STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

V. Effect of Electrolytes on the Rate of Inactivation of Bacteriophage by Alcohol.

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In an earlier paper[1] we have shown that upon the addition of an excess of alcohol to bacteriophage, the activity of the latter is rapidly reduced. This period of rapid inactivation seems roughly to coincide with the process of precipitation occurring when alcohol is added to the lytic filtrate. Further exposure to alcohol affects the residual lytic activity comparatively slowly.[2]

It is known[3–7] that lytic agent is easily adsorbed by various colloids. In our own experience, for instance, mere increase in the concentration of agar in the medium to 1.5 per cent caused loss of over 99 per cent of the activity of lytic filtrate as compared with that observed in the presence of 0.05 per cent of agar.[8,9] It thus seemed possible that the rapid initial inactivation referred to above could be due to the adsorption of lytic agent during precipitation occurring upon the addition of alcohol. In order to see if there exists any relation between the precipitation of the filtrate and the disappearance of its lytic activity, we attempted to vary the intensity of precipitation by controlling the

[1] Bronfenbrenner, J. J., and Korb, C., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 177.

[2] Bronfenbrenner, J. J., and Korb, C., *J. Exp. Med.*, 1925, xlii, 419.

[3] Doerr, R., *Klin. Woch.*, 1922, i, 1493.

[4] Doerr, R., and Berger, W., *Z. Hyg. u. Infectionskrankh.*, 1923, xcvii, 422.

[5] Nakamura, O., *Arch. Hyg.*, 1923–24, xcii, 61.

[6] Brutsaert, P., *Compt. rend. Soc. biol.*, 1924, xc, 1292.

[7] Hauduroy, P., *Compt. rend. Soc. biol.*, 1924, xc, 1463.

[8] Bronfenbrenner, J. J., and Korb, C., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 315.

[9] Bronfenbrenner, J. J., and Korb, C., *J. Exp. Med.*, 1925, xlii, 483.

concentration of electrolytes, and to follow the change in the rate of inactivation of the lytic filtrate caused thereby.

Effect of Excess of Sodium Chloride on the Rate of Inactivation of Bacteriophage by Alcohol.

The general procedure followed in these experiments consisted in mixing, in a series of tubes, 0.5 cc. of active filtrate (prepared in 0.5 per cent NaCl broth pH = 7.4) with equal amounts of sodium chloride solution of varying concentrations. These mixtures were cooled to 7°C.; to each tube except two were added 10 cc. of cooled 95 per cent alcohol; and the whole series was placed in the ice box. Of the two tubes not receiving alcohol, one served as control of the original activity of the filtrate (A), and the other, receiving salt solution of maximum concentration, but no alcohol, was held to indicate the effect of the salt alone (B) on the activity of the filtrate. After 3 hours at 7°C., the contents of the respective tubes were thoroughly mixed and 0.1 cc. samples removed for titration by serial dilution in broth. The contents of Tubes A and B were diluted with 10 cc. of 0.5 per cent NaCl solution, previous to titration, in order to render the concentration of lytic filtrate in them comparable to that in the remaining tubes of the series.

The results of this titration are indicated in Protocol 1 both in terms of the actual amount of the respective mixtures exhibiting lytic activity, and in terms of the approximate amount of the original lytic filtrate present in each. As in the earlier experiments,[2] readings were taken at the end of 24 and 40 hours, and the final readings were checked by means of transfer to broth seeded with susceptible bacteria.

Effect of Precipitation on the Activity of the Alcoholic Solution of the Lytic Agent.

The results of this experiment show that sodium chloride alone does not affect the activity of the lytic agent during 3 hours exposure, even when its concentration in the filtrate reaches 2.59 M (Protocol 1, B). On the other hand, addition of 10 volumes of alcohol in the presence of 0.085 M NaCl reduces the activity of the filtrate about 100 times (Protocol 1, C). As the concentration of sodium chloride in the filtrate increases the precipitation is more complete and inactivation of the lytic principle is more marked; and when the concentration of sodium chloride in the filtrate reaches 1.79 M, its activity is almost completely destroyed (Protocol 1, G and H) during 3 hours incubation

Protocol 1.

Increase in the Rate of Inactivation of Bacteriophage by Alcohol, Due to Addition of NaCl to the Filtrate.

