V. THE REACTION OF SERA AS A FACTOR IN THE SUCCESSFUL CONCENTRATION OF ANTITOXIC SERA BY THE METHODS AT PRESENT IN USE.

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In a previous communication [1916, 1] I suggested that the practical difficulties so often encountered in the filtration of concentrated antitoxic sera might be due to the fact that hitherto no cognisance had been taken of the reaction of the sera before being concentrated.

With this object in view experiments have been carried out in which the effect of variations in the reactions of the sera have been considered with reference to:

(i) The precipitation of serum proteins by 30 per cent. of saturation with ammonium sulphate.

(ii) The increased precipitation of the serum proteins during the shortened heating process in the Banzhaf (1913) method. [Banzhaf, 1913.]

(iii) The increased precipitability of the serum proteins during the preliminary heating of the plasma as suggested in a previous paper. [Homer, 1916, 2.]

(iv) The practical application of the results obtained from the above investigations.

During the investigation of these points there has been accumulated a considerable amount of data which shows that the reaction of the serum is an important factor in the successful precipitation and heat denaturation of the serum proteins and that, unless the reaction be in some way controlled, apparently inexplicable irregularities are apt to occur in the course of the concentration.

I. THE EFFECT OF CHANGES IN THE REACTION OF SERUM ON THE PRECIPITATION OF THE SERUM PROTEINS BY 30 PER CENT. OF SATURATION WITH AMMONIUM SULPHATE.

Several series of experiments were conducted with oxalated plasma from different horses and from batches of pooled plasma or serum made up for concentration.

In each series, separate volumes of the test plasma were treated with known volumes of acid and of alkali. The plasma, thus treated, was brought up to 30 per cent. of saturation with ammonium sulphate by the addition of the necessary volume of a saturated solution of ammonium sulphate. The hydrogen ion concentration of the plasma and of the plasma-ammonium-sulphate mixtures was measured, wherever possible, by means of indicators and Sørensen's solutions in Walpole's colorimeter. The determinations, thus made, were subsequently checked by the electrical method as it was found that many of the indicators were rendered unreliable in the presence of so much protein as there is in serum. The protein contents of the filtrates from the serum mixtures were measured by means of the Zeiss immersion refractometer.

TABLE I.

Showing the influence of changes in the hydrogen ion concentration of the serum-ammonium-sulphate mixtures on the precipitation of serum proteins by 30 % of saturation with ammonium sulphate. (Protein content of the plasma = 7.92 %. Hydrogen ion concentration of the saturated solution of ammonium sulphate added to the plasma = $p_H^+ 6.480$.)

Volume of normal acid (or equivalent) added to 100 cc. of the diluted plasma	Volume of normal alkali (or equivalent) added to 100 cc. of the diluted plasma	p_H^+ of the diluted plasma to which the acid or alkali has been added, measured by the electrical method	p_H^+ of the filtrate from the serum-ammonium-sulphate mixtures, measured by the electrical method	Residual percentage of protein in solution in the filtrates from the serum-ammonium-sulphate mixtures, measured by means of the refractometer
—	19.6 cc. N. NH_3	—	8.754	5.75
—	13.1 "	—	8.551	5.80
—	6.55 "	9.945	8.455	5.90
—	3.28 "	9.789	8.022	6.00
—	1.96 "	9.304	7.745	6.00
—	0.98 "	8.500	7.400	6.30
0.00 cc. N. HAc	0.00 "	7.430	7.077	6.30
0.38 "	—	—	7.000	6.52
1.25 "	—	6.450	6.438	6.60
2.80 "	—	5.921	6.021	6.60
3.60 "	—	5.440	5.579	6.25
3.76 "	—	5.225	5.327	6.20
3.99 "	—	5.025	5.145	5.87
4.99 "	—	4.600	4.972	5.02
6.00 "	—	4.560	4.712	4.52

The data with respect to one of the series of experiments have been embodied in Table I; comparable results were obtained with all the samples of serum and plasma investigated.

A study of Table I shows that within certain limits (p_{H}^+ 5.3 to 7.4) the reaction of the mixtures has little effect on the extent of the precipitation of the serum proteins. Beyond these limits in both directions there is an increased precipitation of protein by the sulphate. It was also noticed that the consistency of the precipitated euglobulin was greatly influenced by the reaction of the mixtures. The agglutination of the precipitate into a granular and readily filterable form proceeded most satisfactorily at a hydrogen ion concentration of about p_{H}^+ 5.3 to 5.0, i.e. on the slightly acid side of the isoelectric point of euglobulin (p_{H}^+ 5.5).

The state of aggregation of the Second Fraction precipitate (50 % of saturation with ammonium sulphate) was also dependent on the reaction of the mixture: the conditions most favourable for this precipitation are in the course of investigation.

II. THE EFFECT OF CHANGES IN THE REACTION OF THE SERUM ON THE INCREASED PRECIPITATION OF THE SERUM PROTEINS INDUCED DURING THE SHORTENED HEATING PROCESS IN BANZHAF'S (1913) METHOD.

