VI. ON THE SEPARATION OF ANTITOXIN AND ITS ASSOCIATED PROTEINS FROM HEAT-DENATURATED SERA.

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It is recognised that in unheated antidiphtheritic and antitetanic sera the antitoxins are associated with the "salt-soluble" globulins precipitated between 30 and 50 % of saturation with ammonium sulphate. For this reason, in the routine concentration of such sera, it has been customary to isolate and dialyse the protein precipitated between these limits and, recently, observations have been published in regard to the factors limiting the degree of concentration thereby obtained [Homer, 1917, 1].

Further work has shown that, in heated sera, the association of the antitoxin with these so-called Second Fraction Precipitates is affected by the extent of the denaturation of the proteins induced during the preliminary heating of the serum. The heat-denaturation is evidenced by an increased precipitability of each of the individual serum proteins by ammonium sulphate, a phenomenon which is accompanied by an increase in the ratio of "salt-insoluble" to "salt-soluble" globulins and by an association of antitoxin with the "salt-insoluble" protein thus formed [Homer, 1917, 2, 1918, 1].

In the light of these observations it was necessary to extend the scope of the previous investigations so as to ascertain:

I. To what extent the limits for the precipitation with ammonium sulphate can be narrowed so as to include, in the Second Fraction Precipitates from heated sera, only those proteins with which the antitoxin is definitely associated.

II. Whether or no the antitoxin is evenly distributed throughout the protein fractions precipitated at successive stages between the limits indicated in (I).

I. THE LIMITS FOR THE PRECIPITATION OF THE SECOND FRACTION PRECIPITATES.

The gradient for the curves representing the precipitation of the serum proteins at progressively increasing percentages of saturation with ammonium sulphate indicates that there are no critical points for the precipitation of the eu- or of the pseudo-globulin or of the serum albumin.

The protein precipitated from unheated sera between 30 and 50 % of saturation with this salt consists mainly of pseudoglobulin admixed with small amounts of euglobulin and of albumin, whereas that similarly precipitated from heated sera contains a lower proportion of admixed euglobulin and a greater proportion of albumin. As was to be surmised, the relative proportion of the proteins precipitated from heated sera at this stage was influenced by the extent of the denaturation induced during the heating of the serum.

Experimental work was therefore undertaken to ascertain to what extent (a) the upper, and (b) the lower limit for the degree of saturation with ammonium sulphate could be changed so as to retain in the Second Fraction Precipitates only those proteins with which the antitoxin is definitely associated.

(a) The upper limit for the precipitation of the Second Fraction Precipitates.

The following observations show that the extent of the precipitation of albumin with the Second Fraction is a function of the heat-denaturation induced in the serum.

To separate volumes of plasma were added the requisite volumes of acid or of alkali to adjust the $[H']$ to suitable values. The separate sera, after being heated at a temperature of 57° for six hours, were made 50 % of saturation with ammonium sulphate and filtered. From the gravimetric determinations of the albumin content of the filtrates was calculated the percentage of albumin precipitated with the Second Fraction Precipitates. It was found that, where the heat-denaturation had been of the order of 60, 50, 45, 35 and 22 % respectively the Second Fractions contained 75, 55, 45, 40 and 22 % of the total albumin of the plasma.

As the antitoxin is attached to the pseudoglobulin and not to the albumin fraction of the serum proteins, the presence of albumin in the Second Fraction Precipitates must reduce the degree of concentration; its exclusion is, therefore, desirable.

It was found that the precipitation of heat-denaturated albumin could be eliminated by a sufficient lowering of the degree of saturation with ammonium sulphate. But, in the concentration of sera, where the main object is to ensure the complete recovery of the antitoxin in association with a minimum percentage of the proteins of the original plasma, such a procedure is not practicable. For, as there is no sharp line of demarcation between the precipitation of pseudoglobulin and of albumin from heat-denaturated sera, the lowering of the upper precipitation limit to the extent required for the complete exclusion of the albumin leads to considerable losses of antitoxin; the concentration of ammonium sulphate thereby adopted is insufficient to precipitate the whole of the pseudoglobulin and its associated antitoxin.