Tube	A	B	C	D	E	F	G	H
	Control.	Control.	Control.					
Lytic filtrate in 0.5 per cent NaCl broth, *cc.*	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
NaCl solution. Amount, *cc.*	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
NaCl solution. Concentration	0.085 M	5.1 M	0.085 M	0.35 M	0.85 M	1.7 M	3.5 M	5.1 M
Resulting concentration of NaCl (about)	0.085 M	2.59 M	0.085 M	0.21 M	0.46 M	0.89 M	1.79 M	2.59 M
95 per cent alcohol, *cc.*	0	0	10	10	10	10	10	10
Contents of tubes thoroughly mixed and placed at 7°C. for 3 hrs.								
Precipitate	0	0	+	+	+	++	+++	+++
0.5 per cent NaCl solution, *cc.*	10	10	0	0	0	0	0	0

Contents of each tube titrated by serial dilution in broth.

Lytic filtrate present in each dilution (approximate).	Actual amount of mixtures transferred to broth.	A 24 hrs.	A 40 hrs.	A Final transfer.	B 24 hrs.	B 40 hrs.	B Final transfer.	C 24 hrs.	C 40 hrs.	C Final transfer.	D 24 hrs.	D 40 hrs.	D Final transfer.	E 24 hrs.	E 40 hrs	E Final transfer.	F 24 hrs.	F 40 hrs.	F Final transfer.	G 24 hrs.	G 40 hrs.	G Final transfer.	H 24 hrs.	H 40 hrs.	H Final transfer.
cc.	*cc.*																								
5×10^{-3}	10^{-1}	±	±	+	+	±	+	+	±	+	+	±	+	+	±	+	±	±	+	±	+	+	−	−	−
5×10^{-4}	10^{-2}										+	±	+	+	±	+	+	±	+	±	−	−	−	−	−
5×10^{-5}	10^{-3}	+	±	+	+	±	+	±	+	+	+	±	+	+	±	+	+	+	+	−	−	−	−	−	−
5×10^{-6}	10^{-4}				±	+	+	±	+	+	±	+	+	±	+	+	±	−	−	−	−	−	−	−	−
5×10^{-7}	10^{-5}	±	+	+	±	+	+	±	+	+	±	+	+	±	+	+	±	−	−						
5×10^{-8}	10^{-6}	±	+	+	±	+	+	±	+	+	±	+	+	±	−	−	−	−	−						
5×10^{-9}	10^{-7}	±	±	+	±	+	+	−	−	−	±	−	−	−	−	−	−	−	−						
5×10^{-10}	10^{-8}	±	+	+	±	+	+	−	−	−	−	−	−	−	−	−	−	−	−						
5×10^{-11}	10^{-9}	−	−	−	−	−	−	−	−	−	−	−	−												

The amount of precipitate in the respective tubes was recorded as 0 = no precipitate; + = precipitate present; ++ = moderate precipitate present; +++ = copious precipitate present.

with alcohol at 7°C. The fact that inactivation of the lytic principle in this experiment appears to be of the nature of adsorption secondary to precipitation of the medium, and not analogous to the process of disinfection, as claimed by d'Hérelle, is further suggested by the experiment which follows.

It was seen in earlier experiments[2] that most of the inactivation of lytic agent occurs within a few minutes following the addition of alcohol. Under the conditions of this reaction, apparently not all of the coagulable material is precipitated (Protocol 1 of the present work, C and D), since addition of sodium chloride to the filtrate results in more copious precipitation, with correspondingly greater inactivation of the lytic agent (Protocol 1, F, G, H). It was to be expected, therefore, that if lytic filtrate is mixed with alcohol, and the precipitate is removed, the clear supernatant fluid which is still active[2] will still contain some coagulable material which should come down if sodium chloride is added to it. If inactivation of lytic agent is due to adsorption, this precipitation must be followed by a greater inactivation, as compared with that occurring in the mixture to which no salt was added.

To test the point 1 cc. of cold lytic filtrate (7°C.) was thoroughly mixed with 10 cc. of cold 95 per cent alcohol (7°C.). This mixture was placed on ice, and after 30 minutes the precipitate which was formed was separated by thorough centrifuging at 7°C. (Protocol 2). The supernatant fluid, now containing only a part of its original lytic activity (the greater part being present in the sediment[2]), was placed in two tubes, 5 cc. in each. To one were added 2.5 cc. of cold distilled water (Protocol 2, A), and to the other, 2.5 cc. of cold 5.1 M sodium chloride (Protocol 2, B); both were placed on ice. At intervals, indicated in Protocol 2, portions of each of the mixtures were removed and titrated for lytic activity by serial dilution in broth. The results were read and recorded exactly as in the earlier experiments.[2]

It will be seen from the results recorded in Protocol 2, A that while the activity of the lytic principle in the supernatant fluid (diluted alcohol) remained practically unchanged for hours, inactivation quickly resulted upon addition of sodium chloride to the mixture (Protocol 2, B). The final concentration of sodium chloride did not exceed 1.7 M, and this concentration did not of itself cause inactivation

(Protocol 1, B). However, when the salt solution was added to the clear supernatant fluid, the solution immediately became opalescent, and a fine precipitate soon appeared. Thus it seems that neither the

Protocol 2.

