XXVII. ON THE INFLUENCE OF THE HEAT DENATURATION OF PSEUDOGLOBULIN AND ALBUMIN ON THE NATURE OF THE PROTEINS APPEARING IN CONCENTRATED ANTITOXIC SERA.

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In previous communications [Homer, 1916, 1917, 1, 2] it has been demonstrated that, as a result of the heating of serum, considerable changes are effected in the precipitability of the serum proteins by 30 % of saturation with ammonium sulphate, the extent of the changes being a function of the reaction of the serum.

The practical application of these results to the concentration of antitoxic sera has led to the preparation of end products (the residue from the dialysis of the protein precipitated between 30 and 50 % of saturation with ammonium sulphate) containing from 20 to 30 % only of the total proteins of the original serum. These results show an improvement on those obtained by the methods hitherto published.

Recent work, however, has shown that the final products obtained by methods involving a heat denaturation of the serum proteins contain albumin as well as pseudoglobulin and, moreover, that the relative amount of albumin increases with the extent of the heat denaturation of the proteins.

The presence of albumin in the final product is to be avoided for two reasons; the one that, since the antitoxin is associated with the pseudoglobulin and not the albumin constituent of the serum proteins, the presence of albumin in the final product must lower the degree of concentration; the other that the inclusion of albumin may be undesirable from the clinical point of view [Dale and Hartley, 1916].

Before attempting to ascertain whether an alteration of the precipitation limits for the Second Fraction precipitate would obviate the precipitation of heat denaturated albumin with the pseudoglobulin, it was necessary to furnish

experimental evidence with regard not only to the extent to which the denaturation of the serum proteins was affected by the time of heating but also to the extent to which the denaturation of the individual serum proteins was affected by the reaction of the serum.

Owing to the necessity for the immediate practical application of the results of the investigation to routine work, the experiments have, for the present, been confined to a study of the behaviour of pseudoglobulin and of albumin in this latter respect.

A. THE INFLUENCE OF THE TIME OF HEATING OF SERA ON THE EXTENT OF THE HEAT DENATURATION OF THE SERUM PROTEINS.

During the progress of the experimental work involved in the study of the influence of the reaction of sera on the extent of the heat denaturation of the serum proteins it was noticed that, with alkaline sera, the changes were a gradual function of the time of heating while, with acid sera, the changes were complete within a comparatively short time.

In order to furnish detailed evidence on this point the following experiments were undertaken. Antitoxic serum to which had been added 2 % of sodium chloride was diluted with one-third its volume of water. To separate volumes, each of 100 cc. of the diluted plasma, were added varying amounts of acid and of alkali and the hydrogen ion concentration $[H^+]$ of the various experimental liquids was measured by the electrical method. The stoppered bottles containing the experimental liquids were then placed in a water bath at 57.5° and were heated for several hours at that temperature.

Samples of the experimental sera, previous to their being heated, were made 30 % of saturation with ammonium sulphate and filtered. The protein content of the filtrates was measured by means of the Zeiss refractometer. Similar measurements were made with samples of the heated liquids withdrawn from time to time from the various bottles of sera in the bath. From the data thus obtained was calculated the percentage increase in the precipitability of the serum proteins after the heating of the sera for the stated periods of time.

The results have been incorporated in Table I and indicate that:

(1) During the heating of alkaline sera the extent of the heat denaturation gradually increases with the prolongation of the time of heating.

The maximum value is obtained after four hours' heating at 57.5° .

(2) In the acid sera the extent of the heat denaturation has practically reached its maximum value after one hour at the specified temperature.

(3) The nature of the changes involved in the alkaline sera is different from that in the acid sera, for at no time during the heating of the alkaline sera does acidification of the clear heated liquids to a value P_H 5.0 produce the precipitate of denaturated protein so characteristic of heated acid sera.

During the course of the work it has also been observed that both with acid and alkaline sera the maximum effects are produced:

(a) more quickly if the temperature to which the serum is heated be raised beyond 57.5° , and

(b) more slowly if the sera be heated at temperatures lower than 57.5° .

TABLE I. *The influence of the time of heating the sera at 57.5° on the extent of the heat denaturation of the serum proteins.*

[Protein content of the plasma = 7.15 %.]