Thus, from sera in which a heat-denaturation of 35 % had been induced, there was a slight precipitation of denaturated albumin at 40 % of saturation

with ammonium sulphate; the extent of the precipitation rapidly increased with the further addition of ammonium sulphate. In order, therefore, to avoid the precipitation of albumin in the Second Fraction Precipitates it was necessary to lower the upper precipitation limit from 50 to 38 % of saturation.

On the other hand the precipitation of the pseudoglobulin and antitoxin from the same serum was not complete until the concentration of the sulphate had reached 44 % of saturation. Thus, the precipitate isolated between 30 and 42 % of saturation contained only about 90 % of the antitoxin, while rather less than 80 % of the antitoxin was associated with the protein precipitated between 30 and 40 % of saturation with the salt.

This difficulty was even more pronounced in the fractional precipitation of the antitoxin and its associated protein from sera showing a more extensive heat-denaturation. Thus, from sera showing a heat-denaturation of 50 % or more there was a marked precipitation of albumin even at 26 % of saturation with ammonium sulphate. It is obvious that in such cases the elimination of albumin from the end products is impracticable.

In dealing with the routine sera in which the heat-denaturation was of the order of 35 % or less, it was decided that the upper limit should not be reduced to less than 44 % of saturation. Even this reduction led to the exclusion of a considerable proportion of the denaturated albumin which would have been precipitated at 50 %, and, as a result, the end products in the former case showed a greater degree of concentration than was obtained in the latter. Thus, from a given serum, while the fraction isolated between 30 and 50 % of saturation with ammonium sulphate contained 33.3 % of the total serum proteins, that between 30 and 44 % of saturation contained only 25 % of the proteins. The load of antitoxin carried by the protein fraction thus isolated between the wider limits was of the order of 16,000 units and that between the narrower limits of 17,500 units per g. of protein.

(b) *The lower limit for the precipitation of the Second Fraction Precipitates.*

Having demonstrated that the degree of concentration is improved by the lowering of the upper limit for the precipitation of the Second Fraction by ammonium sulphate, the next step was to ascertain to what extent the lower limit could be raised so as to ensure the exclusion of that portion of the heat-denaturated globulin to which no antitoxin is attached.

The preliminary precipitation and separation of the First Fraction Precipitates (30 % of saturation with ammonium sulphate) from unheated sera remove most of the euglobulin together with a small amount of pseudoglobulin and antitoxin. The pseudoglobulin being "salt-soluble" can be recovered by the extraction of the precipitates with brine. On the other hand the corresponding precipitates from heated sera contain relatively greater amounts of heat-denaturated pseudoglobulin of which a certain proportion

has been converted into a "salt-insoluble" condition; the extent of the conversion is a function of the denaturation.

In sera showing a heat-denaturation of 40 % or less, the denaturated pseudoglobulin precipitated with the First Fraction Precipitates also carried down appreciable amounts of antitoxin but, as the latter was associated with salt-soluble protein, it could be recovered by an extraction of the precipitates with brine.

The precipitation of antitoxin from sera showing a more extensive heat-denaturation began at, and was completed at, a much lower concentration with ammonium sulphate than in the above. The proportion of antitoxin carried down with the First Fraction Precipitates increased progressively with the heat-denaturation. At the same time, owing to the increasing insolubility of the denaturated protein, there was a corresponding decrease in the percentage recovery of antitoxin and its associated proteins in a form suitable for clinical use. In fact, under some conditions, *e.g.* in sera more acid than P_H 4.5 or in sera containing excessive amounts of phenol and its homologues, or of ether, chloroform, etc., while practically the whole of the antitoxin is precipitated with the First Fraction Precipitates, only a small percentage of it can be recovered in solution, as the protein to which it is attached has become almost entirely "salt-insoluble."

From these observations it is clear that, in such sera, the lower limit for the precipitation of the Second Fraction with ammonium sulphate must be reduced rather than raised.

The attempts to increase the degree of concentration by raising the limit for the precipitation of the First Fraction Precipitates were, therefore, confined to such sera as showed a denaturation of less than 40 % and the problem was accordingly studied in respect of sera showing a denaturation (1) of from 25 to 40 %, and (2) of less than 25 %.

