AN IMPROVED METHOD FOR THE CONCENTRATION OF ANTITOXIC SERA.

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Gibson (1905) was the first to introduce a method of commercial value for the concentration of antitoxic horse sera.

He treated plasma with an equal volume of saturated ammonium sulphate solution, filtered off the precipitated globulins and suspended the latter in saturated salt solution. The pseudoglobulin and antitoxin entered into solution while the euglobulin remained in suspension. The solution of pseudoglobulin and antitoxin was freed from euglobulin by filtration and the clear filtrate was then acidified with acetic acid. The precipitated pseudoglobulin and antitoxin were dialysed. The finished product had a potency two and a half times greater than that of the original plasma.

In 1907 Banzhaf and Gibson, by fractional precipitation with ammonium sulphate, separated the pseudoglobulin-antitoxin combination from the other serum proteins as follows:

The plasma or serum, diluted with one-third its volume of water, was heated to a temperature of 57° C. for 15 hours or of 58° C. for 7 hours. The liquid was then made 30 % saturation with ammonium sulphate[1] and the ensuing precipitate (Fraction 1) separated by filtration.

The Fraction 1 precipitate was suspended in saturated brine. The brined liquid was filtered from the insoluble euglobulin and the filtrate, containing in solution the pseudoglobulin and antitoxin which had been present in the Fraction 1 precipitate, was made 0·25 % with glacial acetic acid. The ensuing precipitate was filtered, pressed and dialysed.

[1] The phrase "made x % saturation with ammonium sulphate" has been used to indicate the following: to $100-x$ parts of the serum by volume there was added x parts by volume of a saturated aqueous solution of ammonium sulphate.

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The filtrate from Fraction 1 was made 54 % of saturation with ammonium sulphate and the precipitate (Fraction 2) thus formed was filtered, pressed and dialysed. The residue from the dialysis of the second Fraction precipitate is the more potent and may be used separately. In practice the dialysis residues from Fractions 1 and 2 were usually mixed and the requisite amount of salt and preservative was added. The finished product by this method had a protein content of about 18 to 20 % and a unitage per c.c. about 4 to 5 times greater than that of the original plasma.

In 1912–13 Banzhaf introduced his One Fraction method in which serum or plasma, diluted with half its volume of water and made 30 % of saturation with ammonium sulphate, is brought up to a temperature of 60° C. and kept at that temperature for a few minutes only, the entire heating process taking about 2 hours in all.

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

The protein content of the finished product is about 20 % while the unitage per c.c. is 4 to 5 times that of the original serum.

Banzhaf's new method certainly possesses the following advantages over the former methods:

(1) There is only one finished product for dialysis.

(2) There is a considerable saving of time as the lengthy process of extracting the first fraction precipitate with brine is obviated.

(3) The expenses incurred in the process are considerably less, both as regards labour and the use of precipitating materials, etc.

But unfortunately practical experience shows that the method is not always easy to work as the production of a readily filterable end product which will remain clear is dependent upon the condition of the serum mixtures after the preliminary heating process. If the latter do not filter rapidly and easily the end product will not be satisfactory. In such cases it is necessary to allow the dialysis residues to stand in the ice chest for weeks so that the opalescent suspension therein may settle, otherwise filtration through filter candles is accomplished with great difficulty and the formation of a solid deposit in the antitoxin in its vial containers occurs.

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

In this laboratory we have ascertained that the irregularities in the filtration can be obviated by the addition of $1\frac{1}{2}$ to 2 % of solid sodium chloride to the serum mixtures thus:

The serum or plasma is diluted with one-half its volume of water, made 30 or 31 % of saturation with ammonium sulphate and to the mixture is added $1\frac{1}{2}$ to 2 % of solid sodium chloride. The temperature of the mixture is gradually raised to 61° or 63° C. and kept at the chosen temperature for not more than five minutes. The mixture is cooled to 40°–45° C. before filtration.

The filtration of the hot mixture and of the subsequent 50 % saturation mixture proceeds rapidly. Moreover the end product has, up to the present, proved satisfactory.

Banzhaf considers that in his new process 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 the operation. In a recent communication (1916), however, I have demonstrated that there is no appreciable conversion of soluble into insoluble protein under the heating conditions advocated in his One Fraction process. The value of the heating process lies in the 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 particles of euglobulin not sufficiently aggregated to be retained by the filter paper will ultimately be an impediment to the filtration of the final product through filter candles. Any suspensoid particles of euglobulin passing through the filter candles will impart an opalescent appearance to the finished product, and will gradually be deposited.

