XXIV. ON THE CONCENTRATION OF ANTITOXIC SERA.

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Of the various methods which have from time to time been proposed for the concentration of sera, those developed by Banzhaf have been most widely adopted on a commercial scale. In this communication it is only necessary to refer to:

A. *The Banzhaf-Gibson* [1907] *Two Fraction Method*, in which serum or plasma, diluted with one-third its volume of water, was heated to a temperature of 57° for 15 hours, or of 58° for 7 hours. The liquid was then made 30 per cent. of saturation with ammonium sulphate¹ and the ensuing precipitate (Fraction I) separated by filtration.

The Fraction I precipitate was suspended in saturated brine solution for 48 hours or longer. The liquid was filtered from the insoluble euglobulin and the filtrate, containing in solution the pseudoglobulin and antitoxin which had been present in the Fraction I precipitate, was made 0.25 per cent. with glacial acetic acid. The ensuing precipitate was filtered, pressed and dialysed.

The filtrate from Fraction I was made 54 per cent. of saturation with ammonium sulphate and the precipitate (Fraction II) thus formed was filtered, pressed and dialysed.

The dialysis residues from Fractions I and II were mixed, the requisite amount of salt and preservative added, and finally filtered ready for distribution. This method has been used in these laboratories for some considerable time and it has been found that by this process a good end product can be

¹ Throughout this paper the phrase "made x per cent. of saturation with ammonium sulphate" has been used to indicate the following: To $100-x$ parts of the serum by volume there were added x parts by volume of a saturated aqueous solution of ammonium sulphate.

obtained which remains clear for years. Care must, however, be taken in the first precipitation, as otherwise some of the insoluble globulin passes on and appears in the final product which, in this case, does not remain clear.

B. *Banzhaf's* [1913] *One Fraction Method*, in which serum or plasma, diluted with half its volume of water and made 30 per cent. of saturation with ammonium sulphate, is brought up to a temperature of 60°, the entire heating process taking about two hours.

According to Banzhaf the filtration of the hot serum mixture should proceed rapidly. The precipitate of euglobulin is pressed well or is washed with 33 per cent. saturated ammonium sulphate; the washings are filtered and added to the main bulk of the filtrate which is then made 50 per cent. of saturation with ammonium sulphate. The ensuing precipitate, consisting of pseudoglobulin and antitoxin, is filtered, pressed, dialysed, and the dialysis residues treated in the same way as in the Banzhaf-Gibson process.

Banzhaf considers that, during the shortened heating of the mixture in the new method, a certain amount of pseudoglobulin is converted into euglobulin, and bases this conclusion on his own experiments [1908]. The effect of heating the serum mixtures is thus to ensure the precipitation of an amount of euglobulin which ordinarily would require a higher percentage of ammonium sulphate for precipitation, and which does not, as would happen in the latter case, carry down with it an appreciable amount of pseudoglobulin and antitoxin.

Banzhaf claims that the new process possesses the following advantages over the former Banzhaf-Gibson process.

- (1) There is only one finished product for dialysis.
- (2) There is a considerable saving of time for the reason that the first precipitate (30 per cent. of saturation with ammonium sulphate), instead of being subjected to the more lengthy process of extraction with saturated brine and subsequent precipitation of the brine extract with acetic acid, is washed with 33 per cent. ammonium sulphate and the washings added to the main filtrate.
- (3) The expenses incurred in the process are considerably less, both as regards labour and the use of precipitating materials, etc.

This new method certainly possesses the advantage of being a much shorter process than the former Banzhaf-Gibson method, but unfortunately it is not always easy to work. The difficulty that sometimes arises in carrying out the process is that the serum will not filter well. This difficulty has arisen in the course of my work both in an English and in a Canadian laboratory; in

the latter laboratory I had the advantage of consulting Dr Banzhaf personally about the matter. He suggested that a slight alteration in temperature or in the concentration of ammonium sulphate might be tried to overcome the difficulty, but these suggestions, although of value in some cases, did not always provide a satisfactory solution to the problem. The majority of workers on the subject with whom I have discussed the question have been faced with the same irregularity in concentrations by Banzhaf's new method.

Practical experience shows that the production of a readily filterable end product which will remain almost permanently clear, goes hand in hand with the rapid filtration of the serum mixtures after the preliminary heating process. When the heated mixtures filter with difficulty it is in some cases necessary to allow the dialysed pseudoglobulin-antitoxin precipitate to stand in the ice chest for weeks in order that the opalescent suspension therein may settle—otherwise filtration through filter candles is accomplished with great difficulty and, moreover, the final product will not remain clear.

This delay is a great disadvantage, and is one which nullifies any gain in time which should accrue from the fact that there is only one fraction to be considered.

It was obvious that the technique of the new process called for improvement or for enlargement, and in this laboratory we have ascertained that the above mentioned irregularities in the filtration can be obviated by the addition of sodium chloride to the serum mixtures, as described below.

The serum or plasma is diluted with one-half its volume of water, made 30 or 31 per cent. of saturation with ammonium sulphate and to the mixture is added 1.5 per cent. of solid sodium chloride. The temperature of the mixture is gradually raised to 61°, 63° or even 65° and kept at this point for a few minutes only. The mixture is allowed to cool to 40°–45° and filtered.

In every case the filtration of this hot mixture and of the subsequent 50 per cent. saturation proceeds rapidly. The liquid obtained after dialysis of the pseudoglobulin-antitoxin precipitate shows no sign of the presence of an opalescent colloidal suspension. After filtration through muslin it is as clear to the naked eye as though it had already been passed through paper pulp. On standing in the ice box these dialysis residues remain clear as there is nothing in opalescent suspension to be deposited.

On the addition of cresylic acid or tricresol to the dialysis residues a certain amount of precipitate is formed in all cases. In the batches of concentrated serum to which the 1.5 per cent. of solid sodium chloride has been

added this deposit is exceedingly slight, even after the serum has stood many weeks. Such a serum may undergo its final filtration through pulp and filter candles almost at once: the end product will remain perfectly clear.

By the addition of 1.5 per cent. solid sodium chloride to the serum mixtures we have so far obtained a satisfactory end product with certainty and the loss of antitoxic units has been very small; a most important item to be considered in assessing the value of any method. From incomplete experiments we have reason to believe that by a further study of the dilution of the plasma, of the concentration of the ammonium sulphate and of sodium chloride and of the general treatment of the mixture, we may be able to reduce the loss of antitoxin to a minimum and at the same time produce a much better end product.