Volume of acid or of alkali added to 100 cc. of the diluted plasma	P_H of the diluted plasma measured by the electrical method	Percentage increase in the precipitability of the serum proteins by 30 % of saturation with ammonium sulphate after heating the serum at the specified temperature for a period of				
		1 hr	2 hrs	3 hrs	4 hrs	6 hrs
4 cc. $N NH_3$	9.4	23.1	37.2	—	44.0	45.0
2	9.1	23.0	32.0	—	40.0	40.0
1	8.8	22.2	30.0	—	40.0	40.0
0.6	8.2	17.7	27.0	—	34.6	35.6
0.5 cc. $N HAc$	7.4	12.0	17.0	—	22.0	22.6
0.75	7.1	9.0	11.0	—	16.0	16.8
2.5	5.9	13.0	12.9	13.0	—	—
3.0	5.7	13.0	16.6	16.6	—	—
3.5	5.5	15.0	16.0	16.0	—	16.0
4.0	5.3	20.0	23.5	24.2	—	—
4.5	5.1	30.0	32.8	33.0	—	33.0
5.0	5.0	34.8	40.0	40.0	—	—
6.0	4.8	60.9	67.5	67.0	—	—
6.5	4.7	64.6	79.8	79.8	—	80.0

These results will be of service to those engaged in the concentration of antitoxic sera. They show that, in order to get the maximum effect, in the case of sera more alkaline than P_H 7.4 the time of the preliminary prolonged heating of the serum at 57.5° must not be less than four and need not be greater than six hours after the liquid has reached the specified temperature. In the case of acid sera the full effect is produced within from one to two hours of the temperature reaching 57.5° .

To what extent variations in the temperature at which the serum is heated affect the order of the maximum value for the heat denaturation of the serum proteins at a particular $[H^+]$ of the serum is under investigation.

B. THE EFFECT OF CHANGES IN $[H^+]$ ON THE DENATURATION OF PSEUDOGLOBULIN INDUCED DURING THE HEATING OF ITS SOLUTIONS AT A TEMPERATURE OF 57.5° FOR A PERIOD OF SIX HOURS.

A solution of pseudoglobulin was prepared as follows:

To four litres of antidiphtheritic serum were added two litres of water and six litres of a saturated solution of ammonium sulphate. The liquid was allowed to stand for one hour and was then filtered. The precipitate was washed with four litres of a half-saturated solution of ammonium sulphate and, after being thoroughly drained, was thrown into four litres of a saturated solution of salt containing an excess of solid salt. The salted liquid was allowed to stand for two days, care being taken to macerate the precipitate thoroughly. The liquid was then filtered and to the filtrate was added 0.25 % of glacial acetic acid. The ensuing precipitate was filtered, pressed and, after the addition of 3 % of solid crystallised sodium carbonate, was dialysed. The residue from dialysis was diluted until the protein content was of the order of 8 %, and 1.5 % of solid salt was then added.

Aliquot parts of the solution were measured into stoppered glass bottles; varying amounts of acid and of alkali were added and the $[H^+]$ of each of the liquids was determined by the electrical method. The bottles containing the liquids were then heated in a water bath at a temperature of 57.5° for a period of six hours. The protein content of the unheated liquids and also of the filtrates from samples of the unheated liquids brought up to 30 % of saturation with ammonium sulphate were measured by means of the Zeiss immersion refractometer. The values thus obtained were compared with the corresponding values for the heated liquids. From the data thus furnished were calculated the percentage precipitation of pseudoglobulin itself and also the percentage increased precipitability of the pseudoglobulin by 30 % of saturation with ammonium sulphate induced during the heating of the pseudoglobulin solution. The results which have been incorporated in Table II (p. 297) have also been plotted in Figure 1, Curves 1 and 2.

The appearance of the heated liquids was characteristic throughout the ranges of acidity and alkalinity investigated. On the alkaline side the liquids were clear or were characterised by an opalescence. On the acid side the liquids were converted during the heating into thin suspensions, the semi-solid consistency of which became more marked as the acidity increased.

From a study of the data given in Table II, Column A, it will be seen that the conversion of soluble pseudoglobulin into insoluble protein was of the

order of 5 % or less in the liquids of which the reaction lay between the ranges P_H 5.5 and 9.7. On the acid side of P_H 5.5 there was a marked precipitation of insoluble protein; thus, at P_H 5.34 it was of the order of 17.9 % while at P_H 4.6 it was of the order of 78 %.