Several series of experiments were undertaken with samples of oxalated plasma from individual horses and from batches of pooled plasma from different horses.

In each series, to separate volumes of the test plasma contained in stoppered bottles, was added a known amount of acid or of alkali together with the volume of a saturated solution of ammonium sulphate necessary to bring the sulphate content to 30 % of saturation. The hydrogen ion concentration and the protein content of the clear filtrates from each of the experimental liquids were measured in the usual way. Experiments were carried out both with undiluted plasma and with plasma diluted with one-third its volume of water.

The mixtures were heated and then filtered. The protein content of the filtrates was estimated and compared with the corresponding value for the unheated liquids. From the data thus obtained was calculated the percentage increased precipitation of the soluble proteins due to the heating of the mixtures.

Comparable results have been obtained with each of the sera examined

irrespective of their protein content and of their dilution with water. The data with respect to one of the series of experiments have been embodied in Table II and represented graphically in the curve in Fig. 1.

TABLE II.

Showing the influence of changes in the reaction of the sera on the increased precipitation of protein during the heating of the serum-ammonium-sulphate mixtures to a temperature of 61° and keeping them at that temperature for 7 to 10 minutes. (Protein content of the plasma = 8.72 %. The saturated solution of ammonium sulphate added to the plasma had a hydrogen ion concentration p_H^+ 6.480.)

Volume of normal acid (or equivalent) added to 100 cc. of plasma diluted with one-third its volume of water	Volume of normal acid (or equivalent) added to 100 cc. of the diluted plasma	p_H^+ of the serum-ammonium-sulphate mixtures determined by the electrical method	Percentage increase in the precipitation of the soluble proteins induced during the heating of the serum-ammonium-sulphate mixtures	Remarks on the filtration of the heated serum-ammonium-sulphate mixtures
—	19.64 cc. N. NH_3	8.754	17.7	Readily, filtrate clear
—	13.04 "	8.577	16.4	" " "
—	6.52 "	8.455	13.4	" " "
—	3.28 "	8.022	10.0	" " "
—	1.96 "	7.745	7.0	" " very slightly opalescent
—	0.98 "	7.489	9.0	" " " "
—	0.49 "	7.312	7.0	Less readily, filtrate slightly opalescent
0.00 cc. N. HAc	0.00 "	7.077	6.3	" " " "
0.98 "	—	7.000	6.8	Slowly, filtrate opalescent
1.96 "	—	6.428	3.0	" " "
2.94 "	—	6.021	2.8	" " "
3.60 "	—	5.579	0.5	" " "
3.76 "	—	5.327	18.0	Less slowly, filtrate slightly opalescent
3.92 "	—	5.145	20.7	Readily, filtrate clear
4.58 "	—	5.001	39.0	" " "
4.88 "	—	4.972	52.6	" " "
5.88 "	—	4.712	72.2	" " "

It was noticed that where the hydrogen ion concentration of the mixtures was between the values p_H^+ 5.3 and 7.0, the filtration of the heated liquids was slow and the filtrates were markedly opalescent. Throughout these ranges the conversion of soluble into insoluble protein was least. In those cases in which the reaction had been more acid or more alkaline than in the above the filtration of the mixtures was rapid and the filtrates were clear, while the conversion of soluble into insoluble protein was of an appreciable order. On the alkaline side between the ranges p_H^+ 7.0 and 8.7 the conversion gradually rose from 5 to 17 %. On the acid side, as is seen at a glance in the

curve in Fig. 1, the rate of increase was much more marked; it rose from 0.5 to 72 % between the ranges p_{H}^+ 5.5 and 4.7.

No trouble was experienced with the filtration of the final products obtained in the concentrations of sera in which the reaction of the serum mixtures previous to heating had been adjusted to p_{H}^+ 5.2 or p_{H}^+ 5.0. As was however to be anticipated from the character of the filtration of the hot serum mixtures, difficulties were encountered with the end products where the reaction of the serum mixtures had been between the limits p_{H}^+ 5.3 and 7.0. In these cases the unsatisfactory filtration of the serum mixtures may be ascribed to one of two possible causes; either that between these limits the

Fig. 1. Curve representing the influence of the reaction of the serum mixtures on the percentage increased precipitation of soluble proteins during the heating of serum-ammonium-sulphate mixtures by Banzhaf's method.

hydrogen ion concentration is unfavourable for the complete agglutination of the precipitated proteins: or that, since near the isoelectric point the heat denaturation of the serum proteins is least, it is possible that in this region the proteins which, under more acid or more alkaline conditions, would be appreciably denaturated are in a transitory, semi-emulsoid, semi-rigid stage and as such impart a slimy consistency not only to the serum mixtures but also to the final products.

