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Investigations dealing with the Phenomena of "Clot" Formations. Part II.—*The Formation of a Gel from Cholate Solutions having Many Properties analogous to those of Cell Membranes.*

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(Communicated by Prof. V. H. Blackman, F.R.S. Received January 22,—Read February 19, 1914.)

In the first communication under the above title,* attention was called to the fact that solutions of sodium cholate, on warming in the presence of calcium salts, set to a gel, which is not reversible on cooling. It has since been found that calcium salts can be replaced by other salts, such as sodium chloride, magnesium chloride, ammonium sulphate and potassium fluoride, and the clot formation is not therefore due to double decomposition between calcium salts and sodium cholate. The concentrations of sodium, potassium, and magnesium salts necessary to produce the "clot" are, however, much higher than that of calcium chloride, which even in the concentration of N/40 can cause 1-per-cent. sodium cholate to set to a solid gel in about a quarter of an hour at 50°. Sodium and magnesium chlorides produce clot formation at 50°, when their

* 'Roy. Soc. Proc.,' B, vol. 86, p. 460 (1913).

concentration is of about the order of half saturation. It is proposed to investigate the relative clotting power of salt in greater detail later; the present communication deals mainly with the question of the influence of one particular factor on gel formation, viz., on the surface tension of the solution.*

A preliminary account of the clot production by various calcium salts has been given in the first paper (*loc. cit.*). It was then shown that in the case of calcium salts which increase the surface tension of water, increase in the concentration of the salt caused a diminution of the clotting time. In the case of the calcium salts which lowered the surface tension, however, the accelerating effect of the increase in the concentration of the salt was counterbalanced by the diminished surface tension of the solution. In two cases (those of calcium dichloracetate and of the sulphocyanide) an optimal point was found. Increase in the concentration of the calcium salt above and below this point caused a lengthening of the time required for gel formation. In the case of calcium trichloracetate, the length of clotting time progressively increased with increasing concentration within the limits investigated.†

Method of Experiment.—The following was the method of experiment adopted:—1 c.c. of a 4-per-cent. sodium cholate solution and 3 c.c. of the salt solution in the required concentration were heated in separate quartz tubes of 10 c.c. capacity in a thermostat at 50°. As soon as the liquids had attained the temperature of the thermostat, they were rapidly mixed; the salt solution was poured into the cholate solution, and the mixture was then poured back into the tube originally containing the salt alone. This was then clamped in the (transparent) thermostat and watched. The formation of oily globules was the first sign of clot formation. These at first moved rapidly in the liquid, but as they increased in size, motion became less rapid, until, finally, movement was hardly visible. At this point, at short intervals, steel balls of 3/32 inch diameter, such as are used for ball-bearings, were dropped into the tube at short intervals. The time of complete gel formation was taken as that at which a ball stopped dead before it had fallen half-way through the tube. The time was taken by a stop-watch, which was started at the moment of mixing the solutions. Even when the clotting time was 5 minutes, control determinations seldom differed by more than 10 seconds—they usually agreed with one another within 5 seconds. In the first series of experiments on calcium salts, the cholate solution was made by saturating N/100 sodium hydroxide with cholalic acid‡

* Throughout this communication by "surface tension" is meant surface tension measured against air.

† The surface tension of the salt solutions is affected mainly by the anions. The series of anions employed was that used in the investigations on aggregation ('Roy. Soc. Proc.,' B, vol. 83, p. 96 (1910)).

‡ Prepared pure by the author's method ('Journ. Physiol.,' vol. 44, p. 265 (1912)).

until a solution neutral to neutral red was obtained. An approximately 4-per-cent. solution was obtained. It was found, however, that cholalic acid is soluble in sodium cholate solution, the amount dissolving varying with the temperature. As this free acid inhibits the clotting, it was found that the clotting time of a solution varied from day to day. For all subsequent experiments, sodium cholate was prepared and a 4-per-cent. solution was made directly from this. The cholate was made by dissolving cholalic acid in 20 times its weight of alcohol, neutralising this solution with sodium ethoxide, heating for a short time on a water-bath and filtering off the first separation of solid, and then evaporating the filtrate. Sodium cholate rapidly separated after a short time, and was filtered off, washed with acetone, and then dried, first on a water-bath, and then over sulphuric acid in a desiccator. It gave a solution in water, acid to phenolphthalein, but slightly alkaline to neutral red.