At Dr MacConkey's suggestion, an effort has been made to control the heating of the serum by means of the immersion refractometer, in order to see if some easy indication might be obtained as to the best point at which to interrupt the process. This appeared hopeful in view of Banzhaf's explanation of the effect of heating. However, the results obtained from the use of this instrument, of which the reliability and accuracy have been well established, have demonstrated that no useful indication was forthcoming, for, contrary to Banzhaf's hypothesis, during the heating process there is no appreciable change in the amount of protein in solution in the serum mixture. This result, not entirely unsuspected, has led to some interesting investigations which have been carried out with routine and with experimental batches of serum in order to elucidate:

- I. The value of the heating process in the new One Fraction Method.
- II. The part played by the sodium chloride in improving the technique of Banzhaf's new process.

I. The value of the heating process in the Banzhaf new One Fraction Method.

As previously stated, it was originally hoped that a study of the refractive indices of the filtrates of samples taken from the serum mixtures from time to time during the heating process would indicate the point at which optimum conditions for filtration had been reached. With this object in view the following procedure was adopted.

Routine concentrations were carried out in which serum or plasma, diluted with one-half its volume of water, and made 30 per cent. of saturation with ammonium sulphate, was heated to a temperature of 61°, 63°, 65° and

kept at the specified temperature for a few minutes only, the process taking from 2 to 3 hours in all. Samples of the original plasma and of the plasma mixtures at various stages during the heating process were taken. The samples were filtered and the refractive indices of the clear filtrates were measured by means of the Zeiss Immersion Refractometer. In each case the clear liquid, thus examined, was boiled and filtered and the refractive index of the second filtrate which contained the serum salts and ammonium sulphate in solution, was noted. The amount of protein in solution in the serum mixture was calculated from the difference between the values of the refractive index of the whole serum mixture and of the filtrate containing the serum salts.

TABLE I.

Oxalated plasma diluted with half its volume of water and made 31 per cent. of saturation with ammonium sulphate. The mixture was heated to 63° and kept at that temperature for about five minutes.

Protein Content of the Original Plasma = 7.45 per cent.

Duration of time of heating of plasma mixtures	Temperature of mixtures	Scale Divisions of Refractometer Measurements of:		Per cent. of protein remaining in solution expressed per 100 cc. of the original plasma
		(a) The filtrates from the mixtures	(b) The filtrates (a) diluted with an equal volume of water, boiled and filtered	
0	16°	98.65	51.50	5.11
1 hr 50'	53.5°	98.72	51.50	5.15
2 hrs 5'	57°	98.55	51.40	5.16
2 hrs 20'	59°	98.56	51.50	5.16
2 hrs 40'	61°	98.50	51.60	5.09
2 hrs 45'	62°	98.85	51.70	5.11
2 hrs 55'	63°	98.90	51.60	5.13

These results have been calculated from the values given in Reiss' table [1904]. For purposes of comparison it has been assumed throughout this research that in serum mixtures containing from 0 to 4 per cent. of protein the difference in protein content for 1 scale division = 0.220 per cent.; from 4 to 8 per cent. the difference for 1 scale division = 0.2175 per cent.

Brailsford Robertson [1915] considers that Reiss' figures are somewhat high, but since in the present investigation the *relative change* only in the protein content of the serum mixture is to be investigated it follows that it is quite immaterial whether Reiss' values are above or below the true value.

An examination of the results from a typical concentration given in Table I shows that during the heating process there was no appreciable change in the refractive indices of filtrates of the serum mixtures heated from room temperature to 63° or 65° and kept at these latter temperatures for

5 or 10 minutes. The very slight changes observed may have been due to the effect of heating the serum itself (*post* p. 301 and Table XIV).

Banzhaf seems to consider that the value of the heating lies in the conversion of soluble pseudoglobulin into a further amount of euglobulin, and that the rapid filtration of the serum mixture depends upon the success of this conversion. Had there been any such change in the amount of protein remaining in solution in the serum mixture, there should have been corresponding variations in the refractive indices of the solution. But, as no change occurred in this physical property of the mixtures one must conclude that a certain amount of euglobulin is thrown out of solution on the addition of the ammonium sulphate at room temperature, and that this amount is not further increased during the heating process.

Since the conversion of pseudoglobulin to euglobulin apparently does not occur during the heating process, it is not improbable that the value of the heating lies in its complete aggregation of the particles of precipitated euglobulin into conglomerates of such dimensions that they are readily retained on the surface of filter paper. The advantage of rapid over slow preliminary heating of the serum mixture—*i.e.* up to 57°—in producing an easy filtering fluid, is most readily explained on the same ground. If the heating were insufficient the smallest particles would pass the filter, while the slightly larger would actually engage and block the pores, producing an ultra filter which would impede or even stop filtration.

With the object of testing the correctness of these hypotheses, the following experiments were accordingly instituted.

Experiment I. Separate volumes of plasma, undiluted or diluted, were made 20, ... 54 per cent. of saturation with ammonium sulphate. The refractive indices of these mixtures were measured and the protein still remaining in solution after the addition of the various amounts of ammonium sulphate solution was calculated, the results, for the purposes of comparison, being expressed in terms of the original plasma.

The various mixtures were then heated to a temperature of 61° or 63° according to the directions given in Banzhaf's paper, and the protein contents of the filtrates from samples of the mixtures were measured at the end of the heating process. The results have been embodied in Tables II, III, IV, and V and in Figure 1.

(a) The curve plotted in Figure 1, giving the relation between the concentration of ammonium sulphate in the mixtures and the amount of protein remaining in solution, shows that precipitation occurs mainly at

percentages of saturation with ammonium sulphate greater than 28. However the gradient of the curve indicates no critical points for the precipitation of the eu- or pseudo-globulin fraction or of the serum albumins.

(b) After heating the mixtures containing concentrations of ammonium sulphate of less than 28 per cent. of saturation, clear liquids could not be obtained—the filtrates remained permanently opalescent and even on long standing the particles of the suspension did not coagulate sufficiently to be retained by filter paper.

Fig. 1. Curves showing changes induced in the solubility of the proteins in serum mixtures made various percentages of saturation with ammonium sulphate during the process of heating the mixtures just to a temperature of 61° and keeping them at that temperature for a few minutes only.

The curve — represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(\text{NH}_4)_2\text{SO}_4$ at room temperature.

The curve - - represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(\text{NH}_4)_2\text{SO}_4$ and heated just to 61°.

(c) If the mixtures were kept at a temperature of 61° or 63° for a few minutes only, as advocated by Banzhaf, there was, during the heating process, no appreciable change in the protein content of the solution indicated by the refractometer (Tables II and III).

