VII. ON THE INCREASED PRECIPITABILITY OF PSEUDOGLOBULIN AND OF ITS ASSOCIATED ANTITOXIN FROM HEAT-DENATURATED SOLUTIONS.

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SOME time ago an investigation was undertaken in order to furnish data as regards the increased precipitability of pseudoglobulin relatively to that of its associated antitoxin from solutions of which, prior to their being heated, the reaction had been adjusted to values between P_H 4.5 and 9.5.

In view of the need for the immediate application of the results to routine work, the data obtained with regard to the precipitability of the protein from solutions of which the reaction had been adjusted to about P_H 8.0 were included in a previous paper [Homer, 1917, 1]; the data furnished from the wider aspect of the problem form the subject matter of the present communication.

The study of the changes in the precipitability of the pseudoglobulin presented no difficulty, but, unfortunately, the scarcity of experimental animals has considerably limited the scope of the investigation as regards the fate of the antitoxin.

(a) THE INCREASED PRECIPITABILITY OF PSEUDOGLOBULIN FROM ITS HEAT-DENATURATED SOLUTION BY AMMONIUM SULPHATE.

The solution of pseudoglobulin and antitoxin used in the experiment was prepared as follows from unheated oxalated antidiphtheritic plasma pooled from the bleedings from several horses.

To 4 litres of plasma was added an equal volume of a saturated solution of ammonium sulphate; the mixture was allowed to remain at room temperature for 4 hours before being filtered. The precipitate, after being well drained, was thrown into 8 litres of a half saturated solution of ammonium sulphate, care being taken both to break up the lumps of precipitate and to keep the mixture stirred from time to time. After two days of this treatment the mixture was filtered. The well drained precipitate was gently pressed between cloths and boards to express as much of the adhering fluid as possible; it was then dissolved in 4 litres of water and the solution was submitted to a repetition of the above described process.

To the solution of globulins finally obtained were added 4 litres of a saturated solution of sodium chloride together with sufficient solid salt to ensure complete saturation. The brined liquid was allowed to remain at room temperature for seven days and was then filtered. To the filtrate, containing the "salt-soluble" globulin in solution, was added 0.25 % of glacial acetic acid; the ensuing precipitate of pseudoglobulin was filtered, pressed and dialysed in the usual way. The residue from dialysis was diluted so as to reduce the protein content to the same order as that of the original plasma.

To twelve separate volumes of the experimental liquid were added varying amounts of acetic acid or of ammonia to adjust the reaction to values between P_H 4.5 and 9.5; the $[H^+]$ of each of the liquids was measured by the electrical method. The stoppered bottles containing the adjusted liquids were placed in a water bath at 57.5° and were kept at this temperature for a period of six hours.

Samples of the unheated solutions were made respectively 26, 30, 33, 36, 38, 40, 42.5, 44.5 and 47 % of saturation with ammonium sulphate and were then filtered. The protein content of the filtrates was measured by means of the Zeiss Immersion Refractometer, and the data thus obtained were compared with those derived from similar determinations of the heated liquids.

Table I. *Showing the influence of heat-denaturation on the precipitability of pseudoglobulin from its solutions at increasing concentrations of ammonium sulphate.*

The solutions which contained 7.48 % of pseudoglobulin were heated at 57.5° for 6 hours.

Experi- mental liquid No.	P_H of the liquids prior to their being heated	Residual percentage of pseudoglobulin in the filtrates from the heated liquids respectively brought to the following degrees of saturation with ammonium sulphate								
		26	30	33	36	38	40	42.5	44.5	47
0 control	unheated	7.40	7.33	5.16	2.48	1.88	1.48	1.04	0.40	0.20
1	9.5	3.47	3.08	1.37	0.60	0.20	0.00	0.00	0.00	0.00
2	9.3	4.38	3.61	1.74	0.64	0.34	0.14	0.00	0.00	0.00
3	8.7	7.04	4.31	2.14	1.40	0.53	0.36	0.00	0.00	0.00
4	8.3	7.36	4.68	2.68	1.64	1.26	1.05	0.21	0.00	0.00
5	7.5	7.48	5.33	3.24	1.54	1.50	—	0.15	0.02	0.00
6	6.4	7.13	5.64	3.56	2.47	1.22	0.75	0.21	0.02	0.00
7	5.9	7.17	5.46	3.18	2.28	1.26	0.80	0.20	0.00	0.00
8	5.1	5.05	4.62	2.95	1.79	0.95	0.75	0.21	0.00	0.00
9	4.9	3.36	2.77	1.87	0.18	0.20	0.00	0.00	0.00	0.00
10	4.65	1.85	1.85	1.62	0.00	0.00	0.00	0.00	0.00	0.00
11	4.5	1.05	1.05	0.46	0.00	0.00	0.00	0.00	0.00	0.00

The results embodied in Table I indicate the percentages of pseudoglobulin remaining in solution in the filtrates from the respective pseudoglobulin-ammonium-sulphate mixtures. From these data was calculated the percentage precipitation of pseudoglobulin at the respective stages: the latter results have been depicted in Table II.

