XVII. ON THE CONCENTRATION OF ANTITOXIC SERA BY THE SALTING OUT OF THE HEAT-DENATURATED SERUM PROTEINS WITH SODIUM CHLORIDE.

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A RECENT investigation of the factors influencing the concentration of antitoxic sera by methods involving the use of ammonium sulphate has shown that the degree of concentration attained is regulated by the extent of the denaturation of the serum proteins induced during the heating of the serum. There is, however, a limit to the degree of concentration. In cases where the heat-denaturation is greater than about 50 %, the attempted separation of the antitoxin from the heated sera by means of ammonium sulphate leads to the production of end products showing a considerable loss of antitoxin. The missing antitoxin is carried down with the First Fraction precipitates (i.e. at 30 % of saturation with ammonium sulphate) and for its recovery it is necessary to extract those precipitates with brine [Homer, 1917, 1].

Even in our routine work where the heat-denaturation of the serum proteins is of the order of 30 %, we have found it advisable, for reasons based on the above observations, to resort to the extraction of the First Fraction precipitates with brine. As the adoption of this procedure necessitates the use of two "salting out" agents it seemed worth while to ascertain whether the process of concentration could be shortened by treating the heated sera directly with sodium chloride, thus dispensing with the use of ammonium sulphate.

In this connexion it was necessary to make a further study, not only of the factors influencing the heat-denaturation of the serum proteins, but also of the extent to which the denaturation can be carried without rendering the protein-antitoxin complex insoluble in brine. The work, which is still in progress, has

shown that the extent of the heat-denaturation of the serum proteins depends on:

(1) The reaction of the serum or plasma previous to its being heated [Homer, 1917, 2].

(2) The temperature at which the heating is conducted.

In all cases the maximum values are more quickly reached in acid than in alkaline sera and, irrespective of the $[H^+]$ of the sera, the time taken is far less at high than at low temperatures.

It was also found that the maximum values for the heat-denaturation at a specific reaction and at a specific temperature could be increased by the addition of substances such as phenol and its homologues, ether, chloroform, or electrolytes to the serum previous to its being heated.

The knowledge thus gained has been applied to the present investigation. Pending the completion of a series of experiments dealing with the effect of heating sera at temperatures ranging from 58 to 72° on the extent of the heat-denaturation of the serum proteins, and the bearing of the latter on the solubility of the thus denaturated proteins and on the inactivation of antitoxin, my experiments have been conducted at temperatures between 57° and 58°, for, it has been shown that during the prolonged heating of sera at this temperature there is no actual destruction of antitoxin even in those cases in which an extensive heat-denaturation of the serum proteins has been induced.

In order to ensure the desired heat-denaturation, the plasma, previous to being heated at 57–58°, was subjected to one of the following methods of treatment:

(I) The reaction was suitably adjusted by the addition of acid or alkali;

(II) To the plasma was added 2 % of sodium chloride followed by fixed amounts of phenol or its homologues; or

(III) To the plasma were added various amounts of electrolytes.

The treated plasma was then heated at 57–58° for the necessary time. At the end of the heating period (*vide infra*) there were added to the hot serum twice its volume of a saturated solution of sodium chloride and a sufficient amount of solid salt to ensure complete saturation of the brine-serum mixture¹. The liquids were allowed to stand at room temperature for several days.

It was found that after the mixtures had stood for two to three days there was then, practically speaking, a complete extraction of the antitoxin

¹ Tested by means of a hydrometer.

from those sera which, during the heating, had remained clear or had become opalescent, or of which the consistency had been changed into that of a thin suspension unfilterable through paper. In those cases in which the consistency of the serum had been changed to that of a thick suspension, due to the actual precipitation of particles of heat-denaturated protein, a much longer time was required for the extraction of the antitoxin by the brine. Thus, with the sera showing a heat-denaturation of 40 to 45 %, about 6 to 8 days' extraction was sufficient for the extraction of the antitoxin. On the other hand, even after several weeks' extraction, the recovery of antitoxin was far from complete from those sera in which a more extensive heat-denaturation and correspondingly increased precipitation of heat-denaturated protein had been induced.

After the brined liquids had stood at room temperature for the necessary length of time they were filtered. Except where phenol and cresylic acid had been added, there was slow filtration of the brined extract of those sera of which the reaction, previous to the heating, lay between P_H 5.5 and 7.2. The slow filtration was due to the presence of heat-denaturated protein in a semi-gelatinous condition. The difficulty in filtration was obviated by gradually warming the liquids to a temperature of 45°, thereby inducing the coagulation of the particles of protein into aggregates of sufficient dimensions to enable their separation to be effected by filtration through paper.

The precipitates were washed with brine and the washings, after filtration, were added to the main bulk of the filtrates to which was then added 0.25 % of its volume of glacial acetic acid. The ensuing precipitate was filtered, drained, pressed and after the addition of 3 % of washing soda was dialysed until the salt content was the same as that of the water in the dialysing tank. To the residue from dialysis was added 1 % of sodium chloride and 0.35 % of cresylic acid.