(d) If the mixtures were kept at a temperature of 61° or 63° for three-quarters of an hour, or longer (Table IV), then there was, during the heating process, a considerable change in the amount of protein in solution.

(e) If *undiluted* plasma were made various percentages of saturation with ammonium sulphate and the mixtures heated to 63° and kept at that temperature for a few minutes only, there was no appreciable change in the protein content of the solutions during the heating process (Table V).

TABLE II.

Oxalated plasma was diluted with half its volume of water and made various percentages of saturation with ammonium sulphate. The mixtures were then heated to a temperature of 61° and kept at that temperature for a few minutes, the heating process taking two hours in all.

Percentage of saturation with ammonium sulphate	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates of the mixtures and expressed, for purposes of comparison, in terms of the original plasma ¹		Percentage conversion of soluble into insoluble protein during the heating process
	(a) Before heating	(b) After heating	
Original plasma	6.49		
20	6.30	6.10	3.2
24	5.91	5.80	1.9
26	5.89	5.79	1.6
28	5.84	5.61	4.0
30	5.85	5.76	1.6
31	5.53	5.36	3.1
32	5.18	5.12	1.1
33	5.18	5.04	2.7
34	4.78	4.69	1.9
36	4.25	4.12	3.1
38	3.84	3.74	2.6
40	2.80	2.86	-2.1
44	2.02	1.90	6
48	1.90	1.90	0

¹ Throughout these tables it has not been deemed necessary to make corrections for the fact that the addition of x cc. of ammonium sulphate solution to $100 - x$ cc. of serum does not necessarily yield a volume of 100 cc. of mixture.

TABLE III.

Oxalated plasma was diluted with half its volume of water and made various percentages of saturation with ammonium sulphate. The mixtures were heated to a temperature of 63° and kept at that temperature for a few minutes.

Percentage of saturation with ammonium sulphate	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates of the mixtures and expressed, for purposes of comparison, in terms of the original plasma		Percentage conversion of soluble into insoluble protein during the heating process
	(a) Before heating	(b) After heating	
Original plasma	8.36		
25	7.35	(Too opalescent to be read)	
28	7.33	6.98	5.0
29	6.81	6.52	4.3
30	6.08	5.92	2.7
32	5.92	5.61	5.3
34	5.48	5.44	0.74
35	5.24	5.11	2.5
40	4.83	4.68	3.1
46	3.39	3.31	2.4
48	2.08	2.14	-3
50	1.31	1.31	0

TABLE IV.

Oxalated plasma diluted with half its volume of water was made various percentages of saturation with ammonium sulphate. The mixtures were heated to 61° or 63° and kept there for three-quarters of an hour.

Percentage of saturation with $(\text{NH}_4)_2\text{SO}_4$	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates of the mixtures and expressed, for purposes of comparison, in terms of the original plasma			Percentage conversion of soluble into insoluble protein during the heating process in periods	
	(a) Before heating	(b) After heating just to 61°	(c) After 0.75 hr further heating at 61° or 63°	(b)	(c)
Original plasma	6.49				
30	5.76	5.63	4.28 ¹	2.3	25.7
33	5.19	5.02	3.78 ²	3.3	27.2
34	4.81	4.67	3.55 ³	3.1	26.1

¹ Heated to 61° for 1 hour.

² Heated to 62° for 0.75 hour.

³ Heated to 63° for 0.75 hour.

TABLE V.

Undiluted oxalated plasma was made various percentages of saturation with ammonium sulphate. The mixtures were heated to a temperature of 63° and kept at that temperature for a few minutes only.

Percentage of saturation with $(\text{NH}_4)_2\text{SO}_4$	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates of the mixtures and expressed, for purposes of comparison, in terms of the original plasma		Percentage conversion of soluble into insoluble protein during the heating process
	(a) Before heating	(b) After heating	
Original plasma	7.14		
28	6.23	5.97	4.2
30	5.98	5.83	2.4
31	5.73	5.43	5.3
32	5.33	4.98	6.6
33	5.17	4.90	5.3

Experiment II. That there was no appreciable change in the pseudoglobulin content of the mixtures during the process of heating was demonstrated gravimetrically as follows.

Several volumes of plasma, both diluted and undiluted, were taken and to them was added respectively ammonium sulphate solution in quantity to make a series of percentage solutions of varying concentration. Each series was duplicated. The same process was carried out with serum.

Of the duplicates of each mixture the one was dialysed without heating and the other previous to dialysis was heated to a temperature of 61° or 63°, and kept there for a few minutes only. After dialysis the residues were made up to a certain volume, aliquot parts of the clear liquid were taken and gravimetric estimations were made on the protein contained therein. Two protein estimations were made on each dialysis residue:

- (a) The total protein present soluble in saturated brine;
- (b) The total amount of protein present not precipitated by 50 per cent. saturated ammonium sulphate.

In some cases the total protein in solution in the dialysis residues was estimated gravimetrically and also by means of the refractometer. Gravimetric estimations were invariably slightly higher than those obtained from the refractometer readings, thus indicating that in the dialysis residues there is, in colloidal suspension, a certain amount of insoluble euglobulin, the particles of which are too small to be retained by filtration through paper.

The gravimetric estimations, collected in Table VI, confirm the evidence obtained from the use of the refractometer that there has been no appreciable conversion of pseudoglobulin into euglobulin during the heating process.

The evidence thus afforded in Experiments I and II indicates that Banzhaf's supposition that the heating process in his new method serves to convert some of the pseudoglobulin into euglobulin does not provide such an explanation of the changes taking place as can be verified by quantitative examination.

At first sight it is difficult to reconcile the conclusions thus to be drawn from a study of these data with the obvious fact that, during the heating process of the serum mixture the bulk of the precipitate visibly increases. The following experiment, however, serves to throw light on this apparent paradox.