It was also found that, with serum mixtures more alkaline than $p_{\text{H}}^+ 8.0$, there was difficulty with the filtration of the final product even though the filtrates from the heated serum mixtures were clear. In these cases, when these clear alkaline filtrates were acidified to a point $p_{\text{H}}^+ 5.5$ an opalescence was produced. The presence of a certain amount of precipitated protein which had been kept in the disperse phase by the alkali was thus indicated. The colloidal aggregates thus dispersed escaped precipitation with the First Fraction precipitate but were subsequently precipitated with the Second Fraction precipitate. They impart to the residue from the dialysis of the latter a slimy consistency and thereby considerably impede filtration through filter candles.

III. THE EFFECT OF CHANGES IN THE REACTION OF SERA ON THE INCREASED PRECIPITABILITY OF THE SERUM PROTEINS INDUCED DURING THE HEATING OF SERUM TO A TEMPERATURE OF 57° FOR 6 HOURS.

During the course of our practical experience with the method suggested by me [Homer, 1916, 2] for the concentration of antitoxic sera it was observed that the heat denaturation of the serum proteins induced during the preliminary heating of the sera varied from 13 to 45 %. Throughout the winter and spring months the values with plasma varied from 28 to 35 % while with carbolised sera values as high as 45 % were reached. During the summer months when the horses were being given a considerable amount of green grass, the denaturation varied from 13 to 20 %.

After a considerable amount of experimental work it was ascertained that these variations were due to differences in the hydrogen ion concentration of the different plasmas and sera. During the summer months, when the low values for the heat denaturation were obtained, the oxalated plasma was slightly less alkaline than that obtained during the winter and spring months.

Samples of plasma from individual horses and from batches of the pooled plasmas from different horses were respectively diluted with one-third their volume of water. The hydrogen ion concentration of the diluted plasma was measured in each case. The reaction of the various samples was adjusted to the value of about $p_{\text{H}}^+ 5.6$ to 5.9 and also to the value $p_{\text{H}}^+ 8.5$. The original samples and the adjusted samples were heated in stoppered bottles to a temperature of 57° for 6 hours. The percentage increased precipitability of the serum proteins by 30 % of ammonium sulphate was measured with the aid of the Zeiss refractometer.

The data with respect to these observations have been embodied in Table III. They show that the variations in the extent of the heat denaturation of the serum proteins of individual sera can be obviated by adjusting the reaction of the sera to some fixed value. Thus, when the reaction was adjusted to about p_H^+ 5.6 to 5.9 the denaturation was of the order of 10 %, while at the value p_H^+ 8.5 it was of the order of 40 %¹.

TABLE III.

Showing that the variation in the heat denaturation induced during the heating of different samples of plasma at 57° for 6 hours can be overcome by an adjustment of the reaction of the sera to a fixed degree of acidity or of alkalinity.

Sample No.	Percent- age pro- tein con- tent of the plasma	Value for p_H^+ of the diluted plasma	Percentage increased precipitability of the serum proteins during the heating of		
			undiluted plasma	diluted plasma brought, by the addition of ammonia, to the hy- drogen ion concentra- tion p_H^+ 8.5*	diluted plasma brought, by the addition of acetic acid, to the hydrogen ion concentra- tion p_H^+ 5.9*
1	8.59	7.648	31.1	—	—
2	8.12	7.503	28.9	44.0	13.0
3	7.80	7.460	26.4	41.0	9.0
4	8.59	7.563	28.4	40.0	12.0
5	6.56	7.430	22.3	41.0	13.0
6	7.57	7.346	23.5	40.5	—
7	7.92	7.346	22.0	—	8.7
8	6.55	7.863	39.0	43.0	13.0
9	6.62	7.346	21.0	41.0	9.0
10	6.60	7.346	23.0	42.0	—
11	8.40	6.813	13.5	40.5	—
12	10.03	6.990	13.5	40.5	—
13	7.15	7.000	17.0	40.0	—

[Similar results were obtained with undiluted plasma.]

* Measured by means of indicators and Walpole's colorimeter.

In order to elaborate the point still further experiments were instituted in which samples of different sera, previous to their being heated, were treated with acid and with alkali to ensure a gradation in the reaction of the sera from p_H^+ 4.2 to 9.9.

Comparable results were obtained with each series of experiments. The typical results from one of these series have been embodied in Table IV and

¹ These adjustments were made with the aid of indicators and Sørensen's solutions in Walpole's colorimeter. A certain amount of difficulty was experienced by me in the matching of colours in serum, especially so, with the indicators available, on the acid side. I subsequently ascertained that, owing to my defective matching, the real values of the hydrogen ion concentration varied from the values aimed at by amounts sufficient to account for the slight variations in the heat denaturation recorded in the Table.

TABLE IV.

Showing the influence of the hydrogen ion concentration of sera on the extent of the heat denaturation of the serum proteins induced during the prolonged heating of serum at a temperature of 57° for 6 hours.