Inactivation of the Alcoholic Solution of Lytic Agent by the Addition of NaCl.

Cold lytic filtrate ..... 1 cc.
Cold 95 per cent alcohol ..... 10 cc.
Placed on ice and after 30 min. centrifuged at 7°C.*
Supernatant fluid used further as indicated below:

	A	B
Supernatant fluid (7°C.), *cc.*	5	5
Distilled water (7°C.), *cc.*	2.5	
5.1 M NaCl (7°C.), *cc.*		2.5

Placed on ice and samples removed for titration at intervals.

Time of removal of samples, *hrs.*	1			3			8			1			3			8		
Amount of precipitate observed	−			−			−			+			+			+		
Absolute amount of filtrate present in each tube.	Actual amounts transferred to broth.	24 hrs.	40 hrs.	Final transfer.	24 hrs.	40 hrs.	Final transfer.	24 hrs.	40 hrs.	Final transfer.	24 hrs.	40 hrs.	Final transfer.	24 hrs.	40 hrs.	Final transfer.	24 hrs.	40 hrs.	Final transfer.
cc.	*cc.*																		
7×10^{-3}	10^{-1}	+	±	+	+	−	+	+	+	+	±	−	−	−	−	−	−	−	−
7×10^{-4}	10^{-2}	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
7×10^{-5}	10^{-3}	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
7×10^{-6}	10^{-4}	+	+	+	+	+	+	±	+	+	−	−	−	−	−	−	−	−	−
7×10^{-7}	10^{-5}	+	+	+	±	+	+	±	+	+	−	−	−	−	−	−	−	−	−
7×10^{-8}	10^{-6}	±	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−
7×10^{-9}	10^{-7}	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
7×10^{-10}	10^{-8}	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

* It is important to keep the temperature as low as possible during centrifuging to avoid an excessive destruction of lytic principle (compare Tables II and III in the second paper of the series[2]).

alcohol nor the salt alone causes inactivation of lytic agent; but when the solution of lytic agent in alcohol is caused to precipitate by the addition of salt, a rapid inactivation of lytic agent is the result.

Effect of Valency of Electrolytes on the Rate of Inactivation of Lytic Agent in the Presence of Alcohol.

If the observed increase in the rate of inactivation of lytic agent by alcohol in the presence of sodium chloride is due, as suggested, to a more complete precipitation of the medium, replacement of sodium chloride by salts with polyvalent ions might be expected to increase further the rate of precipitation and thus to result in a more marked change in the same direction.[10] Or, the same degree of inactivation as that produced with a given concentration of NaCl should be brought about by correspondingly lower concentrations of salts with polyvalent ions.

Since precipitation of colloids may be affected by both anions and cations, depending on their charge,[11,12] we attempted in a preliminary experiment to establish which of the ions of a salt are most active under the conditions of our experiment. Accordingly, we compared the effect of an arbitrarily chosen equimolar solution of three salts: sodium chloride, sodium sulfate, and calcium chloride. The procedure was exactly the same as that illustrated in Protocol 1, except that only one concentration of each salt (0.2 M) was used instead of different concentrations of one salt as before (Protocol 3).

Since sodium chloride and sodium sulfate behaved in the same way, it appears that the valency of the anion was of no significance. On the other hand, the calcium salt showed a considerably greater effect on inactivation than did sodium chloride, although the anion in both cases was the same. It was thus obvious that, in the case of the substances studied at least, the nature of the cation was important in this reaction. In the subsequent study of the effect of valency of ions on the rate of inactivation of the lytic principle, the valency of the cation only was considered.

In the preceding experiments, the duration of exposure of the lytic agent to the combined action of alcohol and salt, before titration, was arbitrarily set at 3 hours. Since one salt might be expected to act more quickly than another, in the following experiment samples of the mixtures were removed from time to time, as indicated in the protocol.