Experiment III. Serum mixtures were made from 25 to 54 per cent. of saturation with ammonium sulphate and, after standing at room temperature in stoppered flasks for 2 or 3 hours, were filtered. The clear filtrate was divided into two portions and kept in stoppered flasks. In the one portion, which stood at room temperature for several days, the clear liquid, within a few hours, became cloudy, and there was then a deposition of solid protein which gradually increased in bulk. The other portion was heated to 63° (two and a half hours being taken just to reach that temperature) and in every case a precipitate was formed, although at dilutions below 28 per cent. of saturation with ammonium sulphate the precipitate was so

TABLE VI.
*Gravimetric estimation of the protein "soluble in saturated brine" in solution in the serum mixtures
 before and after being subjected to the heating process.*

Volume of plasma or serum previously diluted with half its volume of water	Percentage protein content of original plasma	Electrolytes added	Treatment before dialysis	Proportion of mixtures taken for dialysis	Total protein soluble in saturated brine solution		Protein not precipitated by making dialysis residue 50 % saturation with $(\text{NH}_4)_2\text{SO}_4$	
					(a) in the dialysis residue	(b) in 100 cc. of original plasma	(a) in the dialysis residue	(b) in 100 cc. of original plasma
A. PLASMA (oxalated) 7.14								
(i) 50 cc.	—	made 30 % of saturation with $(\text{NH}_4)_2\text{SO}_4$	unheated	whole = ppt + liquid	g. 1.324	g. 3.972	g. 0.650	g. 1.950
(ii) 50 cc.	—	"	heated	whole	1.325	3.975	0.649	2.060
B. PLASMA (oxalated) 7.14								
(i) 50 cc.	—	made 32 % of saturation with $(\text{NH}_4)_2\text{SO}_4$	unheated	"	1.424	4.272	0.635	1.905
(ii) 50 cc.	—	"	heated	"	1.576	4.728	0.6365	1.910
C. PLASMA (oxalated) 5.76								
(i) 50 cc.	—	"	unheated	"	0.995	2.984	0.602	1.806
(ii) 50 cc.	—	"	heated	"	1.015	3.045	0.602	1.805
D. SERUM (carbolised) 5.51								
(i) 50 cc.	—	"	unheated	"	0.678	2.034	—	—
(ii) 50 cc.	—	"	heated	"	0.600	1.800	—	—

finely divided that the liquid could not be filtered, clear for refractometer readings. In all cases, whether the filtrates had been heated or not, although there was the visible formation of solid matter from the previously clear filtrate, there was no change in the refractive indices of the solutions, thus indicating no further change in the protein content of the solution after the immediate effect due to the addition of the electrolyte at room temperature. Moreover, this delayed, slowly forming, precipitate was not a special feature of the mixtures containing a percentage of saturation with ammonium sulphate insufficient to precipitate completely the euglobulin from solution, *i.e.* up to 33 per cent. of saturation with ammonium sulphate, for it occurred in all of the ammonium sulphate-serum mixtures.

We are therefore led to no other conclusion than that the effect of heating the serum mixtures just to 61° or 63° merely serves to accelerate a change which takes place slowly at ordinary room temperature, and that whatever this change may be, it is not concerned with the conversion of the pseudoglobulin into the euglobulin.

Light may however be thrown on the question by a consideration of the following observations. Proteins belong to the hydrophile type of colloids, and in solution form an emulsoid system. It has been demonstrated by Chick and Martin [1913] and others that if a high concentration of electrolytes be added to a solution of a protein, there is withdrawal of water from the internal phase of the emulsoid system, and if the withdrawal be sufficient, this phase becomes rigid and is eventually precipitated. In the salting out of proteins the emulsoid is thus first converted into a suspensoid system. Applying these observations to the salting out of serum proteins, it is probable that in the fractional precipitation, with additions of ammonium sulphate to less than 33 per cent. of saturation, the euglobulin is first changed from the emulsoid to the suspensoid type of colloid: the suspensoid is in part converted into a suspension and the latter settles as a precipitate. The protein thus converted into a suspensoid or suspension will no longer have a marked effect on the viscosity or on the refractive index of the solution—even though the particles be neither visible to the naked eye, nor retained by filter paper.

The experimental evidence given above (Experiment III) shows that the complete agglutination of the particles of the suspensoid to those of a perceptible precipitate takes place slowly at ordinary temperatures, and that the effect of heating the liquids in the new process merely serves to increase the velocity of this change. Clearly then, the change thus taking place is not that suggested by Banzhaf, *viz.* the conversion of pseudoglobulin

into euglobulin, but rather a more simple physical process involving the agglutination of the particles of euglobulin already thrown out of emulsoid suspension by the ammonium sulphate. This agglutination permits of easy separation of the euglobulin by filtration through paper.

If, for some reason or other, in the routine concentrations of batches of sera or plasma, the precipitated euglobulin is not completely agglutinated during the heating process, then difficulties will be encountered in

TABLE VII.

Experiment to demonstrate the power of insufficiently agglutinated particles in a badly filtering heated serum mixture to convert an ordinary filter paper into an ultra filter.

Oxalated plasma, diluted with half its volume of water and made various percentages of saturation with ammonium sulphate had been heated just to 61°. The serum mixtures filtered badly; at first there was an opalescent filtrate but the filtration soon slackened and almost ceased.

The protein content of the first opalescent filtrate was estimated and compared with that of the slowly forming clear drops of filtrate collected a short time before the filtration ceased.

Percentage of saturation with $(\text{NH}_4)_2\text{SO}_4$	Residual percentage of protein in solution calculated from the refractometer readings of the filtrates of the mixtures and expressed, for purposes of comparison, in terms of the original plasma			
	(a) Before heating	(b) After heating		The self made ultra filter has effected the removal of soluble protein to the extent of
		(i) First filtrate slightly opalescent	(ii) Clear drops just before filtration ceased	
Original plasma	6.49			
24	5.91	5.80	4.68	20.8 %
26	5.89	5.80	4.69	20.3 %
28	5.84	5.61	4.86	16.8 %
30	5.85	5.76	5.30	9.5 %
31	5.53	5.36	5.22	5.6 %

the subsequent process of filtration. Some of the insufficiently agglutinated aggregates readily pass through the pores of the filter paper, while others, not sufficiently large to be retained on the surface of the paper, either partially or wholly block the pores of the paper and so retard the process of filtration. (Table VII shows that the partially blocked paper may act as an ultra filter.) The euglobulin thus passing into the filtrate will be precipitated with the pseudoglobulin and antitoxin at one-half saturation with ammonium sulphate. Theoretically speaking, when the precipitated pseudoglobulin and antitoxin

containing a small amount of euglobulin is dialysed until the salt content approaches that of tap water, the pseudoglobulin and antitoxin only should be in solution, but it is generally accepted that the presence of one protein in solution protects another from precipitation.

We have seen (Experiment II) that even in dilute dialysis residues a small amount of euglobulin remained in colloidal suspension. Since, in routine concentrations, the dialysis residues may contain up to and even more than 20 per cent. of pseudoglobulin, the protecting power of the latter in respect of the euglobulin is enhanced. The euglobulin thus in colloidal suspension will greatly retard, if not render impossible, the filtration of the final product through filter candles.