Also, I drew attention to the variability of the precipitating power of 30 and 31 % of saturation with ammonium sulphate. It was noticed that with sera in which there had been a comparatively slight removal of protein by 30 or 31 % of saturation with ammonium sulphate, the heated serum mixtures without exception filtered badly. The addition of $1\frac{1}{2}$ to 2 % of sodium chloride to the serum mixtures before heating remedied this defect.

From these and from other observations it was concluded that the reaction of the sera and of the serum mixtures must be taken into account before the Banzhaf New One Fraction method could be depended

on to furnish a permanently satisfactory end product. In this connection an investigation is being pursued as to whether the sodium chloride improves the filtration mainly by virtue of its specific action on globulins or by decreasing the hydrolysis of the phosphate of sodium to which the alkalinity is due. If the latter be the case then the desired improvement in filtration should be obtained by the addition to the medium of the amount of acid necessary to adjust the reaction to that required for optimum precipitation.

As the experimental work involved in this investigation must of necessity extend over a considerable period of time, I have, in the meantime, sought for some better method of removing from the serum mixture a relatively greater amount of protein without increasing the loss of antitoxin and without adding to the labour involved: the idea being to diminish as far as possible the amount of euglobulin in opalescent suspension in the final product.

The necessary clue was furnished by a study of the changes taking place in the solubility of serum proteins during the heating of serum or plasma (Table I). The changes may be summarised as follows:

(1) If serum or plasma be heated just to a temperature of 61° or 63° C. and kept at that temperature for a few minutes only, there is no appreciable change in the solubility of the serum proteins neither is there any change in the precipitating power of 30 % of saturation with ammonium sulphate. The heating process merely serves to completely aggregate the particles of precipitated euglobulin into a filterable form.

(2) If diluted serum or plasma be heated to a temperature of 61° or 63° C. and be kept at those temperatures for about 1 hour there is a considerable change, about 25 %, in the precipitating power of 30 % of saturation with ammonium sulphate. There is also a corresponding change if the serum or plasma be heated just to 67° (about 15 to 20 %).

(3) If serum or plasma diluted with one-third its volume of water be heated to a temperature of 55° C. for 15 hours there is only a slight change in the solubility of the serum proteins. But there is a 10 % increase in the precipitating power of 30 % saturation with ammonium sulphate.

(4) If diluted plasma or serum be heated to a temperature of 56°–57° for 15 hours, or to 58° for 7 to 8 hours, there is again only a slight change in the solubility of the serum proteins. But, the precipitating power of 30 % ammonium sulphate is increased from 30 to 49 %.

The changes in (4) are comparable with those observed by Banzhaf (1908). He regards them in the light of a conversion of pseudo- into

euglobulin, but in view of the work of Hardy (1899), Chick and Martin (1912) it is more probable that the changes taking place in (1), (2), (3) and (4) are of the nature of a heat denaturation of proteins.

In devising a new method for the concentration of antitoxic sera there were, in view of my experimental observations, the following factors to be borne in mind:

(1) The complete aggregation under the influence of heat of the suspensoid particles of precipitated euglobulin into conglomerates sufficiently large to be retained on the surface of filter paper.

(2) The heat denaturation of serum proteins whereby their precipitability by 30 % saturation with ammonium sulphate is considerably increased.

(3) The stimulating effect of the addition of 1·5 to 2 % of solid sodium chloride on the process in (2)[1].

Experimental work was then undertaken in order to ascertain the most favourable conditions under which to ensure the denaturation of the proteins without appreciable loss of antitoxin. It was found that the more rapid denaturation induced by heating the serum or plasma at 61° or 63° C. for an hour was followed by an appreciable loss of antitoxin. With the prolonged heating of the serum or plasma at 55° C. there was a slight loss only of antitoxin but at the same time the denaturation of the proteins was only 10 %. At temperatures between 56°–58° C. there was, comparatively speaking, a slight loss only of antitoxic units while there was a considerable denaturation of protein.

The technique adopted.

The serum or plasma, diluted with one-third or even with only one-fifth its volume of water, and made 1·5 to 2 % with solid sodium chloride, is heated to a temperature of 56°–57° C. for 15 hours or to 57°–58° C. for 8 hours[2] (Stage I).

During the prolonged heating a considerable change in the precipitability of the proteins occurs (Tables II *a* and *b*).

[1] Our experiments so far are in favour of the adjustment of the medium by means of salt rather than by the addition of acid.

[2] We prefer to heat the serum or plasma at the lower temperature for the longer period. But, in the routine work it is sometimes more convenient to make arrangements for an 8 hours' than for a 15 hours' heating.