Table I.—Clotting Time of 1-per-cent. Sodium Cholate Solution (in seconds) in presence of Varying Concentrations of different Calcium Salts.

	3N/4.	N/2.	3N/8.	N/4.	N/8.
Chloride	3·0	14	14·0	21	34
Bromide	10·5	14	15·5	21	47
Nitrate	16·0	17	37·0	40	62
Iodide	18·0	17	20·0	24	41
Sulphocyanide	87·5	54	**47·0**	**47**	70
Formate	11·0	14	14·0	19	38
Acetate	8·0	10	12·0	19	34
Monochloracetate	24·0	26	33·0	36	77
Dichloracetate	97·0	47	33·0	**30**	52
Trichloracetate	∞	Incomplete clot in 25 minutes	710·0	170	122

Influence of Organic Compounds on the Gel Formation.

Many attempts have been made within recent years to correlate physical properties of organic substances with their different biological actions, such as the production of exosmosis, hæmolysis, narcosis, etc.; more especially, their effect on the surface tension of water has been stated to be intimately associated with the production of changes in the cell. Czapek* (in his monograph, January, 1911) claims to have determined the surface tension of the membrane of certain plant cells by showing that exosmosis of tannin takes place, whenever they are immersed in solutions the surface tension of which falls below 0·681 (water = 1). The smaller the amount of a given substance necessary to reduce the surface tension to this figure, the lower is

* See also the numerous papers by Traube.

the concentration of that substance which will produce exosmosis. The conceptions of Czapek have been subjected to severe criticism, principally by Vernon, who has shown that there are many exceptions to Czapek's rule. Several substances, especially the narcotics and acetonitrile, produce exosmosis in solutions, the surface tensions of which are appreciably greater than Czapek's critical point. Czapek accounts for the exceptions to his rule by ascribing to them some specific toxic property.

Vernon has shown that a marked parallelism exists between hæmolytic and narcotic actions and the inhibitory action on indophenol oxydases of animal tissues. Batelli and Mlle. Stern support Vernon's views in their investigations on the so-called oxydones and correlate the various toxic properties with the property of precipitating nucleoproteins.*

In view of the above-mentioned facts and the preliminary experience obtained from the study of the calcium salts as to the action of surface tensions, it was a matter of considerable interest to study the inhibitory action of various organic substances on the formation of the cholate gel, which is derived from chemically pure crystalline products, and which, in thin films, might be regarded as a structure analogous to the cell membranes. As a result it was found that whilst generally those substances which have the greatest power in lowering the surface tension of water have a greater inhibitory action on the gel formation, this rule is by no means absolute, and the chief exceptions are the narcotics and acetonitrile, *i.e.* the very substances which deviate from Czapek's generalisation. There is, moreover, a very close parallelism between the inhibitory action on gel formation and narcotic and other biological reactions. In the following experiments 0·5 c.c. of calcium chloride was diluted to 3 c.c. with water containing a given amount of the substance under investigation, and this was then mixed with 1 c.c. of 4-per-cent. sodium cholate, and the clotting time was determined by the method given above. The concentration of cholate in the mixture was, therefore, 1 per cent., and of the calcium chloride, N/8. By determining the weights as well as the volumes of the organic substances, and also the specific gravity of the solutions, the concentrations in gramme molecules per litre could be calculated.

* 'Biochem. Zeitsch.,' vol. 51, p. 1 (1913).

Table II.—Clotting Time without addition of Organic Substance, 17 secs.