Fig. 1

Curve 1 — represents the influence of changes of reaction on the conversion of soluble pseudoglobulin into an insoluble condition during the heating of solutions of pseudoglobulin at 57.5° for 6 hours.

Curve 2 - - represents the accompanying percentage increased precipitability of the heat-denatured pseudoglobulin.

The data given in Column B, Table II show that there was, throughout the whole range investigated, a marked increase in the precipitability of the serum proteins by 30 % of saturation with ammonium sulphate: the extent of the increase was a function of the reaction of the liquid.

The increased precipitability was least between the range P_H 5.5 and 8.0. On either side of these limits the rate of increase was appreciable: the gradient for the increase being more gradual on the alkaline than on the acid side. As the degree of alkalinity decreased from P_H 9.7 to 8.0 the increased precipitability decreased from 57.6 to 32.6 %; between P_H 8.0 and 5.5 it varied from 25 to 30 %, while between P_H 5.3 and 4.6 it rapidly rose from 40.0 to 75.5 %.

TABLE II. *Showing the influence of the hydrogen ion concentration on the denaturation of pseudoglobulin induced during the prolonged heating of a 7.95 % solution of pseudoglobulin at a temperature of 57.5° for 6 hours.*

Volume of normal acid or alkali added to 100 cc. of the pseudoglobulin solution	P _H of the solutions measured by the electrical method	Appearance of the heated liquids	A		B	
			Percentage conversion of soluble into insoluble protein during the heating of the pseudoglobulin solutions		Percentage increase in the precipitability of the pseudoglobulin by 30 % of saturation with ammonium sulphate	
5.0 cc. N NH ₃	9.72	clear	...	5.5	...	57.6
4.0	9.51	"	...	5.5	...	57.0
3.0	9.40	"	...	4.2	...	50.4
2.0	9.30	"	...	1.3	...	44.0
1.50	9.03	very slightly opalescent	...	1.3	...	43.7
1.00	8.63	slightly opalescent	...	1.3	...	42.4
0.50	7.97	"	...	0	...	32.6
0.25	7.40	opalescent "	...	0	...	29.2
0.00	6.81	very thin emulsion	...	0	...	25.6
0.50 cc. N HAc	6.43	thin emulsion, filtrate opalescent	...	1.3	...	26.3
1.00	5.99	thin emulsion, filtrate slightly opalescent	...	4.2	...	27.6
1.60	5.34	thick emulsion, filtrate clear	...	17.9	...	40.0
2.50	5.06	" " "	...	43.0	...	48.2
3.30	4.80	" " "	...	60.9	...	58.9
4.25	4.64	almost solid, filtrate clear	...	66.0	...	64.2
5.0	4.60	" " "	...	78.0	...	75.5

C. THE EFFECT OF CHANGES IN [H⁺] ON THE HEAT DENATURATION OF ALBUMIN INDUCED DURING THE HEATING OF ITS SOLUTIONS AT 57.5° FOR A PERIOD OF SIX HOURS.

The solution of albumin was prepared as follows:

The filtrate from the precipitated globulins (ante, p. 295) was saturated with ammonium sulphate and the ensuing precipitate was filtered off. The precipitate was washed from the filter paper into three litres of a half-saturated solution of ammonium sulphate. The liquid after standing for twenty-four hours was filtered, and the filtrate was saturated with ammonium sulphate and allowed to stand for two days at room temperature. The precipitated albumin was filtered off, thoroughly drained, pressed and dialysed until the residue in the bags was practically free from sulphate. The residue from dialysis was diluted until the protein content was of the order of 6 % and 1.5 % of solid sodium chloride was then added.

To aliquot parts of the above solution of albumin were added varying amounts of acid and alkali so as to bring the [H⁺] of the series of experimental liquids within the ranges P_H 9.7 and 4.5; the [H⁺] of each of the solutions being measured by the electrical method previous to the liquids being heated at a temperature of 57.5° for a period of six hours.

The protein content of each of the above liquids and of the filtrates from

Fig. 2

Curve 1 — represents the influence of changes of reaction on the conversion of soluble albumin into an insoluble condition during the heating of solutions of albumin to 57.5° C. for 6 hours.

