XXV. FURTHER OBSERVATIONS ON THE INFLUENCE OF PHENOL AND OF CRESYLIC ACID ON THE CONCENTRATION OF ANTITOXIC SERA BY THE BANZHAF (1913) PROCESS.

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It has been already pointed out [Homer, 1917] that in the Banzhaf [1913] process for the concentration of antitoxic sera the difficulties encountered in filtration can be obviated by (1) an adjustment of the reaction of the sera, and (2) by the addition of phenol, cresylic acid or "trikresol" to the ammonium sulphate mixtures previous to their being heated.

The first of these methods of procedure requires more care and attention than can always be afforded in a laboratory where concentrations are carried out on a commercial scale. The second is much simpler and gives good results as may be seen from the details of the further experimental work which has been carried out in this connexion.

Phenol and its homologues not only increase the heat denaturation of the serum proteins but also so influence the precipitability of the particles of heat denaturated protein that they are retained with the First Fraction precipitate. This phenomenon, presumably brought about by virtue of the effect of the above mentioned substances on the surface tension of the protein particles in colloidal solution, leads to the production of a final product which is clear and which filters readily through filter candles.

Experience has shown us that, in order to obtain the desired results, the percentage of these substances added to the serum, must fall within certain limits. Should the amount not reach the lower limit then the end products are more unsatisfactory than if no phenol, etc., had been added. Should the upper limit be exceeded then some of the antitoxin is transferred to the First Fraction Precipitate from which it cannot be recovered by extraction with 30 % of saturation with ammonium sulphate; for this purpose prolonged extraction with brine is necessary.

Experimental concentrations illustrating this point, each with not less than one litre of plasma, were carried out as follows:

To oxalated plasma were added 2 % of solid sodium chloride and one-fifth of its volume of water¹. To the diluted plasma were then added varying amounts of phenol or of cresylic acid followed by the volume of a saturated solution of ammonium sulphate necessary to bring the ammonium sulphate content of the mixture to 30 % of saturation.

The phenol or cresylic acid-serum-ammonium sulphate mixtures were distributed in stoppered glass jars and placed in a bath containing water at a temperature of from 62 to 64°. The temperature of the serum mixtures gradually rose to 60°. As soon as this was reached the jars were taken from the bath and allowed to cool to 45° and filtered (First Fraction Precipitate).

The precipitates were washed with a solution containing ammonium sulphate to the extent of 30 % of saturation. The filtered washings were added to the main bulk of the filtrates which were then brought up to 50 % of saturation with ammonium sulphate. The ensuing precipitate (Second Fraction Precipitate) was filtered, pressed and dialysed in the usual way. To the residue from dialysis was added 1 % of sodium chloride and 0.35 % of cresylic acid.

A comparison was then made between the various end products thus obtained as regards their clearness and their tendency to become cloudy on standing. At the same time data were furnished as to the percentage removal of the total serum proteins and also as to the percentage of the total antitoxic units of the original serum appearing in the end product in those cases in which the higher percentages of phenol and cresylic acid had been used. The antitoxic content of the end products, where lower percentages of these substances had been used, were not estimated in the published series of experiments owing to our shortage of experimental animals. From other work we have satisfied ourselves that in the latter cases there is no appreciable loss of antitoxin during the process of concentration.

The results which have been embodied in the accompanying table (p. 281) indicate that:

¹ Banzhaf recommends that the plasma be diluted with one half its volume of water previous to the addition of the ammonium sulphate. However, in our routine work, we no longer consider the dilution of the plasma of any advantage, for it is our experience that the heat denaturation and precipitation of the serum proteins is a factor of the reaction and not of the dilution of the sera.

In my experimental work the dilution of the plasma with one-fifth its volume of water is a matter of personal convenience for the reading of the scale of the Zeiss Refractometer.

(1) Cresylic acid has a greater effect on the heat denaturation and precipitation of the heat denaturated proteins than has phenol. Moreover, the effect of the former takes place at a lower temperature than that of the latter.

The end products obtained from concentrations in which 0.30 % of cresylic acid had been added to the plasma were more clear and filtered better than when 0.50 % of phenol had been used.

(2) The addition of progressively increasing amounts of phenol or of cresylic acid to samples of the same sera previous to the heating of the serum mixtures was followed by a correspondingly increased percentage removal of the serum proteins of the original plasma.

(3) For the production of clear and readily filterable end products which would remain clear after filtration it was found necessary to add a minimum of 0.30 % of cresylic acid to the diluted plasma.

With a smaller amount of cresylic acid it was found that the end products were cloudy and far more unsatisfactory than if the Banzhaf process had been carried out without the addition of this substance.

(4) While the percentage of cresylic acid may be increased to 0.35 %, it is inadvisable to go beyond this limit, as the ensuing increased denaturation induces a transference of antitoxin to the First Fraction Precipitate from which it can only be recovered by long extraction with brine.

(5) The degree of concentration is considerably increased by the addition of 0.30 to 0.35 % of cresylic acid to the plasma previous to concentration.

Thus, whilst by the Banzhaf (1913) technique during the process of concentration, there is usually a 4 to 5 times increase in potency per cc., with the above modification the potency per cc. is increased 7 to 8.5 times¹.

In view of the above experimental data the concentration of routine batches of 50 and 100 litres of sera was conducted in the same way and similar results ensued. The protocol of the concentration of a 50 litre batch of antitetanic serum is given below.

T. R. 65.

Volume of oxalated plasma taken	...	...	...	...	...	...	...	50 litres
Volume of water added	...	...	...	...	...	...	...	10 "
Weight of NaCl added	...	...	...	...	...	...	...	1200 g.
Volume of cresylic acid shaken with 1 litre of water and added to the diluted plasma	...	...	...	...	...	...	...	180 cc.
Volume of a saturated solution of ammonium sulphate added	...	...	...	...	...	...	...	25,700 cc.