The end products from (1) were clear and readily filtrable. Those from (2) were cloudy and, owing to the suspension of heat-denaturated protein, could only be filtered with difficulty; they also showed a lower degree of concentration and a lower percentage removal of the serum proteins than was obtained from the sera in (1).

(1) *Sera in which a heat-denaturation of 25 to 40 % had been induced.*

It was found that in such sera the amount of protein precipitated with the Second Fractions isolated between 30 and 44 % of saturation with ammonium sulphate was not appreciably less than in those isolated between 33 and 44 % of saturation. On the other hand by raising the lower limit for the precipitation of the First Fraction Precipitates to 36 % of saturation, the amount of protein isolated between 36 and 44 % of saturation was only 70 % of that obtained between the wider limits. At the same time, however, there was a corresponding proportional increase (30 %) in the percentage of the antitoxin precipitated with the protein.

It is evident that no material gain resulted from the raising of the lower precipitation limit. On the contrary, the method of procedure was disadvantageous. It transpired that, while the antitoxin carried down with the First Fraction precipitated from the serum in question at 30 % of saturation with ammonium sulphate could be completely recovered by extraction of the precipitates with brine, that carried down with the precipitates at 33 and at 36 % of saturation with the sulphate could not be recovered to the same extent. In the latter cases it seems as though the greater concentration of *sulphions*, presumably by an electrical charging of the protein molecules, favours the conversion of the heat-denaturated pseudoglobulin and its associated antitoxin into a "salt-insoluble" condition; the antitoxin attached to the protein thus rendered "salt-insoluble" cannot be recovered in a form suitable for clinical use even though its activity has in no way been impaired.

(2) *Sera in which a heat-denaturation of less than 25 % had been induced.*

As was to be expected the raising of the lower limit for the isolation of the Second Fraction Precipitates from sera showing a heat-denaturation of 25 % or less led to a more marked diminution in the amount of protein precipitated than was shown in the sera discussed in (1).

Thus, in the cases investigated, the precipitate between 30 and 44 % of saturation with ammonium sulphate contained 15 and 48 % more protein than where the limits for precipitation had been 33-44 and 36-44 % of saturation respectively. Moreover, the products obtained from the dialysis of the protein precipitated between the widest limits were cloudy and unsatisfactory; those from the precipitates isolated between the narrower limits were clear, as the increased concentration of ammonium sulphate had given the particles of suspended heat-denaturated protein the necessary charge to ensure their aggregation, precipitation, and separation with the First Fraction Precipitates.

The determination of the antitoxin associated with the Second Fraction Precipitates thus isolated showed that while those precipitated between 30 and 44 % of saturation with ammonium sulphate contained the whole of the antitoxin of the original plasma, those between 33 and 44 % of saturation contained 95 % of the antitoxin, while only 65 % was associated with the fraction isolated between 36 and 44 % of saturation with the salt.

Hence, notwithstanding the increased precipitation of antitoxin with the First Fraction Precipitates induced by the raising of the lower limit of saturation with ammonium sulphate to 33 and 36 % respectively, the proportionally greater removal of protein thereby effected led to an increase in the degree of concentration shown by the end products. But, in no case was the concentration increased beyond that which would have been obtained between the wider limits of precipitation had the serum been subjected to the preliminary adjustment recently advocated [Homer, 1918, 2].

These observations show that the cloudy end products of unadjusted sera can be avoided by precipitating the First Fraction at 33 or 36 % of saturation with ammonium sulphate. Such an expedient will probably appeal to those engaged in routine work as being a simpler process than the preliminary adjustment of the serum. However, for the reason given above (p. 49), those who adopt the procedure must be prepared to face losses considerably greater than were recorded in my previous communication [Homer, 1918, 2], where the First Fraction was precipitated at 30 % of saturation with the sulphate.

It is thus evident that, in the routine concentration of sera, in which a complete recovery of antitoxin is to be desired, the Second Fraction should not be precipitated within narrower limits than 30 to 44 % of saturation with ammonium sulphate irrespective of whether the serum has been suitably adjusted previous to its being heated.

II. IS THE ANTITOXIN EVENLY DISTRIBUTED THROUGHOUT THE PROTEIN FRACTIONS SUCCESSIVELY PRECIPITATED AT STAGES BETWEEN THE LIMITS FIXED IN (I)?