Curve 2 - - - represents the accompanying percentage increased precipitability of the heat denaturated albumin by 30 % of saturation with ammonium sulphate.

Curve 3 — x — x — represents the accompanying percentage increased precipitability of the heat denaturated albumin by 50 % of saturation with ammonium sulphate.

them made 30 % and also 50 % of saturation with ammonium sulphate were estimated before and after their having been heated.

The data thus obtained have been incorporated in Table III and have been plotted in Curves 1, 2 and 3 in Figure 2.

From a study of these data it will be seen that, as in the case of solutions of pseudoglobulin, the heated liquids on the alkaline side were either clear or else showed only a slight opalescence, whereas on the acid side the liquids were converted into thin suspensions the consistency of which became more markedly solid as the degree of acidity increased.

From the results given in Column *A* of Table III it will be seen that the actual conversion of soluble albumin into an insoluble condition was less than 5 % between the ranges P_H 9.5 and 5.5. On the acid side of P_H 5.5 it rapidly rose from 6.0 % at P_H 5.5 to 57.6 % at P_H 4.87 and to 100 % at P_H 4.40.

From the results given in Column *B* of the same table it will be seen that the precipitability of albumin by 30 % of saturation with ammonium sulphate is not appreciably affected during the heating of solutions of which the

TABLE III. *Showing the influence of the hydrogen ion concentration on the denaturation of albumin induced during the prolonged heating of a 5.20 % solution of albumin at a temperature of 57.5° for a period of 6 hours.*

Volume of normal acid or alkali added to 100 cc. of the solution of albumin	P_H of the solutions measured by the electrical method	Appearance of the heated liquids	Column A		Column B		Column C	
			Percentage conversion of soluble into insoluble protein during the heating of the albumin solutions		Percentage increase of the precipitability of the albumin by 30 % of saturation with ammonium sulphate		Percentage increase of the precipitability of the albumin by 50 % of saturation with ammonium sulphate	
4.3 cc. $N NH_3$	9.53	slightly cloudy	...	2.2	46.0	75.0		
3.0	9.11	" "	...	1.0	36.7	58.0		
2.2	8.70	clear	...	0.0	8.0	50.0		
1.00	8.41	"	...	0.0	0	44.0		
0.50	7.81	"	...	0.3	0	31.5		
0.25	7.40	"	...	0	0	22.7		
0.00	6.90	"	...	0	0	21.3		
0.70 cc. $N HAc$	6.27	slightly opalescent, filtrate opalescent	...	0	0	26.1		
1.40	5.53	cloudy, filtrate opalescent	...	6.0	14.2	34.2		
2.20	5.18	thin emulsion, filtrate clear	...	30.0	36.7	44.0		
3.0	4.87	thick	...	57.6	55.0	57.0		
6.0	4.52	"	...	89.0	Too thick to be measured in a pipette	Too thick to be measured in a pipette		
8.5	4.40	almost solid, filtrate clear	...	100				
6.0 cc. $N H_2SO_4$	3.40*	"	...	100				

* Opalescent after the addition of acid.

reaction falls between the limits P_H 8.4 and 5.5. On either side of these limits there is a rapid rise in the precipitability of the albumin.

In Column C have been given the data with regard to the increased precipitability of the solution of albumin by 50 % of saturation with ammonium sulphate. It will be seen that the increased precipitability of the albumin is a function of the $[H^+]$ of the solutions. Thus, as the $[H^+]$ increased from P_H 9.5 to 7.8 so the increased precipitability decreased from 75 % to 31 %; between the values of P_H 7.8 and 5.5 it varied from 20 to 30 % while on the acid side of P_H 5.5 the percentage increased precipitability was practically equivalent to the percentage conversion of soluble into insoluble protein.

The results recorded above for pseudoglobulin and for albumin respectively show certain similarities with those previously obtained from corresponding experiments with antitoxic sera [Homer, 1917, 1]. Thus it was noticed that:

(1) The extent of the heat denaturation of the individual proteins, pseudoglobulin and albumin, is a function of the $[H^+]$ of the solutions.

(2) There is coincidence between the curves representing the conversion of soluble into insoluble protein during the heating of whole serum, of solutions of albumin and of pseudoglobulin respectively.

