'S SOLUTION.

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(Received February 18th, 1921.)

As the author had recently been investigating the turbidity concentration relations of suspensions of the crystalline substance, calcium oxalate [1921, 3], and of suspensions of red corpuscles [1921, 2], he deemed it advisable to extend the work to the study of the emulsoid colloid, serum. In the routine of a pathological laboratory serum occupies an important position, forming, as it does, the basis of immunity reactions. For example, the Widal test for agglutinations by typhoid serum, and the Wassermann test for syphilitic serum, are reactions which are very frequently employed. Moreover it is customary to dilute the patient's serum with physiologically normal saline (0.85 % NaCl) with the object of keeping the osmotic pressure constant. In this way an impression has been created that, by keeping the osmotic pressure of serum constant, no other changes occur on dilution. The author has long doubted the correctness of this impression, and as a sensitive apparatus for measuring the opacity of liquids had been devised [1921, 1], it was decided to make use of this characteristic of colloids in the present investigation.

The general method employed was as follows. Serum was used which had been "inactivated" by heating at 56° for 20 minutes. From the inactivated serum was prepared a series of dilutions in Jena glass test-tubes, which were then allowed to stand for four hours at 40°, but were shaken every hour. It was found by preliminary experiments that four hours were sufficient for the opacity to become constant. In order to differentiate between normal and pathological conditions, sera were taken which were negative or positive to the Wassermann reaction.

In the first series of experiments, negative serum was diluted with distilled water, physiologically normal saline, normal saline plus calcium chloride, and Ringer's solution. Most of the results are plotted in Fig. 1, and the remainder, for dilutions less than 1/7.5, are given in Table I.

Table I.

The opacity of *negative* serum diluted with:

- (1) distilled water,
- (2) physiologically normal saline,
- (3) physiologically normal saline plus 0.022 % calcium chloride,
- (4) Ringer's solution (NaCl 0.85 %, CaCl_2 0.022 %, KCl 0.056 %).

Dilution of serum	Concentration of serum	Opacity of dilutions of serum			
		Distilled water	NaCl 0.85 %	NaCl 0.85 % CaCl_2 0.022 %	NaCl 0.85 % CaCl_2 0.022 % KCl 0.056 %
Undiluted	100 %	42	42	42	42
1/1.5	66.6	40	...	50	53
1/2	50.0	37	28	46	50
1/3	33.3	35	27	43	47
1/4	25.0	33	26	39	44
1/5	20.0	32	25	37	42

Fig. 1.

In the second series of experiments a pathological serum was taken which gave a positive reaction to the Wassermann test of + 1/10 but delayed lysis when diluted 1/20. The results are given partly in Table II, and partly in Fig. 2.

Table II.

The opacity of *positive* serum (+ 1/10, delayed lysis 1/20) diluted with:

- (1) distilled water,
- (2) physiologically normal saline,
- (3) physiologically normal saline plus 0.022 % calcium chloride,
- (4) Ringer's solution (NaCl 0.85 %, CaCl_2 0.022 %, KCl 0.056 %).

Dilution of serum	Concentration of serum	Opacity of dilutions of serum			
		Distilled water	NaCl 0.85 %	NaCl 0.85 % CaCl_2 0.022 %	NaCl 0.85 % CaCl_2 0.022 % KCl 0.056 %
Undiluted	100 %	50	50	50	50
1/1.5	66.6	...	...	...	...
1/2	50.0	...	37	...	...
1/3	33.3	...	35	...	...
1/4	25.0	...	35	...	...
1/5	20.0	38	34	42	47

Fig. 2.

In the third series of experiments a pathological serum was taken which gave a strongly positive reaction to the Wassermann test of + 1/40. The results are collected together in Table III.

Table III.

The opacity of a strongly positive serum (+ 1/40) diluted with:

- (1) distilled water,
- (2) physiologically normal saline,
- (3) physiologically normal saline plus 0.022 % calcium chloride,
- (4) Ringer's solution (NaCl 0.85 %, CaCl₂ 0.022 %, KCl 0.056 %).

Dilution of serum	Concentration of serum	Opacity of dilutions of serum			
		Distilled water	NaCl 0.85 %	NaCl 0.85 % CaCl ₂ 0.022 %	NaCl 0.85 % CaCl ₂ 0.022 % KCl 0.056 %
1/15	6.6 %	40	33	48	53
1/20	5.0	36	29	44	48
1/25	4.0	35	28	43	47

It will be seen that whether the serum is normal or pathological, or whether it is diluted with distilled water or with salt solution, the turbidity concentration curve is not a straight line such as is produced in diluting a suspension of red corpuscles, but that for all dilutions the turbidity is greater than that which would be produced if the protein particles scattering the light had remained the same in size and had merely been proportionally reduced in number. For example, if the turbidity of the negative serum had varied directly with the concentration, then a dilution of ten times would have given an opacity of $42/10 = 4.2$. But with distilled water the opacity was 26, with "normal" saline it was 19, with normal saline plus calcium chloride 30, and