Volume of normal acid (or equivalent) added to 100 cc. of the plasma diluted with one-third its volume of water	Volume of normal alkali (or equivalent) added to 100 cc. of the plasma diluted with one-third its volume of water	p_H^+ of the plasma determined by the electrical method	Appearance of the heated plasma	Percentage conversion of soluble protein during the heating of the diluted plasma	Percentage increase in the precipitability of the soluble proteins by 30% of saturation with ammonium sulphate	Percentage destruction of antitoxin during the heating process
				A	B	
—	6.55 cc. N. NH_3	9.945	clear	2.2	55.7	nil
—	4.92 "	9.910	"	2.0	53.5	
—	3.275 "	9.789	"	1.5	46.6	
—	2.62 "	9.477	"	1.5	45.5	
—	1.96 "	9.304	"	2.2	46.5	nil
—	1.31 "	8.870	"	3.7	41.5	
—	0.65 "	8.249	clear liquid and slight deposit	2.2	36.8	
0.00 cc. N. HAc	0.00 "	7.430	" " " "	3.3	22.3	nil
0.63 "	—	6.990	opalescent liquid and filtrate	5.2	10.2	
1.25 "	—	6.450	" " " "	5.2	12.0	nil
1.87 "	—	5.921	" " " "	3.7	13.1	
2.49 "	—	5.440	thin suspension, clear filtrate	6.3	13.1	
3.74 "	—	5.225	" " " "	20.8	25.7	nil
2.50 cc. N. H_2SO_4	—	5.160	" " " "	26.0	35.8	nil
4.17 cc. N. HAc	—	5.025	thick " " "	30.0	45.0	
2.52 cc. N. HCl	—	5.000	" " " "	34.0	44.2	
4.99 cc. N. HAc	—	4.600	" " " "	54.0	70.0	nil
3.60 cc. N. HCl	—	4.635	" " " "	54.0	74.0	
3.60 cc. N. H_2SO_4	—	4.643	" " " "	50.0	74.0	
6.74 cc. N. HAc	—	4.560	" " " "	68.0	77.5	nil
4.40 cc. N. H_2SO_4	—	4.262	almost solid	98.0	not fluid enough to be measured	
4.36 cc. N. HCl	—	4.251	" "	92.0		

With the sera ranging from p_H^+ 9.9 to 5.4 the extent of the changes recorded in B was a function of the time of heating, whereas with the more acid sera the change was practically completed within one hour's heating at the specified temperature.

have also been represented graphically in the curves in Fig. 2. Of the two, Curve 1 illustrates the changes in the solubility of the serum proteins during the heating of the serum, while Curve 2 indicates the accompanying changes in the precipitability of the serum proteins by 30 % of saturation with ammonium sulphate.

It was found that, with each of the sera examined, the extent of the changes involved was a function of the hydrogen ion concentration of the serum irrespective of the protein content: the use of undiluted serum or plasma gave rise to results similar to those obtained from experiments with plasma diluted with one-third its volume of water.

The appearance of the heated sera was characteristic throughout the ranges of acidity and alkalinity investigated.

It was found that the heated sera were clear when the reaction of the unheated serum had been more alkaline than $p_H^+ 8.3$. When the reaction had been between the limits $p_H^+ 8.2$ and 5.5 the heated sera were characterised by an opalescent appearance or by the presence of a slight deposit of protein. On the acid side of $p_H^+ 5.5$ the sera were converted, during the heating, into thin suspensions, the semi-solid consistency of which became more marked as the degree of acidity increased, until at $p_H^+ 4.2$ the serum became practically solid.

Fig. 2. The curve — — — represents the influence of changes in the reaction of sera on the percentage conversion of soluble into insoluble proteins induced during the heating of sera at 57° for 6 hours.

The curve ——— represents the influence of changes in the reaction of sera on the percentage increased precipitability of the serum proteins by 30 % of saturation with ammonium sulphate induced during the heating of sera at 57° for 6 hours.

From a study of the data given in Table IV, Column A, it will be seen that the conversion of soluble into insoluble protein was of the order of 5 % or less in the sera of which the reaction lay between the ranges $p_H^+ 9.9$ and 5.5 . On the acid side of $p_H^+ 5.5$ there was marked precipitation of the soluble proteins; thus at a hydrogen ion concentration of $p_H^+ 5.4$ it was of the order of 6 %, at $p_H^+ 5.0$ it was 30 %, while at $p_H^+ 4.2$ it was over 90 %.

From a consideration of Column B in the same table it will be seen that, irrespective of any accompanying conversion of soluble into insoluble protein, there was throughout the whole range investigated a marked increase in the precipitability of the serum proteins by 30 % of saturation with ammonium sulphate: the extent of the increase was a function of the reaction of the serum previous to its being heated.

This increased precipitability was least between the ranges $p_{\text{H}}^+ 5.4$ and 7.0. On either side of these limits the rate of increase became apparent: the gradient for the increase was more gradual on the alkaline than on the acid side (Curve 2, Fig. 2). As the degree of alkalinity decreased from $p_{\text{H}}^+ 9.9$ to 8.0 the increased precipitability gradually fell from 45 to 30 %. Between the ranges $p_{\text{H}}^+ 7.0$ and 5.5 it was of the order of 10 to 15 %; on the acid side it rapidly rose from 25 % at $p_{\text{H}}^+ 5.2$ to 90 % at $p_{\text{H}}^+ 4.2$.

