The Purification of Antitoxic Plasmas by Enzyme Treatment and Heat Denaturation

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The work of Parfentjev (1936) refocused attention on the possibility of refining antitoxins contained in immune plasmas by the use of enzymes (originally claimed in British Patent No. 18340, Imray, 1902). Later work by Pope (1938), confirmed by the ultra-centrifuge studies of Peterman & Pappenheimer (1941), showed that the molecule of diphtheria antitoxic protein may be split by treatment with certain enzyme preparations into two portions of approximately equal size but having different properties, one fragment containing the whole of the antitoxic property of the original molecule. These smaller protein units could still be heat denatured, and Pope (1939*a*) showed that the rate of denaturation of the non-antitoxic portion was very much greater than that of the portion which carried the antitoxic properties of the original unmodified antitoxic molecules. Pope used these findings as the basis of a method for the purification of antitoxic plasmas, consisting essentially of two stages: (1) The antitoxic plasma is treated with pepsin; this splits the antitoxic molecule into two portions, only one of which bears the antitoxic property of the original. (2) This enzymic splitting is followed by critical heat denaturation in the presence of salts and chemical denaturants to precipitate non-antitoxic protein, leaving the modified antitoxic protein in solution.

Pope's original work was carried out on diphtheria and other antitoxin solutions (Pope, 1938) and the results obtained gave information on which a large-scale process could be based. There was, however, little detailed quantitative knowledge relating to the numerous factors which might influence the yield and the degree of purification of the antitoxic protein.

The object of this paper is to describe the large-scale process eventually evolved for refining horse-blood plasmas containing antitoxins and for the concentration and preparation of the refined products for therapeutic use. The process has an intrinsic interest since it involves reactions with labile biological materials of fundamental importance. The development from the original observations which formed the basis of the method to the large-scale process gave rise to numerous problems not encountered in the laboratory. Further problems arose from the increase in the scale of operation which

necessitated different ways of handling (involving heating and filtration) during processing. Since some of the problems encountered may arise from fundamental properties of the plasma constituents they should be recorded, as they may suggest new fields for experimental study. Furthermore, the increase in size of the operations magnifies the effect of some plasma constituents which might otherwise be unobserved; processes in which blood plasma is routinely handled in volumes measured in hundreds of litres are not common.

EXPERIMENTAL

Methods and materials

In outline, the purification process comprises two stages: (a) pepsin treatment; (b) critical denaturation of non-antitoxic protein on samples of antitoxin solution (obtained by the fractional precipitation of antitoxic plasma with $(\text{NH}_4)_2\text{SO}_4$, followed by dialysis to remove the $(\text{NH}_4)_2\text{SO}_4$) or antitoxic plasma, varying one or more of the different factors involved in separate experiments. Thus, the method used in each experiment, unless otherwise specified, was as follows:

(a) *Pepsin treatment.* Antitoxin solution or antitoxic plasma was diluted with the requisite volume of cold tap water, the dilution adjusted to the required pH with solid citric acid and pepsin powder added. After the action of the pepsin a sample was taken (stage A), the sample being neutralized to c. pH 7 with 40% (w/v) NaOH, filtered and the filtrate analyzed for total protein and antitoxin unitage.

(b) *Denaturation.* After the action of the pepsin the required quantity of solid $(\text{NH}_4)_2\text{SO}_4$ was added and dissolved, the pH adjusted as required with 40% (w/v) NaOH or solid citric acid. Portions of 100 ml. were then heated in a water bath at $55^\circ (\pm 0.5^\circ)$ in 120 ml. rubber-stoppered glass bottles. The duration of this heat denaturation was measured from the time of immersion in the bath. Samples were taken after the addition of the $(\text{NH}_4)_2\text{SO}_4$ and adjustment to the pH required for heat denaturation (stage B), filtered and the total protein and antitoxin estimated in the filtrates. Finally samples were taken after heat denaturation (stage C) and similarly analyzed.

Total protein. This was estimated by a refractometric method (Pope, 1939*b*).