It is not really necessary to dilute the plasma or serum in order to ensure the required change during the prolonged heating, for the heat denaturation takes place to the same extent in the undiluted as in the diluted fluid. But in order to obtain the best conditions

The heated plasma is then made 30 % of saturation with ammonium sulphate and the mixture is heated to a temperature of 61° C. and kept at that temperature for a few minutes only (Stage II).

By raising the temperature of the serum-ammonium-sulphate mixtures to 61° the aggregation of the particles of suspensoid protein into a precipitate which can be readily separated by filtration through paper takes place without the precipitation of a further amount of soluble protein and without loss of antitoxin.

The mixture after cooling to 40°–45° C. is filtered. The precipitate is washed with 33 % of saturation with ammonium sulphate. The washings, after being filtered, are added to the main bulk of the filtrate which is then made 50 % of saturation with ammonium sulphate.

The resulting precipitate is filtered off, pressed and dialysed. The pressed precipitate has a yellowish colour and not the bluish green colour of Banzhaf's product.

To the residues from dialysis are added the necessary amounts of salt and preservative. The final products in bulk are of a reddish brown colour and do not exhibit even a trace of an opalescent suspension. In layers of about 1 to 2 inches thick the liquids are transparent and are of a yellowish brown colour.

The filtration of these products through pulp and through Pasteur-Chamberland filters, up to the time of writing, has presented no difficulty. Moreover the dilution of the dialysates with two or three times their bulk of 1 % saline does not impair their ease of filtration. In this respect alone the final product is superior to that obtained in the Banzhaf One Fraction process.

In the method thus advocated in this paper there have been incorporated the important factors in the Banzhaf-Gibson Two Fraction method, the Banzhaf One Fraction method and in my modification of the latter.

The method was carried out at first on an experimental scale and promised success: it was then used with a batch of 10 litres of oxalated plasma. It was found that, during the process the removal of about

for a satisfactory precipitation and subsequent heat aggregation of the protein precipitated by 30 % saturation with ammonium sulphate it is, in our opinion, necessary to make the above dilution with water before adding the necessary amount of ammonium sulphate solution.

So far as the denaturation without loss of antitoxin is concerned the same results may be obtained by making the plasma or serum 30 % of saturation with ammonium sulphate previous to Stage I of the heating process. But in this case the end product is not so satisfactory: it is distinctly opalescent.

70 % of the total proteins of the original plasma was effected, and at the same time, even though the precipitate from the 30 % saturation with ammonium sulphate had not been extracted, there was only a loss of 10 % of antitoxic units. The end product was so satisfactory that routine concentrations were carried out by this method.

Several batches of plasma and of serum, each of 100 litres, have been concentrated so far with excellent results both for tetanus and for diphtheria antitoxic sera. The details of typical concentrations carried out by this method and by the Banzhaf One Fraction process are given in Table III.

The denaturation of the serum proteins during the prolonged heating of the serum and their consequent increased precipitation by 30 % of saturation with ammonium sulphate has led to the preparation of a final product in which the protein content is about 17–19 % while the unitage per c.c. has been increased to 8–9 times that of the original serum. Moreover if the process be carried out with the customary precautions against undue loss and if all precipitates, filter papers, etc., used in the process be carefully washed with 33 % ammonium sulphate and added to the main filtrates from the first precipitate, the loss of antitoxic units need not be greater than 10 %; in fact it can be reduced to as little as 5 %.

Furthermore, the final product is a clear limpid fluid which, even on dilution with 2 or 3 parts of a 1 % salt solution, shows no trace of opalescence and therefore should remain permanently clear.

The method given above is an improvement on

A. *The Gibson-Banzhaf Method for four reasons.*

(1) There is only one fraction to consider and the extraction of the first precipitate with brine is no longer necessary. This involves the saving of time, labour and materials.

(2) The heating of the serum made 30 % of saturation with ammonium sulphate serves to agglutinate the suspensoid particles of protein into conglomerates sufficiently large to be retained by filter paper. In the Gibson-Banzhaf process, in order to ensure the retention of these particles, it was often necessary to increase the percentage of ammonium sulphate to 35.

(3) The potency of the finished product is nearly *twice* as great as that obtained by the Banzhaf-Gibson process while the protein content is only 17 to 19 %.

(4) The total loss of antitoxic units need not be greater than 10 %.

And on

B. *The Banzhaf One Fraction Process for three reasons.*

(1) By the removal of a much greater amount of protein, as a result of the heat denaturation of serum proteins and their consequent increased precipitability by 30 % of saturation with ammonium sulphate, there is less likelihood of filtration difficulties.