It was also interesting to note that, in all cases, there was no appreciable loss of antitoxin during the heating of sera the reaction of which had been adjusted to degrees of acidity or alkalinity within the limits dealt with in the accompanying tables, although under some conditions the extraction of the antitoxin from the heat-denatured proteins proved a matter of difficulty.

From a study of the curves in Fig. 2 it is apparent that while on the alkaline side the configuration of Curve 1 is quite distinct from that of Curve 2, on the acid side of $p_{\text{H}}^+ 5.5$ there is practical coincidence. There is, moreover, a marked similarity between the configuration of Curve 2 and that of the curve in Fig. 1. The extent of the increased precipitability on the alkaline side is not so marked in the latter as in the former, but on the acid side the curves, if superimposed, would be almost identical.

The question then arose as to whether the individuality of Curves 1 and 2 (Fig. 2) on the alkaline side indicated some radical difference between the changes respectively involved during the heating of alkaline and acid sera.

In order to elucidate this point samples of the clear heated alkaline sera which showed a 40 % increased precipitability by the sulphate were brought to an acidity of $p_{\text{H}}^+ 5.0$. Now, had the changes involved during the heating of the alkaline sera been the same as those induced in the acid sera which showed a corresponding percentage increased precipitability, acidification of the clear alkaline sera should have changed the consistency of the latter to that of a thin suspension (*vide ante*, p. 29). A slight opalescence only was formed and the refractometer readings demonstrated that the quantity of protein thus precipitated was inappreciable. The opalescence therefore

probably indicated that a small amount of heat changed protein had been kept in the disperse phase by the excess of alkali (*vide ante*, p. 26).

It was also noticed that the increased precipitability of the proteins of the heated alkaline sera was a function of the duration of the heating process. On the other hand the changes recorded in the acid sera were practically complete after one hour's heating at the specified temperature.

From these observations it is evident that the changes taking place during the heating of alkaline sera are of a different nature from those which occur during the heating on the acid side of the isoelectric point of the globulins. For this reason it is possible that if the heat denaturation be conducted on the acid side, there may be prepared concentrated products which will be less likely to give rise to the troubles of anaphylaxis and of serum sickness than the present products concentrated by methods involving a denaturation of the serum proteins by heating the slightly alkaline sera.

PRACTICAL APPLICATION OF THE RESULTS OBTAINED IN SECTIONS I, II AND III ABOVE.

The results of the investigation detailed above have led us to a better understanding of the causes underlying the irregularities encountered in the concentration of sera by methods involving the heating of the serum previous to the fractional precipitation of the serum proteins by ammonium sulphate.

We are finding that the production of clear and readily filterable end products can only be assured by controlling the reaction of the serum and of the serum mixtures unless certain expedients described below be resorted to.

An account of the investigation undertaken to overcome the causes of irregularities in the results obtained in (A) the Banzhaf (1913) method and later in (B) the method suggested by me [1916, 2] may be of service to those engaged in the concentration of antitoxic sera.

(A) *The regulation of the factors causing irregular results in the concentration of sera by the Banzhaf (1913) method.*

It seems probable that the difficulties in filtration so often encountered in this method are due to the presence of incompletely precipitated euglobulin and also of the small amount of protein denaturated during the heating of the serum.

As an outcome of the work recorded above it has been concluded that,

with the published technique, there can be no guarantee for the complete elimination of these disturbing factors, and this for two reasons:

- (1) That no cognisance has been taken of the reaction of the serum-ammonium-sulphate mixtures previous to their being heated.
- (2) That there is no increased precipitation of the serum proteins during the heating of the serum mixtures.

It is generally recognised that rapid and clear filtration of the hot serum mixtures is one of the essential conditions for the production of readily filterable end products. In this laboratory it has been shown that, for the successful filtration of the hot serum mixtures, there must be assured the agglutination of the particles of precipitated euglobulin into aggregates sufficiently large for their retention on the surface of filter paper. From Tables I and II it is obvious that the success of this operation can only be guaranteed if the reaction of the mixtures be suitably adjusted.

It has been demonstrated that during the heating process advocated in this method the precipitating power of 30 % of saturation with ammonium sulphate is not appreciably enhanced. It is evident, therefore, that the small amount of euglobulin which must always escape precipitation with the First Fraction precipitate will appear in colloidal suspension in the final product. Unless the latter be allowed to stand for a considerable time until this colloidal suspension has settled as a deposit the unprecipitated protein proves a hindrance to filtration and is the cause of the marked clouding of so much of the antitoxic serum concentrated by this method.

The irregularities in the filtration of the serum mixtures can be obviated by an adjustment of the hydrogen ion concentration to the optimum required for the complete agglutination of the particles of "salted out" euglobulin and of the particles of protein denaturated during the heating of the serum. Good results can be obtained by adjusting the reaction to an acidity of about $p_{\text{H}}^+ 5.0$. As however the determination of the hydrogen ion concentration of the sera by the electrical method is not always practicable in a routine laboratory, an endeavour is being made to work out a simple practical method by which the reaction can be adjusted by the use of suitable indicators.