Table II. *Showing the relation between the extent of the heat-denaturation of pseudoglobulin and its increased precipitability by ammonium sulphate.*

The solutions which contained 7.48 % of pseudoglobulin were heated at 57.5° for 6 hours.

Experimental liquid No.	P_H of the liquids prior to their being heated	Percentage denaturation of the pseudoglobulin	Percentage of the total pseudoglobulin precipitated from the solutions by the addition of ammonium sulphate to the following degrees of saturation									
			26	30	33	36	38	40	42.5	44.5	47	
0 control	unheated	—	0.0	1.6	30.1	67.0	75.7	80.0	86.0	94.7	97.3	
1	9.5	53.5	53.4	58.8	82.4	92.0	97.3	100.0	100.0	100.0	100.0	
2	9.3	47.3	41.2	51.5	76.4	92.5	95.7	98.1	100.0	100.0	100.0	
3	8.7	38.1	5.5	42.1	71.3	80.1	92.9	95.9	100.0	100.0	100.0	
4	8.3	34.0	1.0	37.6	62.7	78.0	84.0	86.0	97.3	100.0	100.0	
5	7.5	25.0	0.0	27.5	56.2	79.3	80.0	—	98.0	100.0	100.0	
6	6.4	21.8	3.0	24.3	51.0	67.0	84.0	90.0	98.0	99.0	100.0	
7	5.9	22.7	3.1	26.4	55.6	69.4	84.2	89.0	97.5	99.0	100.0	
8	5.1	33.3	32.2	38.0	60.5	76.0	87.2	89.5	97.5	100.0	100.0	
9	4.9	60.0	55.0	63.0	75.7	98.0	98.0	100.0	100.0	100.0	100.0	
10	4.65	70.0	75.5	75.5	78.0	100.0	100.0	100.0	100.0	100.0	100.0	
11	4.5	83.0	86.0	86.0	94.0	100.0	100.0	100.0	100.0	100.0	100.0	

From a study of the tables it will be seen that, in the unheated liquid, the precipitation of pseudoglobulin begins at about 28 % and is not quite completed at 47 % of saturation with the sulphate.

In the heated liquids there was complete precipitation of the protein at a much lower concentration of sulphate than that required in the unheated liquid. Thus, where a heat-denaturation of from 20 to 30 % had been induced, the precipitation of protein was complete at about 44 % of saturation with the sulphate; where the denaturation was of the order of 50 % there was complete precipitation of the protein between 38 and 40 % of saturation with the sulphate; as the denaturation was further increased, so the concentration of ammonium sulphate necessary for the complete precipitation of the pseudoglobulin diminished.

It will also be seen that the precipitability of the protein, at each degree of saturation with the sulphate, was considerably greater in the heated than in the unheated liquids, the increase being most pronounced where the heat-denaturation was greatest.

The latter point is more clearly brought out by the plotting of the results given in Table II in the form of curves. For the sake of clearness it was necessary to present the curves in two figures. In Fig. 1 have been included the curves representing the precipitation of protein by ammonium sulphate from the unheated liquid and from the heated acid liquids of which the reaction lay between the values P_H 7.4 and 4.5: the curves in Fig. 2 represent the precipitation of protein from the heated alkaline liquids of which the reaction lay between P_H 7.1 and 9.5.

From the relative positions of corresponding points on the curves it is evident that, at the respective concentrations of ammonium sulphate, the

precipitation of protein is greater from the heated than from the unheated liquids.

To take a case in point: in the unheated solution 40 % of protein was converted from the emulsoid to the suspensoid condition at 34 % of saturation with ammonium sulphate: the same percentage conversion was obtained in the heated liquids at lower concentrations with ammonium sulphate. Thus, in the heated liquids Nos. 1, 2, 3, 4, 5, 6, 7 and 8 in which the denaturation had been of the order of 53.5, 47.3, 38.1, 34, 25, 21.8, 22.7 and 33.3 % the above precipitation of protein was respectively achieved at 25, 28.8, 30.5, 31, 31.5, 31.5, 31 and 30 % of saturation with the sulphate.

Fig. 1.

Curves representing the percentage precipitation of pseudoglobulin from unheated and from heated solutions by increasing concentrations of ammonium sulphate.