The end products were clear and readily filterable, for, in contrast to those obtained by methods involving the use of ammonium sulphate they were not liable to contain particles of protein in colloidal suspension.

On the other hand they showed a lower percentage removal of the serum proteins and a consequent lower degree of concentration than is now obtained in the Banzhaf [1913] and the Homer [1916] methods.

The less complete removal of the serum proteins can be attributed mainly to the fact that the addition of 0.25 % of glacial acetic acid to the brined sera leads to the precipitation of a considerable amount of albumin with the pseudoglobulin. As the antitoxin is attached to the latter, the presence of the former must reduce the degree of concentration to a value considerably less

than would have been obtained had the precipitation of albumin at this stage been avoided.

The observations that were made during the progress of the work have presented further points of interest with regard to the properties of heat-denaturated serum proteins. They are dealt with in detail below and may be of assistance to those engaged in problems connected with the concentration of sera.

I. THE SALTING OUT OF SERA IN WHICH THE HEAT-DENATURATION HAS BEEN REGULATED BY AN ADJUSTMENT OF THE REACTION.

The hydrogen ion concentration $[H]$ was adjusted to values ranging from P_H 8.7 to 4.2 by the addition of the necessary amounts of ammonia, acetic or sulphuric acids respectively, the determinations being made in a Walpole's colorimeter with the indicators suitable for the purpose [Homer, 1917, 4].

The alkaline sera were heated to a temperature of 57–58° for a period of not less than four hours; the acid sera were kept at this temperature for two hours only.

At the conclusion of the heating the amounts of acid or of alkali initially added to the sera were respectively neutralised. The liquids were then treated with solid sodium chloride and with brine in the manner described above.

It was found that the success of the operation was influenced by the nature of the changes induced during the heating of the serum. As the extent of the change was primarily regulated by the $[H]$, comparisons were made of the results from the concentration of those sera of which previous to their being heated the $[H]$ had been adjusted to values between P_H 8.7 and 5.0, 5.0 and 4.7 and to values more acid than P_H 4.7.

(a) *The concentration of sera of which the $[H]$ had been adjusted to values between P_H 8.7 and 5.0.*

In Table I have been embodied the results obtained from the concentration of such sera.

It will be seen that, where the $[H]$ had been adjusted by the addition of acetic acid or of ammonia, there was a sufficient recovery of antitoxin in the final products to warrant the application of the method to the routine concentration of sera. As was to be expected a further recovery of antitoxin could be made by a second extraction of the precipitates with brine.

However, in those cases in which sulphuric acid had been employed to adjust the $[H]$ to values between P_H 5.2 and 5.0, the recovery of antitoxin in

the end product was considerably less than where the reaction had been adjusted approximately to the same values by the addition of acetic acid. It was found that, although, in the two sera, the extent of the heat-denaturation was approximately the same, the percentage removal of the serum proteins was considerably more from those sera acidified with sulphuric acid than from those to which acetic acid had been added.

TABLE I.

Showing the results obtained from the concentration of antitoxic sera by the direct treatment of the heated sera with salt. The extent of the heat-denaturation had been regulated by an adjustment of the $[H^+]$ to values between P_H 8.7 and 5.0.

Reaction of the plasma adjusted by the addition of:—	P_H of the plasma previous to its being heated	Percentage denaturation of the serum proteins induced during the heating of the plasma	Percentage of the total proteins of the original serum removed during the process of concentration	Percentage of the total antitoxic units of the original serum recovered in the residue from the dialysis of the proteins precipitated by the addition of acetic to the filtered brined sera	Increased potency per c.c. shown by the end products calculated, for the purpose of comparison, on the basis that they contain 20% of protein
Acetic acid	5.4	16	26	—†	—
"	5.2	27	35	> 90	4
"	5.15	30	40	—†	—
"	5.1	33	43	—†	—
"	5.08	36	43	86	4.5
"	5.05	40	58*	75	4
"	5.00	43	54	88	5
Sulphuric acid	5.18	33	54	65	5
"	5.05	43	80	< 50	< 3
Ammonia	8.3	35	46	84	5
"	8.5	40	55	88	5
"	8.7	42	54	—*	—

* Brined plasma which had stood at room temperature for two days only.

† Not tested owing to the scarcity of experimental animals.

It is thus evident that there are factors other than the $[H^+]$ which affect the nature of the changes induced during the heating of sera. It is possible that the above increased precipitation was due to the more marked effect of the divalent "sulphions" than of the monovalent "acetions" on the *electrical* relationship between the heat-denaturated protein and the solution; a relationship which is recognised as a determining factor in the precipitation of a colloid.

(b) *The concentration of sera of which the $[H^+]$ had been adjusted to values between P_H 5.0 and 4.7.*

In these cases the consistency of the heated sera was that of a thick suspension due to the precipitation of heat-denaturated protein in a filterable form.

It was ascertained that, during the process of concentration, the recovery of antitoxin in the end products decreased as the acidity of the sera was increased. At the same time there were indications that the recovery in brine was more satisfactory from those sera in which the reaction had been adjusted by means of acetic acid than where sulphuric acid had been used.