Antitoxin. Diphtheria antitoxin has been used except where otherwise stated. The antitoxic content was estimated by the flocculation method (accurate to $\pm 5\%$). From time to time these results have been checked by intracutaneous injection in guinea pigs (Römer & Sames, 1909).

Degree of purification of antitoxin protein. This was evaluated in terms of antitoxic units/g. protein (u./g.); the increase in purity is denoted by *R*, which represents the ratio of the final u./g. to the initial u./g. (Pope, 1939b).

Percentage yields. These have been corrected for the dilution involved when solid $(\text{NH}_4)_2\text{SO}_4$ is added to a given volume of liquid.

Pepsin. Throughout the work pepsin refers to a powder preparation of B.P. 1 : 2500 quality (Chas. Zimmerman and Co. Ltd., London).

The large-scale process

The process finally evolved, using the data obtained from numerous experiments carried out as just described in order to choose the optimal conditions for each operation, can be divided into the following operations:

- (1) Bulking of plasma followed by dilution.
- (2) Adjustment of pH and addition of pepsin.
- (3) Readjustment of pH and addition of $(\text{NH}_4)_2\text{SO}_4$.
- (4) Heat denaturation and filtration.
- (5) Recovery of purified antitoxic protein by precipitation with $(\text{NH}_4)_2\text{SO}_4$.
- (6) Dialysis followed by addition of antiseptic.
- (7) Ultrafiltration.
- (8) Clarification and adjustment of tonicity.
- (9) Sterilization.

Experimental details of the above operations are given below.

(1) *Bulking of plasma followed by dilution.* Bulk plasmas of similar antitoxic value (to a total volume of 140 l.) were used whenever possible as this provided a more constant starting material and gave greater flexibility in the final blending for issue for therapeutic use. Since plasma is viscous it is preferable for handling on a large scale that it should flow freely through pipelines and valves. Experimental study of various dilutions both for pepsin treatment and heat denaturation indicated that a dilution of plasma with 2 vol. of water was optimal (protein concentration 2.5–3.0%) although it was feasible to treat with pepsin at a 1 : 2 dilution and then to add a solution of $(\text{NH}_4)_2\text{SO}_4$ to give a 1 : 3 dilution for the heat denaturation stage. Dilution was carried out with hot water (55°) to give a final temperature in the mixture of 30°.

(2) *Adjustment of pH and addition of pepsin.* Experiment showed that the period usually required for considerable enzymic digestion (24 hr. at 37°) was quite unnecessary for the splitting of the antitoxin molecule to the degree needed to allow the differential heat denaturation. Provided the pH was low, either 2 hr. at 20° or 30 min. at 30° produced sufficient disaggregation to prevent subsequent heat denaturation of the resulting modified antitoxic protein. The pH optimum was 3.2; higher pH values produced insufficient disaggregation over a short period, while lower pH values caused destruction of antitoxin. Although in experimental work citric acid was used, to avoid volume changes, it caused unnecessary buffering of the solution and on a large scale HCl, which could be added either after dilution (as 1 : 4 dilution of conc. HCl), or more conveniently during dilution (as 1 : 15 dilution of conc. HCl), was found preferable. The quantity of pepsin required was small as shown in Table 1; 5 g./l. of plasma were sufficient.

Table 1. *The effect of varying the concentration of pepsin*

(Antitoxic plasma containing 0.70% (v/v) 'tricrosol'* + ether mixture (i.e. 0.35% 'tricrosol') was diluted 1 : 3. Samples taken after 1, 2 and 3 hr. of pepsin treatment at 19° and pH 3.2, were heated for 2 hr. at pH 4.1 in the presence of ammonium sulphate (140 g./l.). Original material (diluted): protein 2.57%; antitoxin 164 u./ml., 6400 u./g.)

