XXIX. A COMPARISON BETWEEN THE PRECIPITATION OF ANTITOXIC SERA BY SODIUM SULPHATE AND BY AMMONIUM SULPHATE.

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My previous work on the factors influencing the concentration of sera has led me to the conclusion that the preliminary heat-denaturation so generally advocated prior to the fractional precipitation of the serum proteins by ammonium sulphate and by sodium chloride does not of necessity materially contribute to the refining and concentrating of antitoxic sera. Recent experiments have shown that, with the technique adopted for the routine work, the heat-denaturation merely induces the precipitation of the antitoxin-bearing protein at lower concentrations of the electrolyte than would be required in the unheated plasma; apparently the heating does not affect the nature of the antitoxin-protein combination.

Further corroborative evidence on this point has been furnished by a study of the fractional precipitation of the serum proteins and of the antitoxin from unheated and from heated antidiphtheritic plasma by sodium sulphate¹.

Several series of experiments were undertaken with samples of oxalated plasma from individual horses and from batches of pooled plasma from different horses. In all cases the plasma was adjusted, either by bringing the $[H^+]$ to definite values, or by the addition of cresylic acid in stated amounts to the plasma. The method of procedure for each of the series of experiments was as follows: equal volumes of the adjusted plasma were measured into stoppered bottles each of which contained the amount of anhydrous sodium sulphate necessary to bring the proportion of the latter in solution in the plasma to values ranging from 4 to 34 per cent. Owing to the slight solubility

¹ Some years ago Dr C. J. Martin (unpublished observations), worked out a method for the concentration of antitoxic sera by the fractional precipitation of the serum proteins with sodium sulphate rather than with the more toxic ammonium sulphate. Taking advantage of the slight solubility of sodium sulphate at 0° he was able to freeze out the excess of this salt from the Second Fraction precipitates thereby dispensing with the lengthy process of dialysis. For some reason the end products sometimes showed too high a viscosity. The factors underlying this variability were not fully investigated as, in the meantime, Gibson and Banzhaf published their methods involving the heat-denaturation of the proteins.

of anhydrous sodium sulphate in water¹ the respective liquids were kept at 35–40° and constantly stirred until the solution of the sulphate had been effected. Samples were then taken from the bottles and filtered.

The filtration of the hot liquids containing percentages of sodium sulphate in solution greater than those required for saturation at room temperature should be conducted in funnels surrounded with jackets at a temperature of from 30–35°. However, owing to the tendency of solutions of sodium sulphate to remain supersaturated, the filtration of plasma containing 18 per cent., or less, of the anhydrous sulphate could be conducted at the ordinary spring and summer temperature of our London laboratories (about 17°) without the crystallisation of $\text{Na}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$ from the mixtures or from the filtrates. It was always advisable to filter large quantities of the plasma containing the higher concentrations of the sulphate through funnels jacketed at 30–35°. The bottles containing the sulphated plasmas were heated in a water bath at 57·5–58° for five to six hours and the hot liquids were filtered, the filtering funnels being jacketed at 35–40° when necessary.

The antitoxin and the protein contents of the filtrates from these heat-denaturated sulphated plasmas were estimated in the usual way, and, after the necessary volume corrections, the data thereby obtained were expressed in the form of curves which were compared with those obtained from similar estimations of the filtrates of the unheated liquids and also with those from the precipitation of samples of the same plasmas by ammonium sulphate.

In accordance with the recent request for the curtailment of the subject matter of communications, curves expressing the experimental data from only three separate batches of antidiphtheritic plasmas are included in this paper.

I. THE PRECIPITATION OF THE SERUM PROTEINS BY SODIUM SULPHATE AND BY AMMONIUM SULPHATE.

Gravimetric estimations of the percentage composition of the respective plasmas were made as regards total protein, pseudoglobulin and albumin with the following results.

	A	B	C
Total protein	7·92 %	8·90 %	7·60 %
Pseudoglobulin	3·18	3·52	2·74
Albumin	2·75	2·68	2·13
Euglobulin, fibrin (by difference)	2·00	2·68	2·73

¹ 100 parts of water at t° dissolve a parts of anhydrous Na_2SO_4 :

t°	a parts Na_2SO_4
0	5
13	11·8
16	14·5
20	19·5
30	43

The variations in the proportionate composition of the proteins of the respective plasmas can doubtless be ascribed to variations in the stage of immunisation or of resting at which the horses were bled.

