ON FACTORS LIMITING THE EXTENT OF THE CONCENTRATION OF ANTITOXIC SERA BY THE FRACTIONAL PRECIPITATION METHODS AT PRESENT EMPLOYED.

By ANNIE HOMER.

(*From the Lister Institute, Elstree.*)

The use of concentrated antitoxic sera in the treatment of diphtheria and tetanus has been generally recommended with a view to minimising the troubles of serum sickness. For, during the process of concentration of sera, there is a removal of the proteins not associated with antitoxin. It is thus possible to administer large doses of antitoxin with far less discomfort to the patient and with the introduction of a considerably smaller amount of the proteins of horse serum than if the original unconcentrated sera had been used.

The aim of any concentration process is therefore the preparation, on a commercial scale for therapeutic uses, of antitoxic sera which shall contain a minimum amount of attendant protein.

In a previous communication (Homer, 1916) it has been shown that, in the method recently adopted in this laboratory for the concentration of sera, a removal of from 65 to 70 % of the total proteins of the original serum is effected with a consequent increased potency eight to nine times that of the original serum.

The new method thus presented a step towards the desired goal, for by previous methods there had been a removal of about 40 % of the total proteins with a consequent increased potency of from four to five times. From experimental observations it was evident that the heat denaturation[1] of the serum proteins was the primary factor influencing the removal of protein beyond that obtained by the methods previously in vogue.

[1] Throughout this paper the term "heat denaturation" is used as a convenient term for the increased precipitability of the serum proteins by 30 % of saturation with ammonium sulphate induced by the prolonged heating of serum.

https://doi.org/10.1017/S0022172400006434 Published online by Cambridge University Press

Some definite relationship thus exists between the extent of the removal of unnecessary protein from the serum and the degree of concentration of antitoxin in the final product. Therefore the question arose as to whether the heat denaturation of the proteins could not be induced to an extent greater than that obtained in our new process so as to yield a final product in which the percentage of protein had been still further reduced.

In the course of experimental work I have ascertained that the heat denaturation of the serum proteins is considerably influenced by the presence of acid and of electrolytes. But, as was anticipated, the presence of acid during the prolonged heating of sera, even at 57–58° C., under some conditions has a deleterious effect on the antitoxin: a loss of as much as 50 % of the antitoxin has been measured. On the other hand, as is shown in Table I, in the presence of the electrolytes employed there was no appreciable destruction of antitoxin at a temperature of 57–58° C. Heating the serum in presence of 30 % of ammonium sulphate to a temperature of 61° and 63° for 2 hours resulted in the destruction of 20 % of the antitoxin. With 10 to 20 % of sodium chloride there was no loss after $2\frac{1}{2}$ hours' heating at 62° C. But, when the serum was saturated with salt and heated to 62° for $2\frac{1}{2}$ hours the mixture coagulated; the residue after dilution with an equal bulk of 1 % saline, was tested for its antitoxin value and showed a loss of about 50 %.

In view of these results the use of sodium chloride as the denaturating agent was more carefully investigated.

Serum or plasma was diluted with one-third its volume of water, definite amounts of the electrolyte were added and the diluted serum was then heated to a temperature of 57–58° C. for periods of 7 to 8 hours. The heated liquid was made 30 % of saturation with ammonium sulphate, the temperature raised just to 60° C., and the mixture cooled to 50° C. and filtered. The precipitate (*First Fraction Precipitate*) was washed with 30 % saturated ammonium sulphate and the washings added to the main bulk of the filtrate. The filtrate and washings were then brought up to 50 % of saturation with ammonium sulphate and filtered. The precipitate (*Second Fraction Precipitate*) was pressed and dialysed. The residue from the dialysis of this precipitate was made 0·35 % with cresylic acid and the necessary amount of salt was added (*Final Product*).

The protein and the antitoxic contents of the final products were estimated in the usual way and were compared with those of the original serum.

TABLE I.

The loss of antitoxic units during the prolonged heating of antitoxic sera to which electrolytes have been added.

In each case the serum or plasma was diluted with one-third its volume of water previous to the addition of the electrolyte.