[10] Schulze, H., *J. prakt. Chem.*, 1881–82, xxv, N.S., 431; 1884, xxvii, 320.
[11] Hardy, W. B., *Z. physik. Chem.*, 1900, xxxiii, 385.
[12] Pauli, W., and Flecker, L., *Biochem. Z.*, 1912, xli, 461.

Thus we obtained not only an idea of the total effect after 3 hours, but also curves representing the rates of inactivation of the lytic agent due to each salt. In order to avoid unnecessary repetition, only one such experiment (with $MnCl_2$) will be described in detail. It is under-

Protocol 3.

Comparative Effect of Cations and Anions on Inactivation of Bacteriophage by Alcohol.

	A	B	C	D
	Control.	NaCl	Na_2SO_4	$CaCl_2$
Lytic filtrate, *cc*.	0.5	0.5	0.5	0.5
0.4 M solution of salts, *cc*.		0.5	0.5	0.5
Distilled water, *cc*.	0.5			
Resulting concentration of salts	0.04 M NaCl	0.24 M NaCl	(0.04 M NaCl) 0.2 M Na_2SO_4	(0.04 M NaCl) 0.2 M $CaCl_2$
Mixtures were cooled to 7°C.				
Cold 95 per cent alcohol, *cc*.	10	10	10	10
Placed on ice for 3 hrs. and titrated.				

Absolute amount of lytic agent present in each tube.	Actual amounts transferred to broth.	A 24 hrs.	A 40 hrs.	A Final transfer.	B 24 hrs.	B 40 hrs.	B Final transfer.	C 24 hrs.	C 40 hrs.	C Final transfer.	D 24 hrs.	D 40 hrs.	D Final transfer.
cc.	*cc.*												
5×10^{-3}	10^{-1}	+	+	+	+	+	+	+	+	+	+	+	+
5×10^{-4}	10^{-2}	+	+	+	+	+	+	±	+	+	±	+	+
5×10^{-5}	10^{-3}	+	±	+	+	±	+	±	+	+	±	+	+
5×10^{-6}	10^{-4}	±	+	+	±	+	+	±	+	+	−	−	−
5×10^{-7}	10^{-5}	±	+	+	±	+	+	−	+	+	−	−	−
5×10^{-8}	10^{-6}	±	+	+	−	−	−	−	−	−	−	−	−
5×10^{-9}	10^{-7}	−	−	−	−	−	−	−	−	−	−	−	−
5×10^{-10}	10^{-8}	−	−	−	−	−	−	−	−	−	−	−	−

stood that in the case of each salt exactly the same procedure was followed throughout, with variation only in the concentration of salts until such concentrations were found for each as caused an inactivation of the lytic filtrate comparable with that produced by alcohol in the presence of 1.7 M sodium chloride.

Protocol 4.

Comparative Effect of Different Concentrations of $MnCl_2$ on the Rate of Inactivation of the Lytic Agent in the Presence of Alcohol.

Salt used	NaCl					MnCl₂										
Laudman Shiga filtrate, *cc.*	1					1				1				1		
Concentration of salt used	3.4 M					1 M				0.3 M				0.1 M		
Amount of salt solution used, *cc.*	1					1				1				1		
Approximate resulting concentration of salt	1.7 M					0.5 M				0.15 M				0.05 M		
Amount of the above mixture (A), *cc.*	1				1 (control).	1				1				1		
Cold water, *cc.*					10											
Cold alcohol, *cc.*	10					10				10				10		
Duration of exposure at 7°C.	5 min.	15 min.	1 hr.	3 hrs.	3 hrs.	5 min.	15 min.	1 hr.	3 hrs.	3 hrs.	5 min.	15 min.	1 hr.	3 hrs.	3 hrs.	5 min.	15 min.	1 hr.	3 hrs.	3 hrs.
Amount of original filtrate in each tube. / Amount of mixtures used.																				
cc. / *cc.*																				
5×10^{-3} / 1×10^{-1}	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+
5×10^{-4} / 1×10^{-2}	+	+	−	−	+	+	−	−	−	+	+	+	−	−	+	+	+	+	+	+
5×10^{-5} / 1×10^{-3}	+	−	−	−	+	−	−	−	−	+	+	−	−	−	+	+	+	+	+	+
5×10^{-6} / 1×10^{-4}	+	−	−	−	+	−	−	−	−	+	+	−	−	−	+	+	+	+	−	+
5×10^{-7} / 1×10^{-5}	+	−	−	−	+	−	−	−	−	+	+	−	−	−	+	+	+	+	−	+
5×10^{-8} / 1×10^{-6}	+	−	−	−	+	−	−	−	−	+	−	−	−	−	+	+	+	−	−	+
5×10^{-9} / 1×10^{-7}	−	−	−	−	+	−	−	−	−	+	−	−	−	−	+	+	−	−	−	+
5×10^{-10} / 1×10^{-8}	−	−	−	−	+	−	−	−	−	+	−	−	−	−	+	−	−	−	−	+
5×10^{-11} / 1×10^{-9}	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