¹ The potency can be still further increased (viz. to the order of 10 times) by making the precipitation limit for the Second Fraction Precipitate 45 per cent. of saturation with ammonium sulphate instead of 50 per cent. as hitherto used [Homer, 1917].

Temperature to which the cresylic-plasma-ammonium sulphate mixture was just heated	60°
Percentage increased precipitation of the serum proteins induced during the heating of the mixtures to 60°	28

Original Plasma

Volume, 50 litres

Potency per cc., 200 (slightly +)¹

Protein content, 8.26 %

Final Product

Volume, 6.5 litres

Potency per cc., 1650

Protein content, 18.6 %

We thus see that, in the above concentration :

- (1) there has been a removal of 70 % of the total proteins of the sera taken for concentration,
- (2) the loss of antitoxic units during the process has been negligible,
- (3) the potency of the serum has been increased eight times.

The final product was clear and limpid. On dilution with saline it showed no trace of the presence of a colloidal suspension of euglobulin or of heat denaturated protein. Its filtration through filter candles proceeded with ease and the filtered product showed no signs of becoming cloudy on standing.

From these results it will be seen that, by the addition of 0.30 to 0.35 % of cresylic acid to the diluted plasma before concentration by the Banzhaf method, the degree of concentration and the percentage removal of the serum proteins thereby induced are of the same order as those obtained by the method recently published from these laboratories [Homer, 1916]. Such a simple modification of the technique of the former process entailing the production of a highly concentrated product will probably be welcomed by those laboratories in which my double heating process has not yet been adopted.

SUMMARY.

The difficulties encountered in the Banzhaf (1913) technique and the tendency of the end products to become cloudy can be obviated by the addition of 2 % of sodium chloride and of 0.30 to 0.35 % of cresylic acid to the plasma previous to the heating of the plasma-ammonium sulphate mixtures.

There will not be undue loss of antitoxin in presence of the specified amounts of cresylic acid provided that:

- (a) the addition of 2 % of sodium chloride be made a matter of routine,

¹ The experimental guinea-pig to which 100 m.l.d.'s of U.S.A. Standard Tetanus Toxin and 1/2000 cc. of the above plasma had been given died within 120 to 136 hours after the injection.

A comparison between the end products obtained from the concentration of antitoxic sera by the Banzhaf (1913) Process and by a modification of the same process in which to the plasma diluted with one-fifth its volume of water have been added varying amounts of phenol and of cresylic acid previous to the heating of the serum mixtures.

Experi- mental No.	Percentage of protein in the original plasma	Percentage of phenol or of cresylic acid added to the diluted plas- ma	Percentage increase in the precipitation of the soluble proteins during the raising of the temperature of the phenol or cresylic acid- serum-ammonium sulphate mixtures to		Percentage of the total pro- tein of the original plas- ma appearing in the final product	Percentage of the total anti- toxic units of the original plasma ap- pearing in the final product	Increased po- tency per cc. over that of the original plasma	Appearance of the final product standing in bulk in the cold room within (a) 24 hours, and (b) three months of being taken from the dialysing bags		The effect of dilution of the end product with twice its volume of saline	
			55°	60°				(a) after 24 hours	(b) after 3 months		
Antidiphtheritic serum											
49 a	7.81	0.00 phenol	2.1	5.2	64.5	—	—	slightly turbid	turbid	opalescent	
49	7.81	0.35 "	11.0	17.0	45.0	>84*	4.5	"	"	slightly opalescent	
50	7.81	0.50 "	15.5	20.8	40.0	>83*	5.5	very slightly turbid	slightly turbid	very slightly opalescent	
51	7.81	0.35 cresylic acid	24.6	26.0	30.0	82	7.5	clear	clear	clear	
Antitetanic serum											
75 a	6.49	0.00 cresylic acid	—	2.1	65.0	—	—	slightly turbid	turbid	opalescent	
75	6.49	0.15 "	—	15.0	45.0	—	—	"	"	"	
80	6.49	0.20 "	—	17.0	36.7	—	—	"	"	slightly opalescent	
81	6.49	0.25 "	—	22.5	35.0	88.0	6.5	"	slightly turbid	"	
82	6.49	0.30 "	—	25.0	28.1	88.0	8.5	clear	clear	clear	
Antidiphtheritic serum											
85	7.61	0.00 cresylic acid	—	3.0	63.1	—	—	clear	turbid	opalescent	
86	7.61	0.25 "	—	26.0	42.9	—	—	turbid	"	"	
87	7.61	0.30 "	—	30.0	37.1	—	—	clear	clear	clear	

* Not tested further on account of the shortage of experimental guinea pigs. For the same reason tests were not carried out on 49 a, 75 a, 75, 80, 85, 86, and 87 as we have (in other work) satisfied ourselves that with as much as 0.5 and 0.35 % of cresylic acid there is no undue loss of antitoxin provided that the temperature of the serum mixture be not raised beyond 60°.

(b) the mixtures be neither heated beyond 60° nor allowed to remain at this temperature for more than two or three minutes.

The addition of the cresylic acid has considerably enhanced the degree of concentration that can be obtained by the Banzhaf method. It has also increased the percentage removal of the serum proteins.

REFERENCES.

- Banzhaf (1913). *Collected Studies Research Dep. Health, New York*, 4, 230.
Homer (1916). *J. Hygiene*, 15, 388.
—— (1917). *J. Hygiene*, 15, 580.