Thus, it was found that with sera acidified with acetic acid, and showing a heat-denaturation of about 50 %, the extraction of the heated sera with brine for two days led to the recovery of about 50 % of the antitoxin; after seven days the recovery amounted to 66 %. Where the heat-denaturation had been of the order of 70 %, the treatment of the heated sera with brine for seven days led to the recovery of about 30 % of the antitoxin. In neither case was there a further increase in the percentage recovery of the antitoxin by a prolongation of the time of extraction to a period of five weeks.

On the other hand with sera acidified with sulphuric acid, in which a heat-denaturation of 35 to 40 % had been induced, there was a recovery of only 66 % of the total antitoxin after seven days' extraction with brine. Where the denaturation had been of the order of 65 % the recovery was less than 10 % in the same time. In neither case was the extent of the recovery of the antitoxin appreciably improved by a prolongation of the time of extraction to five weeks.

The more marked insolubility of the heat-denaturated proteins in brine induced during the heating of sera to which sulphuric acid had been added, is shown by the following experiment.

The reaction of antitoxic plasma was adjusted by means of acetic acid and of sulphuric acid respectively so as to ensure a heat-denaturation of about 60 %. The adjusted sera were then heated for two hours at 57–58° by which time they had become semi-solid in consistency. In each case after the neutralisation of the acid there was added an equal volume of a saturated solution of sodium chloride, and the mixture was well shaken and filtered at once. The precipitates were well drained and, after being freed from adhering fluid by gentle pressure between cloths and boards, were triturated in a mortar with 50 cc. of a saturated solution of salt. The thin paste was then diluted with the salt solution to a volume six times that of the original plasma. The liquids were kept at room temperature in stoppered flasks and were well shaken from time to time.

After standing for stated periods of time, samples of the liquids were filtered and the protein and antitoxin contents of the filtrates were measured. The results showed that at the end of one week the brine had redissolved an

appreciable amount of the heat-denaturated protein precipitated from those sera to which acetic acid had been added. Animal tests showed that, to this redissolved protein, was attached about 50 % of the total antitoxin taken for concentration. The amount of protein and antitoxin thus recovered from the precipitate was not appreciably increased after a further twenty-one days' extraction.

On the other hand none of the filtered brine extracts of the protein precipitated from the sera acidified with sulphuric acid contained more than a trace of protein even after four weeks' extraction; the antitoxin recovered was considerably less than 10 % of the original.

(c) *The concentration of sera of which the $[H']$ was more acid than P_H 4.6.*

To separate volumes of antitoxic sera were added the requisite amounts of acetic and of sulphuric acids respectively to bring the reaction to the value P_H 4.4. The sera were then heated for two hours at $57-58^\circ$, and, after the neutralisation of the acid, the semi-solid liquids were treated with salt and with brine in the manner described above. Samples of the brined sera were taken at stated periods and the antitoxic content of the filtrates were compared.

After ten days traces only of the protein had been redissolved; the amount of antitoxin in solution was considerably less than 10 % of the original. There was no improvement in the recovery of antitoxin effected by a prolongation of the time allowed for extraction to four weeks.

The precipitated heat-denaturated proteins from the sera thus acidified, were triturated with brine in the manner described above. Even after several weeks' extraction in a volume of brine six times that of the original serum, there was redissolved practically none of the protein precipitated from the sera acidified with sulphuric acid, and not more than a trace where acetic acid had been used. In both cases the filtered brine extracts contained considerably less than 10 % of the original antitoxin of the serum.

The observations recorded under (a), (b) and (c) thus indicate that there is a greater production of "salt-insoluble" protein during the heating of sera of which the $[H']$ has been adjusted to values more acid than P_H 5.4 by the addition of sulphuric acid than by that of acetic acid. They also demonstrate that, irrespective of the acid used, an increase in the degree of acidity is followed by an increase in the precipitation of heat-denaturated protein and a corresponding decrease in the recovery of antitoxin in a form in which it can be rendered suitable for use clinically.

It is thus evident that, for the successful concentration of sera by the method under discussion, it is advisable to adjust the reaction of the plasma to values about P_H 8.3 or about 5.0. For this purpose a routine method has been suggested [Homer, 1917, 4] in which use is made of the indicators *α -naphtholphthalein* and *dibromo-o-cresolsulphonephthalein*.

Since slight variations in the reaction round about P_H 8.3 are not followed by the marked variations in the heat-denaturation and precipitation of the serum proteins so characteristic of sera more acid than P_H 5.5, preference has been given to the adjustment of the $[H^+]$ to the former value.

II. THE "SALTING OUT" OF SERA IN WHICH THE HEAT-DENATURATION HAD BEEN CONDUCTED IN PRESENCE OF CRESYLIC ACID.

As the use of trikresol¹ has been of such practical value in improving the concentration of sera by the Banzhaf [1913] and the Homer [1916] processes, experiments were undertaken to ascertain whether its use in the method under consideration would be followed by similar satisfactory results.

Experimental concentrations were carried out as follows.