A

B

In each case, to several 1 cc. portions of Laudman Shiga lytic filtrate (prepared in unbuffered broth), was added 1 cc. of varying concentrations of salts (in this case $MnCl_2$); after thorough mixing and cooling to 7°C., two portions of 1 cc. each of this Mixture A were removed. To one of these were added, in each case, 10 cc. of cold alcohol, to the other 10 cc. of cold water, and both sets of tubes were placed in the ice box. At intervals indicated in Protocol 4, B, the contents of the tubes containing alcohol were thoroughly mixed, after which samples were removed for immediate titration by serial dilution in broth, and the tubes were replaced in the ice box. After the last titration (3 hours after the beginning of the experiment), the second tube of each series which received no alcohol was also titrated to show what effect, if any, the salt alone had on the activity of the filtrate during the entire period of the experiment. The results of

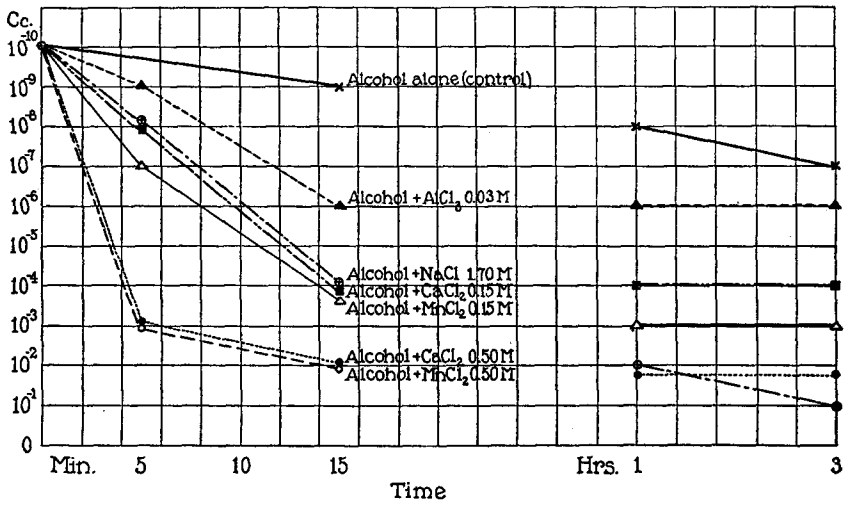

CHART 1. Effect of electrolytes on inactivation of the lytic filtrate by alcohol.

these titrations were read exactly as before (Protocol 1), that is after 24 hours, 40 hours, and after a subsequent control transfer to fresh broth,—but for the sake of simplicity only the final reading has been recorded in Protocol 4.

As can be seen from Protocol 4, the rate of inactivation of lytic agent in the presence of 1.7 M NaCl was most closely approached in the tube containing 0.15 M $MnCl_2$. On the composite Chart 1 are plotted the results of several such experiments. This chart shows again that immediately after the addition of alcohol there occurred a rapid diminution in the activity of the filtrate. This inactivation proceeded

at a fairly constant rate for about 15 minutes. Later, the curves show a distinct tendency to flatten, possibly coincident with the completion of precipitation. This chart shows further that approximately the same rate of inactivation resulted in the presence of 1.7 M sodium chloride as in that of 0.15 M calcium chloride or 0.15 M manganese chloride. In the case of aluminum chloride or lanthanum chloride, a concentration of a little over 0.03 M would accomplish approximately the same inactivation.[13]

Thus, in general, salts with polyvalent cations increase the rate of inactivation of lytic filtrate in the presence of alcohol much more than does sodium chloride under similar conditions. The greater the valency of the cation, the greater is the rate of inactivation. Although there were some minor deviations, which will be discussed in detail in one of the later papers of this series, the nature of cation as apart from its valency influenced the respective rates of inactivation of the lytic agent but little; in many instances no measurable difference could be detected. Thus, for instance, the increase in the rate of inactivation of the lytic agent resulting from the increase in the concentration of calcium chloride from 0.15 M to 0.5 M was duplicated by a corresponding change in the concentration of manganese chloride (Chart 1). Similar parallelism was observed when the concentrations of $AlCl_3$ and $LaCl_3$ were varied.