(3) The curve for the increased precipitability of albumin by 50 % of saturation with ammonium sulphate has a configuration very similar to those for the increased precipitability of pseudoglobulin and of the proteins of whole serum respectively by 30 % of saturation with ammonium sulphate.

(4) The values for the increased precipitability of the serum proteins by 30 % of saturation with ammonium sulphate are somewhat lower than those for solutions of pseudoglobulin at the same $[H^+]$. This is probably due, partly to the diluent effect of the accompanying albumin in whole serum and partly to the "buffer" action of the latter.

(5) In all cases the minimum change takes place between the ranges P_H 5.5 and 8.0 and there is coincidence of the curves on the acid side of P_H 5.5.

(6) The changes taking place during the heating of alkaline sera and of alkaline solutions of pseudoglobulin and of albumin are not of the same type as those induced in the acid solutions, for acidification of the heated alkaline liquids to P_H 5.0 does not produce the thin suspension so characteristic of the heated acid liquids.

(7) The increased precipitability of the heated alkaline sera and of alkaline solutions of albumin and of pseudoglobulin only attains its maximum value after several hours' heating. On the other hand the changes in the acid liquids are practically complete within one hour's heating at the specified temperature.

From the work of Chick and Martin [1910, 1911, 1912, 1, 2] it was anticipated that while a certain similarity would exist between the configuration of the curves for the heat denaturation of pseudoglobulin and of albumin respectively at hydrogen ion concentrations on either side of their isoelectric points, there would be a shift in the position of corresponding portions of the curves due to the fact that the isoelectric point of pseudoglobulin is at P_H 5.52 while that of albumin is at P_H 4.70. Curiously enough not only was the region of least change the same in both cases, viz., from P_H 5.5 to 8.0, but there was absolute coincidence of the curves on the acid side of P_H 5.5.

These phenomena present points of interest in connexion with the views held both as to the justification for regarding the protein constituents of serum as well defined entities and as to the possible conversion of pseudo- into euglobulin and of albumin into pseudoglobulin.

The results of an investigation of the nature of the heat denaturated proteins obtained from albumin and pseudoglobulin will be dealt with in a later paper.

D. THE PRECIPITATION OF HEAT DENATURATED PSEUDOGLOBULIN AND HEAT DENATURATED ALBUMIN WITH THE FIRST AND SECOND FRACTION PRECIPITATES.

From a consideration of the above results it is obvious, that in sera which have been subjected to prolonged heating the First and Second Fraction precipitates respectively comprise the various proteins in proportions which are somewhat different from those in the corresponding precipitates in the unheated serum.

An investigation was therefore undertaken in order to furnish data with regard to the composition of the First and Second Fraction precipitates in heat denaturated sera throughout the ranges of acidity and alkalinity previously considered above in sections A, B and C.

The wider aspect of the problem has not yet been completed but, in view of the need for the immediate application of my results to routine concentration work, data are being published with regard to the precipitability of heat denaturated pseudoglobulin and albumin by ammonium sulphate from solutions the reaction of which had been adjusted to that of the routine batches of sera taken for concentration. The application of those results to the routine work has led to the production of end products in which the proportion of albumin has been lessened while the degree of concentration has been increased.

Solutions of pseudoglobulin and of albumin (prepared as in B and C), previous to their being heated to a temperature of 57.5° for six hours, were adjusted to about P_H 8.0. Samples of the heated and of the unheated liquids were made varying percentages of saturation with ammonium sulphate and were then filtered, the protein content of the filtrates being estimated by means of a Zeiss refractometer. From the data thus obtained was calculated the percentage conversion of pseudoglobulin and of albumin respectively from the emulsoid to the suspensoid condition induced by the addition of the various amounts of the electrolyte to the heated and to the unheated solutions.

The results obtained with pseudoglobulin have been embodied in Table IV. They show that in the unheated solutions the extent of the precipitation of pseudoglobulin increases with the concentration of ammonium sulphate, the change being complete at about 50 % of saturation with the electrolyte. In the heated solutions the precipitability of the protein by concentrations of ammonium sulphate beyond 20 % of saturation is considerably enhanced, the precipitation being practically complete at 42.5 % of saturation.