Per cent. (vol.).	Grm. mol. per litre.	Clotting time, secs.
Ethyl Alcohol.		
2·5	0·437	20
3·75	0·656	31
5·0	0·875	47
6·25	1·093	85
7·5	1·312	218
8·75	1·532	514
10·0	1·749	805*
11·25	1·968	1190*
Propyl Alcohol.		
1·25	0·171	21
1·875	0·256	38
2·5	0·342	72
3·125	0·432	198
3·75	0·513	405
4·375	0·600	794
5·0	0·684	1200
5·625	0·770	†
Isopropyl Alcohol.		
2·5	0·334	24
3·75	0·561	99
5·0	0·668	272
6·25	0·835	675
7·5	1·002	1710
Butyl Alcohol (Normal).		
1·25	0·137	38
1·5625	0·171	76
1·875	0·205	185
2·1875	0·239	333
2·5	0·273	442
2·8125	0·308	665
3·125	0·342	Inc.
Secondary Butyl Alcohol.		
1·25	0·139	27
2·5	0·277	195
3·125	0·346	645
3·75	0·416	Inc.‡
Tertiary Butyl Alcohol.		
1·25	0·136	18
3·125	0·339	60
3·75	0·407	105
4·375	0·468	270
5·0	0·543	375
5·625	0·611	510
6·25	0·679	790
7·5	0·814	Inc.
Amyl Alcohol.		
0·9375	0·096	71
1·25	0·129	210
1·5625	0·161	480
Secondary Amyl Alcohol.		
1·25	0·113	56
1·875	0·169	267
2·1875	0·197	810
2·5	0·225	Inc.
Tertiary Amyl Alcohol.		
1·25	0·115	28
1·875	0·173	78
2·1875	0·202	136
2·5	0·230	237
2·8125	0·259	352
3·125	0·288	808
3·25	0·346	Inc.
Allyl Alcohol.		
1·25	0·188	21
2·5	0·376	38
3·125	0·470	45
3·75	0·564	135
5·0	0·752	382
6·25	0·940	810
7·5	1·128	Inc.
Acetonitrile.		
2·5	0·495	47
3·75	0·703	114
4·375	0·867	144
5·0	0·991	333
5·625	1·115	607
6·125	1·239	780
7·5	1·406	§
Methyl Ethyl Ketone.		
2·5	0·288	59
3·125	0·360	107
3·75	0·432	173
4·375	0·505	362
5·0	0·577	695
5·625	0·650	910
6·25	0·721	1257
7·5	0·864	Inc.
Methyl Propyl Ketone.		
1·25	0·121	38
1·875	0·182	46
2·5	0·242	260
2·8125	0·272	496
3·125	0·302	607*
Chloral Hydrate.		
Per cent. (weight).		
0·625	0·038	29
1·25	0·076	109
1·5625	0·094	210
1·875	0·113	915
2·1875	0·132	‖
Methyl Carbaminate.		
Per cent. (weight).		
2·5	0·333	33
3·75	0·500	50
5·0	0·666	79
6·25	0·833	255
7·5	1·000	645
8·75	1·166	>1800

* With separation of crystals.
† Not complete in half hour.
‡ Inc. indicates incipient clotting in half an hour.
§ Inc. with separation of crystals.
‖ Not quite complete in half hour.

Table II—*continued.*

Per cent. (vol.).	Grm. mol. per litre.	Clotting time, secs.	Per cent. (vol.).	Grm. mol. per litre.	Clotting time, secs.	Per cent. (vol.).	Grm. mol. per litre.	Clotting time, secs.
Ethyl Carbaminate.			Propyl Carbaminate.			Witte's Peptone.		
Per cent. (weight).								
1·25	0·140	26	1·25	0·121	36	1·875		213
1·875	0·210	38	1·875	0·182	93	2·5		360
2·5	0·281	56	2·1875	0·212	248	3·125		570
3·75	0·421	147	2·5	0·242	360	3·75		1530
4·875	0·491	296	3·125	0·303	910			
5·0	0·561	564				Phenol.		
5·625	0·632	750						
6·25	0·702	1448				0·625		165
						0·9375		550

Chloroform.