The Second Fraction Precipitates from heat-denaturated sera were further fractionated in successive stages between the limits suggested in (I) in order to ascertain whether the antitoxin was evenly distributed throughout the protein fractions respectively isolated, or whether it was mainly precipitated at a definite concentration with ammonium sulphate.

The consideration of these points was of importance for, in the former case, the evidence would indicate that, in order to isolate antitoxin as a separate entity, means other than the fractional precipitation of the serum with salts must be employed. In the latter case then, for special clinical purposes such as for the intravenous or intrathecal injection of antitoxin, where a high unitage of antitoxin per gram of protein would be advantageous, it might be advisable to precipitate the antitoxin and associated protein within the narrow limits thus indicated, even though by so doing the percentage recovery of the antitoxin were considerably reduced.

Experimental work was accordingly instituted in regard to the successive fractionation of the end products from the concentration of sera by the Homer (1916, 1918) methods. The investigation was confined to the examination of:

(a) *Clear End Products*, i.e. those from sera in which the reaction had been adjusted so as to ensure a heat-denaturation of about 35 %.

(b) *Cloudy End Products*, i.e. those from sera in which the heat-denaturation was of the order of 25 % or less.

In each case the end product was diluted with normal saline so as to reduce the protein content of the liquid to the order of 5 %.

To a known volume of the diluted liquid was added a sufficient volume of a saturated solution of ammonium sulphate to bring the concentration of this

salt in the mixture up to 30 % of saturation. If a precipitate were formed at this stage the liquids were filtered. To an aliquot part of the filtrate was added the volume of the saturated solution necessary to raise the concentration of the sulphate in the filtrate to 33 % of saturation. The liquids were again filtered and by a similar method of procedure the ammonium sulphate content of the filtrates was successively raised to the order of 36, 40, 45 and 50 % of saturation.

The precipitates isolated at each of the successive stages were pressed and dialysed in the usual way. The protein content of the respective residues from dialysis was estimated with the aid of the Zeiss Refractometer; determinations were also made of the percentage of the total antitoxin units appearing in the respective end products.

Owing to the scarcity of experimental animals the research could not be carried out so extensively as originally planned. Nevertheless the results, so far as could be obtained, have provided sufficient data to elucidate the problem under discussion.

(a) *The successive fractionation of the Clear End Products obtained from sera showing a heat-denaturation of about 35 %.*

Antidiphtheritic plasma was concentrated by the Homer (1918) method, the heat-denaturation of the plasma being regulated by the preliminary adjustment of the reaction.

The end products, obtained from the dialysis of the protein precipitated from the heated plasma between 30 and 45 % of saturation with ammonium sulphate, were clear and remained clear on dilution, for they were free from any opalescent suspension of heat-denaturated protein; subsequent tests showed that they were also free from "salt-insoluble" protein.

The end product, under consideration, contained 15,000 units of antitoxin per gram of protein. After the necessary dilution with saline it was precipitated in the manner described above; the results are embodied in Table I.

Table I. *The successive fractionation of the Clear End Products from the concentration of antitoxic sera in which a heat-denaturation of 35 % had been induced.*

(The end product taken contained 15,000 units of antitoxin per gram of protein.)

Percentages of saturation with ammonium sulphate marking the limits for the precipitation of the various fractions	The residues from the dialysis of the respective fractions showed			
	Appearance	Percentage of the total proteins	Percentage of the total antitoxic units	Number of units of antitoxin associated with 1 g. of protein
0-30	—	0.0	0.0	—
30-33	clear	traces	traces	—
33-45	clear	80.0	73.5	13,800
33-40	clear	62.5	59.5	14,500
40-45	clear	18.5	16.5	13,800
45-50	clear	2.5	2.8	16,000

From a study of the table it will be seen that there was no appreciable precipitation of protein when the concentration of ammonium sulphate in the diluted end product was successively raised to 30 and to 33 % of saturation respectively.

Between 33 and 40 % of saturation with the sulphate there was precipitated 62.5 % of the total pseudoglobulin of the end product, and with this protein there was associated 59.5 % of the total antitoxin.