To plasma containing 2 % of solid sodium chloride were added fixed amounts of cresylic acid. The reaction of the plasma was then adjusted to values ranging between P_H 7.2 and 8.5 previous to its being heated. The heated plasma, after being treated with solid salt and with brine in the manner described above, was allowed to stand at room temperature for at least six days to ensure the extraction of the antitoxin.

It was found that the antitoxin could be completely recovered by the brining of sera containing 0.35 % of cresylic acid or less which had been heated to 57–58° for periods of from four to six hours. The addition of cresylic acid in a greater amount led to an increased production of "salt-insoluble" protein; during the process of concentration, the antitoxin attached to this insoluble protein was lost. Thus, the filtered brine extracts of those sera containing

¹ Since the publication of the papers on this subject, we have read in the current literature an abstract of a paper by Banzhaf in which, for the improvement of his technique, he recommends the use of trikresol in an amount somewhat greater than that advocated by us.

We have recently ascertained that the amount of trikresol necessary for the production of clear end products, without inducing undue losses during the process of concentration, depends on the composition of the sample used. Thus with that in use during the years 1916–1918, it was necessary to add 0.30 % to the serum; with a recent consignment of the substance only half this amount was required. It is therefore advisable for those in charge of the concentration of sera to ascertain experimentally, with the trikresol at their disposal, the minimum amount necessary to give the desired results.

In our routine work we have not found it advisable to raise the temperature of the serum mixtures to the high limit now suggested by Banzhaf [1918].

0.5 % of cresylic acid showed a loss of about 15 % of the antitoxin; with 0.75 % of this substance the loss was somewhat greater. The percentage recovery of antitoxin was in no way increased by a prolongation of the time allowed for extraction or by an increase in the volume of brine added to the sera.

Appreciable losses were also experienced in the concentration of sera containing 0.30 to 0.35 % of cresylic acid in which the heating had been conducted at temperatures above 60°.

The losses were, in part, due to the precipitation of antitoxin with the "salt-insoluble" protein and, in part, to an actual inactivation of antitoxin. The extent to which the cresylic acid influences these factors is in the course of investigation and will be dealt with in a future communication.

During the course of the concentration of sera containing cresylic acid it was noticed that, irrespective of the [H'] of the batches of sera, the brined extracts of the heated sera filtered readily. The ease of filtration was probably due to the stimulating effect of the cresylic acid on the aggregation of the heat-denatured protein into particles of sufficient dimensions to ensure their separation by filtration through paper.

TABLE II.

Showing the results obtained from the concentration of sera by the direct treatment of the sera, heat-denatured in presence of cresylic acid, with salt.

Percentage of cresylic acid added to the plasma	pH of the plasma previous to its being heated	Percentage denaturation of the serum proteins induced during the heating of the plasma	Percentage of the total proteins of the original plasma removed during the process of concentration	Percentage of the total antitoxic units of the original plasma appearing in the residue from the dialysis of the proteins precipitated by acetic acid from the filtered brined sera	Increased potency per c.c. shown by the end products, calculated for purposes of comparison on the basis that these contain 20 % of protein
A { 0.00	8.3*	40	46.8	about 85†	5
0.30	7.4	22.3	51.1	„ 85†	4
0.30	8.0*	42.0	46.8	„ 85†	4
B { 0.00	7.6	18.2	55.4	> 90†	5
0.25	7.6	36.5	48.0	> 88†	4.6

* Reaction adjusted by the addition of ammonia.

† Not tested out because of the shortage of experimental animals.

The data with regard to concentrations of plasma containing 0.30 % of cresylic acid have been embodied in Table II. They show that, in spite of the increased heat-denaturation induced by the addition of cresylic acid, there was no appreciable increase in the percentage removal of the serum proteins. For this reason the degree of concentration did not show any improvement on that obtained in control experiments with the same sera containing no cresylic acid.

This anomaly was eventually shown to be due to the fact that, while the cresylic acid increases the precipitation and removal of heat-denaturated pseudoglobulin, it also induces an increased precipitation of albumin at the stage at which the pseudoglobulin and antitoxin are precipitated.

III. THE "SALTING OUT" OF SERA IN WHICH THE HEAT-DENATURATION HAD BEEN INDUCED IN THE PRESENCE OF ELECTROLYTES.

It has been demonstrated [Homer, 1916] that there is no appreciable destruction of antitoxin during the prolonged heating of antitoxic sera, to which electrolytes have been added, at 57-58°. As a detailed study has already been made of the effects produced by the addition of definite amounts of sodium chloride to the serum previous to its being heated, attention has been confined in the present investigation to the addition of sulphates to the plasma. For, in view of the more marked tendency of the "sulphion" (*ante*, p. 194) to render the particles of heat-denaturated protein insoluble in brine, it was thought that their addition to the plasma might lead to a greater removal of the serum proteins, and consequent increased degree of concentration, than had been obtained in sections I and II.

Experimental concentrations were accordingly undertaken in which to fixed volumes of the plasma were added definite amounts of the sulphates of ammonium, sodium, potassium, magnesium and zinc respectively. The liquids after being heated at 57-58° for periods of from four to six hours were treated with salt and with brine according to the directions given above; the brined sera were allowed to stand at room temperature for periods of from ten to fourteen days.