with Ringer's solution, 36. Hence dilution of serum leads to an increase of the surface of particles scattering light, due probably to an increase both in the size and number of the particles. To account for the relative increase in surface of the protein particles of serum on dilution, it would be better first to endeavour to form some idea of the physico-chemical condition of the protein. Proteins are for the most part amino-derivatives of fatty acids. Hence their salts may be considered as amino-derivatives of soaps. Hence also their salts will exhibit the phenomenon of hydrolysis. At all dilutions of serum therefore there will be an equilibrium between the hydrolysed and the non-hydrolysed portions of the salts and the degree of hydrolysis will increase with dilution and with temperature. Moreover as serum is slightly alkaline in reaction, only minute traces of free acid can exist as such in the products of hydrolysis, and the insoluble portion of the protein must therefore be of the nature of an acid soap. The proteins of diluted serum therefore exist probably in the following forms: (1) non-hydrolysed protein salts, of which those with sodium are soluble, while those containing calcium are soluble, if the acid is derived from a lower fatty acid such as propionic acid, but insoluble if the acid comes from the higher group; (2) hydrolysis products which are acid in character, though combined with a certain amount of sodium for the most part, and with calcium to a slight extent, in both cases the higher products being insoluble and tending to form aggregates; and (3) a minute quantity of free amino-acid which will ionise with the production of (4) a very minute quantity of amino-acid anions. It seems therefore that when serum is diluted, hydrolysis of the protein salts occurs with the consequent relative increase in the particles scattering light. It will be seen in referring to Table I and Fig. 1, that the turbidity of small dilutions of serum is even greater than that of the undiluted serum. Such a phenomenon also occurs in the hydrolysis of sodium palmitate, in which the hydrolysis alkalinity first rises to a maximum and then falls away on further dilution. On referring to the curves it will be seen that at a dilution of 1/20 (5 % concentration) there is a definite discontinuity in all the curves, which is most marked with distilled water, and least marked with Ringer's solution. The significance of this discontinuity seems to be that in serum there are two distinct types of protein, one of which only begins to produce insoluble particles at dilutions below 1/20. Possibly these two types are represented by globulin and albumin.

The action of the ions of sodium, calcium and potassium is well shown in the curves. With 0.85 % sodium chloride not only is hydrolysis thrown back, but the non-hydrolysed portion of the salts is soluble. With "normal" saline plus 0.022 % calcium chloride, the insoluble products are increased because the calcium salts are insoluble. With Ringer's solution the products are still further increased. These antagonistic actions of sodium and calcium are comparable to the results obtained by Ringer on perfusing the excised heart. With "normal" saline the heart continued to beat feebly for some time, and then stopped in a condition of relaxation. On adding a trace of calcium chloride

the heart commenced to beat again, but more strongly, then stopped in a condition of tonic contraction. On further adding a trace of potassium chloride the heart beat much more strongly for many hours. In view of the above results with serum, it would appear that when the heart stopped under perfusion with saline the colloids of the cardiac muscle fibres contained relatively few insoluble particles. On the other hand when the calcium chloride was added the number of insoluble particles was increased, and finally, when the potassium chloride was added the number of insoluble particles was increased still further to a point corresponding to the condition found in the cardiac muscle of the living animal.

With regard to the pathological side of the investigation, it will be seen that syphilitic serum contains more insoluble matter than normal serum, and that the stronger the Wassermann reaction the greater is the quantity of insoluble matter. Correspondingly, of course, there will also be an increase in the "soluble" portion of the protein in syphilitic sera. It may be pointed out here that the strength of the Wassermann reaction is measured in practice by the greatest dilution to which the serum can be carried and still prevent the specific haemolysis of red corpuscles. For example the + 1/40 serum used in the present investigation ceased to prevent complete haemolysis after a dilution of 1/40, while the + 1/10 serum failed to prevent complete haemolysis beyond a dilution of 1/10. At a dilution of 1/40 (2.5 %) in "normal" saline, the strongly positive serum had a turbidity of 27. At a dilution of 1/10 (10 %) in normal saline the weaker positive serum had a turbidity of 28. But it was shown [1921, 2] that turbidity is a measure of the surface of the particles scattering light. Therefore the available adsorptive surfaces of the sera at their respective greatest dilutions were the same. This result therefore provides evidence for the theory that the Wassermann reaction depends on surface action.

In serological investigations the serum, after dilution and mixing with the antigen, is allowed to stand for some time at a definite temperature. It was advisable therefore to test the effect of change of temperature on the opacity. For this purpose negative serum was diluted five times with distilled water, with physiologically normal saline, and with Ringer's solution, and then allowed to stand in an incubator at different temperatures for four hours. The following were the results.

Table IV.

The effect of temperature on the opacity of negative serum diluted five times with:

- (1) distilled water,
- (2) physiologically normal saline (0.85 % NaCl), and
- (3) Ringer's solution (NaCl 0.85 %, CaCl_2 0.022 %, KCl 0.056 %).

Temperature	Opacity of diluted serum		
	Distilled water	NaCl 0.85 %	Ringer's solution
5° C.	22	22	22
15	24	23	25
24	28	24	30
30	30	24	31
40	32	25.5	33
57	32	27	35

The results if plotted show that the temperature curve for the limits 5° to 57° is a straight line in the case of physiologically normal saline and that the total effect is slight, increasing only from 22 at 5° to 27 at 57°. The slow increase in turbidity of diluted serum with increase of temperature is probably due to the increased solubility of the hydrolysis products at higher temperatures. On the other hand with both distilled water and Ringer's solution, the curve shows an initial lag up to 10° and then rises somewhat sharply up to 30°. Beyond 30° the curves are nearly flat. In the case of dilutions with distilled water, there is a progressive diminution in the quantity of salts present, with a consequent increase in the quantity of acid hydrolysis products, which are only slightly more soluble at higher temperatures. In the case of dilutions with Ringer's solution, the small quantity of calcium present (0.022%) is more than sufficient to counterbalance the effect of the sodium. The action of calcium in this case can scarcely be accounted for by its bivalency. It seems more probable that it is rather a question of decreased solubility of the calcium salts. Such a phenomenon is also observed with caseinogen salts, the sodium salts being soluble and the calcium salts relatively insoluble.

REFERENCES.

- Holker (1921, 1). *Biochem. J.* 15, 216.
— (1921, 2). *Biochem. J.* 15, 226.
— (1921, 3). *Biochem. J.* 15, 232.