	Electrolytes added to the diluted plasma	Temperature to which the plasma, after the addition of the electrolyte, was heated	Duration of the time of heating at the specified temperature	Unitage of the original serum	Percentage loss of antitoxic units during the heating process
Tetanus:					
Adelaide 1/6/16	1½ % NaCl	57–58° C.	8 hours	150	negligible
Adelaide 1/6/16	4 ,,	,,	8 ,,	150	,,
Diphtheria:					
△ R. 41	2 ,,	,,	8 ,,	250	,,
△ R. 42	5 ,,	,,	4 ,,	250	,,
△ R. 40	2 ,,	,,	8 ,,	500	,,
Lille 26/6/16	8 ,,	,,	8 ,,	150	less than 10 %*
Lille 26/6/16	20 ,,	,,	8 ,,	150	about 10 %
Ethel 2/5/16	20 ,,	,,	8 ,,	350	negligible
Lille 26/6/16	Saturated with NaCl	,,	8 ,,	150	,,
Ethel 2/5/16	Saturated with NaCl	,,	8 ,,	350	,,
Lille 26/6/16	2 % NaCl + 30 % AM_2SO_4	,,	8 ,,	150	,,
Ethel 2/5/16	2 % NaCl + 30 % AM_2SO_4	61°	2 ,,	350	20 %
Ethel 2/5/16	2 % NaCl + 30 % AM_2SO_4	63°	2 ,,	350	,,
Ethel 10/10/16	10 % NaCl	62°	2½ ,,	300	negligible
Ethel 10/10/16	20 % NaCl	,,	2½ ,,	300	,,
Ethel 10/10/16	Sat. NaCl	,,	2½ ,,	300	about 50 %

* Not tested out.

TABLE II *a*.

The precipitation of serum proteins by the addition of sodium chloride to plasma (1) *previously diluted with half its volume of water, and* (2) *previously diluted with half its volume of water and made* 30 % *of saturation with ammonium sulphate.*

Percentage of NaCl added to the diluted plasma.	(1) Percentage of Residual Protein in solution in the plasma after the addition of the various percentages of NaCl. Expressed in terms of the original plasma	(2) Percentage of Residual Protein in solution after making the salted plasma 30% saturation with ammonium sulphate. Expressed in terms of the original plasma
0	7·70	6·20
1	7·68	6·20
2	7·68	5·50
3	7·65	5·38
4	7·37	5·37
5	7·32	5·32
6	7·32	5·30
7	7·32	4·15
8	7·19	3·75
9	6·99	3·70
10	6·86	3·70

TABLE II *b*.

Showing the influence exercised by sodium chloride on the precipitation of antitoxin along with the First Fraction Precipitate *both* (1) *before and* (2) *after the prolonged heating of the serum.*

Serum	Unitage	Percentage of salt added to the diluted plasma	Unitage of the filtrate from the First Fraction Precipitate
(1) *The unheated plasma:*			
Adelaide 25/5/16 Tetanus	150	4	150 (guinea pig used in testing the serum for this unitage died in 90 hours)
Adelaide 25/5/16 ,,	150	7	150 (guinea pig used in testing the serum for this unitage died within 48 hours)
(2) *The heated plasma:*			
Adelaide 1/6/16 Tetanus	150	2	145 nearly (guinea pig used in testing the serum for this unitage died in 90 hours)
Adelaide 1/6/16 ,,	150	4	145 just
Adelaide 1/6/16 ,,	150	8	100 (guinea pig used in testing the serum for this unitage died within 90 hours)

Where the percentage of sodium chloride added to the serum had been greater than 5 it was found advisable to dilute the heated liquid before the addition of ammonium sulphate for the first precipitation. Otherwise as is shown in Tables II*a* and II*b* there is an increased precipitation of protein and of antitoxin in the first precipitate both in the unheated and in the heated plasma.

In Table III*a* have been incorporated the results of those concentrations in which the antitoxic units of the original serum were, within the limits of experimental error, fully accounted for in the final product.

In Table III*b* have been embodied the results of concentrations in which, although there had been no loss of antitoxin during the heating process, the final product showed an appreciable loss of antitoxic units.