The observations made during the progress of the experiments were as follows.

(a) *The effect of the addition of sulphates on the changes induced during the heating of the sera.*

In all cases it was evident that the addition of sulphates increased the precipitation of heat-denaturated protein.

With the aid of the Zeiss immersion refractometer determinations were made of the actual precipitation of protein from the heated sera and also of the increased heat-denaturation induced in the presence of the respective sulphates. The results which were compared with those obtained from control experiments with the original plasma have been embodied in Table III.

TABLE III. *Showing the changes induced during the heating of sera containing sulphates.*

Percentage of electrolyte added to the plasma previous to its being heated	pH of the plasma previous to its being heated	Appearance of the plasma to which the electrolyte has been added		Percentage increased precipitation of the serum proteins during the heating of the plasma	Percentage increased precipitability of the serum proteins by 30 per cent of saturation with ammonium sulphate induced during the heating
		(a) Before being heated	(b) After being heated		
MgSO₄·7H₂O					
0	7.4	clear	opalescent and slight deposit	2.0	17.7
15	7.4	"	slight precipitate	2.7	17.0
20	7.4	slight precipitate	increased precipitation	3.7	17.7
30	7.4	"	"	13.0	24
20 and ammonia	8.0	"	thin emulsion	28	32.8
K₂SO₄					
0	7.0	clear	opalescent and slight deposit	0	10
5	7.0	very slight precipitate	very slight precipitation	1	11
10	7.0	slight precipitate	precipitation more marked	0	10
15	7.0	"	"	3	13
10 and ammonia	8.3	"	almost solid	36	40
Na₂SO₄					
0	7.0	clear	opalescent and slight deposit	0	10
5	7.0	slight precipitate	slight precipitation	2	10.6
10	7.0	"	precipitation more marked	2	10
15	7.0	thick	thick precipitate	15	—
10 and ammonia	8.3	slight	"	33	38
ZnSO₄·7H₂O					
0	7.4	clear	opalescent	0	17.7
15	more acid than p _H 4.5	thick precipitate	almost solid	protein completely precipitated during the heating	
20	"	"	"	"	"
(NH₄)₂SO₄					
0	6.8	thin suspension	thin suspension	0	—
30 saturation	6.8	"	precipitation increased	10	—
30	7.1	"	"	12	—
30	7.6	"	"	20	—
30 and 0.3 cresylic acid	7.2	"	"	30	—
30 and ammonia	8.5	"	"	44	—

In those cases where the sulphates of ammonium, potassium, magnesium or sodium had been used, the determination of the refractive indices of the heated and unheated sera of which the $[H']$ lay between P_H 5.5 and 7.6, showed that the increased precipitation was due to an agglutination of colloidal particles of suspensoid protein into a suspension which ultimately settled as a perceptible precipitate. During the heating there had been no conversion of protein from the emulsoid to the suspensoid condition beyond that experienced in the control sera.

Where the reaction of the plasma had been more alkaline than P_H 8.0 the consistency of those sera containing sulphates in the proportions given in the Table, was invariably changed to that of a thick suspension; in the control experiments with plasma adjusted to the same $[H']$ the heated liquids remained clear. In these cases the determination of the refractive indices of the sera containing sulphates showed that, during the heating, there had been an actual conversion of the emulsoid protein into a suspension. In the control experiments the changes induced had been insufficient to effect this conversion; the extent of the changes was detected by an estimation of the increased precipitability of the proteins.

From the data furnished with regard to the increased precipitability of the serum proteins by ammonium sulphate it will be seen that, except at concentrations of the electrolyte nearing those required for the precipitation of one or other of the serum proteins, the extent of the heat-denaturation was not directly affected by the addition of those sulphates which caused no appreciable change in the $[H']$ of the plasma.

Thus, the extent of the heat-denaturation induced in plasma containing the sulphates of ammonium, potassium, magnesium or sodium, was practically the same as in the control experiments and was obviously regulated by the $[H']$ of the plasma.

The addition of 15 and 25 % of zinc sulphate to the plasma changed the $[H']$ from a value P_H 7.4 to values more acid than P_H 4.5. As was to be expected, there was complete precipitation of the serum proteins during the heating of plasma to which this sulphate had been added.

It is interesting to note that in the case of sera more alkaline than P_H 8.0 the increased precipitation of protein from the heated sera was approximately a measure of the extent of the heat-denaturation induced; the latter was in no way increased beyond that of the control sera adjusted to the same $[H']$. The addition of sulphates to these alkaline sera had thus induced a precipitation of heat-denatured protein similar to that observed during the

heating of acid sera, or of sera to which either phenol and its homologues, or ether or chloroform had been added.

The results so far obtained show that while the addition of sulphates to the plasma does not directly affect the extent of the heat-denaturation of the serum proteins, it considerably improves the aggregation of suspensoid particles of protein into a filterable form. Moreover, in alkaline sera showing a sufficiently high denaturation, they induce the precipitation of the heat-denaturated protein which would otherwise have remained in the disperse phase.

