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PHENOL AND TRICRESOL EFFECTS IN TOXIN AND ANTITOXIN SOLUTIONS.

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The antiseptics generally employed as preservatives for bacterial toxins, antitoxins and toxin-antitoxin mixtures are phenol and tricresol (a mixture of ortho-, meta- and para-cresols). At these laboratories during certain periods phenol has been used in a concentration of 0·5 per cent. for diphtheria toxin and toxin-antitoxin mixtures, and tricresol in a concentration of 0·3 per cent. to 0·4 per cent. for antitoxic serum. In such concentrations and at normal temperatures these antiseptics appear to have little or no immediate destructive effect on the toxin or antitoxin.

Under certain conditions however changes occur. Kelly (1924) reported that toxin-antitoxin mixtures, which had become frozen during a spell of extremely cold weather in America, with the thermometer well below zero, caused severe reactions when injected into children. The experimental work carried out on this point by White and Robinson (1924), Kirkbride and Dow (1924) and Anderson and Leonard (1924) produced very conflicting results. In some cases an increase in toxicity was found after freezing, while in others no increase and in some cases a decrease in toxicity occurred. It was noticed however by White and Robinson (1924) that the presence of phenol or tricresol in the toxin-antitoxin mixture usually caused a precipitation of insoluble protein after freezing.

By the addition of increasing concentrations of phenol or tricresol to toxin-antitoxin mixtures, Glenny Pope Waddington and Wallace (1925) showed that at a fairly sharply defined concentration of the antiseptic relatively more antitoxin than toxin was destroyed. Similar results were also obtained by the evaporation at low temperatures of toxin-antitoxin mixtures containing the normal concentration of antiseptic.

The object of the present paper is to show in more detail the conditions under which the antiseptics may produce undesired effects.

Alteration in the concentration of the antiseptic on freezing.

When a dilute solution of phenol in water is frozen, water is withdrawn in the form of ice crystals and an increase in the concentration of phenol occurs.

Experiment I.—To two 10 c.c. volumes of (*a*) 1·0 per cent. saline and (*b*) 0·5 per cent. phenol in 1 per cent. saline were added 0·1 c.c. of "universal indicator." This, prepared by British Drug Houses Ltd., when added to solutions of phenol in water is a brownish orange, but when added to solutions of water in phenol or to pure phenol is very bright red: brom-cresol-purple can be used with equally satisfactory results. The tubes containing the mixtures were then placed in a salt and ice freezing mixture at a temperature of −8° C. In a short time the mixtures appeared to be frozen solid and the colour of the indicator in both tubes had become a dull brown. On continued immersion in the freezing bath it was found that the colour of the indicator in the solution containing phenol had changed to bright red, while that in the solution containing no phenol remained dull brown. If the tube containing the phenol saline were removed from the freezing bath and broken into two as soon as the colour had changed to red, it was found that the central portions of the frozen mass were still brown. Prolonged freezing caused the colour to change to red throughout the mixture.

This method has been employed to determine the effect of certain variables on the separation of the antiseptic in toxin solutions, antitoxin solutions and prophylactic mixtures when these are frozen. It may be pointed out as evidence of the reliability of the method, that no mixture containing "universal indicator" and either antiseptic which failed to give a red colour on freezing has ever been found to contain precipitated protein, and that no mixtures which showed the red colour on freezing have ever been found free from precipitated protein. The precipitation of protein is due to the separation of almost pure antiseptic; under the conditions which obtain when mixtures are frozen, the existence of a solution of these antiseptics in water of sufficient strength to cause precipitation of the protein is impossible. It is hoped to give a detailed account of the precipitation of serum protein by phenol and tricresol in a later paper.

The variable factors which must be taken into account when considering the changes in the concentration of the antiseptic which occur on freezing are (*a*) the temperature at which the mixtures are frozen, (*b*) the length of time the mixtures are allowed to remain frozen, (*c*) the protein content of the mixtures and (*d*) the salt content of the mixtures.

Variation in the freezing temperature.

When a dilute solution of sodium chloride is frozen, water separates in the form of ice crystals and it is obvious that the concentration of salt in the unfrozen portion must increase. The relation between the amount of ice that separates and the concentration of salt in the unfrozen portion is governed by the temperature. There is however a limiting temperature, *i.e.*, when the unfrozen liquid is saturated with salt. Any further reduction of temperature will now cause the separation of both ice and salt. This limiting point is reached at a temperature of −21·85° C.