(2) By the addition of $1\frac{1}{2}$ to 2 % of sodium chloride to the serum mixture there is an adjustment of the reaction of the medium towards the conditions required for more complete precipitation of the particular proteins thrown out of emulsoid solution by the 30 % saturation with ammonium sulphate. This factor has a beneficial effect on filtration.

(3) The finished product is nearly *twice* as potent per c.c. as that obtained by the concentration of the same sera by Banzhaf's One Fraction method without containing a higher percentage of protein.

SUMMARY.

The method for the concentration of antitoxic sera suggested in this communication and now being used in this laboratory, presents a further step towards the desired goal, viz. the preparation, on a commercial scale for general therapeutic use, of antitoxic sera with a minimal amount of attendant protein.

In the concentration of sera on a large scale by the routine methods hitherto published the final products have shown a protein content of about 18 to 20 % with a potency per c.c. four to five times that of the original serum. The above described method yields, as a matter of routine, a final product with a protein content of about 17 to 19 % and with a potency per c.c. as much as nine times that of the original serum. With the use of better processes the potency might be higher still.

It is worth while to concentrate by this method plasma or sera in which the unitage is so low that hitherto they would have been discarded as having too low a potency for use even after concentration.

The patient will receive a relatively higher percentage of antitoxic units per gram of protein than if the sera had been concentrated by other methods, for by this method the removal of 70 % of the total proteins is ensured.

The heat denaturation of the proteins during the prolonged heating of the serum and consequent diminution of protein relatively to antitoxin may serve to minimise troubles with serum sickness.

In conclusion I desire to express my thanks to Dr A. T. MacConkey for the interest he has taken in the work and for the facilities accorded to me for the furtherance of the investigation.

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TABLE I.

The effect of the heating of serum or oxalated plasma (a) *on the solubility of the serum proteins and* (b) *on their precipitability by* 30 % *of saturation with ammonium sulphate.*

	Percentage protein content of serum or plasma	Proportion of water added to the serum or plasma before heating	Temperature to which diluted serum or plasma was heated	Duration of the time of heating at the chosen temperature	(a) Percentage of protein in solution, calculated from the Refractometer Readings and expressed in terms of the original serum or plasma	(a) Percentage conversion of soluble into insoluble protein during the heating process	(b) Percentage of protein in solution in the filtrates from the serum or plasma in (a) made 30 % of saturation with ammonium sulphate. Calculated from the Refractometer Readings and expressed in terms of the original plasma or serum	(b) Percentage increase in the precipitation of protein by 30 % of saturation with ammonium sulphate
I	7·78	½	Room temperature		7·78	—	6·11	
	7·78	½	63° C.	5 minutes	7·52	3·3 %	5·92	3·0 %
II	6·49	½	Room temperature		—	—	5·76	
	6·49	½	63° C.	¾ hr	—	—	4·28	24·0 %
III	6·23	½	Room temperature		—	—	4·98	
	6·23	½	67° C.	5 minutes	—	—	4·00	19·8 %
IV	7·39*	⅕	Room temperature		7·39	—	5·35	
	7·39	⅕	55° C.	15 hrs	6·89	6·7 %	4·81	10·0 %
V	7·38	⅓	Room temperature		7·38	—	5·12	
	7·38	⅓	56°–57° C.	4 hrs	7·05	4·5 %	—	
	7·38	⅓	56°–57° C.	8 hrs	7·01	5·0 %	4·82	5·5 %
VI	6·13	⅓	Room temperature		6·13	—	5·78	
	6·13	⅓	56°–57° C.	6 hrs	5·99	2·3 %	5·48	5·2 %
	6·13	⅓	56°–57° C.	15 hrs	5·79	5·5 %	3·52	39·1 %
VII	7·10	⅓	Room temperature		7·10	—	5·52	
	7·10	⅓	58° C.	7–8 hrs	6·99	1·5 %	3·76	31·9 %
VIII	7·59*	⅕	Room temperature		—	—	4·88	
	7·59	⅕	58° C.	7–8 hrs	—	—	3·56	28·7 %

* In these cases an addition of 1·5 % of sodium chloride had been made.

TABLES II *a* AND *b*.

Changes in the serum proteins during the two stages of the heating process adopted in the method described in this paper.

In Table II *a*, during Stage I, the diluted oxalated plasma or serum, to which had been added 2 % of salt, was heated to 56°–57° for 15 hours. In Table II *b* this preliminary heating was conducted at a temperature of 58° for 7–8 hours.