The impediment to filtration in the new process caused by the suspensoid particles of euglobulin was in practice removed by allowing the dialysis residue to stand for a prolonged period. This tedious method has had to be resorted to frequently.

II. *An investigation of the rôle of Sodium Chloride in improving the Banzhaf new One Fraction Method.*

Having ascertained the value of the heating process in the new method, the question then arose as to the part played by the sodium chloride in improving the concentration process. Did the addition of salt to the serum mixtures serve to induce the desired conversion of pseudoglobulin into euglobulin or did it merely exert some stimulating effect on the agglutination of the aggregates of the euglobulin, a process which, from the experimental work in Part I, seems to take place slowly?

In order to elucidate this point, routine concentrations in which 1·5 per cent. of solid sodium chloride had been added to serum or plasma mixtures which had been made 30 per cent. or 31 per cent. of saturation with ammonium sulphate were carefully examined at different stages of the heating process. As before, samples were taken from the mixtures and filtered, and the protein content of the clear filtrates was estimated from the difference between the refractometer readings of the clear filtrate before and after coagulation of the protein therein. For purposes of comparison the protein content of the mixtures at various stages in the heating process was expressed in terms of the original serum or plasma.

Up to the present the concentration of 20 batches each of not less than 50 litres of plasma or serum has been followed; the data with regard to

three typical concentrations are given in Table VIII. In no case has any difficulty been experienced in the filtration of the hot liquids or of the final product so far as this latter has up to the present been filtered. In no case has there been any appreciable change in the protein content of the mixtures during the heating process.

From these observations we are led to the conclusion that the effect of the heating process is to agglutinate the particles of euglobulin already converted from the emulsoid to the suspensoid state. This process, which seems to be incomplete in some serum mixtures, is stimulated by the addition of sodium chloride to the same mixtures.

TABLE VIII.

Changes taking place during the heating process to 63° or 65° in the protein content of routine concentration serum mixtures which have been made 30 or 31 per cent. of saturation with ammonium sulphate and 1.5 per cent. with solid sodium chloride.

Amount of oxalated plasma concentrated	Protein content of original plasma	Percentage of saturation with ammonium sulphate	Duration of time of heating of mixtures	Temperature of mixtures at which samples were taken	Residual percentage of protein in solution in the filtrates of the serum mixtures, calculated from the refractometer readings and expressed in terms of the original plasma
Diphtheria 50 litres	7.00	31	0	Room temp.	5.11
			2 hrs	57°	5.00
			2 hrs 35'	61°	5.02
			3 hrs	63°	4.93
Tetanus 50 litres	7.47	30	1 hr 55'	57°	6.82
			2 hrs 25'	61°	6.60
			2 hrs 55'	63°	6.60
Tetanus 50 litres	6.73	30	0	Room temp. = 16°	5.19
			1 hr 35'	57°	5.28
			2 hrs 15'	61°	5.28
			2 hrs 35'	65°	5.12

Additional evidence on this point was furnished by the following experiments.

Experiment IV. Serum or plasma (diluted or undiluted) was made from 25–50 per cent. of saturation with ammonium sulphate and solid sodium chloride was added to the mixture to the extent of 1.5 per cent. The protein content of the filtrates of samples of the mixtures was estimated from the refractometer readings as in the previous experiments. The mixtures were then subjected to various treatments.

(a) The mixtures were heated to 63° and kept at that temperature for a few minutes, the process in all taking from 2 to 2.5 hours. (Table IX.)

It will be seen from the measurements of the refractive indices of the solutions and from the curves in Figure 2 that there was no appreciable change in the pseudoglobulin content of the mixtures during the process of heating.

(b) The mixtures were heated to a temperature of 67° and kept at that temperature for 10 minutes. (Table X and Figure 3.)

TABLE IX.

Oxalated plasma diluted with half its volume of water, made various percentages of saturation with ammonium sulphate and 1.5 per cent. solid sodium chloride added to the mixtures. The mixtures were heated to a temperature of 63° and kept at that temperature for a few minutes.

Percentage of saturation with ammonium sulphate	Residual percentage of protein in solution, calculated from the refracto- meter readings of the filtrates from the mixtures and expressed, for purposes of comparison, in terms of the original undiluted plasma		Percentage conversion of soluble into insoluble protein during the heating process
	(a) Before heating	(b) After heating	
Original plasma	8.30		
29	6.93	6.66	4.0
30	6.73	6.52	3.1
31	6.38	6.14	3.8
32	5.97	5.89	1.36
33	5.09	4.85	0.8
34	4.81	4.70	2.96
36	3.73	3.64	2.5
39	2.44	2.39	2.1
40	2.19	2.19	0
46	2.15	2.15	0
54	0.574	0.582	-1.6
Original plasma	7.14		
29	5.68	5.44	4.3
30	5.30	5.25	1
31	5.12	5.08	0.8

From the results it is clear that heating the serum mixtures to this higher temperature is accompanied by a considerable change in the protein content of the solutions.

(c) The mixtures were heated to a temperature of 63° and kept at that temperature for 1.25 hours. (Table XI and Figure 3.)

Obviously the prolongation of the time of heating at 63° also gives rise to considerable change in the protein content of the solution.

Fig. 2. Curves showing changes induced in the solubility of the proteins in serum mixtures made various percentages of saturation with ammonium sulphate and 1.5 per cent. with solid sodium chloride during the process of heating the mixtures just to a temperature of 63° and keeping them at that temperature for a few minutes only.

The curve ——— represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(\text{NH}_4)_2\text{SO}_4$ at room temperature.

The curve - - - represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(\text{NH}_4)_2\text{SO}_4$ heated to 63° and kept at that temperature for a few minutes only.

Fig. 3. Curves showing changes induced in the solubility of the proteins in serum mixtures made various percentages of saturation with ammonium sulphate, during the process of heating (a) just to a temperature of 67° and (b) to a temperature of 63° and keeping at that temperature for 1½ hours.

The curve ——— represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(\text{NH}_4)_2\text{SO}_4$ at room temperature.

The curve - - - represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(\text{NH}_4)_2\text{SO}_4$ and heated just to 67°.

The curve - x - x - represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(\text{NH}_4)_2\text{SO}_4$ and heated to 63° and kept at that temperature for 1½ hours.

Experiment V. That there was no appreciable change in the pseudoglobulin content of the mixtures during the heating process adopted in the new method was also demonstrated gravimetrically.

Several volumes of serum or plasma, diluted or undiluted, were respectively made various percentages of saturation with ammonium sulphate, and 1·5 per cent. of solid sodium chloride was then added. Each series was duplicated and of the duplicates the one was dialysed without heating and the other, previous to dialysis, was heated to a temperature of 63° and kept at that temperature for a few minutes only—the heating process taking 2 to 2·5 hours in all.