Effect of Partial Removal of Salts by Dialysis on the Rate of Inactivation of Bacteriophage by Alcohol.

The results of the preceding experiments consistently indicated an intimate relation between the rate of precipitation of lytic filtrate and the loss of its activity resulting from the addition of alcohol. It has been shown that addition of electrolytes increases this inactivation, and that the rate of increase depends on the valency of the cation. Since the lytic filtrate, consisting of beef infusion broth, already contains various salts in addition to 0.5 per cent sodium chloride added to it in the process of preparation, it was thought that perhaps even the initial inactivation of bacteriophage by alcohol[2] (prior to the addition

[13] The two latter salts could not have been used in concentrations higher than 0.03 M on account of the fact that the change in hydrogen ion concentration in the solution attained thereby was detrimental to the activity of the lytic agent.

Protocol 5.

Activity of Dialyzed Lytic Filtrate.

Actual amount of fluid having lytic activity.	A	B										C		D
	Original lytic filtrate.	Outer fluid after first 24 hrs. of dialysis.		Outer fluid after second 24 hrs. of dialysis.		Outer fluid after third 24 hrs. of dialysis.		Outer fluid after fourth 24 hrs. of dialysis.		Outer fluid after fifth 24 hrs. of dialysis.		Dialyzed lytic filtrate (after 5 days of dialysis).		Original lytic filtrate kept on ice for 7 days.
		Lytic power.	Chloride.	Lytic power.	Chloride.	Lytic power.	Chloride.	Lytic power.	Chloride.	Lytic power.	Chloride.	Lytic power.	Chloride.	Lytic power.
cc.														
1×10^{-1}	+	+	Present.	+	Present.	+	Present.	+	Present.	+	Tr.	+	Present.	+
1×10^{-2}	+	+		+		+		+		+		+		+
1×10^{-3}	+	+		+		+		+		+		+		+
1×10^{-4}	+	+		+		+		+		+		+		+
1×10^{-5}	+	+		+		+		+		+		+		+
1×10^{-6}	+	+		+		+		−		+		+		+
1×10^{-7}	+	−		−		−		−		−		+		+
1×10^{-8}	+	−		−		−		−		−		+		+
1×10^{-9}	+	−		−		−		−		−		+		+
1×10^{-10}	−	−		−		−		−		−		−		−
1×10^{-11}	−	−		−		−		−		−		−		−

of electrolytes) might be due largely to this presence of salts in it. We therefore attempted to reduce the salt content of the lytic filtrate by dialysis, and to compare the rate of inactivation (by alcohol) of the resulting lytic filtrate with that of the original filtrate.

For this purpose, 50 cc. of ordinary beef infusion broth containing 1 per cent of peptone and 0.5 per cent of sodium chloride, and adjusted to neutral reaction, were seeded with 0.1 cc. of a suspension of young *B. dysenteriæ* Shiga (grown for 18 hours on an agar slant) containing 100,000,000 microorganisms, and incubated at 37°C. for 1 hour. At this time, 0.1 cc. of a dilution of Laudman Shiga lytic principle 1:10,000 in broth was added to the culture. The latter was further incubated for 40 hours, filtered, and the filtrate placed on ice after having been tested for sterility and for its lytic action (Protocol 5, A). 2 days later, when the tests showed the filtrate to be sterile, a 5 cc. portion of it was removed and placed in a sterile collodion thimble surrounded by 200 cc. of sterile distilled water. Both the dialyzing thimble and the remaining filtrate were replaced on ice. The next day, as well as on each succeeding one, the liquid surrounding the dialyzing thimble was removed, tested for the presence of chlorides, and titrated for its lytic activity (Protocol 5, B). At the same time, 200 cc. of fresh sterile distilled water were introduced in place of the liquid removed, and the flask was returned to the ice chest. After 5 days of dialysis against distilled water, the contents of the collodion bag were likewise subjected to the qualitative test for chlorides, and to titration for their lytic power (Protocol 5, C) as well as for sterility. At the same time, a portion of the original filtrate was also titrated for comparison (Protocol 5, D). It will be observed that the lytic filtrate remained sterile; that it was not entirely freed of chlorides by the end of 5 days of dialysis (Protocol 5, C); also, together with chlorides, a certain portion of lytic agent dialyzed and thus was removed daily (Protocol 5, B). This amount was so small, however, that it was not detected when the contents of the bag were titrated after the 5 days of dialysis (compare C and D in Protocol 5) by the usual method of tenfold dilution.