Duration of pepsin treatment (hr.)	Pepsin added (g./l. plasma)	Protein present after denaturation (%)	<i>R</i> value	Yield of antitoxin (%)
1	1	0.230	4.21	41.1
	2	0.281	4.56	54.3
	5	0.339	4.23	60.9
	10	0.385	4.01	64.9
2	1	0.254	4.29	46.4
	2	0.332	4.14	58.3
	5	0.411	4.03	70.3
	10	0.403	3.95	67.6
3	1	0.352	4.19	62.3
	2	0.405	3.78	64.9
	5	0.442	3.75	70.2
	10	0.433	3.82	70.2

* 'Tricrosol' (Schering) is a liquid mixture of the three isomeric cresols, and is usually added to plasma mixed with an equal volume of diethyl ether, to give 0.35% of 'tricrosol' in the plasma.

(3) *Readjustment of pH and addition of $(\text{NH}_4)_2\text{SO}_4$.* The quantity of protein rendered insoluble by heat denaturation and therefore the degree of purification were found to depend on both the pH and concentration of $(\text{NH}_4)_2\text{SO}_4$ (see Figs. 1, 2). The quantity of antitoxin denatured was also dependent on these two factors as shown by the curves for '% yield'. The maximum yield is of primary importance on a large scale (provided an *R* value of at least 3.0 can be obtained), and for this the essential requirements are high pH and low $(\text{NH}_4)_2\text{SO}_4$ concentration. It was found, however, impossible to obtain efficient filtration if the pH were above 4.3 and the $(\text{NH}_4)_2\text{SO}_4$ concentration below 130 g./l.; these values were therefore chosen. (The use of salts other than $(\text{NH}_4)_2\text{SO}_4$ has not given any improvement and has invariably led to slower rate of filtration. Various adsorbents of the kieselguhr type and also activated charcoal have been added during heat denaturation, but in no case has an increase in purity of the antitoxin been observed, nor have filtration rates been substantially improved.)

At this stage of the process 0.1% toluene is added, which later removes lipid material by adsorption on the heat denatured protein, thus preventing its appearance as a precipitate in the final product. Sodium pyrophosphate (0.2%) is also added to raise the pH and to inhibit the formation of coloured compounds from the 'tricrosol', previously added (0.35%) to the plasma as a preservative. The first batches of material processed in stainless steel tanks developed a marked red colour due, as was known from earlier work (Pope, unpublished), to the presence of iron which catalyzed the production of quinones from the 'tricrosol'; these react with the proteins to give coloured

products. This catalytic action of iron can be inhibited by pyrophosphate (Pope, personal communication), and in all subsequent batches sodium pyrophosphate has been added.

Fig. 1. The effect of pH on the % yield of antitoxin and the degree of purification (R). Antitoxic plasma, diluted 1 : 3 and treated with pepsin powder (5 g./l.) for 2 hr. at pH 3.2 and 18°; heated for 2 hr. in the presence of ammonium sulphate (100 g./l.). Original material (diluted): protein 2.32%; antitoxin 156 u./ml.; 6720 u./g.

Fig. 2. The effect of ammonium sulphate concentration (g./l.) on the % yield of antitoxin and the degree of purification (R) when antitoxic plasma is treated as in Fig. 1, but heating was at pH 4.1.

(4) *Heat denaturation and filtration.* The mixture prepared as just described and adjusted to pH 4.3, is heated in spray-heated stainless steel vessels for 1 hr. at 55°, during which approximately 65% of the original protein is rendered insoluble. It is important that the temperature should be raised to 55° as quickly as possible (20 min.) otherwise peptic action continues, causing the heat-denatured precipitate to become difficult to filter. Heating may be pro-

longed, leading to increased purity with further slight loss of antitoxin, but higher temperatures, above 60°, markedly increase the rate of destruction of antitoxin.

The concentration of 'tricrosol' present during heat denaturation has a marked effect on the purity as shown in Table 2. The concentration of 'tricrosol' usually used in these laboratories as a preservative and which gives a concentration of 0.12% on dilution of the plasma 1 : 3 as above is therefore suitable for obtaining a high yield and degree of purification. Many other compounds such as phenol, chloroform, ethanol, acetone and urea were tested in the denaturation stage but none was found superior to 'tricrosol'.