In the meantime, pending the search for suitable and reliable indicators, it has been shown that the irregularities in the filtration of the serum mixtures can also be obviated by the simple expedient of adding 1.5 to 2 % of sodium chloride to the mixtures previous to their being heated.

It is important to know whether the salt improves the filtration of the hot serum mixtures by virtue of a specific action on the particles of precipi-

tated protein or by an adjustment of the reaction of the medium more nearly to the desired optimum.

In order to elucidate this point duplicate samples of several plasmas were taken. To one of each of the duplicate pairs was added 1.5 to 2 % of sodium chloride. The hydrogen ion concentration of the duplicates was measured. The liquids were then made 30 % of saturation with ammonium sulphate and gradually heated to 61°.

From the results given in Table V it will be seen that the addition of sodium chloride decreases the alkalinity of the serum and of the serum

TABLE V.

Showing the influence of the addition of sodium chloride to the serum on the hydrogen ion concentration of the serum and of the serum-ammonium-sulphate mixtures.

p_H^+ of the plasma diluted with one-third its volume of water = A	p_H^+ of A made 30 % of saturation with ammonium sulphate	Percent- age of NaCl added to the diluted plasma	p_H^+ of the salted plasma = B	p_H^+ of B made 30 % of saturation with ammonium sulphate	Remarks on the filtrate from the hot serum-ammonium-sulphate mixtures from	
					A	B
7.485	—	1.5	7.216	—	opalescent	clear
7.408	—	2	7.200	—	"	"
7.701	—	1.5	7.485	—	slightly opalescent	"
7.701	—	4	7.485	—	"	"
7.701	—	8	7.485	—	"	"
7.347	—	2	7.223	—	opalescent	"
—	7.250	1.5	—	7.010	"	"
—	7.220	2	—	7.000	"	"

mixtures. From a comparison with the data given in Table II, it is however obvious that the change in the hydrogen ion concentration induced by the addition of the salt is insufficient to account for the marked improvement in filtration and for the absence of an opalescent suspension in the filtrates.

It seems justifiable, therefore, to conclude that the improvement in filtration is induced by a specific action of the sodium chloride on the particles of precipitated protein whereby their satisfactory agglutination is assured.

Although the salt greatly improves the filtration of the serum mixtures, it cannot completely obviate the difficulties with the final product for it is powerless to ensure the complete precipitation of the euglobulin by 30 % of saturation with ammonium sulphate. Even in the presence of salt the desired increased precipitation of the serum proteins during the heating process does not take place.

In order to guarantee the elimination of euglobulin from the final products there were three courses open, viz.

(i) *The old method of extraction with brine.*

The Second Fraction precipitate containing the pseudo-globulin and antitoxin together with the small amount of attendant euglobulin and heat-denaturated protein was thrown into brine. The pseudo-globulin-antitoxin combination passed into solution while the euglobulin remained undissolved and was separated by filtration. The pseudo-globulin and antitoxin were precipitated by the addition of 0.25 % of glacial acetic acid to the clear filtrate. The precipitate was pressed and dialysed: the end product was crystal clear and its filtration presented no difficulty as it contained no trace of euglobulin or of heat-denaturated protein.

From the practical standpoint this method is not favoured as it involves a considerable amount of extra time and labour.

(ii) *A combination of the prolonged heating process of the Gibson-Banzhaf method with the shortened heating process of the Banzhaf (1913) method.*

By the adoption of this course it has been possible to obtain end products which, while showing an increased potency of from 8 to 10 times that of the original serum, do not exhibit the same tendency to form the cloudy deposit so often seen in the sera concentrated by Banzhaf's method. However, recent observations on the factors limiting the extent of the concentration of sera by the fractional precipitation methods at present in use [Homer, 1917], have demonstrated that there is a limit to which the concentration of sera can be carried. It was found that in all cases where the load of antitoxin attached to one gram of protein in the final product threatened to be greater than of the order of 25,000 units, the excess of units beyond this limit were transferred to the First Fraction precipitate.

From these observations it is evident that, for high grade sera (over 600 units per cc.), the above modification, yielding under ordinary circumstances a concentration of 8 to 10 times, cannot be used without apparent loss of antitoxin. The missing antitoxin can be recovered by extraction of the First Fraction precipitate with saturated salt solution.

To provide for such cases a third method of procedure has been employed, viz.

(iii) *The addition of substances such as phenol and its homologues to the serum mixtures previous to their being heated.*

Some years ago MacConkey [1914] observed that, during the heating of carbolised and of etherised sera there was a considerable loss of antitoxin. He subsequently found that the loss was negligible if 1.5 to 2 % of salt had previously been added to the serum.

In this laboratory it has also been demonstrated that, in the presence of salt, there is usually no appreciable loss of antitoxin during the concentration of carbolised sera by Banzhaf's method, if the temperature of the serum mixtures be not raised beyond 61°. At temperatures of 63° and 65° the loss becomes appreciable.