(b) The increased production of proteins insoluble in brine during the heating of sera containing sulphates.

Samples of the unheated and heated sera of which the reaction lay between P_H 6.6 and 7.6 were filtered. To a fixed volume of each was added twice the volume of a saturated solution of salt and sufficient solid salt to ensure saturation. From comparative tests it was seen that, in all cases, the amount of "brine-insoluble" protein was considerably increased during the heating of the sera; the increase was more marked in those sera containing sulphates than in the controls with the original plasma.

In the preceding sections of the paper it has been shown that the addition of acid or of trikresol to the plasma increased the production of "salt-insoluble" protein during the heating of the plasma; under certain conditions, the "salting out" of the thus denaturated proteins ceased to be of a reversible nature.

It has been established that sulphates also favour an increased precipitation of heat-denaturated protein by salt. In this case, however, the following evidence shows that in sera of which the reaction was of the order of P_H 7.8, the increased salting out of the denaturated proteins, presumably the result of an electrical charging of the protein molecules by the sulphions, can be reversed, to a certain extent, by sufficiently reducing the active mass of the latter.

A precipitate of heat-denaturated pseudoglobulin, which in the control sera was "brine-soluble," but which had been rendered insoluble in brine by the presence of sulphates, was suspended in a volume of brine twice that of the original serum taken for concentration. After several weeks' extraction it was found that not more than a trace of the protein had been redissolved.

The volume of the brine was then increased to eight times that of the original serum. After a week the amount of protein in solution was not

visibly improved; after three weeks there was an appreciable improvement; by the end of three months a considerable amount had been dissolved, although, even after five months, the recovery was not complete.

The same phenomena are exhibited by the proteins precipitated by the addition of ammonium sulphate in excess of that required to precipitate "salt-soluble" heat-denaturated pseudoglobulin from solution. The ease with which the heat-denaturated pseudoglobulin, thus precipitated, is redissolved in brine depends partly on the extent to which the protein has been denaturated during the heating, and partly on the percentage of sulphate present. Thus, in a case under investigation, while the denaturated pseudoglobulin and antitoxin attached thereto precipitated by the addition of ammonium sulphate to the extent of 30 % of saturation, was readily dissolved by brine, that precipitated from the same solution by the addition of ammonium sulphate in greater amounts was not completely redissolved after six months' extraction with a large volume of brine. Moreover, it was found that, as the heat-denaturation was increased, so the reversibility of this precipitation was either diminished or delayed. Experimental work is in progress to settle this point.

(c) *The percentage removal of the serum proteins and the degree of concentration experienced during the concentration of sera containing sulphates.*

The data with regard to the percentage removal of the serum proteins and the increase in potency obtained during the process of concentration have been recorded in Table IV. A study of the Table shows that in those cases in which the percentage removal of the serum proteins had been greater than 50 % there were appreciable losses of antitoxin. Without doubt the losses were due to the increased insolubility of heat-denaturated protein and associated antitoxin in brine. For the recovery of the antitoxin thus precipitated it was necessary to submit the precipitates to a prolonged extraction in a large volume of brine.

In all cases the end products were more deeply coloured and, generally speaking, neither the percentage removal of the serum proteins nor the degree of concentration experienced was in any way better than that obtained in the preceding sections.

It was subsequently shown that the failure of the sulphates to improve the degree of concentration, in spite of the increase in "salt-insoluble" proteins which they favour, was due to the increased precipitation of albumin at the stage at which pseudoglobulin and antitoxin are precipitated.

TABLE IV.

Showing the results obtained from the concentration of sera by the direct treatment of the sera, heat-denatured in presence of sulphates, with brine.

Electrolyte added to the plasma previous to its being heated	pH of the plasma	Percentage denaturation of the serum proteins induced during the heating of the plasma	Percentage removal of the total proteins of the original serum induced during the process of concentration	Percentage of the total antitoxic units of the original serum appearing in the residues from the dialysis of the protein precipitated by the addition of acetic acid to the filtered sera	Increased potency per cc. shown by the end product, calculated for purposes of comparison on the basis that they contain 20% of protein
Controls { —	7.0	10	27	—	—
—	7.4	18	35	—	—
—	7.6	28	48	> 90	4
(NH ₄) ₂ SO ₄ (30 % of saturation)	6.8	10	35	> 85	3.5
do.	7.2	10	35	> 85	3.4
do.	7.4	20	48	88	4
do.	8.3	30	51.8	70	4
MgSO ₄ ·7H ₂ O (30 %)	7.2	13.5	60	70	5.9
Na ₂ SO ₄ (10 %) and 0.3 % cresylic acid	7.6	40	57	70	5
Na ₂ SO ₄ (7 %)	7.4	18	42.6	—	—

IV. THE INVESTIGATION OF THE REASON FOR THE LOWER DEGREE OF CONCENTRATION OBTAINED FROM THE "SALTING OUT" OF THE HEATED SERA WITH SODIUM CHLORIDE THAN FROM THE USE OF AMMONIUM SULPHATE.