The protein precipitated from the diluted end product between 33 and 45 % of saturation with ammonium sulphate comprised 80 % of the total protein and 73.5 % of the total antitoxic units. Further fractionation indicated that 62.5 % of the protein and 59.5 % of the antitoxin were precipitated between 33 and 40 % of saturation with ammonium sulphate; 18.5 % of the protein and 16.5 % of the antitoxin were precipitated between 40 and 45 % of saturation with the sulphate; 2.5 % of the total protein and 2.8 % of the total antitoxin were precipitated between 45 and 50 % of saturation with the sulphate.

Unfortunately owing to the shortage of experimental animals the fractionation was not continued further.

An examination of these results shows that, within the limits of experimental error, the percentage precipitation of antitoxin in the respective sub-fractions is directly proportional to the percentage precipitation of protein. Moreover, it will be seen that, at each stage of the precipitation, the load of antitoxin per gram of protein in solution was practically the same as that exhibited in the original liquid taken for fractionation, viz. of the order of 15,000 units.

Similar results were obtained from the fractionation of other clear end products.

The data thus obtained have furnished sufficient evidence for the conclusion that the antitoxin precipitated with its associated proteins from the heat-denaturated sera under discussion is proportionately distributed between the protein fractions successively precipitated at increasing concentrations of ammonium sulphate.

(b) The successive fractionation of the Cloudy End Products from sera showing a heat-denaturation of 25 % or less.

It has been demonstrated that the concentration of antitoxic sera which, prior to their being heated, contained an unsuitable percentage of phenol and its homologues or of which the reaction lay between P_H 5.5 and 7.4, led to the production of cloudy end products. The latter yield a lower degree of concentration than that furnished by sera adjusted so as to ensure not only a more extensive denaturation but also better conditions for the precipitation of the heat-denaturated protein with the First Fraction Precipitates at 30 % of saturation with ammonium sulphate.

Some of these cloudy products were taken and fractionally precipitated in stages in the manner described above (p. 50).

The data with regard to the percentage precipitation of the total protein and of the total antitoxin with the fractions precipitated at the various stages have been included in Table II.

Table II. *The successive fractionation of the Cloudy End Products from the concentration of antitoxic plasma in which a heat-denaturation of 25 % had been induced.*

(The end product under consideration contained 12,000 units of antitoxin per gram of protein in solution.)

Percentages of saturation with ammonium sulphate marking the limits for the precipitation of the respective fractions	The residues from the dialysis of the various protein fractions showed			
	Appearance	Percentage of the total protein	Percentage of the total antitoxin	Number of units of antitoxin associated with 1 g. of protein
0-30	—	0.0	0.0	—
30-33	thick suspension	16.0	6.0	4,550
30-36	markedly opalescent	48.8	35.0	8,600
33-36	clear	32.0	28.0	10,500
33-40	clear	57.0	58.0	12,300
40-45	clear	8.9	10.0	13,500
36-40	clear	24.5	30.0	14,500

In the case recorded, the addition of ammonium sulphate to the diluted end product to the extent of 30 % of saturation did not cause a precipitation of protein.

Between 30 and 33 % of saturation there was precipitated 16 % of the total protein and with this was associated only 6 % of the total antitoxin. The fraction between 30 and 36 % of saturation contained 48.8 % of the total protein and 35 % of the total antitoxin. The percentage of the total protein precipitated in both of these fractions is considerably greater than that of the antitoxin, the disparity being more marked in the former fraction.

An analysis of the respective results shows that, by difference, there was a direct proportionality between the percentage of protein and antitoxin in the fraction precipitated between 33 and 36 % of saturation with the sulphate. A separation of the protein precipitated at 33 % of saturation with the sulphate was made and the filtrate was sub-fractionated further, the details being given in Table II.

These results indicate that, after the preliminary separation of the protein precipitated at 33 % of saturation with the sulphate, the sub-fractions successively isolated show the same proportionality as regards the relative precipitation of antitoxin and protein as was found in the sera discussed in (I).