In the routine work, however, it has sometimes happened that, during the concentration of carbolised sera, there were appreciable losses of antitoxin even in the presence of salt. The records showed that in some of these cases the serum had been heated to a temperature of 65°, a procedure which in itself was sufficient to account for the loss. In other cases the records showed that during the concentration there had been removed more than 80 % of the total proteins of the original serum. With such a high removal of the serum proteins a certain proportion of the antitoxic units must be carried down with the First Fraction precipitate instead of with the Second Fraction precipitate. The antitoxin thus brought down with the First Fraction precipitate can only be extracted therefrom with great difficulty; hence the apparent and inexplicable loss of antitoxin under the conditions of the concentration.

It having been previously noticed that the heat-denaturation induced in the prolonged heating of sera to 57° is considerably increased by the addition of phenol, trikresol and cresylic acid to the serum, an experiment was made to ascertain whether the same phenomenon was experienced in the shortened heating process of Banzhaf's method. As this was found to be the case the investigation was carried further to ascertain whether the increased precipitation was due to changes in the hydrogen ion concentration caused by the addition of these substances.

In Table VI have been embodied data to illustrate the influence of phenol and cresylic acid on the precipitation of the serum proteins during the heating of the serum-ammonium-sulphate mixtures by the Banzhaf (1913) method. Similar results have been obtained by other workers in this laboratory from the use of trikresol, ether and chloroform.

From a study of the table it will be seen that while the addition of the above-mentioned substances had no appreciable effect on the hydrogen ion concentration of the sera, there was a marked increase in the precipitation

of protein during the heating and at the same time a marked improvement in the filtration of the hot serum mixtures. The end products were characterised by their clearness and their ease of filtration; they contained no euglobulin.

TABLE VI.

Showing the increased precipitation of serum proteins induced by the addition of phenol and its homologues to the serum-ammonium-sulphate mixtures previous to their being subjected to the shortened heating process of the Banzhaf (1913) Method.

Percentage of phenol or its homologues added to the plasma diluted with one-third its volume of water	p_H^+ of the carbollised plasma measured by the electrical method	p_H^+ of the carbollised plasma made 30% of saturation with ammonium sulphate	Percentage conversion of soluble into insoluble protein during the heating process	Remarks on the filtration of the hot serum-ammonium-sulphate mixtures	Loss of antitoxin during the heating process
I. Plasma containing 8.74% of protein.					
0.0 phenol	7.748	7.347*	6.3	slow, filtrate opalescent	nil
0.1 "	7.730	7.035†	16.5	more rapid, filtrate slightly opalescent	"
0.2 "	7.730	7.034	23.0	rapid, filtrate clear	"
0.3 "	7.730	7.085	24.0	" " "	slight
0.4 "	7.730	7.085	26.0	" " "	nil
0.5 "	7.730	7.061	33.0	" " "	slight
II. Plasma containing 7.92% of protein.					
0.0 phenol	7.460	—	2.0‡	slow, filtrate opalescent	
0.1 "	7.347	—	17.0	rapid, filtrate slightly opalescent	
0.2 "	7.347	—	24.4	rapid, filtrate clear	
0.4 "	7.347	—	27.9	" " "	
0.5 "	7.347	—	30.8	" " "	
III. Plasma containing 10.03% of protein.					
0.0 cresylic acid	7.000	—	1.0	slow, filtrate opalescent	nil
0.35 " "	7.000	—	27.0	rapid, filtrate clear	"
IV. Plasma containing 7.88% of protein.					
0.0 cresylic acid	7.648	—	5.0	slow, filtrate opalescent	
0.30 " "	7.648	—	26.0	rapid, filtrate clear	

* p_H^+ of the saturated solution of ammonium sulphate added to the plasma = 6.480.

† " " " " " " " = 6.010.

‡ " " " " " " " = 5.900.

As the addition of phenol, etc., to the serum mixtures previous to their being heated does not cause any alteration in the hydrogen ion concentration it is obvious that the improvement in filtration is due to the specific action of these substances on the proteins themselves, although the mode of action is different from that of sodium chloride. The latter merely serves to stimulate the aggregation of the particles of already precipitated protein, the former induce a considerable increase in the precipitation of the serum

proteins by 30 % of saturation with ammonium sulphate, thereby assuring the complete precipitation of euglobulin at this stage. It is quite possible that the increased heat-denaturation of the serum proteins is brought about by an effect of these substances on the surface tensions of the colloidal particles of dissolved protein whereby their precipitability is considerably increased. Whatever may be the fundamental cause of this change the fact remains that the effect is so marked that, not only is the precipitating power of 30 % of saturation with ammonium sulphate considerably increased, but, in the cases investigated, the agglutination of the aggregates of the precipitated protein is so well defined that the reaction of the serum mixtures is of secondary importance. Moreover, the proteins involved do not seem to enter the intermediate ill-defined stage between the emulsoid and the suspensoid state which proves so great a hindrance to the filtration of the serum mixtures and of the final products.