The data obtained for the sodium sulphate precipitation of the plasma A ($P_H 8.3$) and of the cresylised plasmas A and B, and for the ammonium sulphate precipitation of the plasma A ($P_H 8.8$) and of the cresylised plasma B, have been represented in the curves in Figs. 1, 2, and 4, and 3 and 5 respectively. In each figure the continuous and the intermittent curves represent the data for the unheated and the heated sulphated plasmas respectively.

Fig. 1. The precipitation of plasma A (adjusted to $P_H 8.3$) by sodium sulphate

The continuous Curve I ————— represents the precipitation of the plasma A adjusted to $P_H 8.3$

The intermittent Curve II — — — represents the precipitation of the plasma A adjusted to $P_H 8.3$ and subsequently heated to 57.5°C . for 5 hours

From the relative positions of the points on the curves can be deduced (a) the amount of protein remaining in every 100 c.c. of the filtrates from the sulphated plasmas, and (b) the percentage precipitation of the serum proteins at each stage with sodium sulphate.

It will be seen that the gradients of the curves for the precipitation of the serum proteins from unheated plasma by sodium sulphate are similar to those for the precipitation of the same plasma by ammonium sulphate; with neither salt are there critical points for the precipitation of eu- or pseudo-globulin or of the serum albumin.

It was found that at all stages in the precipitation of the heated plasmas with sodium sulphate there was a marked increase in the precipitability of

the serum proteins similar to that shown by the precipitation of the same plasmas with ammonium sulphate. Moreover, the increased precipitability of the pseudoglobulin by sodium sulphate, registered by the relative shift in the position of corresponding points on the pairs of curves in Figs. 1, 2, and 4 was regulated by the reaction of the plasma, by the temperature at which the plasma was heated and by the duration of the heating at the chosen temperature.

Fig. 2. Showing the precipitation of the serum proteins from plasma A, containing 0.30 per cent. of cresylic acid, by sodium sulphate

The continuous Curve 1 ————— represents the precipitation of the unheated cresylised plasma A

The intermittent Curve 2 — — — represents the precipitation of the same cresylised plasma after it had been heated to 57.5° C. for 5 hours

On the other hand, in the euglobulin-pseudoglobulin zone the extent of the shift of corresponding points on the curves for the precipitation of the respective heated and unheated plasmas by the two sulphates is greater than that shown in the pseudoglobulin area and than that shown by the data from the precipitation of similarly adjusted solutions of pseudoglobulin [Homer 1919].

No doubt this differentiation is due to differences in the relative susceptibility of the individual serum proteins to heat-denaturation.

As was to be expected from the general behaviour of colloids the precipitation of the individual proteins from plasma began at and was completed at lower concentrations of ammonium or of sodium sulphate than was

required for their precipitation from their respective separate solutions in saline; there was also a more marked overlapping of the precipitation limits for the individual proteins than was found for their separate solutions.

Thus, from solution in normal saline, the bulk of the pseudoglobulin is precipitated between the limits of 32 and 50 per cent. of saturation with ammonium sulphate, and between 13 and 23 per cent. of anhydrous sodium sulphate; the albumin under similar conditions is mainly precipitated between 54 and 76 per cent. of saturation with ammonium sulphate and between 20 and 34 per cent. of sodium sulphate.

Fig. 3. The precipitation of plasma A (adjusted to P_H 8.8) by ammonium sulphate

On the other hand there was an appreciable precipitation of pseudoglobulin from unheated unadjusted plasma (P_H 7.4) on the addition of ammonium sulphate to the extent of 28 per cent. of saturation or of 11.5 per cent. of anhydrous sodium sulphate. Moreover, while the precipitation of the pseudoglobulin from the plasma was complete at 46 per cent. of saturation with ammonium sulphate or on the addition of 18.5 per cent. of sodium sulphate, there was, even at these concentrations of the sulphates, an appreciable precipitation of albumin. The precipitation of the latter from the plasma was complete at 67.5 per cent. of saturation with ammonium sulphate or at a concentration of 32 per cent. of anhydrous sodium sulphate.