Table 2. The effect of various concentrations of 'tricrosol' on the yield and degree of purification of antitoxin

(Antitoxic plasma diluted 1 : 4 was treated with pepsin (5 g./l.) for 2 hr. at 18°, pH 3.2. Ammonium sulphate (100 g./l.) was then added and the pH adjusted to 4.1. Various percentages of 'tricrosol' were then added and the solution heated for 2 hr. Original material (diluted): protein 2.32%; antitoxin 156 u./ml., 6720 u./g.)

'Tricrosol' (%)	Protein in filtrate (%)	R value	Yield of antitoxin (%)
0.00	0.541	3.24	82.2
0.0125	0.531	3.20	79.4
0.025	0.503	3.38	79.4
0.05	0.442	3.84	79.4
0.10	0.368	4.38	75.2
0.15	0.315	4.83	71.0
0.25	0.252	5.09	59.9

After cooling the liquor to 50° the denatured protein is filtered off in a washing-type ebonite plate and frame filter press and the precipitate washed with (NH₄)₂SO₄ solution. By carefully controlling the conditions under which the precipitate is formed it is possible to build up a cake 1 in. thick and still obtain filtration in a reasonable time.

On a large scale an average yield of 65% of the original unitage is obtained in the filtrate plus washings, which are quite clear and colourless.

(5) *Recovery of purified antitoxic protein by precipitation with (NH₄)₂SO₄.* The purified antitoxic protein is precipitated from the filtrate obtained as described in section (4) by a further addition of between 160–190 g. (NH₄)₂SO₄/l., the quantity added being dependent on the type and potency of the original antitoxic plasma. It is important that the amount of (NH₄)₂SO₄ added should be as low as possible in order to minimize concurrent precipitation of protease, formed in the initial stages of the process by peptic digestion of the serum albumin.

This precipitate of modified antitoxic protein is very difficult to filter and a filter press can only be used with a gravity feed having a head of less than 6 ft. It is then possible to build up a cake 0.5 in. thick. For efficient filtration, the precipitation should be carried out while the filtrate is still warm, but if it is allowed to cool the suspension formed by the addition of (NH₄)₂SO₄ may be carefully reheated to 50° before filtering. It is usual to allow this filtration to proceed slowly overnight.

If the filtrate is fractionated by the addition of successive portions of $(\text{NH}_4)_2\text{SO}_4$ it is found that a first fraction, containing about 10% of the total antitoxin, is of lower purity than the remainder. When processing low-grade antitoxic plasma, it is convenient to add an extra 40 g./l. of $(\text{NH}_4)_2\text{SO}_4$ to the suspension after heat denaturation in order to precipitate this antitoxin. This is then recovered from the filter press by washing and can be worked up as a recovery batch. If other methods for increasing the degree of purification, such as lowering the pH or increasing the $(\text{NH}_4)_2\text{SO}_4$ concentration for heat denaturation are adopted the additional lost antitoxin cannot be recovered.

(6) *Dialysis followed by addition of antiseptic.* The cakes of antitoxic protein obtained from the filter press contain a large amount of 30% $(\text{NH}_4)_2\text{SO}_4$ solution. This is removed by dialysis in cellophan sacs against running tap water, until 0.1% $(\text{NH}_4)_2\text{SO}_4$ remains; this operation takes approximately 48 hr. After bulking the contents of the sacs 0.35% of 'tricresol' is added as a 10% emulsion in water, which is sprayed into the solution through a perforated stirring tube.

(7) *Ultrafiltration.* It was customary in the early days of this process to press the filter-press cakes of antitoxic protein between sheets of blotting paper in order to remove excess $(\text{NH}_4)_2\text{SO}_4$ solution before dialysis. It was found however that precipitates of denatured protein sometimes appeared in the final product after candling, possibly due to excess pressure during the pressing. When pressing was discontinued the solution of antitoxic protein obtained after dialysis contained only 9% protein, whereas for therapeutic purposes it is desirable to have the solutions as concentrated as possible, without undue viscosity.