Having thus established the fact that lytic filtrate, partially dialyzed against distilled water, retained practically its full activity in so far as the method of titration permitted estimation, we next proceeded to inquire into the effect of alcohol upon it.

Two portions of 3 cc. each of dialyzed lytic filtrate were placed in tubes;[14] to one of them (Protocol 6, A) was added 0.05 cc. of water, and to the other 0.05 cc. of 30 per cent sodium chloride solution (Protocol 6, B). At the same time, a 5 cc.

[14] The filtrate, during dialysis, became diluted through osmosis, so that instead of the original volume of liquid placed in the bag (5 cc.), the bag contained by the end of the 5th day approximately 11 cc. of fluid.

Protocol 6.

Effect of Partial Dialysis on Resistance of Bacteriophage to Alcohol.

	A	B	C
Dialyzed filtrate (diluted 5:11 during dialysis), *cc.*	3	3	
Original filtrate (diluted 5:11), *cc.*			3
0.5 per cent NaCl solution, *cc.*			0.05
Distilled water, *cc.*	0.05		
30 per cent NaCl solution, *cc.*		0.05	
Resulting concentration of NaCl	Definite traces.	About 0.5 per cent.	About 0.5 per cent.
Placed on ice for 3 hrs.			
From the above mixtures (cold), *cc.*	1	1	1
95 per cent alcohol (cold), *cc.*	10	10	10

Placed on ice and titrated at intervals.

Duration of exposure to alcohol		15 min.	1 hr.	3 hrs.	24 hrs.	48 hrs.	15 min.	1 hr.	3 hrs.	24 hrs.	48 hrs.	15 min.	1 hr.	3 hrs.	24 hrs.	48 hrs.
Amount of original filtrate in each tube.	Amount of mixtures used.															
cc.	*cc.*															
5×10^{-2}	1×10^{-0}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5×10^{-3}	1×10^{-1}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5×10^{-4}	1×10^{-2}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5×10^{-5}	1×10^{-3}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
5×10^{-6}	1×10^{-4}	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−
5×10^{-7}	1×10^{-5}	+	+	+	+	+	+	+	+	−	−	+	+	+	−	−
5×10^{-8}	1×10^{-6}	+	+	+	+	−	+	−	−	−	−	+	+	−	−	−
5×10^{-9}	1×10^{-7}	+	+	+	−	−	+	−	−	−	−	+	−	−	−	−
5×10^{-10}	1×10^{-8}	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

portion of the original filtrate (not subjected to dialysis) was diluted to 11 cc. with a 0.5 per cent NaCl solution (in order to duplicate the dilution of the dialyzed portion), 3 cc. of the resulting mixture were placed in a third tube (Protocol 6, C), and to this was further added 0.05 cc. of 0.5 per cent NaCl solution. The contents of the tubes were thoroughly mixed, and now 1 cc. portions of each were put into cooled tubes and placed on ice. To the contents of each tube, after cooling, were added 10 cc. of cold 95 per cent alcohol, the tubes were placed on ice, and after thorough mixing of the contents samples were titrated at intervals, as indicated in Protocol 6.

The findings of this experiment indicate that removal of a part of the salts from the lytic filtrate resulted in its greater resistance to alcohol (Protocol 6, A), and that replacement of sodium chloride in the dialysate to approximately the same concentration as that in the original broth results in the increase in its sensitiveness to alcohol (Protocol 6, B). That in the latter case (Protocol 6, B) the rate of destruction does not quite attain the level observed in the control (Protocol 6, C) is possibly due to the fact that, during dialysis, in addition to sodium chloride some other salts were also removed from the filtrate.

DISCUSSION.

The results of the experiments reported in this paper suggest, in our opinion, that the inactivation of the lytic principle by alcohol is not due to the virucidal action of the latter, but to the adsorption of dissolved lytic substance on the surface of the precipitate occurring when alcohol is added to the lytic filtrate. However, our results lack the character of indisputable proof. Thus, for instance, those in favor of the parasitic nature of bacteriophage might take the view that the addition of electrolytes in our experiments increased the disinfecting action of alcohol by forcing it out of the water, and concentrating it in the lipoid constituents of the supposed cell membrane of the "*bacteriophagum intestinale*."