TABLE X.

Oxalated plasma diluted with half its volume of water was made various percentages of saturation with ammonium sulphate and 1·5 per cent. of solid sodium chloride was added to the mixtures which were then heated to a temperature of 67°.

Percentage of saturation with (NH ₄) ₂ SO ₄	Residual percentage of protein in solution, calculated from the refracto- meter readings of the filtrates from the mixtures and expressed, for purposes of comparison, in terms of the original plasma		Percentage conversion of soluble into insoluble protein during the heating process
	(a) Before heating	(b) After heating	
Original plasma	6·23		
28	5·34	4·66	12·9
29	5·14	4·24	17·7
30	4·98	4·00	20·0
31	4·97	4·25	14·5
32	4·62	3·99	13·6
33	4·54	4·05	11·0
34	4·56	3·96	10·4

The protein estimations were made on each dialysis residue, viz.:

- (a) The total protein present soluble in saturated brine solution.
- (b) The total amount of protein not precipitated by 50 per cent. of saturation with ammonium sulphate.

The results showed that, during the heating process, there was no appreciable conversion of pseudoglobulin into euglobulin.

From the results given in Tables VI and XII it is interesting to note that the amount of protein precipitated from the dialysis residues by saturation with brine depends upon the original dilution of the serum or plasma. This is a generally accepted fact (Banzhaf-Gibson).

In certain cases the whole serum mixture, *i.e.* precipitate and liquid, as well as controls of the filtrate alone were dialysed, when it was observed that the pseudoglobulin was much greater in amount in the former than in the latter case: that is to say that the precipitate brought down by the ammonium sulphate was in reality a mixture of eu- and pseudo-globulin.

Further, in those instances in the above experiment in which oxalated plasma was used, the dialysis residues of the unheated plasma mixtures contained the insoluble fibrin and euglobulin formed into a jelly-like clot. In the dialysis residues of the heated mixtures these insoluble proteins were present as a flocculent and readily subsiding precipitate.

Experiment VI. Further experimental work was then undertaken to ascertain the effect of sodium chloride on the solubility of serum proteins under different conditions.

TABLE XI.

Oxalated plasma, diluted with half its volume of water, was made various percentages of saturation with ammonium sulphate and 1.5 per cent. solid sodium chloride added to the mixtures which were then heated to 63° and kept at that temperature for 1.25 hours.

Percentage of saturation with $(\text{NH}_4)_2\text{SO}_4$	Residual percentage of protein in solution, calculated from the refracto- meter readings of the filtrates from the mixtures, and expressed, for purposes of comparison, in terms of the original plasma		Percentage conversion of soluble into insoluble protein during the heating process
	(a) Before heating	(b) After heating	
Original plasma	6.23		
28	5.44	2.81	48.6
30	5.29	2.89	45.5
32	4.56	2.80	39.0
34	4.62	2.86	38.4

(a) *The effect of heating a saturated brine solution of pseudoglobulin.*

The globulin precipitate thrown down by making plasma 50 per cent. of saturation with ammonium sulphate was suspended in saturated brine solution and after standing at room temperature for 48 hours was gradually heated to a temperature of 61°, the process taking 2 hours in all.

From the use of the refractometer it was ascertained that at room temperature (after 48 hours) the saturated brine contained protein in solution corresponding with a value of 6.3 per cent. in the original plasma. After

TABLE XII.

Gravimetric estimation of the protein "soluble in saturated brine" present in serum mixtures made various percentages of saturation with ammonium sulphate and 1.5 per cent. with sodium chloride, before and after being heated to 63° and kept at that temperature for a few minutes only.

Percentage protein content of citrated plasma taken	Dilution of plasma	Volume of diluted plasma taken	Percentage of saturation with $(\text{NH}_4)_2\text{SO}_4$	Treatment of mixtures previous to dialysis	Total protein soluble in saturated brine		Protein not precipitated at 50 per cent. of saturation with $(\text{NH}_4)_2\text{SO}_4$	
					(a) the dialysis residue	(b) 100 cc. of original serum or plasma	(a) in the dialysis residue	(b) in 100 cc. of original plasma or serum
A. 7.14	undiluted	100 cc.	29	The mixture, after standing 2 hrs, was filtered. Of the clear filtrate (i) 50 cc. were unheated (ii) 50 cc. were heated	1.074 1.098	3.024 3.091	0.633 0.620	1.880 1.745
B. 7.14	undiluted	100 cc.	31	The mixture, after standing 2 hrs, was filtered and of the clear filtrate (i) 50 cc. were unheated (ii) 50 cc. were heated	1.596 1.700	4.62 4.92	0.624 0.600	1.800 1.714
C. 7.14	undiluted	2 lots of 50 cc. each	31	The whole mixtures, i.e. ppt. and liquid, were respectively taken and (i) unheated (ii) heated	2.68 2.56	5.36 5.12	1.17 1.13	2.34 2.26
D. 5.76	diluted with $\frac{1}{2}$ its vol. of H_2O	100 cc.	31	The mixture, after standing 2 hrs, was filtered and of the clear filtrate (i) 50 cc. were unheated (ii) 50 cc. were heated	0.234 0.227	0.641 0.621	— —	— —

raising the temperature of the brine solution just to 61° , the protein in solution had decreased to the value of 2.48 per cent.

During the heating process there had thus been a conversion of 60.6 per cent. of the soluble into insoluble protein; at the same time there was considerable loss of antitoxic units from the solution.

TABLE XIII.

The effect of the prolonged heating of plasma, diluted with one-third its volume of water and containing 1.5 per cent. of sodium chloride, upon (a) the solubility of the serum proteins and (b) the precipitating power of 30 per cent. of saturation with ammonium sulphate.

Length of time diluted plasma or serum was heated	Temperature of heated liquids	Residual percentage of protein in solution		Percentage increase in the amount of soluble protein precipitated by 30 per cent. of saturation with (NH ₄) ₂ SO ₄
		(a) in the diluted plasma or serum at various stages in the heating process calculated from the refractometer readings of the heated liquids and expressed, for purposes of comparison, in terms of the original plasma	(b) after making the heated liquids 30 per cent. of saturation with (NH ₄) ₂ SO ₄ , calculated as in (a)	
<i>Plasma (oxalated)</i>				
0 hrs	Room temperature	7.38	5.12	—
3 hrs	57°	7.05		
7 hrs	57°	7.01	4.84	5.5
<i>Serum (carbolised)</i>				
(1) 0 hrs	Room temperature	6.47	5.82	
15 hrs	57°	5.99	3.81	34.6
(2) 0 hrs	Room temperature	6.95	5.41	
15 hrs	57°	5.77	3.13	42.2

(b) *Prolonged heating of serum or plasma to which 1.5 per cent. sodium chloride had been added.*

Plasma and serum were each diluted with one-third of their volume of water, 1.5 per cent. of salt added, and heated to a temperature of 57° for periods varying from 6 to 15 hours.