Curve 0 for the unheated liquid; Curves 6, 7, 8, 9, 10 and 11 for the heated liquids of which the reaction, prior to their being heated, had been adjusted to P_H 6.4; 5.9; 5.1; 4.9; 4.65 and 4.5 respectively.

It will be seen that the group of curves 0, 3, 4, 5, 6 and 7, representing the precipitation of protein from those liquids in which a heat-denaturation of 40 % or less had been induced, is marked by a similarity of configuration throughout the whole of the range investigated; the shift in the relative positions of corresponding points on the curves is constant and is a measure of the increased precipitation induced by the heat-denaturation of the protein.

On the other hand, while there is a marked similarity between the con-

figuration of the individual curves in the group 1, 2, 8, 9, 10 and 11 the composite type of curve differs from that of the former group. The shift in the relative positions of corresponding points is not constant, the magnitude of the increased precipitation being relatively greater for the lower concentrations with the sulphate, a phenomenon which is accompanied by an increasing "salt-insolubility" of the denaturated protein precipitated at stages in the flat portion of the curves.

Fig. 2.

Curves representing the percentage precipitation of pseudoglobulin from unheated and from heated solutions by increasing concentrations of ammonium sulphate.

Curve 0 for the unheated solution. Curves 1, 2, 3, 4 and 5 for the heated solutions of which, prior to their being heated, the reaction had been adjusted to P_H 9.5; 9.3; 8.7; 8.3 and 7.5 respectively.

(b) THE PRECIPITATION OF ANTITOXIN WITH HEAT-DENATURATED PSEUDOGLOBULIN.

It had been my intention to ascertain the limits of saturation with ammonium sulphate between which the antitoxin was precipitated from each of the twelve solutions dealt with in (a). But, owing to the long continued scarcity of experimental animals, it was impossible to carry out the scheme on such an extensive and comprehensive scale, and, therefore, the scope of the work has been restricted to a determination of the extent of the association of antitoxin with the protein precipitated at a conveniently chosen concentration with ammonium sulphate, viz. at 30 % of saturation with the salt.

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The method of procedure was as follows. To each of the twelve solutions dealt with in (a) was added the necessary volume of a saturated solution of ammonium sulphate to bring the concentration of the latter in the mixture up to 30 % of saturation. The mixture was filtered; the protein and the antitoxin content of the filtrates were estimated in the usual way; the values obtained were compared with those for the experimental solution. From these data was calculated the percentage of the total antitoxin precipitated with the protein from the respective solutions.

The data thus furnished were incorporated in Table III and in the curve in Fig. 3. They show that while in the unheated liquid the precipitation of antitoxin at 30 % of saturation with the sulphate is negligible, in the heated liquids the proportion precipitated at this stage is considerable and increases with the extent of the denaturation.

Fig. 3.

Curve representing the relative precipitations of pseudoglobulin and antitoxin at 30 % of saturation with ammonium sulphate from solutions of which, prior to their being heated, the reaction had been adjusted to values between P_H 4.5 and 9.5.

Table III. *Showing the relation between the extent of the heat-denaturation of pseudoglobulin and the precipitation of antitoxin with the denaturated protein by 30 % of saturation with ammonium sulphate.*

The solution of pseudoglobulin showed a potency of 450 units per cc.

Experi- mental liquid No.	P _H of the liquid prior to its being heated	Percentage de- naturation of the pseudo- globulin	Percentage of the total pseudoglo- bulin precipitated at 30 % of satura- tion with ammon- ium sulphate	Percentage of the total antitoxic units precipi- tated with the pseudo- globulin at 30 % of saturation with ammonium sulphate
0 control	7.1	unheated	1.6	—
1	9.5	53.5	58.8	50
2	9.3	47.3	51.5	40
3	8.7	38.1	42.1	30
4	8.3	34.0	37.6	20
5	7.5	25.0	27.5	20
6	6.4	21.8	24.3	12
7	5.9	22.7	26.4	15
8	5.1	33.3	38.0	20
9	4.9	60.0	63.0	55
10	4.65	70.0	75.5	>66 <80
11	4.55	83.0	86.0	>80 <90
12	4.4	too solid to measure		>90

The precipitation of antitoxin was least from those liquids showing the least extensive denaturation (Nos. 4, 5, 6, 7 and 8), and in these cases the proportion of the total protein precipitated by the sulphate was not a linear measure of the proportion of the total antitoxin associated with the precipitate. In the more acid and in the more alkaline liquids, there was a marked increase in the precipitation of protein and of antitoxin, but it was found that the increased precipitation of protein beyond that shown in Nos. 4, 5, 6, 7 and 8 was a measure of the accompanying increased precipitation of antitoxin at this stage.