In another case, not recorded in the Table, in which the heat-denaturation was less than 20 %, the sub-fraction precipitated between 30 and 33 % of saturation with ammonium sulphate contained about 20 % of the total protein

and about 5 % of the total antitoxin; that between 33 and 36 % of saturation with the sulphate contained 36 % of the total protein and about 16 % of the total antitoxin. In such sera the sub-fractions isolated from the filtrates after the preliminary removal of the protein precipitated at 36 % of saturation showed the proportionality exhibited after the preliminary removal of protein precipitated at 33 % in the previous case and at 30 % of saturation in the sera discussed in (a).

A study of Table II also shows that in the First Sub-fraction (30–33 % of saturation) the load of antitoxin per gram of protein is considerably less than in the subsequent fractions and in the original solution. Moreover, as was to be expected, the sub-fractions subsequently precipitated after the preliminary removal of this First Sub-fraction contained a load of antitoxic units per gram of protein slightly greater than that shown by the original liquid. However, the load of antitoxin per gram of protein was in no case greater than it would have been had the reaction been suitably adjusted as in (a).

From these results it is obvious that in heat-denaturated sera showing a denaturation of 25 % the concentration of the end products and their degree of clarity can be increased by raising the precipitation limit for the First Fraction Precipitates to 33 % of saturation with ammonium sulphate. In sera showing a lower degree of denaturation it is necessary to raise the limit to 36 % of saturation in order to achieve the maximum advantage. In these cases the increased concentration of the sulphate ensures the precipitation and separation of heat-denaturated globulin to which a small portion only of the antitoxin is attached: this protein would have been precipitated at 30 % of saturation in sera suitably adjusted as in (a).

After the preliminary removal of the denaturated globulin to which a comparatively low percentage of the antitoxin is attached, the remainder of the antitoxin was found to be evenly distributed throughout the successive sub-fractions precipitated by successively raising the concentration of ammonium sulphate.

It was also observed that, in the unadjusted sera under discussion, the protein fractions respectively precipitated between 30 and 33, and between 30 and 36 % of saturation with the sulphate did not dialyse completely. The increased concentration of sulphates had induced an apparent irreversible precipitation of heat-denaturated protein. The precipitate thus formed was only partially redissolved by long saturation in about ten times its bulk of a saturated solution of salt.

A study of the data given in Tables I and II shows that, while from the whole serum the proteins associated with the antitoxin were precipitated between 30 and 45 % of saturation with ammonium sulphate, the precipitation of the refined products within the same limits yielded only 80 % of the protein-antitoxin combination. It has been my experience that the percentage addition of ammonium sulphate necessary for the complete precipitation of a particular fraction of an individual protein is influenced by the concentration

of the latter and by the presence of other proteins in solution; it is also affected by the degree of purity of the protein and its rarity in solution. The relationships hereby involved are being more fully investigated.

SUMMARY.

1. For the complete recovery of antitoxin during the concentration of sera, showing a heat-denaturation of 35 % or less, by fractional methods employing the use of ammonium sulphate it is advisable to precipitate the Second Fraction between 30 and 45 % of saturation with the sulphate.

If the upper limit be reduced there will be incomplete precipitation of pseudoglobulin and antitoxin and a certain percentage of the latter will be discarded with the "albumin" filtrates.

If the lower limit be raised then antitoxin will be precipitated with the First Fraction Precipitates in a form not readily soluble in brine, and, therefore, to all intents and purposes lost.

2. In the precipitation of the Second Fraction from sera in which a denaturation of 25 % or less has been induced, the raising of the lower limit to 33 or to 36 % of saturation leads to the production of clearer and more concentrated end products than those obtained by the adoption of the lower limit of 30 % of saturation.

However, for the reasons given in (1) the benefit thus gained is minimised by the greater losses of antitoxin thereby incurred.

Moreover, the degree of concentration is not increased beyond that which would have been obtained had the serum been suitably adjusted and subsequently treated as in (1).

3. In heated sera showing a denaturation of 35 % or less the bulk of the antitoxin is associated with the proteins precipitated between 36 and 45 % of saturation with ammonium sulphate.

4. The further fractionation of the protein isolated between the limits indicated in (3) showed that the percentage of the total antitoxin precipitated between progressively increasing percentages of saturation with the sulphate is directly proportional to the percentage precipitation of protein at the respective stages.

5. From these observations it is clear that, in order to isolate antitoxin, means other than the fractional precipitation of the serum proteins must be employed.

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