From the data embodied in Table VI we see that, by the addition of phenol or its homologues, the heat-denaturation of the serum proteins can be regulated to the extent suitable for a particular serum.

For those who prefer to use the Banzhaf method this expedient is recommended until a simple routine method for the adjustment of the reaction of the serum mixtures previous to their being heated has been worked out. The judicious addition of 0.35 to 0.5 % of phenol or of 0.25 to 0.35 % of its homologues to the batches of plasma for concentration will result in the production of clear and readily filterable end products. If the addition of salt is also made a matter of routine, the concentration of the carbolised plasma or sera will not involve undue loss of antitoxin, although a considerable loss may result if the substances in question are added in amounts greater than those suggested.

(B) *The regulation of the factors causing irregular results in the concentration of sera by the more recent methods.* [Homer, 1916, 2.]

It has been found that there was slight trouble with the filtration of the end products from the concentration of sera in which the heat-denaturation had been less than 20 %, that is to say from the concentration of sera the reaction of which lay between the limits p_{H}^+ 5.4 and 7.0. In these cases the end products contained a small amount of heat-denaturated protein in colloidal suspension.

The occurrence of this difficulty could be prevented by a preliminary adjustment of the serum, previous to its being heated, to a hydrogen ion

concentration of about p_{H}^+ 7.8 to 8.0. It was inadvisable to increase the alkalinity further for the reasons given (*vide ante*, pp. 30, 31).

Trouble with the final filtration of end products from the concentration of sera of which the reaction fell within the limits p_{H}^+ 5.4 to 7.0 could also be obviated by the judicious addition of from 0.25 to 0.35 % of phenol or its homologues to the serum previous to the preliminary heating.

It is inadvisable to add the latter substances to more alkaline or to more acid sera than those falling between the aforesaid limits as there is the danger of thereby inducing so great an increased precipitability of the serum proteins that transference of antitoxin to the First Fraction precipitate will occur. Under these conditions there will be an apparent loss of antitoxin during the concentration as the extraction of the antitoxin thus carried down with the First Fraction precipitate is a matter of great difficulty.

It has also been found advisable to adjust the reaction of the serum-ammonium-sulphate mixtures previous to the second stage of the heating process (*ante*, p. 32). It may also be stated that at this stage of the heating the temperature of the serum mixtures is not raised beyond 59°.

From the consideration of the work detailed above it is evident that the reaction of the sera must be taken into account by those engaged in the concentration of antitoxic sera. On the reaction of the serum depends, not only the extent of the heat-denaturation of the serum proteins, but also the successful precipitation of the serum proteins by ammonium sulphate. Unless the reaction be regulated the necessary increased precipitation of the proteins cannot be ensured except by the addition of phenol, etc., to the serum. The latter course, in the hands of inexperienced workers may prove somewhat costly, as these substances favour the destruction of antitoxin.

Although the regulation of the reaction of the serum is proving of material value the possibility must not be lost sight of it being shown at some future date, that, in the concentration of antitoxic sera, there are other, hitherto, unsuspected factors which also influence the operations involved. In any case it is clear that the standardisation and regulation of a concentration process can only be attained by a thorough knowledge of the factors involved.

SUMMARY.

During the course of the investigation it has been demonstrated that:

(1) In the Banzhaf (1913) method the uncertainties in filtration are due to two causes:

(a) That no cognisance has been taken of the reaction of the serum.

(b) That as the precipitating power of 30 % of ammonium sulphate is not appreciably increased during the heating process, a certain amount of euglobulin escapes precipitation with the First Fraction precipitate and appears in colloidal suspension in the final product.

(2) The uncertainties in the filtration of the hot serum-ammonium-sulphate mixtures in the above method can be obviated by an adjustment of the hydrogen ion concentration. The filtration can also be improved by the addition of sodium chloride to the mixtures, but, in this case, the improvement is due to a specific action of salt on the globulins.

(3) The complete elimination of euglobulin can be assured by:

(a) An adjustment of the hydrogen ion concentration of the serum mixtures to the point at which the desired increased precipitation is assured.

(b) Brine extraction of the Second Fraction precipitate containing the pseudo-globulin-antitoxin combination together with the small amount of euglobulin which has escaped precipitation with the First Fraction precipitate.

(c) Subjecting the serum to a preliminary prolonged heating at a temperature of 57—58° whereby the precipitability of the serum proteins is considerably enhanced.

(d) The addition of organic substances such as phenol and its homologues, ether or chloroform which increase the precipitation of the serum proteins, presumably, by virtue of their effect on the surface tension of the protein aggregates in colloidal solution.

(4) The extent of the heat-denaturation of the serum proteins during the heating of serum at 57° for several hours is also influenced by the hydrogen ion concentration of the serum and can be controlled by the adjustment of the latter.

(5) The denaturation induced by heat in alkaline sera apparently does not involve the same type of change as that induced in acid sera.

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