Experimental work was next undertaken to ascertain the cause underlying the lower degree of concentration obtained by the direct treatment of the heated sera with salt as in sections I, II and III, than that which is usually obtained as a result of their fractional precipitation with ammonium sulphate.

The concentration of volumes of antitoxic sera, ranging from one to sixty litres, were carried out by the method under discussion and by the Homer [1916, 1918] method. A comparison was made between the respective end products as regards their protein content, the increased potency over that of the original serum and their antitoxic content. The results of the investigation have been embodied in Table V.

The use of ammonium sulphate as the salting out agent in the latter method resulted in end products which showed a potency per cc. about eight times that of the original serum; their protein content indicated that there had been a removal of about 75 % of the serum proteins during the process of concentration.

In the concentration of sera by the former method in which sodium chloride was used as the "salting out" agent the maximum increased

TABLE V. A comparison between the composition of the end products obtained from the concentration of sera by the methods described in sections I, II and III of this paper and by the Homer [1916, 1918] method.

Series No.	Substances added to the plasma previous to its being heated	pH of the plasma being heated	Method of concentration adopted	Percentage of the total antitoxin units of the original plasma appearing in the end product	Increased potency per cc. shown by the end product, calculated for purposes of comparison on the basis that they contain 20 % of protein	Percentage removal of serum proteins induced during the process of concentration	Percentage of the antitoxin admixed with pseudo globulin in the end product
Antitetanic sera							
57	—	7.6	Homer [1916]	> 90*	8.5	73.5	1.5
57	acetic acid	5.05	the method given in section I	88	5	55	21.0
57	ammonia	8.30	" "	88	5	54	22.0
87	ammonia	8.3	Homer [1918]	circ. 85*	8	78.5	2.0
80	0.30 % crealylic acid	7.2	" "	90	12	78.0	5.0
85	0.30 % "	7.1	" "	circ. 85*	7	78.3	5.0
85	(NH ₄) ₂ SO ₄ (30 % of saturation)	6.8	method given in section III	> 85*	3.5	35	36.5
85	" "	7.2	" "	> 82*	3.4	35	33.5
85	30 % MgSO ₄ ·7H ₂ O	7.2	" "	70	5.9	60	38.0
85	10 % Na ₂ SO ₄ and 0.30 % crealylic acid	7.2	" "	70	5	57	50.0
85	7.5 % Na ₂ SO ₄	7.2	" "	—*	—	42.6	40.0
Antidiphtheritic sera							
55	0.30 % crealylic acid	7.6	Homer [1918]	circ. 88*	8	78.0	4.9
55	0.30 % "	7.6	method given in section II	88	4	51.0	35.5
55	ammonia	8.3	" "	88	4	46.3	23.5
55	0.30 % crealylic acid and ammonia	8.3	" "	88	4	46.8	35.5
73	(NH ₄) ₂ SO ₄ (30 % of saturation)	7.1	Homer [1916]	88	10.5	71.0	—
73	" "	7.1	method given in section III	> 90*	4	48.0	25
73	(NH ₄) ₂ SO ₄ (30 % and 0.30 % of crealylic acid	7.1	" "	70	4	51.8	50.3

* Not tested further because of the lack of experimental animals.

potency that was obtained without undue loss of antitoxin was of the order of five times; no improvement in this respect was effected by the addition of substances such as cresylic acid which are of proven value in the methods involving the use of ammonium sulphate.

It has been shown that cresylic acid materially improves the latter processes by inducing a greater removal of the serum proteins. However, in the method under consideration, the use of cresylic does not appreciably reduce the percentage of the original proteins appearing in the end product.

It was noticed that the end products from the brining of the heated sera were, without exception, more intensely coloured than those now obtained by the Banzhaf and the Homer methods. The colour was invariably most pronounced where the heat-denaturation has been conducted in presence of cresylic acid or sulphates.

It has been recently shown that in the Banzhaf and the Homer methods the colour of the end products depends to a large extent on the presence of heat-denaturated albumin and adsorbed colouring matter of the serum [Homer, 1917, 3, 5]. These observations together with those on the failure of cresylic acid and of sulphates to improve the percentage removal of the serum proteins during the process of concentration, led me to conclude that the end products from the direct brining of sera contain albumin in considerable amounts.

Experimental evidence in justification of this conclusion was furnished in the following way.

Albumin and pseudoglobulin were respectively precipitated from unheated plasma in the usual way. The precipitated proteins were twice redissolved and reprecipitated in order to free them as much as possible from admixture with the other protein entities of the serum. The precipitates after filtration were drained, pressed and dialysed in the usual way. The residues from dialysis were diluted so as to contain about 5 % of protein in solution.

It was found that there was no precipitation of protein from aqueous and from 0.85 % salt solutions of pseudoglobulin and of albumin respectively, of which the $[H^+]$ had been adjusted to values between P_H 7.0 and 3.0.

After the addition of electrolytes to the solutions, the precipitation of protein was readily induced at a $[H^+]$ between P_H 4 and 5. Thus, the solution of pseudoglobulin saturated with salt began to show slight precipitation on the addition of 0.05 % of glacial acetic acid (circ. P_H 5.5); the precipitation was complete after the addition of 0.30 % of the acid (circ. P_H 4.7). The solution of albumin, similarly treated, showed a slight precipitation on the

addition of 0.15 % of glacial acetic acid (circ. P_H 5.0); while the precipitation was considerable at 0.3 % it was not complete until 0.5 % of glacial acetic acid had been added.