It seems justifiable to conclude that *in the unheated liquid* there is a comparatively small load of antitoxin attached to the fraction of pseudoglobulin which is precipitated at about 34 to 35 % of saturation with ammonium sulphate, that is, with the pseudoglobulin which, in unheated sera, would be precipitated at concentrations of ammonium sulphate slightly greater than that required for the precipitation of the euglobulin. A comparatively low degree of heat-denaturation suffices to ensure the precipitation of this fraction of the pseudoglobulin at 30 % of saturation with the sulphate. A more extensive denaturation leads to the precipitation at this stage of fractions of the pseudoglobulin which, in the unheated liquids, would require considerably greater concentrations of the sulphate. The antitoxin is evenly distributed throughout the range of the latter fractions, a deduction readily made from the study of the curve in Fig. 3 taken in conjunction with the results obtained in a recent investigation of the further fractionation of the proteins of heat-denaturated sera [Homer, 1919].

The data thus obtained also furnish additional evidence that, in the fractional precipitation of antitoxin and pseudoglobulin by ammonium sulphate, an increase in the extent of the denaturation, up to a point, favours the isolation of the antitoxin with a correspondingly decreased percentage of the original protein. But beyond this previously described limit [Homer, 1917, 2, 3; 1919] a more extensive denaturation ceases to be advantageous, for, with the further increased removal of protein there is a correspondingly increased proportional precipitation of antitoxin; moreover, much of the antitoxin thus precipitated is lost, as the protein with which it is associated has been converted into a "salt-insoluble" condition.

The consideration of the above results taken in conjunction with those from my previous investigations leads me to the conclusion that, in order to isolate the bulk of the antitoxin from antitoxic sera in association with a minimum percentage of the total serum proteins, there is no need for the preliminary prolonged heating of the serum originally advocated by Banzhaf and Gibson [1907] and now generally adopted by those engaged in the concentration of sera. For while I have shown that, if the preliminary heating be conducted to the best advantage, the antitoxin can be isolated from *heated* sera in association with a minimum amount of protein in the fraction precipitated between 30 and 44 % of saturation with ammonium sulphate, my more recent work demonstrates that the same results would have been obtained by the isolation of the protein fraction precipitated from the *unheated* serum between (*circ.*) 36 and 50 % of saturation with the sulphate.

The concentration of antitoxic sera by the fractional precipitation of the unheated serum by ammonium sulphate, as indicated above, is obviously a shorter process than those in which a preliminary heating of the serum is adopted. Furthermore, the fractional precipitation of the unheated plasma or serum between the higher limits for the concentration of ammonium sulphate is not attended with the filtration difficulties so often experienced with heated sera fractionated between the lower limits, for, in the former case not only is the complete separation of the euglobulin with the First Fraction assured, but the possibility of trouble arising from the incomplete agglutination of particles of heat-denaturated protein is avoided.

The above considerations seem to indicate that from the point of view of the isolation of antitoxin and its associated protein, there is no practical advantage to be gained by the preliminary heat-denaturation of the serum.

There is, however, some evidence to show that, from a clinical point of view, the heating of the serum is beneficial. For, it has been found that the subcutaneous injection into mice and guinea-pigs of a given amount of cresylic acid causes more pronounced toxic symptoms when administered in solution in presence of unheated serum proteins than when in the presence of serum proteins previously heated to 57.5° for four hours.

My observations with regard to the fractional precipitation and concentration of unheated sera and to the lessened toxicity of cresylic acid in presence of heat-denaturated proteins will be dealt with in later communications.

SUMMARY.

1. The increased precipitability of pseudoglobulin from its heat-denaturated solutions at concentrations of ammonium sulphate ranging from 26 to 47 % of saturation is a function of the heat-denaturation.

2. The increased precipitation of pseudoglobulin thus induced in (1) at 30 % of saturation with ammonium sulphate is accompanied by an increased precipitation of antitoxin. With the least extensive denaturation, the percentage of the total proteins precipitated at this stage is greater than that of the antitoxin; as the denaturation increases so the further increased precipitability of the protein becomes a linear measure of the increased precipitation of the antitoxin.

3. In the concentration of antitoxic sera by the fractional precipitation of the serum with ammonium sulphate there is no need for a preliminary prolonged heating of the serum. The results that are now obtained by the isolation from the heated serum of the protein fraction precipitated between 30 and 44 % of saturation with ammonium sulphate could be obtained from the *unheated* serum between 36 and 50 % of saturation with the sulphate.

4. The heating of the serum reduces the toxicity of the cresylic acid-protein complex.

5. The data furnished in this research also point to the conclusion that, in order to isolate antitoxin as a separate entity, means other than the fractional precipitation of pseudoglobulin solutions by salts must be employed.

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