In both cases the heated liquids were made 30 % of saturation with ammonium sulphate and the mixtures were then heated just to a temperature of 61° (Stage II).

TABLE II *a*. *Batch T.R.* 35.

	(i) Changes in the solubility of the Serum Proteins. Percentage of protein in solution calculated from the Refractometer Readings and expressed in terms of the original plasma	(ii) Changes in the precipitability of proteins by 30 % of saturation with Am_2SO_4. Percentage of protein in solution in the filtrates from making the serum in (i) 30 % of saturation with Am_2SO_4
STAGE I. *The prolonged heating of the diluted serum + 2 % NaCl at 56°–57° for 15 hours.*		
Serum + ⅔ its vol. of H_2O + 2 % NaCl (= A)	6·16	5·90
Serum + ⅔ its vol. of H_2O + 2 % NaCl heated to 56°–57° C. for 15 hours (= B)	5·90	3·90
STAGE II. *The rapid heating of B made 30 % with Am_2SO_4 just to 61° C.*		
Mixture B made 30 % of saturation with Am_2SO_4	—	3·90
do. do. heated just to 61° C.	—	3·90

Thus during the 15 hours' heating in Stage I there has been an *increased precipitation of* 34·5 % of the soluble protein by 30 % of saturation with ammonium sulphate. During the heating process in Stage II there has been no further conversion of soluble into insoluble protein.

TABLE II *b*. *Batch T.R.* 38.

	(i) Changes in the solubility of the Serum Proteins. Percentage of protein in solution calculated from the Refractometer Readings and expressed in terms of the original plasma	(ii) Changes in the precipitability of proteins by 30% of saturation with Am_2SO_4. Percentage of protein in solution in the filtrates from making the serum in (i) 30% of saturation with Am_2SO_4
STAGE I. *The prolonged heating of the diluted serum + 1½ % NaCl at 57°–58° for 8 hours.*		
Serum + ⅓ its vol. of H_2O + 1½% NaCl (=A)	7·10	5·52
Serum + ⅓ its vol. of H_2O + 1½% NaCl after being heated to 57°–58° C. for 8 hours (=B)	6·99	3·76
STAGE II. *The rapid heating of B made 30 % with Am_2SO_4 just to 61° C.*		
B made 30 % of saturation with Am_2SO_4	—	3·76
do. do. and heated just to 61° C.	—	3·76

During the 8 hours' heating of the serum in Stage I there has been *an increased precipitation of* 31·3 % of the soluble protein by 30 % of saturation with ammonium sulphate. During the heating process in Stage II there has been no further conversion of soluble into insoluble protein.

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TABLE III.

A comparison between the results obtained for the concentration of antitoxic sera by the Banzhaf One Fraction method and by the method advocated in this paper.

	Sera concentrated by Banzhaf's One Fraction method	**Sera concentrated by the new method given above**
A.	*Diphtheria Antitoxic Sera.*	
	Batch No. D.R. 31 (50 litres of oxalated plasma)	Batch No. D.R. 33 (100 litres oxalated plasma)
	Before concentration: Unitage per c.c. = 500; Protein content = 7·00 %	*Before concentration:* Unitage per c.c. = 275; Protein content = 7·39 %
	After concentration: Unitage per c.c. = 2000; Protein content = 20·10 %	*After concentration:* Unitage per c.c. = 2200; Protein content = 17·5 %
	Loss of antitoxic units during the process = 12 %	**Loss of antitoxic units during the process = 8 %**
	Amount of protein removed = 25·4 % of total	**Amount of protein removed = 70·4 % of total**
	Potency increased by 4 times	**Potency increased by 8 times**
B.	*Tetanus Antitoxic Sera.*	
	Batch No. T.R. 31 (100 litres)	Batch No. 35 T.R. (100 litres)
	Before concentration: Unitage per c.c. = 175; Protein content = 6·99 %	*Before concentration:* Unitage per c.c. = 100; Protein content = 6·47 %
	After concentration: Unitage per c.c. = 850; Protein content = 20·2 %	*After concentration:* Unitage per c.c. = 900; Protein content = 19·0 %
	Loss of antitoxic units during the process = 20 %*	**Loss of antitoxic units during the process = 10 %**
	Amount of protein removed = 40·4 % of total	**Amount of protein removed = 68·9 % of total**
	Potency increased by 5 times (nearly)	**Potency increased by 9 times**

* In this particular concentration the percentage loss of antitoxic units was greater than usual. Our average loss by the Banzhaf One Fraction process is of the order of 10 %.

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