We shall consider this possibility in later experiments. For the present, however, such an explanation does not seem satisfactory. In the first place, such analogy with the effect of sodium chloride on the bactericidal effect of phenol[15] does not suffice to explain the effect of valency of salts as observed in our experiments. Furthermore, if the

[15] Spiro, K., and Bruns, H., *Arch. exp. Path. u. Pharmakol.*, 1898, xli, 355.

inactivation of lytic agent were due in the final analysis to direct toxic action of alcohol, one would expect the rate of the reaction to be expressed by a straight line, as is the case with the rate of lysis of erythrocytes by alkalies,[16,17] for example, or with the rate of destruction of bacterial spores by mercuric chloride,[18] as calculated by Morawitz.[19] As a matter of fact, we have seen that for the first few minutes the inactivation of lytic agent by alcohol proceeds at a rate consistent with the formula proposed by Ostwald for disinfection, but soon the curves flatten out, in spite of the presence of an excess of both bacteriophage and alcohol in the solution. This break in the rate of inactivation of the lytic agent suggests that the reaction which takes place is not due to the alcohol directly, but to some other factor set into action by the addition of alcohol, but ceasing to operate after the first few minutes. We believe this factor is the formation of a fine precipitate with the concomitant adsorption of the lytic agent.

The adherents of the vitalistic theory of the bacteriophage action might suggest that the filtrate representing, according to their views, a suspension of "*bacteriophagum intestinale*," by analogy with bacteria, might be composed of variants possessing varying degrees of susceptibility to the destructive effects of alcohol. Thus, if the majority of individuals were highly susceptible, and the remainder highly resistant to toxic action, the result would be, in general, similar to that which we observed; namely, the majority of the hypothetical organisms would be destroyed soon after the addition of alcohol, and the filtrate would show a marked and rapid fall in its activity. The remainder of the organisms would be destroyed at a different and much slower rate and consequently the curve expressing the rate of inactivation would show a break.

That such a view is untenable follows from our earlier findings that lytic filtrates are uniform in their susceptibility to the action of alcohol, and contain no fraction possessing a higher degree of resistance.[2]

Still another objection might be raised against our interpretation of

[16] Gros, O., *Biochem. Z.*, 1910, xxix, 350.
[17] Stadler, E., and Kleemann, H., *Biochem. Z.*, 1911, xxxvi, 301, 321.
[18] Krönig, B., and Paul, T., *Z. Hyg. u. Infectionskrankh.*, 1897, xxv, 1.
[19] Morawitz, H., *Kolloidchem. Beihefte*, 1909–10, i, 301.

the results here presented. Lytic agents have been adsorbed on a number of organic and inorganic colloids. Usually they are not permanently inactivated thereby. In the case of the adsorption of the lytic agent by the precipitate resulting from precipitation of the lytic filtrate by alcohol, however, there occurs a marked irreversible reduction in its activity. This destruction, in our opinion, finds its analogy in the similar effect of alcohol upon enzymes and toxins, and is due to denaturation of the protein vehicle on which lytic agent is distributed in a very thin (monomolecular ?) layer.

Somewhat similar observations may be quoted with respect to the destruction of bacteriophage by heat. It has been found by a number of investigators that bacteriophage is relatively heat-resistant, and requires at least ½ hour's exposure at 70–90°C. for complete destruction. However, when it is adsorbed on the surface of bacteria, it is destroyed at 56°C.[20]

Another analogy is seen in our experiments[21] in which bacteriophage is completely inactivated by acetone if electrolytes are added to the filtrate before precipitation, whereas without the addition of electrolytes bacteriophage is found practically unaffected by the acetone.

SUMMARY AND CONCLUSIONS.

Addition of neutral salts to the lytic filtrate results in an increased rate of inactivation of the latter when alcohol is added to it. This effect of salts is the more marked the higher the valency of the cation. Conversely, removal by dialysis of salts originally present in the lytic filtrate tends to render lytic agent less sensitive to alcohol. Restitution of the original salt content to the dialyzed filtrate tends to bring the sensitiveness to alcohol in the dialyzed filtrate to the level of the non-dialyzed control.

It appears, therefore, that inactivation of the lytic agent by alcohol depends directly on the rate of precipitation of the coagulable constituents of the medium, and is not the result of a direct toxic action of alcohol on "*bacteriophagum intestinale.*" Considered in association with our earlier findings, these results speak in favor of the chemical nature of the agent of transmissible lysis.

[20] Seiffert, W., *Med. Klin.*, 1922, xviii, 997, 1093, 1121.

[21] Bronfenbrenner, J. J., *Proc. Soc. Exp. Biol. and Med.*, 1925 (in press).