Fig. 4. The precipitation of cresylised plasma B by sodium sulphate

The continuous Curve I ————— represents the precipitation of the unheated plasma
 The intermittent Curve II - - - - - represents the precipitation of plasma after it had been heated at 57.5° C. for 5 hours

Fig. 5. The precipitation of cresylised plasma B by ammonium sulphate

The continuous Curve I ————— represents the precipitation of unheated cresylised plasma B
 The intermittent Curve II - - - - - represents the precipitation of cresylised plasma B after being heated at 57.5° C. for 5 hours

For some time it has been realised that the denaturation of the serum proteins considerably affects their precipitability and that the extent of the overlapping of their precipitation limits from heat-denaturated plasma depends largely on their respective susceptibilities to heat-denaturation.

Recent work has shown that, even with unheated plasma, there are several factors affecting the extent to which the various serum proteins are precipitated by the sulphates under discussion. In this connexion reference may be made to the results which have accrued from preliminary experiments as regards the influence of dilution, of reaction and of the addition of substances such as phenol and cresylic acid to plasma prior to its precipitation by electrolytes.

Fig. 6. Showing the influence of cresylic acid on the precipitability of the serum proteins from plasma and from heat-denaturated plasma by sodium sulphate

The continuous Curve 1 ————— represents the precipitation of protein from plasma C (P_H 7.3)

The continuous Curve 2 —○—○—○ represents the precipitation of protein from plasma C containing 0.30 per cent. of cresylic acid

The intermittent Curve 1' — — — represents the precipitation of protein from plasma C (P_H 7.3) heated at 57.5° C. for 5 hours

The intermittent Curve 2' —○—○—○ represents the precipitation of protein from cresylised plasma C heated at 57.5° C. for 5 hours

It has been found that the limits stated above for the precipitation of the serum proteins were consistent for all the samples of plasma examined in which the protein content varied from 6.5—10 per cent. Dilution of the plasma with water or with normal salt solution did not necessitate a change in the concentration of the precipitating electrolyte until the percentage of protein in solution was reduced to the order of 2.5. At this stage a concentration of 70 per cent. of saturation with ammonium sulphate or of 34 per cent.

of sodium sulphate was required before complete precipitation of the serum protein was effected. The further dilution of the plasma required a further increase in the concentration of the precipitating salt.

The required concentration of the precipitating sulphate was also affected by the reaction of the plasma. However, the variations in the precipitability of the proteins when the reaction of the plasma lay between P_H 5.2 and 9.3 were slight; in more acid or more alkaline plasmas greater changes were detected and, as was to be expected, those shown in plasma more acid than P_H 5.2 were more marked than those found in the alkaline plasma.

The addition of cresylic acid (0.30 per cent.) to unheated and to heat-denaturated plasma produced marked changes in the precipitability of the serum proteins at all stages with the given sulphates. This phenomenon is readily seen from a comparison of the curves in Fig. 6. The curves 1 and 1' respectively represent the precipitation of unheated and of heat-denaturated plasma C by sodium sulphate; the curves 2 and 2' represent the corresponding treatment of plasma C containing 0.30 per cent. of cresylic acid.

The data included in Table I show that the increased precipitability of the serum proteins in cresylised plasma is a function of the amount of cresylic acid present in the plasma.

Table I. *Showing the effect of the addition of varying amounts of cresylic acid on the precipitability of the serum proteins in unheated antidiphtheritic plasma.*

Percentage addition of cresylic acid to the plasma	Concentration of (A) anhydrous sodium sulphate and (B) ammonium sulphate required for the complete precipitation of the serum proteins from the plasma			
	A		B	
	Na ₂ SO ₄ expressed as		(NH ₄) ₂ SO ₄ expressed as	
	grams per 100 c.c.	gram-molecules per 100 c.c.	Percentage of saturation	gram-molecules per 100 c.c.
0.00	32	0.225	68	0.24
0.15	30	0.21	61.5	0.215
0.30	27	0.19	58.5	0.20
0.45	26	0.18	56	0.19
0.60	25	0.176	54	0.18
0.75	24	0.17	51	0.175

It was also interesting to find that, under the conditions of the experiment, the values for the molecular concentrations of ammonium sulphate required for the complete precipitation of the proteins from the respective plasmas are practically the same as those for sodium sulphate.