The refractometer readings showed that during the heating process there was a slight change in the protein content of the solutions, but this change did not take place with less than three hours' heating. Samples of the heated mixtures, taken after 6 and 15 hours' heating respectively, were made 30 per cent. of saturation with ammonium sulphate and the protein thrown out of solution compared with that precipitated by the same

saturation with ammonium sulphate in the original unheated serum or plasma. (Table XIII.)

The results show that the amount of protein remaining in solution after making the serum 30 per cent. of saturation with ammonium sulphate is practically the same for the unheated serum as for the serum heated to 57° for 6 hours. After 15 hours' heating there is a 30–40 per cent. increase in the precipitation of protein.

Further heating of the respective ammonium sulphate mixtures to a temperature of 61° was not accompanied by any increased change in the protein content of the solutions.

(c) *The heating of serum or plasma without the addition of salt.*

In order to ascertain whether the change thus taking place in (b) during the prolonged heating of plasma or serum with 1·5 per cent. sodium chloride was due to the specific action of the sodium chloride on the proteins of horse serum or was an inherent property of the proteins themselves, plasma and serum diluted with water were heated, *without the addition of salt*, in order to ascertain whether changes had been induced during the heating.

(a) *Changes in the solubility of the serum proteins.*

During the heating process there was slight deposition of proteins and samples of the heated liquids filtered with difficulty.

From Table XIV it will be seen that whether the serum or plasma had been heated at 63° for a short time or kept at lower temperatures for longer periods there was a slight change in the solubility of the proteins.

(β) *Changes in the precipitating power of 30 per cent. of saturation with ammonium sulphate.*

The results depicted in Table XV show that considerable changes may take place in the precipitating power of this reagent.

It has been previously demonstrated that the heating of the serum to 63°, when the liquid was kept at that temperature for a few minutes only, was not followed by any appreciable change in the amount of protein precipitated by 30 per cent. of saturation with ammonium sulphate. But if the liquids were kept at this temperature for 1 hour, or if the temperature were raised to 67°, then considerably more protein was precipitated (from 10 to 20 per cent. increase).

TABLE XIV.

The effect of the heating of serum or plasma on the solubility of the serum proteins
 [No addition of NaCl.]

Dilution of the oxalated plasma or serum	Temperature to which heated	Duration of the time of heating at the specified temperature	Percentage of protein in solution in the plasma—measured by means of the refractometer		Percentage conversion of soluble into insoluble protein during the heating process
			(a) Before heating	(b) After heating	
(1) <i>Plasma</i> with $\frac{1}{2}$ its vol. of H ₂ O	63°	10 min.	7.69	7.43	3.4
(2) <i>Plasma</i> with $\frac{1}{2}$ its vol. of water	57°	0 hrs	5.85	—	9.4
	57°	15 hrs	5.85	5.30	
(3) <i>Plasma</i> with $\frac{1}{2}$ its vol. of water	57°	0 hrs	6.14	—	2.4
	57°	4 hrs	„	5.99	
	57°	15 hrs	„	5.79	
(4) <i>Carbolised serum</i> ¹ with $\frac{1}{2}$ its vol. of water	57°	0 hrs	5.59	—	32.2
	57°	15 hrs	„	3.79	

¹ A considerable change always takes place during the heating of carbolised serum. This point is being investigated.

TABLE XV.

The effect of the prolonged heating of plasma and of serum respectively diluted with one third their volume of water upon the precipitating power of 30 per cent. of saturation with ammonium sulphate. [No addition of NaCl.]

Temperature reached	Duration of the heating at the specified temperature	Percentage of protein in solution at the stage in the heating process. Expressed in terms of the original plasma	Percentage of protein remaining in solution after making the liquid 30 per cent. of saturation with ammonium sulphate. Expressed in terms of the original plasma	Percentage change in the amount of dissolved protein precipitated by 30 per cent. of saturation with (NH ₄) ₂ SO ₄
<i>Carbolised serum</i>				
57°	0 hrs	5.59	4.53	46.0
	15 hrs	3.79	2.42	
<i>Plasma</i>				
57°	0 hrs	5.85	5.80	39.3
	15 hrs	5.30	3.52	
<i>Plasma</i>				
57°	0 hrs	6.14	5.78	5.2
	4 hrs	5.99	5.48	
	15 hrs	5.79	3.52	
<i>Plasma</i>				
55°	0 hrs	7.39	5.35	10.0
	15 hrs	6.89	4.81	

If the plasma or serum were kept at a temperature of 55° for 15 hours there was a 10 per cent. increase in the precipitating power of 30 per cent. saturation with ammonium sulphate.

When the plasma or serum had been kept at 57° for 4 hours there was very little difference between the amounts of protein precipitated from the heated and unheated liquids by the addition of saturated ammonium sulphate to the extent of 30 per cent. of saturation.

After 15 hours' heating at 57° it was found that, on the addition of ammonium sulphate to 30 per cent. of saturation, from 39-47 per cent. more of the soluble protein was precipitated from the heated than from the unheated liquids.

It is thus obvious that the change taking place in the prolonged heating of serum or plasma with 1.5 per cent. sodium chloride in (b) is in no way dependent on the presence of the sodium chloride, as the same phenomenon takes place in the prolonged heating of serum or plasma to which no salt has been added.

That changes take place in the prolonged heating of solutions of pseudoglobulin has been shown by Banzhaf [1908] but from the details given in his paper it is difficult to understand the exact procedure adopted by him. Banzhaf suggests that the change is due to the conversion of pseudoglobulin into euglobulin, but from consideration of the work of Hardy [1899], Chick and Martin [1912], and Chick [1914] it is probable that the change taking place in (b) and (c) above is of the nature of a denaturation of proteins. For, evidently, during the prolonged heating of the serum or plasma, while the actual solubility of the proteins has been little changed their physical properties have become so altered that their precipitability by electrolytes is considerably increased.