A further investigation, however, demonstrated that in the concentrations thus studied the missing antitoxin had been brought down in the First Fraction Precipitate. None was detected in the albumin fraction (filtate from Second Fraction Precipitate). Moreover, the antitoxin thus brought down with the First Fraction Precipitate could not be recovered by washing the precipitate with 30 % saturated ammonium sulphate: for this purpose extraction with saturated salt solution was essential.

A study of the data in Tables III*a* and III*b* reveals the fact that *there is a limit to the extent to which the removal of the serum proteins can be effected without disturbing the precipitation of the antitoxin with the Second Fraction Precipitate.* For, it is apparent that if the percentage removal of the total serum proteins be increased much beyond 80 %, there is a consequent increased precipitation of antitoxin in the First Fraction Precipitate.

It is also obvious from Table III*a* that the order of the increased potency measured in the Final Product depends upon two factors. The one factor is the extent of the denaturation induced during the heating process and its effect on the removal of proteins during the concentration processes. The other factor is the efficacy of the pressing of the Second Fraction Precipitate before dialysis.

From the results thus recorded it will be seen that it has been possible to concentrate sera by as much as 12 times while the percentage protein content of the Final Product has not been greater than 20.

In an experimental concentration in which it was calculated that the potency would be increased by 15 times it was however found to be only $8\frac{1}{2}$ times that of the original. The antitoxin, thus not present

TABLE III *a*.

Showing the percentage of the total antitoxic units of the original serum recovered in the Second Fraction Precipitate *in cases where the percentage removal of the proteins of the original serum during the concentration process has not been greater than* 80.

	Unitage of the original serum	Percentage protein content of the original serum	Percentage of electrolytes added to the plasma	Percentage loss of antitoxic units during the heating process	Extent of the heat denaturation	Percentage removal of the total serum proteins during the concentration	*Second Fraction Precipitate after Dialysis*			Percentage of the total original antitoxic units recovered in the Second Fraction Precipitate
							Percentage protein content	Unitage per c.c.	Increased potency per c.c. over that of the original serum	
Tetanus:										
Adelaide	150	6·16	8 % NaCl	negligible	48 %	79·0	13·3	1800	12 times (nearly)	95
Diphtheria:										
Lille 26/6/16	150	7·26	8 % ,,	,,	35 %	80·0	14·24	1100	$7\frac{1}{3}$,,	76
Lille 26/6/16	150	7·26	12 % ,,	,,	40 %	71·0	17·3	1600	$10\frac{1}{2}$,,	94
Lille 26/6/16	150	7·26	20 % ,,	about 10 %	not estimated	70·0	15·0	900	6 ,,	80
Smyrna* 7/7/16	600	9·68	7 % ,,	not estimated	2 %	68·2	20·0	2750	$4\frac{1}{2}$,,	75
Lille 26/6/16	150	7·26	30 % Am_2SO_4 2 % NaCl	negligible	20·6 %	71·0	10·5	800	5·6	87·5
Tetanus:										
T.R. 35	100	6·61	2 % NaCl	not tested†	42·2 %	68·9	19·5	900	9 ,,	90
T.R. 36	150	7·59	,,	,, †	28·7 %	70·0	19·2	1300	$8\frac{1}{2}$,,	80
T.R. 37	200	7·10	,,	,, †	31·3 %	60·7	18·04	1300	$6\frac{1}{2}$,,	91
T.R. 38	100	6·60	,,	,, †	32·0 %	72·0	15·62	850	$8\frac{1}{2}$,,	85
Diphtheria:										
△R. 33	275	—	,,	,, †	—	70·4	17·5	2200	8 ,,	92
△R. 39	400	8·51	,,	,, †	31·1 %	77·3	19·33	3000	$7\frac{1}{2}$,,	80
△R. 40	500	7·57	,,	negligible	23·5 %	60·0	17·0	2500	5 ,,	85
△R. 42*b*	250	6·60	5 % NaCl	negligible	41·2 %	70·0	18·6	2250	$8\frac{1}{2}$,,	86

* Concentrated by Banzhaf's 1913 Method.