The evidence so far obtained in regard to the precipitability of the proteins of unheated and of heat-denaturated plasma indicate that the addition of cresylic acid or of phenol to the extent of 0.5 per cent. or less prior to the prolonged heating of the plasma at 57.5° for five hours does not increase the extent of the heat-denaturation of the serum proteins. The cresylic acid and the phenol materially aid the precipitation of the heat-denaturated protein

which is apt to be so troublesome in the filtration of concentrated antitoxic sera; in this respect their behaviour is analogous to that shown by electrolytes. [Homer 1918, 1.]

In contrast to the experience with non-cresylised plasma, it was found that the dilution of cresylised plasma to a volume four to five times that of its original volume, provided the percentage of cresylic acid in solution were kept constant, did not affect the precipitability of the serum proteins. On the other hand, if, by the dilution, the percentage of cresylic acid in solution were reduced to a value X , then the amount of sodium sulphate or of ammonium sulphate necessary to precipitate the protein was found to be the same as that required by the original undiluted plasma containing X per cent. of cresylic acid.

The above observations on the variations in the precipitability of the proteins of unheated plasma present many points of interest for discussion and elucidation.

Shortly I hope to publish more extensive quantitative data in this connexion.

II. THE ASSOCIATION OF ANTITOXIN WITH THE PROTEINS PRECIPITATED BY THE ADDITION OF AMMONIUM SULPHATE OR OF SODIUM SULPHATE TO ANTIDIPHtherITIC PLASMA.

Recent work on the precipitation of solutions of pseudoglobulin by ammonium sulphate [Homer 1919], has demonstrated that there is a comparatively small load of antitoxin attached to the proteins precipitated by ammonium sulphate in the pseudoglobulin-euglobulin zone, that the remainder of the antitoxin is evenly distributed throughout the fractions of the pseudoglobulin precipitated at higher concentrations, and moreover that, at all stages in the latter region, the increased precipitation of proteins due to heat-denaturation is a linear measure of the accompanying increased precipitation of antitoxin.

These results together with my previous observations on the factors influencing the concentration of heat-denaturated plasma by ammonium sulphate have led me to the conclusion that the ratio of antitoxin to protein in the final products can only be increased by a removal of the proteins to which little or no antitoxin is attached, viz. those of the euglobulin-pseudoglobulin or of the pseudoglobulin-albumin zones.

Further evidence in this respect is given by the fractional precipitation of the unheated plasma by ammonium sulphate and by sodium sulphate respectively.

In Table II have been included the data obtained from the fractional precipitation of separate volumes of unheated antidiphtheritic plasma A and of the same plasma to which an addition of 0.30 per cent. of cresylic acid had been made. The method of procedure was the same as that used in the fractionation of pseudoglobulin [Homer 1919].

Table II. *The fractional precipitation of unheated antidiphtheritic plasma by ammonium sulphate*.*

Limits of saturation with ammonium sulphate between which the protein fractions were isolated	Percentage of the total protein appearing in the dialysed fraction	Percentage of the total antitoxin appearing in the dialysed fraction	Degree of concentration found in the end products, calculated, for purposes of comparison, on the basis that they contain 20 % of protein
<i>Non-cresylised Plasma.</i>			
33-50	45.5	77	4.2
36-50	37.0	70	4.95
38-50	23.3	48	5.45
36-44	22.4	60	7.1
38-44	10.5	38	9.5
<i>Cresylised Plasma.</i>			
32-45	40.8	70	3.8
33-45	34.1	68	5.1
34-45	25.8	62	5.4
36-45	21.0	51	5.5
38-45	10.4	28	6.0
34-50	27.3	62	5.1

* The antitoxin precipitated with the various First Fraction precipitates was completely recovered by extraction of the precipitates with a saturated solution of salt.