In view of these observations it is at first sight not easy to picture the part played by the sodium chloride in rendering the concentration of antitoxic sera by Banzhaf's new process more certain of success. If it were merely a question of the better salting out of the euglobulin by increasing the dehydrating action exerted by the salt, then why should the addition of 1.5 per cent. sodium chloride to the 29 or 30 per cent. of saturation with ammonium sulphate serum mixtures yield a better and more easily filterable end product than that obtained from 31, 32, 33 per cent. or even 34 per cent. of saturation in the absence of sodium chloride.

Now Banzhaf and Gibson [1907] have stated that the salting out of euglobulin is influenced by the dilution of the plasma and they have

given what is, in their opinion, the optimum dilution for routine concentrations.

But, that factors other than the dehydrating effect of the salts added to the serum and the dilution of the serum proteins have to be considered, is shown by an examination of the data collected during the progress of this research. (Table XVI.)

TABLE XVI.

The percentage of protein remaining in solution in serum mixtures made 30 or 31 per cent. of saturation with ammonium sulphate.

Percentage protein content of the original plasma taken	Treatment of the diluted plasma		Residual percentage of protein remaining in solution in the mixtures and expressed in terms of the original plasma
	(a) Percentage of saturation with $(\text{NH}_4)_2\text{SO}_4$	(b) Percentage of NaCl in the mixtures	
7.14	30	—	4.93
6.47	"	—	5.85
6.47	31	—	5.53
8.10	"	—	6.29
8.36	"	—	6.39
7.14	"	—	5.74
6.71	30	1.5	5.16
6.73	"	"	4.19
7.38	"	"	4.97
7.14	"	"	5.20
7.38	"	"	5.12
6.38	"	"	5.81
5.51	"	"	4.53
5.76	"	"	5.80
6.04	"	"	5.76
6.23	"	"	5.29
8.32	"	"	6.39
8.32	31	1.5	5.97
7.14	"	"	5.12
7.28	"	"	5.11
7.00	"	"	5.11
6.71	"	"	4.61
7.47	"	"	6.80
7.28	"	"	4.42
6.79	"	"	5.59

It will be seen, that though the ammonium sulphate concentration and the protein concentration of some of the samples were practically identical, yet the amount of protein left in solution was considerably different. Evidently factors operated which had not been controlled.

It is believed that colloidal aggregates are kept in solution by their mutual

repulsion which is due to the electric charge they carry. If this charge be sufficiently reduced, then the forces keeping the particles apart diminish; at the iso-electric point the colloids will be most completely precipitated. In other words, the more nearly the hydrogen-ion concentration of the system approaches that of the iso-electric point for a particular protein, the more complete will be the precipitation of that protein.

Therefore, when dealing with the concentration of antitoxic sera on a large scale, we ought not to lose sight of the fact that the reaction of the serum mixtures is probably of more vital importance in the salting out process than has hitherto been recognised in the methods of practical value advocated for the concentration of antitoxic sera. The more nearly the reaction of the serum mixtures approaches the hydrogen-ion concentration at the iso-electric point of euglobulin, not only should there be more rapid and effective filtration, but more constant results should be obtained with regard to the percentage removal of protein from the solution.

In the Banzhaf new One Fraction Method no cognisance is taken of the reaction of the mixture and this may well be the secret of its uncertainty.

The alkalinity of blood is supposed to be due to the partial hydrolysis of certain sodium salts of weak acids, and if this be the case the introduction of a salt like sodium chloride should slightly reduce the alkalinity. It therefore follows that the stimulating effect of the sodium chloride may be due to the fact that it tends to make the hydrogen-ion concentration of the mixtures approach more nearly that required for the optimum precipitation of euglobulin.

In support of this theory it may be mentioned that during the past few months I have had occasion to study the heat coagulation of 350 samples of serum. With some samples the heat coagulation could be readily brought about. With others the formation of a readily filterable coagulum could only be induced after the addition of from 1 to 6 per cent. of sodium chloride, or by the addition of corresponding amounts of a standard solution of acetic acid. The sodium chloride and the acetic acid were interchangeable and apparently had the same effect whether used separately or whether a certain proportion of the one was replaced by the corresponding proportion of the other.

In this connection experiments are being conducted with the object of ascertaining the influence of alteration of the reaction (hydrogen-ion concentration) of the serum mixtures on the precipitation and filtration of the eu- and pseudo-globulin and antitoxin fractions. For, although the

desired improvement in filtration may be obtained by the addition of acetic acid to the more alkaline sera we must not lose sight of the fact that, as salt is known to exert a protecting action on antitoxin, it may be better to adjust the reaction of the medium by means of salt rather than by the addition of acid. The results will be dealt with in a future communication.

SUMMARY.

1. The advantages claimed by Banzhaf for his new One Fraction process are sometimes vitiated by the fact that a final product is obtained which, owing to the presence of an opalescent suspension presumably of euglobulin, cannot be filtered except after long standing.

2. The successful preparation of an easily filterable end product depends on the initial heating of the serum mixtures.

3. The heating process advocated in the new method does not cause a conversion of pseudoglobulin into euglobulin but merely serves to complete the aggregation of the euglobulin into particles sufficiently large to admit of separation by filtration.

4. The uncertainty with regard to the successful heating of the serum mixtures can be obviated by a slight modification of the described technique, viz. by the addition of 1.5 per cent. sodium chloride to the serum mixtures.

5. The following observations have been made with regard to the effect of the addition of sodium chloride to the serum mixtures:

(a) During the process of heating the mixtures to 63° or 65° the addition of salt does not induce changes in the amount of proteins still remaining in solution, but if the temperature be raised to 67° or if the mixtures be kept for one hour or more at 63°, then a considerable change in the protein content of the solution takes place.

(b) If a solution of pseudoglobulin and antitoxin in saturated brine be heated to a temperature of 61° and kept at that temperature for a few minutes only, there is a conversion of 60 per cent. of soluble into insoluble globulin, but at the same time there is loss of antitoxic units.

(c) Prolonged heating of serum or plasma diluted with water and containing 1.5 per cent. sodium chloride leads to an increase in the precipitating power of 30 per cent. of saturation with ammonium sulphate. This change, however, is not due to the presence of the sodium chloride. The prolonged heating of diluted plasma or serum gives rise to the same phenomenon.

Banzhaf considers that the change thus produced in the serum is that of pseudoglobulin into euglobulin, but it seems more probable that the prolonged heating serves to denature the protein in solution and to make it more readily precipitable by saturation with sodium chloride or by 30 per cent. saturation with ammonium sulphate.

6. It is possible that the addition of sodium chloride exerts a favourable influence on the filtration processes by virtue of an adjustment of the hydrogen-ion concentration of the serum mixtures.

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