In the case of a solution containing 0·5 per cent. phenol in addition

to the salt, two factors are tending to cause the separation of the phenol when the solution is frozen, (*a*) lowered solubility of phenol in water at decreasing temperatures, and (*b*) the "salting out" effect of the increasing concentrations of salt produced as the temperature is decreased. Phenol is almost insoluble in a saturated solution of sodium chloride at 0° C. Thus it is seen that the relative amount of phenol which separates depends on the temperature at which the solution is frozen.

Variation in the length of time the mixtures are frozen.

In experiment I, attention was drawn to the fact that if the tube containing 0·5 per cent. phenol saline were withdrawn from the freezing bath immediately the colour had changed to red and broken in two the central portions of the mass were still a dull brown colour. Prolonged freezing caused the colour to change to red throughout. This slow rate with which the central portions changed to red is due to the low thermal conductivity of ice, which acts as an insulating material and so prevents the central portions of the solid mass from attaining rapidly the lowest temperature of the freezing bath. This "time factor" explains the results of White and Robinson (1924) who found with a given toxin-antitoxin mixture "that the increase in toxicity ran parallel with the length of exposure to the freezing temperature within the limits studied."

Variation in the protein content.

In order to determine the effect of varying the protein content on the separation of phenol by freezing, the following experiments were carried out, using the method described in experiment I.

Experiment II.—Mixtures were made consisting of increasing amounts of serum protein in 0·5 per cent. phenol saline, to which were added 0·1 c.c. of "universal indicator" to every 10 c.c. of the mixtures. The tubes containing the mixtures were then placed in a salt and ice freezing mixture at −8° C. They were examined after 30 minutes when all mixtures containing 0·37 per cent. or less protein showed a red colour. In those mixtures containing more than 0·37 per cent. protein no red colour was produced, even after several hours' exposure to the temperature of −8° C. The intensity of the colour produced in those mixtures containing 0·37 per cent. or less protein was not constant; in the limiting concentration (0·37 per cent. protein) the colour produced was only a faint pink. After thawing the mixtures, the extent of protein precipitation was studied. It was found that in mixtures having protein contents up to 0·2 per cent. all the protein had been precipitated. At concentrations greater than this the fraction of the total protein precipitated decreased, the amount precipitated from the mixture containing 0·37 per cent. protein being almost negligible. No mixture having a protein content exceeding 0·37 per cent. showed any trace of precipitate.

From this experiment it is seen that, in a series of mixtures having a given salt content and varying protein concentrations, the amount of

pure phenol which separates when they are frozen at some definite temperature depends on the concentration of protein present in the mixture.

Experiment III.—The mixtures containing protein in a concentration greater than 0·37 per cent., which showed no precipitated protein after freezing at −8° C., were placed in a freezing bath of different composition, the temperature of which was −13° C. At this temperature all mixtures with a protein content of 0·4 per cent. to 1·5 per cent. showed a red colour and after thawing they contained precipitated protein. Here again the limiting concentration (1·5 per cent. protein) showed only a faint pink colour when frozen, and only a trace of precipitated protein on thawing.

By lowering the temperature it will be seen that an increased amount of pure phenol is caused to separate and as a result a correspondingly increased amount of protein is precipitated. The conditions are however rather more complicated than would appear at first sight.

Cooper (1912) studied the distribution of phenol between water and several proteins. He found the ratio $\frac{\text{G phenol in 1 G protein}}{\text{G phenol in 1 G water}} = 3$ provided that the protein was not precipitated by the phenol. From this it follows that the amount of phenol in solution in the water (as distinct from that in solution in the protein) must decrease as increasing amounts of protein are added to the 0·5 per cent. phenol solution.

For example we may take 100 c.c. of a solution containing 0·5 per cent. phenol and 1·0 grm. protein. Assuming the ratio given by Cooper to hold for serum protein,* the water will contain 0·485 grm. and the protein 0·015 grm. phenol. The concentration of phenol in the water is 3 per cent. less than would have been the case had no protein been present, *i.e.*, 0·485 per cent. instead of 0·5 per cent. If now 80 per cent. of the water is removed in the form of ice crystals, 20 c.c. of water will contain 0·434 grm. and the protein 0·066 grm. phenol. Here the water contains 13 per cent. less phenol than would have been the case had no protein been present, *i.e.*, 2·17 per cent. instead of 2·5 per cent.