† We have so often satisfied ourselves that there is no appreciable loss of antitoxin during the heating of serum to which has been added 2 % of NaCl, to a temperature of 57–58° C. for a period of 7–8 hours, that we consider it as established that at this stage in the concentration there is no loss of antitoxin.

TABLE III *b*.

Showing the percentage of the total antitoxic units of the original serum recovered in the Second Fraction Precipitate *in cases where the percentage removal of the proteins of the original serum during the concentration process has been greater than* 80.

	Unitage of the original serum	Percentage protein content of the original serum	Percentage of NaCl added to the diluted plasma	Temperature to which the plasma was heated	Duration of the heating at the specified temperature	Percentage loss of antitoxic units during the heating process	Extent of the heat denaturation	Percentage of the total proteins removed during the process of concentration	Percentage of original antitoxin units appearing in the dialysed Second Fraction Precipitate	Presence or absence of antitoxin in the First Fraction Precipitate which requires to be extracted with brine
Diphtheria:										
Lille 26/6/16	150	7·26•	20	57–58° C.	8 hours	about 10 %	51 %	83·2	60	+ ve*
Ethel 2/5/16	350	8·62	20	,,	,,	negligible	50 %	90†	23	+ ve (found to be 53·5 %)
Lille 26/6/16	150	7·26	saturated with NaCl	,,	,,	negligible	60 %	83·6	50	+ ve*
Ethel 2/5/16	350	8·62	saturated with NaCl	,,	,,	negligible	not tested	86·8	44	+ ve (at least 20 %)*
△ R. 35	300	6·60	10 % NaCl and 0·3% Trikresol	,,		negligible	50 %	89·5	66	+ ve*
Tetanus:										
Adelaide 1/6/16	150	6·16	8 %	,,	,,	negligible	50 %	85·1	70	+ ve*

* Not tested out because of scarcity of experimental guinea-pigs.

† The heated serum was not diluted before precipitating with ammonium sulphate.

in the final product, was found to be in the First Fraction Precipitate. The phenomenon was somewhat unexpected as the percentage removal of total serum proteins was not greater than 66 and was well within the limit indicated in Table III*a*.

This observation, in conjunction with those given in Table III*b*, naturally suggested that there might be a maximum load of antitoxin which could be carried by a fixed amount of protein in the Final Product obtained by the Fractional Precipitation Methods at present in use for the concentration of sera.

In order to elucidate this point experimental concentrations of high potency antidiphtheritic sera were carried out. The sera were concentrated by Banzhaf's One Fraction Method which will yield a concentration of about 4–5 times with a removal of about 40–50 % of the total serum proteins. The same sera were also concentrated by my method which, with a heat denaturation of 30 to 40 % will yield a concentration of about 8 times.

From the results of these concentrations, which have been embodied in Table IV, it appears that *there is a limit to the amount of antitoxin which can be associated with a given weight of protein in the Final Product*: this maximum amount is of the order of 20,000 to 25,000 diphtheria antitoxin units per gramme of protein.

The possible load of tetanus antitoxin units per gramme of protein beyond 13,000 units has not been worked out as high potency tetanus antitoxic sera could not be spared at the present time for experimental work.

In those cases, Table IV, in which the load, by estimation, should have been of the order of 30,000 units per gramme of protein or more, it was found that the excess of units beyond the aforesaid limit had been transferred to the First Fraction Precipitate in spite of the fact that the percentage removal of proteins was less than 80.

We thus see that, by the fractional precipitation methods at present in vogue for the concentration of antitoxic sera, the degree of concentration is limited by two factors:

(1) The extent of the removal of the total proteins of the original serum; and

(2) the maximum load of antitoxic units which can be associated with one gramme of protein in the final product.

There will be transference of antitoxin to the First Fraction Precipitate if the percentage removal of proteins be greater than 80 or if

TABLE IV.