From these observations it follows that the protein precipitated by the addition of 0.25 % of glacial acetic acid to the filtered brined sera contains a considerable amount of albumin admixed with the pseudoglobulin.

Confirmatory evidence on this point was obtained by a gravimetric determination of the proportion of albumin to pseudoglobulin in the respective end products.

The results of the determinations have been included in Table V. They show that, where the $[H]$ had been suitably adjusted to the desired value, the proteins appearing in the end product from the concentration of sera by the Homer [1916, 1918] method consisted of about ninety-eight parts of pseudoglobulin to two parts of albumin: where the alternative treatment of the plasma had been adopted, viz., the addition of 0.3 % of cresylic acid to the plasma previous to its being heated, the proportion of albumin rose to 5 %.

The protein appearing in the end products from the methods involving the brining of the heated sera contained a relatively greater amount of albumin; which latter depended on the extent of the heat-denaturation and on the nature of the substances added to the serum previous to its being heated.

In those cases in which the heat-denaturation had been regulated by an adjustment of the $[H]$ to values between P_H 5.0 and 8.7, 20 to 25 % of the protein appearing in the end product consisted of albumin. Where the denaturation had been induced in presence of cresylic acid or of sulphates the proportion of albumin was considerably increased, in some cases being as high as 50 %.

The evidence thus adduced shows that, by the direct treatment of the heated sera with salt and the subsequent precipitation of the brined extracts with acetic acid, the degree of concentration cannot be so great as that attained by the use of methods in which the separation of the albumin is more or less assured.

From a study of the data given in Table V it will be seen that the best results were obtained from the brining of those sera in which, previous to their being heated, the reaction had been adjusted to about P_H 8.3.

The addition of electrolytes or of cresylic acid to the plasma adjusted in this way was of no material advantage, for while they induced an increased precipitability of heat-denaturated pseudoglobulin, the advantage thus gained

was more than counterbalanced by the increased precipitability of heat-denaturated albumin.

In fact, for this reason, the addition of cresylic acid or of sulphates sometimes induced a lower degree of concentration than would otherwise have been obtained. Thus in experiments (not recorded in the Tables) where a high percentage removal of the serum proteins had been induced, the end products showed a potency of only 2-3 times that of the original serum; they also contained less than 50 % of the antitoxin taken for concentration.

The low degree of concentration was found to be due to the high percentage admixture of denaturated albumin with pseudoglobulin in the end product; the high percentage loss of antitoxin was due to its precipitation with the "brine-insoluble" heat-denaturated pseudoglobulin.

SUMMARY.

1. The concentration of antitoxic sera can be successfully conducted by a regulation of the heat-denaturation of the serum proteins followed by a direct treatment of the heated sera with brine and salt.

2. The end products thus obtained were clear and readily filterable, as they were not liable to contain the colloidal suspension of heat-denaturated protein which is so often found in those resulting from methods involving the use of ammonium sulphate.

3. Where the heat-denaturation had been of the order of 30 to 40 %, the use of this method led to the removal of about 50 % of the serum proteins. The potency of the end products was about five times that of the original serum.

4. The degree of concentration was not improved by a further increase in the extent of the heat-denaturation or by the addition of substances such as cresylic acid or sulphates to the serum previous to its being heated.

It is a matter of experience that the factors which increased the insolubility of the heat-denaturated pseudoglobulin in brine also increased the precipitation of heat-denaturated albumin at the stage at which the pseudoglobulin and antitoxin are precipitated.

5. In all cases the end products were more deeply coloured and contained a greater percentage of albumin than is found in those now obtained by the use of recent modifications of the Banzhaf [1913] and the Homer [1916] methods.

6. Under some conditions the heat-denaturated proteins precipitated

during the heating of sera containing cresylic acid or of which the reaction is more acid than P_H 5.0, do not redissolve in brine, even on long standing.

On the other hand there is evidence that the proteins precipitated during the heating of sera containing electrolytes can be redissolved by the prolonged treatment of the precipitates with a large volume of brine.

7. For the successful concentration of sera, the precipitation of those proteins to which the antitoxin is attached must not be changed from a reversible to a non-reversible type of reaction. In the latter case, even though the animal tests show that there has been no inactivation of the antitoxin attached to the insoluble particles of protein, from the point of view of the concentration of serum for clinical use this antitoxin is hopelessly lost until a method of dealing with the insoluble precipitates is forthcoming.

8. The method under discussion has not furnished end products showing so great a percentage removal of the serum proteins nor a degree of concentration so high as that now obtained in these laboratories by the Banzhaf and the Homer methods. Nevertheless the description of the method with sodium chloride may be of value to those engaged on problems connected with the routine concentration of sera, for, should untoward circumstances arise to prevent the concentration of sera by the methods now in use, there is hereby furnished an alternative method of proven practicability.

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