TABLE IV. *Showing the extent to which the precipitability of pseudoglobulin by ammonium sulphate is affected by the prolonged heating of its solution at 57.5° for 6 hours.*

[Percentage of pseudoglobulin in original solution = 7.10.]

P_H of the solution = 8.0.

Percentage of saturation with ammonium sulphate	Residual percentage of pseudoglobulin in solution in the filtrates from the ammonium-sulphate-pseudoglobulin mixtures		Percentage precipitation of pseudoglobulin by the ammonium sulphate in the	
	(a) before heating	(b) after heating	(a) unheated liquids	(b) heated liquids
20	6.92	6.77	2.5	4.6
25	6.77	5.65	4.6	20.5
28	6.67	4.56	6.0	35.9
30	6.04	3.77	15.0	47.0
33	3.86	2.76	45.5	61.2
37.5	1.65	0.75	79.5	89.5
42.0	1.14	0.19	84.0	97.5
44.5	0.54	0.03	92.5	99.5
47.5	0.26	0.00	96.5	100.0
50.0	0.07	0.00	99.0	100.0

In the case of albumin (Table V) similar results were obtained. Thus, in the unheated liquids at 44 % of saturation with ammonium sulphate about 6 % of the total albumin was converted from the emulsoïd to the suspensoïd state: at 50 % of saturation with the electrolyte the conversion was of the order of 10 %; beyond this point the precipitation of albumin gradually increased, the precipitation being complete at about 75 % of saturation. In the heated solutions the precipitation of albumin at concentrations of ammonium sulphate greater than 40 % of saturation was considerably increased. Thus, at 42 % of saturation the precipitation was of the order of 29 % of the total albumin; at 44 % of saturation it increased to 33, while at 50 % of saturation the precipitation of albumin rose to the order of 52 %.

E. THE PRACTICAL APPLICATION OF THE ABOVE RESULTS TO THE ROUTINE CONCENTRATION OF ANTITOXIC SERA.

While recognising that the values recorded in Tables IV and V for the percentage precipitation of the individual proteins from their respective solutions may be somewhat higher than when they are associated together in serum, it seemed justifiable to conclude that, in the routine work, by lowering the concentration of ammonium sulphate for the precipitation of the Second Fraction from 50 to 44 % of saturation, there would still be complete precipitation of the pseudoglobulin and antitoxin while the proportion of albumin precipitated at this stage would be appreciably lessened.

TABLE V. *Showing the extent to which the precipitability of albumin by ammonium sulphate is affected by the prolonged heating of its solution at 57.5° for 6 hours.*

[Percentage of albumin in the original solution = 6.27.]

P_H of the solution = 8.24.

Percentage of saturation with ammonium sulphate	Residual percentage of albumin in solution in the filtrates from the ammonium-sulphate-albumin mixtures		Percentage precipitation of albumin by the ammonium sulphate in the	
	(a) before heating	(b) after heating	(a) unheated liquids	(b) heated liquids
20	6.27	6.27	0	0
25	6.27	6.27	0	0
28	6.05	6.14	3	2
30	6.14	6.02	2	4
33	6.05	5.83	3	7.1
37.5	5.95	5.61	5	10.6
42	5.92	4.46	5.5	29.0
44.5	5.84	4.20	6.7	32.9
47.5	5.77	3.86	8.0	38.4
50.0	5.64	2.98	10.0	52.5
56.5	4.76	1.10	23.7	82.5
62.5	3.45	0.81	45.0	87.0
66.5	0.73	0.15	88.5	97.5
70.0	0.18	0.00	97.0	100.0

In order to test this point concentrations were carried out with antiphtheritic and with antitetanic sera. In both cases a denaturation of the serum proteins to the extent of about 30 % was induced during the initial heating of the serum. Aliquot parts of the filtrates from the First Fraction precipitates were respectively brought up to 44, 47 and 50 % of saturation with ammonium sulphate and the ensuing precipitates were filtered, pressed and dialysed in the usual way.