The result of increasing the amount of protein in the mixtures is that the initial concentration of phenol present in the water is decreased and a lower freezing temperature is necessary to cause a concentration of phenol in excess of its solubility under the conditions of the experiment. As previously pointed out there is a limiting temperature where the unfrozen portion of the mixture consists of a saturated solution of salt, in which phenol is practically insoluble.

Variation in the salt content.

The results of varying the salt content can easily be shown by making the variation an extreme one. If a mixture containing about 0·1 per cent. protein in 0·5 per cent. phenol solution is saturated with

* The ratio was the same for such widely different proteins as gelatin and egg albumin.

sodium chloride the phenol is "salted out" and protein is irreversibly precipitated. In this case freezing is unnecessary, but with lower concentrations of sodium chloride the increase in concentration depends on the removal of water as ice and is therefore dependent on the temperature. Obviously if the concentration of salt in the mixture is varied, more or less water must be removed as ice to give a salt concentration of sufficient strength to "salt out" the phenol. Thus there is a close relation between the initial salt strength and the temperature which will cause a separation of pure phenol.

Discussion.

When we consider the variables involved it is easy to understand the discrepant results obtained by various workers on freezing toxin-antitoxin mixtures. Since antitoxin cannot be obtained as a pure substance, the protein content of toxin-antitoxin mixtures must obviously depend on the antitoxic value of the serum used. A low value antitoxic serum will give a toxin-antitoxin mixture with a high protein content, and *vice versa*. Anderson and Leonard (1924) pointed out that 3L+ toxin-antitoxin mixtures prepared with heat-concentrated antitoxic serum did not become toxic after freezing; while similar mixtures prepared with antitoxic serum which had been concentrated without heat became very toxic. Since heating the plasma in the concentration process leads to denaturation of some of the protein with an increase in the antitoxin/protein ratio, it follows that 3L+ mixtures prepared with heat-concentrated antitoxic serum may contain less protein than similar mixtures in which antitoxin concentrated without heat is used. Some idea of the variation in the protein content of toxin-antitoxin mixtures may be gathered from a consideration of 3L+ mixtures made from 200 unit per c.c. unconcentrated serum and 2000 unit per c.c. concentrated serum. Assuming a 7 per cent. protein content for unconcentrated serum and 15 per cent. for concentrated serum the mixtures will contain 0·1 and 0·02 per cent. protein respectively.

The work of Glenny Pope Waddington and Wallace (1925) showed that fairly sharply defined concentrations of phenol or tricresol caused a greater relative destruction of antitoxin than of toxin. Excess of phenol caused destruction of both toxin and antitoxin, while insufficient phenol failed to cause any increase in toxicity. It is clear therefore, that the amount of phenol which separates on freezing is the all-important factor, deciding whether or not the mixture will become toxic. The chief variables in practice are (*a*) the freezing temperature and (*b*) the protein content.

Schmidt and Scholz (1925) have stated that an old ripe toxin-antitoxin mixture will not increase in toxicity to a dangerous extent on freezing, but this is not in agreement with our experience. We have obtained a marked increase in toxicity in an over-neutralised

mixture prepared two and a half years previously. Such a statement as that made by Schmidt and Scholz shows the danger of accepting the result obtained by freezing at one temperature or at several widely differing temperatures.

There are probably strictly limited conditions under which a given toxin-antitoxin mixture, containing phenol or tricresol as antiseptic, may become very toxic after freezing, but the conditions will vary for mixtures differing in composition.

Although toxin-antitoxin mixtures have, in view of their importance, received chief consideration in this paper, it is obvious that in bacterial toxins and dilute solutions of antitoxic sera containing these antiseptics the separation of pure phenol or tricresol as a result of freezing may lead to destruction of the specific substances. In the case of undiluted antitoxic sera, the protein concentration is too high to allow any separation of antiseptic on freezing.

Alteration in the concentration of antitoxin on shaking.