Table III. *The fractional precipitation of unheated cresylised antidiphtheritic plasma by sodium sulphate*.*

Limits of percentage addition of sodium sulphate to the plasma between which the protein fractions were isolated	Percentage of the total protein appearing in the dialysed fraction	Percentage of the total antitoxin appearing in the dialysed fraction	Degree of concentration found in the end products, calculated, for purposes of comparison, on the basis that they contain 20 % of protein
8-16.5	59.5	97	3.6
10-16.5	53.0	97	4.3
11-16.5	47.0	97	4.6
12-16.5	36.0	80	5.0
13-16.5	26.0	60	5.5
14-16	14.6	40	6.5
10-18	57.0	97	3.7
10-20	63.0	97	3.45

* The antitoxin precipitated with the various First Fraction precipitates was recovered by extraction with a saturated solution of sodium chloride.

In Table III have been embodied the corresponding data obtained from the fractional precipitation of unheated cresylised plasma B by sodium sulphate. The method of procedure was as follows.

To separate volumes of cresylised plasma (0.30 per cent.) was added the amount of anhydrous sodium sulphate necessary to bring the concentration of the latter to the value chosen for the precipitation of the First Fraction,

viz. X per cent. The respective liquids were kept at a temperature of $35-40^{\circ}$ ¹ and were constantly stirred until complete solution of the sulphate had taken place. The hot liquids were then filtered through paper at room temperature. The First Fraction precipitates were washed with a volume of an X per cent. solution of anhydrous sodium sulphate in water equal to that of the plasma taken for fractionation, the washings were filtered and added to the main filtrate. To the measured volume of filtrate and washings was added the weight of anhydrous sodium sulphate necessary to bring the concentration of the latter in solution up to Y per cent.² The liquids were again heated to $35-40^{\circ}$ and kept at this temperature until complete solution of the sulphate had taken place. The hot liquids were then filtered, and as before (*ante*, p. 279), where the concentration of sulphate was 18 per cent. or less, the filtration was conducted at room temperature; with greater concentrations of the sulphate it was necessary to enclose the filtering funnels in water jackets kept at about 35° .

Where the value for Y was not greater than 18 per cent. the protein fractions, thus isolated in the Second Fraction precipitates, were drained and pressed quickly in the usual way. At greater concentrations of the sulphate, owing to the tendency of the sodium sulphate to crystallise out from the fluid adhering to the precipitate, it was necessary to conduct the pressing operations in the hot room.

The pressed precipitates were dialysed and to the residues from dialysis were added 0.35 per cent. of cresylic acid, and 1 per cent. of sodium chloride. The protein and antitoxin content of the end products were respectively estimated and from the results were calculated the degree of concentration and also the percentages of the total protein and of the total antitoxin associated with the various fractions.

The data included in the tables show that, by a suitable selection of the precipitation limits for the isolation of the antitoxin-protein fraction, results can be obtained from unheated plasma similar to those hitherto obtained from heated plasma, provided that the precipitating limits be chosen so as to exclude albumin, euglobulin and the lower fractions of pseudoglobulin to which a relatively small proportion of the antitoxin is attached.

More detailed work was then undertaken to furnish evidence both as to the proportional distribution of the antitoxin throughout the protein fractions precipitated at progressively increasing concentrations of sodium sulphate, and also as to whether the heat-denaturation (at 58°) effects changes in the nature of this association.

Several batches of plasma were treated in the manner described in Section I (p. 279). Estimations of the protein and the antitoxin content of the filtrates

¹ No appreciable heat-denaturation of the serum proteins takes place during the short time required for the solution of the sulphate.

² To every 100 c.c. of liquid add $Y - X$ grams anhydrous sodium sulphate; the volume changes following the solution of the sulphate in the plasma can be neglected.

from the unheated and from the heated sulphated plasmas were made and from the data thus obtained were calculated (a) the amount of protein remaining in every cc. of the filtrates, and (b) the respective precipitations of the serum proteins and of the antitoxin at each stage with the sulphate.

It was found that in the *unheated* plasmas the preliminary treatment of the plasma affected the limits for the precipitation of the antitoxin-bearing proteins. Thus, in plasma of which the reaction lay between $P_H 7.4$ and 8.3 the main part of the antitoxin-bearing proteins was precipitated in the

Fig. 7. Showing the relationship between the precipitation of the antitoxin and of the serum proteins from cresylised plasma B by sodium sulphate

The circumscribed points $\odot$ represent the data for the unheated plasma.