A solution of water saturated with chloroform at 17° contains 0·710 per cent. When 2·5 c.c. of this solution was mixed with 0·5 c.c. N. calcium chloride and 1 c.c. 4-per-cent. cholate solution, the clotting time was not appreciably longer than when no chloroform was present. By diminishing the concentration of the calcium salt to one half, the clotting time was 230 seconds in the presence of saturated chloroform water (2·75 c.c. in 4 = 0·041 grm. mol. per litre), as compared with 45 seconds, the clotting time in the absence of chloroform. The clotting time in presence of 0·064 grm. mol. per litre amyl alcohol, and the same amount of calcium salt, was 155 seconds, and of 0·038 grm. mol. per litre chloral hydrate 194 seconds. The inhibitory action of chloroform is therefore greater than that of amyl alcohol.

Nitromethane.

The action of nitromethane is anomalous. In the presence of 3·75 per cent. the clotting time is 23 seconds, in the presence of 5 per cent. it is 57 seconds, and of 6·25 per cent. it is 42 seconds. It appears to behave more or less like an acid, for in the presence of hydrochloric acid in N/800 concentration, the clotting time of cholate solutions is 41 seconds, in N/400 it is 104 seconds, 3N/800 it is 185 seconds, and in N/200 it is 74 seconds. At the highest of these concentrations the acid is sufficient to cause precipitation of free cholalic acid; on keeping at 50° the precipitate disappears and a gel then forms. The lower concentrations produce no separation of free organic acid in form visible to the naked eye. The nitromethane possibly forms the salt $CH_3N\begin{matrix}=O\\ -ONa\\ -OH\end{matrix}$ by double decomposition.

Polyhydroxy-Derivatives.

The inhibitory action of these substances is small, as is shown by the following examples :—

Substance.	Per cent. (weight).	Clotting time, secs.
Ethylene glycol	12·5	281
Propylene „	8·75	385 (with separation of crystals)
Glycerol	12·5	148
Sucrose	12·5	29
Dextrose	12·5	30
Dextrin	12·5	258
„	10·0	100

Discussion of Results.

Whilst it cannot be denied that those substances which lower most markedly the surface tension of water have, as a rule, the greatest tendency to exert an inhibitory effect on the formation of the cholate gel, the law is not by any means an absolute one. The exceptions are precisely the ones which deviate from Czapek's generalisation. Acetonitrile, which lowers the surface tension of water but little, has a greater inhibitory power than ethyl alcohol, which lowers it much more. The deviation from the rule is shown in a very marked manner also by the typical narcotics, chloral, chloroform, and (in the experiment on gel formation) by urethane. There is, in fact, a striking parallelism between inhibition of gel formation, narcotic and hæmolytic actions and production of tannin exosmosis, which is well exhibited in the following table. The various substances are arranged

Substances in decreasing order of gel-inhibiting action.	Critical narcotic concentration. Grm. mol. per litre.
Chloroform	0·0012
Chloralhydrate	0·02
Isoamyl alcohol	0·023
Secondary amyl alcohol (methyl propyl carbinol)	—
Tertiary amyl alcohol (dimethyl ethyl carbinol)	0·037
Propyl carbaminate	—
Normal butyl alcohol	0·038
(Methyl propyl ketone	0·019)
Isobutyl alcohol	0·045
Normal propyl alcohol	0·11
Urethane	0·041
Tertiary butyl alcohol	0·13
Isopropyl alcohol	0·13
Allyl alcohol	0·13
Methyl carbaminate	0·27
Acetonitrile	0·36
Ethyl alcohol	0·3

in the order in which they inhibit the gel formation, the more active substances being placed first in the list. The numbers given are the strengths in which they produce narcosis of tadpoles according to Overton.