At a meeting of the Pathological Section of the Royal Society of Medicine (see *Lancet*, 1922, i. 442) it was reported that toxin diluted to "Schick strength" in 0·5 per cent. phenol saline was very susceptible to shaking, under certain circumstances the toxin being almost completely destroyed. Details of the experiments were recorded later (Glenny Pope Waddington and Wallace, 1925) and it was shown that two factors were involved in the destruction of toxin, (*a*) the presence of phenol used as an antiseptic and (*b*) an air/liquid interface. Now phenol is known to lower the surface tension of water and it would therefore tend to concentrate in the surface. Hence it was possible that a concentration of phenol sufficiently high to cause destruction of the toxin might occur on shaking. That the phenol can so concentrate and to a surprising extent is shown by the following experiment.

Experiment IV.—To 200 c.c. of 0·5 per cent. phenol or 0·4 per cent. tricresol solution contained in a 500 c.c. stoppered cylinder were added 0·2 c.c. of 1·2 per cent. brom-cresol purple (Cooper Laboratories). The cylinder was shaken for a few moments and then allowed to stand. It was found that the liquid was purple and the froth an intense yellow. This phenomenon did not occur if the phenol or tricresol were omitted. In course of time the foam disappears leaving a yellow surface layer, which gradually becomes dispersed. The yellow colour can be seen in streaks if the cylinder is moved very slightly. Once the yellow colour has dispersed the experiment may be repeated with the same results.

Since the yellow colour was obtained only when phenol or tricresol was present, an attempt was made to find the concentration of phenol necessary to change the colour of the indicator from purple to yellow. It was found that solutions of phenol in water did not give a yellow colour; on the other hand a yellow colour was obtained on adding the indicator to solutions of water in phenol.

In another experiment, brom-cresol purple was added to pure phenol which was then slowly diluted with water from a burette, the mixture being well

stirred during the experiment. The point at which the colour changed from yellow to purple corresponded to a solution of phenol in water of between 7 per cent. and 8 per cent. At the temperature at which the experiment was carried out, water can dissolve 8 per cent. of phenol, and any phenol present in excess of this amount separates as a "phenol rich phase," *i.e.* a solution of water in phenol.

It is therefore clear that when a 0·5 per cent. solution of phenol in water is shaken in a partially filled bottle, the concentration of phenol which occurs at the air/liquid interface is such that the solubility of phenol in water is exceeded and a solution of water in phenol separates.

When diphtheria toxin of "Schick strength," containing 0·5 per cent. phenol as an antiseptic, is shaken in a partially filled bottle, no visible changes in the condition of the liquid are apparent. If however 0·05 grm. of purified horse pseudoglobulin is dissolved in 100 c.c. of 0·5 per cent. phenol solution, and the solution is shaken in a partially filled bottle, the protein is precipitated irreversibly. No protein is precipitated by shaking in the absence of the phenol. With the dilute toxin solution, the concentration of protein precipitable by phenol is extremely low; but with pseudoglobulin solution the high concentration of phenol produced in the air/liquid interface is capable of causing extreme protein precipitation.

Rideal and Wolf (1924) have shown that the addition of caprylic alcohol prevents the destruction of rennin on shaking Its action on the surface concentration of phenol was therefore investigated. It was found that the addition of three drops of caprylic alcohol to 100 c.c. of 0·5 per cent. phenol saline prevented any concentration of phenol detectable by the indicator method, and toxin diluted in 0·5 per cent. phenol saline containing this amount of caprylic alcohol was not destroyed by shaking. Here it is clear that the caprylic alcohol has occupied the surface and so prevented concentration of the phenol.

Conclusions.

1. A separation of phenol can be shown to occur when a 0·5 per cent. phenol solution is frozen.
2. The extent of the separation of phenol depends on the protein and salt content of the toxin or antitoxin solutions, the freezing temperature and the duration of freezing.
3. When these factors permit, a critical amount of phenol may separate from a toxin-antitoxin mixture, causing a greater relative destruction of antitoxin than of toxin and rendering the mixture toxic.
4. A separation of phenol can be shown to occur at the air/liquid interface when a 0·5 per cent. phenol solution in water or toxin is shaken.

5. This separation of phenol causes marked destruction of the specific substances when dilute solutions of toxin or antitoxin containing these antiseptics are shaken in a partially filled bottle. The use of phenol or tricresol in material for the "Schick test" or "Schultz-Charlton test" is therefore inadvisable.

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