The points at the intersection of two lines $\times$ represent the data for the heat-denatured plasma

The figures by the side of each point indicate the concentration of sodium sulphate in the plasma

fraction isolated between 12 and 18.5 per cent. of sodium sulphate; from the same plasma to which 0.30 per cent. of cresylic acid had been added, the precipitation limits were respectively reduced to 11.5 and 16.5 per cent. of the sulphate¹. Furthermore, in the former case the limits were raised further by the dilution of the plasma; in the latter case they were lowered by an increase in the concentration of the cresylic acid in the plasma.

¹ A similar lowering of the precipitation limits was seen in the precipitation of cresylised plasma by ammonium sulphate.

In the *heated* plasmas the precipitation limits with sodium sulphate were considerably lower than those given above for the unheated liquids, and, as was anticipated, the increased precipitability of the antitoxin proved to be a function of the heat-denaturation of the antitoxin-bearing proteins.

The data furnished from the study of the precipitation of the cresylised plasma B by sodium sulphate have been embodied in the curve in Fig. 7 in which the circumscribed points and those at the junction of a cross respectively denote the values obtained from the unheated and the heated liquids.

A study of the curve shows that, in the *unheated* cresylised plasma, only a small amount of antitoxin (less than 5 per cent.) was precipitated in the pseudoglobulin-euglobulin zone, viz. at concentrations from 9—11.5 per cent. Beyond this stage the points representing the relative precipitation of antitoxin and protein lie on a straight line the slope of which remains constant until the whole of the antitoxin has been precipitated.

In the *heated* cresylised plasma there was an increased precipitation of protein and of antitoxin at each of the stages investigated. Even at a concentration of 8 per cent. of sodium sulphate there was an appreciable precipitation of denaturated pseudoglobulin and its associated antitoxin; the precipitation of antitoxin and pseudoglobulin was complete on the addition of 15.5 per cent. of sodium sulphate. The position of the corresponding points on the curve shows that, in heated plasma also, there is a direct proportionality between the precipitation of antitoxin with the protein at each stage with the sulphate, and, furthermore, that the points representing this relationship fall on the same straight line as that connecting the points obtained for the unheated liquids.

The gravimetric estimations of the relative amounts of the individual proteins precipitated at various stages with the sulphate indicated that the precipitation of antitoxin from the plasma coincided with that of the pseudoglobulin.

Calculations were accordingly made of the percentages of the total antitoxin and of the total pseudoglobulin precipitated at each stage with the sulphate and the data thus obtained were incorporated in the curve in Fig. 8, the positions for the data from unheated and heated plasmas being indicated by circumscribed and by crossed points respectively.

It will be seen that, in the unheated liquids, there is a comparatively small load of antitoxin associated with the fractions of the pseudoglobulin precipitated in the pseudoglobulin-euglobulin zone, and that, beyond this zone *the percentage of the total pseudoglobulin successively precipitated by increasing concentrations of the sulphate is a linear measure of the percentage of the total antitoxin associated with the precipitate.*

In the heated liquids there was no disturbance of this relationship between the relative precipitation of pseudoglobulin and antitoxin. The heat-denaturation merely served to induce the precipitation of a particular pro-

portion of the pseudoglobulin and its associated antitoxin at a concentration of sodium sulphate lower than that required for their precipitation from the unheated plasma.

These results furnish evidence in confirmation of the conclusions drawn from the investigation of the fractional precipitation of the pseudoglobulin by ammonium sulphate, viz. that from the point of view of reducing the proportion of protein associated with the antitoxins of sera, there are no practical advantages to be gained by the preliminary heat-denaturation of the serum proteins [Homer 1919].

Fig. 8. Showing the relative precipitation of antitoxin and pseudoglobulin from cresylised plasma B by sodium sulphate

The points $\odot$ refer to data obtained from the precipitation of unheated plasma
 The points $\times$ refer to data obtained from the precipitation of heat-denaturated plasma
 The figures by the side of each point indicate the concentration of sodium sulphate used for precipitation

III. THE APPLICATION OF THE ABOVE RESULTS TO THE ROUTINE CONCENTRATION OF SERA.

The question as to whether the routine concentration of antitoxic plasma shall be carried out with unheated or with heat-denaturated plasma can only be decided after a careful comparison of the respective end products as regards (a) their ease of filtration through Berkfeld and through white Doulton filter candles, and (b) the clinical after-effects of their administration to the animal or to the human.