The maximum load of antitoxin which can be associated with one gramme of protein in the residues from the dialysis of the Second Fraction Precipitate (*i.e. in the* Final Product), *in the concentration of antitoxic sera by the Fractional Precipitation Methods now in vogue.*

	Unitage of the original serum per c.c.	Protein content of the original serum %	Concentration process adopted	Denaturation induced during the heating process %	Percentage of the total protein of the original serum removed during the concentration process	*Anticipated* unitage of the final product per c.c.	In the Final Product was found: Unitage per c.c.	Protein content %	Number of antitoxic units per gramme of protein	Percentage of the total antitoxic units of the original serum appearing in the Final Product	Antitoxin carried down with the First Fraction Precipitate not extractable with 30% of ammonium sulphate but extractable with brine
Aurora 19/6/16	1100	10·03	Banzhaf (1913)	1·0	47	3500	3500	17·6	20,000	93	negligible
Aurora ,,	1100	10·03	†Homer (1916)	13·0	54·5	4500	4250	19·8	21,500	93	,,
Aurora ,,	1100	10·03	†Homer (1916)	44·0	70	6600	4750	20·2	23,500	65	22·5 %
Aurora 21/8/16	900	8·18	Banzhaf (1913)	2·0	40	3600	3000	17·2	18,300	85·5	negligible
Aurora ,,	900	8·18	†Homer (1916)	34·5	56	5400	4750	21·3	22,300	88	,,
Lloyd George 5/9/16	800	8·74	Homer (1916)	37·0	72	7000	4400	20·8	21,200	59	+ ve*
Aurora 5/9/16	700	7·08	†Homer (1916)	30·0	70	6000	3600	20·6	17,500	57	+ ve*
San 5/9/16	750	7·26	Homer (1916)	41·0	70	6500	4500	21·1	21,300	66·5	+ ve*

* Owing to scarcity of experimental guinea-pigs these products were not tested out

† We have many times demonstrated that there is no appreciable loss of antitoxin during the prolonged heating of sera by my method. This was again verified with the concentrations of the sera thus indicated.

the load of antitoxin threatens to be greater than of the order of 20,000 to 25,000 units per gramme of protein[1].

In a previous communication (Homer, 1916) I have drawn attention to the necessity for a study of the hydrogen ion concentration of the sera to be concentrated. Additional evidence in support of this point has been forthcoming during the progress of the present investigation. It will be seen (Table III*a*) that, in routine concentrations, the heat denaturations varied from 20 to 45 % and as a matter of practical experience it has been found that the most satisfactory end products, from the standpoint of filtration, were obtained where the heat denaturations had been of the order of 30 to 40 %.

I have ascertained that these variations are due to differences in hydrogen ion concentrations of the respective sera. Moreover, by altering the reaction of the sera it is possible to produce wide variations in the heat denaturations, although, apparently, the heat denaturation cannot be induced beyond certain limits without destruction of antitoxin.

The adjustment of the reaction of the batches of sera for concentration to a definite hydrogen ion concentration is proving of much value as it leads to uniform and consistent results. The details of the procedure adopted for the standardisation of the concentration process by an adjustment of the hydrogen ion concentration of the sera will form the subject of a further communication.

SUMMARY.

The following conclusions of practical value to those engaged in serum concentration can be drawn from the experimental work described above:

(1) An end product containing not more than 20 % of protein and having a potency of 8 or 10 times that of the original can be obtained as a matter of routine where the pooled antidiphtheritic sera has a unitage not greater than 500, and where the heat denaturation is of the order of 40 %.

[1] Banzhaf and Gibson (1907) working on an experimental scale with small quantities of unheated serum, state that, by successively fractionally precipitating with ammonium sulphate within narrow limits (50 + and 50–56 % of saturation), they have obtained a high potency fraction of the order of 45,000 units per gramme of protein.

Up to the present, working with heated sera, I have been unable to obtain a fraction with so high a unitage per gramme of protein.

(2) By the fractional precipitation methods at present employed for the concentration of antitoxic sera for therapeutic use the degree of concentration of high potency sera cannot be taken beyond the limit of about 22,500 units of antitoxin per gramme of protein in the end product.

REFERENCES.

BANZHAF and GIBSON (1907). *Coll. Studies Research Lab. Dept. Public Health.* New York, III. 97.

HOMER (1916). *Journal of Hygiene,* XV. 388.

—— —— *Biochemical Journal,* X. 280.

https://doi.org/10.1017/S0022172400006434 Published online by Cambridge University Press