TABLE VI. *Showing the increase in the degree of concentration of antitoxic sera induced by lowering the precipitation limit for the Second Fraction precipitate.*

Precipitation limits for the Second Fraction precipitates (percentage of saturation with ammonium sulphate)	Percentage of the total antitoxic units appearing in the Second Fraction precipitates	Percentage removal of the total proteins of the original serum	Increased potency per cc.	Units of antitoxin per g. of protein in the final product
Antitetanic plasma*				
30-44	95	69	10 times	6,250
30-50	95	64	8 "	5,250
Antiphtheritic plasma*				
30-44	92	72.5	9.5 "	18,750
30-47	96	67	8.0 "	16,500
30-50	96	66	7.5 "	15,000

* Comparable results were obtained irrespective of whether the heat denaturation had been induced by the prolonged heating of serum at 57.5° or by the shortened heating process after the addition of cresylic acid to the serum.

A comparison was then made between the removal of the serum proteins, the increased potency and the relative recovery of antitoxin in the several experiments (Table VI).

From these results it is obvious that for the complete precipitation of the antitoxin and the pseudoglobulin from sera in which a heat denaturation of about 30 % has been induced, the concentration of ammonium sulphate need not be greater than 44 % of saturation.

The adoption of 44 % of saturation with ammonium sulphate in preference to 50 % hitherto recommended presents the following advantages:

(1) The precipitation of heat denaturated albumin with the Second Fraction precipitate is appreciably lessened, thereby ensuring in the end product:

(a) a further reduction in the percentage of the total proteins of the original serum without transference of antitoxin to the First Fraction precipitate, and

(b) a corresponding increase in the degree of concentration.

(2) The colour of the end products is much less pronounced than when the precipitation limit of 50 % of saturation is used. The extra amount of albumin precipitated between 44 and 50 % of saturation adsorbs an appreciable amount of the colouring matter of serum.

(3) The smaller volume of saturated solution of ammonium sulphate thus required for the precipitation of the Second Fraction precipitate entails a slight reduction of labour and expense.

From the above observations it is clear that, if the precipitation limits for the Second Fraction precipitate be altered so as to exclude the precipitation of heat denaturated albumin, the maximum load of antitoxin which can be carried by one gram of protein in the final product will be somewhat higher than the values previously found [Homer, 1917, 2]. Experimental work in this connexion is in progress and will be published later.

SUMMARY.

During the course of the investigation it has been shown that:

1. The heat denaturation of the serum proteins in alkaline sera is a function of the duration of the time of heating. The maximum effect is produced after four hours' heating at 57.5°.

2. In acid sera the change is complete within one hour's heating at the specified temperature.

3. The heat denaturation of pseudoglobulin and of albumin is a function of the $[H^+]$ of their solutions.

4. There is a marked similarity between the curves for the conversion of soluble into insoluble protein during the heating of serum, of solutions of albumin, and of pseudoglobulin throughout the ranges P_H 4.6 to 8.0.

5. The curves for the increased precipitability of the serum proteins and of pseudoglobulin by 30 % of saturation with ammonium sulphate and for the increased precipitability of albumin by 50 % of saturation with ammonium sulphate show a minimum amount of change through the region P_H 5.5 to 8.0 while on the acid side of P_H 5.5 there is absolute coincidence of the curves.

6. In heat denaturated sera the protein precipitated between 30 and 50 % of saturation with ammonium sulphate contains a mixture of pseudoglobulin, antitoxin and heat denaturated albumin.

7. The precipitation of pseudoglobulin from solutions of which the reaction, previous to the liquids being heated, had been adjusted approximately to P_H 8.0 was complete between 42 and 44 % of saturation with ammonium sulphate.

8. The precipitation of albumin from solutions of which the reaction, previous to the liquids being heated, had been adjusted approximately to P_H 8.0 gradually rose from 29 to 52 % between 42 and 50 % of saturation with ammonium sulphate.

9. The practical application of the above observations to the routine concentration of sera has shown us that :

(a) The preliminary prolonged heating of sera of which the reaction is on the alkaline side of P_H 7.0 must be for not less than 4 hours and not more than 5 hours at 57.5° .

(b) In methods involving a heat denaturation of the serum proteins, the amount of heat denaturated albumin appearing in the final product can be appreciably lessened by including in the Second Fraction precipitate the protein precipitated between 30 and 44 % of saturation with ammonium sulphate instead of between 30 and 50 % of saturation as hitherto employed.

(c) This decreased precipitation of albumin entails the production of end products showing a less pronounced colour, a greater removal of the total proteins of the original serum and a degree of concentration considerably greater than if the higher precipitation limit had been used.

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