Unfortunately, through the lack of the necessary training, I cannot produce evidence on the latter point other than that referred to in a previous note [Homer 1918, 2] in which attention is drawn to variations in the toxicity of cresylic acid in plasma according as the serum proteins in the latter have been subjected to heat-denaturation or not.

The conditions regulating the preparation of satisfactory end products from the ammonium sulphate or from the sodium sulphate fractionation of unheated and of heat-denaturated plasma have been studied separately and the following observations have been made.

(i) *The filtration of the end products from the concentration of unheated plasma.*

In order to obtain clear end products it is essential to fix the precipitation limits with ammonium sulphate or with sodium sulphate so as to ensure the elimination of euglobulin. Satisfactory products are obtained from the dialysis of the protein fractions isolated from unheated plasma (P_H 7.4—8.3) between concentrations of ammonium sulphate of which the lower limit is not less than 34 per cent. of saturation. In the cases where sodium sulphate is used the lower limit should not be less than 11.5 per cent.

Where the lower limit for the precipitation with ammonium sulphate was 33 per cent. of saturation or less, or was less than 11.5 per cent. with sodium sulphate, the end products were opalescent and filtered badly; the filter candles during the filtration became coated with a slimy deposit of euglobulin which retarded and ultimately stopped the process.

The Second Fraction precipitates isolated from unheated plasma (P_H 8.3) between 34 and 46 per cent. of saturation with ammonium sulphate or between concentrations of 12 and 18.5 per cent. of sodium sulphate contained the bulk of the antitoxin. The corresponding precipitates from cresylised plasma (0.30 per cent.) contained a lower proportion of the antitoxin for the reason that the presence of the cresylic acid induced an increased precipitation of the antitoxin-bearing proteins with the First Fraction precipitates.

The antitoxin carried down with the First Fraction whether precipitated by sodium sulphate or by ammonium sulphate, was, in all cases, recovered by extraction of the precipitates with a saturated solution of sodium chloride.

Contrary to the experience with heat-denaturated plasma it was found that the end products from the fractionation of unheated cresylised plasma between the above limits with ammonium sulphate were opalescent and filtered less readily than those obtained from the non-cresylised plasma. Further experiment showed that in the cresylised plasma the concentration of the precipitating sulphate required to ensure the agglutination and filtration of the cresylic acid-protein complex with the First Fraction precipitates was greater than that required for the complete precipitation of euglobulin.

The same phenomena were exhibited to a less marked degree in the precipitation of cresylised plasma by sodium sulphate.

These results indicate that, in the concentration of antitoxic plasma by the fractional precipitation of the unheated plasma, the preliminary addition of cresylic acid is disadvantageous. For, not only does it reduce the proportion of antitoxin associated with the Second Fraction precipitates, but it also leads to the production of less readily filterable end products than are furnished from the similar treatment of non-cresylised plasma.

(ii) *The filtration of the end products from heat-denaturated plasma.*

In previous communications I have described techniques [Homer 1917, 1918, 3] which gave the best results for the fractional precipitation of heat-denaturated plasma by ammonium sulphate. I am still convinced that more consistent and more reliable results are to be obtained from the preliminary adjustment of the reaction than from the alternative procedure of adding 0.30 per cent. of cresylic acid to the plasma.

In the present investigation also it has been found desirable to regulate the heat-denaturation of the serum proteins prior to their fractional precipitation with sodium sulphate.

The directions previously given for the suitable adjustment of the reaction of the plasma may be somewhat simplified. Satisfactory values for the heat-denaturation are obtained by the adjustment of the reaction of the plasma, so that 10 c.c. show a very faint green tinge with α -naphthol-phthalein; the colour change is readily detected when the plasma is viewed slantwise.

A further simplification of the routine technique in regard to the heating processes hitherto advocated in order to ensure the required heat-denaturation and the subsequent separation of heat-denaturated protein with the First Fraction precipitate can be effected as follows. To the adjusted plasma, prior to its being heated, is added the amount of sodium sulphate or ammonium sulphate required for the precipitation of the First Fraction precipitates. The sulphated mixtures are heated to 58° and kept at that temperature for four to five hours. They are then filtered and from the combined volume of the filtrates and washings are precipitated the Second Fraction precipitates in the usual way. By this method of procedure the two stages of the heating process are reduced to one.