The concordance between the gel inhibitory action and the narcotic action is striking. Methyl propyl ketone is an apparent exception, but gel-inhibiting action of this substance cannot be accurately determined, as in relatively small concentrations it causes the formation of crystals. The same is true for ethyl alcohol in higher concentrations. Normal propyl alcohol should follow instead of preceding urethane. The substances showing a marked deviation from the surface tension generalisation are indicated in large type.*

General Summary and Conclusions.

The inhibition of gel formation may be assumed to be due to adsorption of various substances from solution, which prevent the formation of larger aggregates, which constitute the gel.† The adsorbability of those substances cannot be determined by their effect alone in lowering the surface tension of water. Czapek has assumed that certain plant cells have a lipoid membrane, with a surface tension of about 0·681 (water = 1), and that, when they are immersed in an aqueous solution, the surface tension of which has been reduced to below this figure, exosmosis of complex molecules takes place, owing to the changes in the lipoid membrane. Czapek found, however, that certain substances deviated from his rule. To these he ascribed a specific toxic action on the cell. In view of the fact that these same substances show a deviation also from a surface tension rule in their inhibitory action on the formation of the cholate gel, a phenomenon from which specific biological action is excluded, the purely mechanical conception of cytolysis, as propounded by Czapek, is clearly no longer tenable. Nor do the results in the above paper support the Overton-Meyer lipoid hypothesis. Although the lipoid soluble substances have, as a rule, the greatest inhibitory action on gel formation, the gel itself cannot, by any extension of the meaning of the term, be described as a lipoid. It is formed from the salt of an acid, which is generally insoluble in organic solvents, in which even the free acid itself is only slightly soluble. The results suggest that the semipermeability of the cell may owe its properties to the presence of some gel-forming substance

* Several estimations of the surface tensions of solutions have been made by different observers. Czapek's own numbers have been adopted. In arranging the above table the approximate dilutions which delay gel formation 15 minutes have been ascertained. The surface tensions of these dilutions in water lies normally between 0·5 and 0·67 (water = 1). The substances indicated in capitals deviated markedly from these numbers.

† Compare Schryver, 'Roy. Soc. Proc.,' B, vol. 83, p. 96 (1910).

which has not yet been isolated, and which need be neither lipoid nor protein. Such a gel need not, furthermore, be continuous, but may simply form a matrix, holding together proteins and lipoids and other cell constituents. The protoplasm itself may exert its normal functions only when its constituents are held in such a matrix. The amount of substance to which the gel formation may be due need be present only in very small quantities. A solid gel has been obtained with $\frac{1}{2}$-per-cent. solutions of sodium cholate, but the author, in conjunction with Dr. E. Graf von Schönborn (in a preliminary communication to the Biochemical Society last May), has shown that solid gels are formed from sodium deoxycholeate (another bile acid), when the concentration does not exceed 1 in 1000.

Various other problems arise from the study of these gels. Attention has been called to the fact that relatively large quantities of sodium and magnesium salts are necessary to produce gel formation as compared with those of calcium salts. These facts offer a suggestion as to the antagonism of calcium salts to the toxic action of sodium and magnesium salts, as has been observed by Loeb, in the case of fundulus eggs, and of which many other biological examples exist. The replacement of a calcium salt by sodium or magnesium salts may render a gel unstable. It is proposed to investigate phenomena of this description. In all the above experiments a large excess of calcium salts has been employed in gel formation, in order to accelerate this phenomenon. To obtain results more analogous to the various biological phenomena, it will be necessary to study the action of various reagents on the gel when in thin membranes, and under conditions under which excess of calcium salts can be readily removed.* Preliminary experiments indicate that under such conditions the gel may be reversed. Work is proceeding in this direction, and it is also proposed to employ the gels for the study of various phenomena of permeability.

* In the above-described experiments the inhibitory action of various substances on a membrane (or gel) formation has been studied. It has been assumed in these arguments that the more powerful this particular action of a given substance is, the greater will be its disaggregating action on an already formed membrane (or gel).