However, in the case of the ammonium sulphate precipitates the advantage thus gained by combining the two stages of the heating is minimised by the greater losses of antitoxin subsequently experienced; the latter are no doubt due to the hydrolysis of ammonium sulphate and its dissociation into its component parts during the prolonged heating.

On the other hand, as sodium sulphate is the salt of a strong acid and a strong base, no such secondary complications arise.

Presumably this difference in the nature of the two sulphates also accounts for the somewhat sharper line of demarcation shown in the precipitation of eu- and pseudo-globulin from plasma by sodium sulphate than when

ammonium sulphate is employed; a phenomenon which favours the use of the former sulphate in the concentration of antitoxic sera.

In the heated plasmas containing sodium sulphate, in which the heat-denaturation had been regulated as above, the precipitation of the euglobulin was complete at a concentration of 8 per cent. of anhydrous sodium sulphate, but the separation of the precipitated protein by filtration through paper was impossible. Just as with ammonium sulphate, the indifferent filtration of the First Fraction mixtures was due to the presence of incompletely agglutinated particles of heat-denaturated protein, and unless the separation of the latter was ensured at this stage, the filtration of the end products was impossible. For this purpose it was found necessary to increase the concentration of sodium sulphate to 10 per cent., but, in order to guarantee the rapid filtration of the First Fraction precipitates it was advisable to use 11 per cent. of the sulphate, a procedure which entailed the precipitation of at least 35 per cent. of the antitoxin with the protein eliminated at this stage. However, provided that the heat-denaturation has not been greater than that recommended for the routine work, the antitoxin thus precipitated with the First Fraction precipitates can always be recovered by the extraction of the precipitate with a saturated solution of salt.

Those engaged in the routine work must be prepared to find that the precipitation limits discussed above may not give quite the same results when applied to the concentration processes carried out in their laboratories. There are many conditions which affect the precipitability of the serum proteins, and in the routine work the important factor, dilution, is apt to be forgotten. Thus, after the removal of the First Fraction, the solution contains considerably less protein than the original plasma; the protein content is further reduced by the addition of the filtered washings, a procedure which tends to modify the precipitation limits for the proteins (*ante*, p. 284). The investigation of practical details of this nature are best carried out by the individual worker under the conditions which prevail in his particular laboratory.

SUMMARY.

The results of the investigation have shown that:

- (1) In the precipitation of the serum proteins by sodium sulphate there are no critical points marking the limits for the precipitation of the individual proteins.
- (2) The concentration of either ammonium sulphate or sodium sulphate required for the precipitation of the serum proteins is affected by the reaction and by the dilution of the plasma and by the addition of cresylic acid to the plasma. In the latter case the extent of the increase is a function of the concentration of cresylic acid in the plasma.
- (3) Within the limits of the error of the experiment, the respective decreases in the molecular concentration of sodium sulphate and of am-

monium sulphate measured under the conditions given in (2) are practically identical.

(4) The percentage precipitation of the antitoxin with the proteins precipitated at various concentrations of sodium sulphate is a linear measure of the percentage precipitation of the antitoxin-bearing proteins. This relationship is undisturbed by the heat-denaturation of the serum proteins induced during the heating of adjusted plasma at 58° for four to five hours.

(5) In the concentration of antitoxic plasma by its fractional precipitation with sodium sulphate or ammonium sulphate, results can be obtained by the suitable fractionation of the unheated plasma similar to those which have hitherto been obtained with heated plasma.

(6) While the addition of cresylic acid materially aids the concentration of heat-denaturated plasma, its use with unheated plasma is to be deprecated.

(7) The agglutination of the particles of precipitated protein in the eu- and pseudo-globulin zone seems to be more satisfactory with sodium sulphate than with ammonium sulphate.

(8) As sodium sulphate, in contradistinction to ammonium sulphate, does not hydrolyse in solution, the sodium-sulphate-plasma First Fraction mixtures can be heated for four to five hours at 58° without loss of antitoxin. This method of procedure favours the production of clearer end products than would result from conducting the heating in the two stages adopted in the ammonium sulphate method.

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