With purified and modified antitoxic protein, 15% solutions are not unduly viscous. The method for raising the concentration to about 15% is ultrafiltration, using collodion membranes deposited on the external surfaces of filter candles. These are immersed in a solution of 8% collodion (Imperial Chemical Industries product) in glacial acetic acid and, after draining, gelled in water. The ultrafiltration takes approximately 7-10 days, but has the merit of removing a portion of the proteose present and also of allowing 'tricresol' to come into equilibrium with the protein. It is carried out by running serum into the stainless steel vessel, which contains five ultrafilters, and applying a vacuum of 29 in. to the bottles connected to the ultrafilters (Fig. 3). Concentrated serum is drawn off from the bottom of the stainless steel vessel every 24 hr.

(8) *Clarification.* It is essential that the ultrafiltered solution should be perfectly clarified to ensure easy filtration through filter candles. This clarification is accomplished by drawing the protein solution through a thick pad of lightly packed paper pulp by means of a low vacuum (5 in. Hg). Autoclaved and finely shredded paper pulp is formed into a pad about 1.5 in. thick in a stainless steel vessel shown in Fig. 4.

(9) *Sterilization.* After clarification, the antitoxin solution is made isotonic by the addition of sterile 20.0% (w/v) NaCl containing 0.1% NaHCO_3 , in order to bring the solution to pH 6.0, which is the optimum for storage and therapeutic use. The antitoxin solution is then drawn through a filter candle (see Fig. 5) using a vacuum not greater than 20 in. Hg in order to prevent frothing, which has a tendency to cause denaturation of protein. Under these conditions for clarification and sterilization, it is

possible to pass 20-30 l. of antitoxin solution (corresponding to two batches) through each pulp and candle.

Fig. 4.

Fig. 5.

Fig. 4. Stainless steel vessel (6 in. diameter) for containing the pad of paper pulp. (A) Paper pulp. (B) Disk of stainless steel gauze. (C) Perforated stainless steel disk.

Fig. 5. Filter candle (10 in.) clamped into glass case (constructed from 2 in. Pyrex pipeline) by means of two stainless steel disks. (A) Filter candle. (B) Porcelain head of candle. (C) Stainless steel supporting bracket.

Applications of the process

Antitoxins. It has been found possible to refine by the process described all true antitoxins, in which the antibody lies largely in the pseudoglobulin fraction of the plasma. A typical series of experimental results is given in Table 3. The *R* values in

Table 3. *Results obtained by processing various types of antitoxic plasma*

(Typical batches of plasma were processed by the method described.)

Type	<i>R</i> value	Overall yield of antitoxin (%)
Perfringens antitoxin, type A	3.28	57.4
Septique antitoxin	5.38	57.6
Oedematiens α -antitoxin	4.53	52.5
Tetanus antitoxin	3.84	65.5
Scarlet fever antitoxin	4.97	55.4
Staphylococcus α -antihaemolysin	4.33	66.1
Shiga dysentery neuro-antitoxin	2.99	54.3
Diphtheria antitoxin	3.87	63.2

general vary between 2.5 and 5.0 and are to a large extent dependent on the antitoxic potency of the original plasma, i.e. the higher the antitoxic value of the original plasma, the lower the *R* value. Thus results from 80 batches of diphtheria antitoxic plasma showed a correlation coefficient between *R* value and antitoxic potency of -0.777 which is statistically very significant.

The final product is a perfectly clear straw-coloured liquid, not unduly viscous and showing no signs of precipitation when stored, even at room temperature, over a period of 5 years. It is also remarkably stable, the rate of loss of antitoxic potency at the pH optimum (5.5–6.0) being negligible between 0° and $+5^{\circ}$, approximately 5.0% per annum at 20° and 10.0% per annum at 37° (Harms, 1945). An extremely low incidence of serum reactions has been reported when antitoxic sera refined in the way described have been used therapeutically (Hutchinson, 1939). It has also been shown by Glenny & Llewellyn-Jones (1938) to be absorbed more rapidly and eliminated more slowly than antitoxin purified only by fractional precipitation with ammonium sulphate.

Antibacterial antibodies. Although the fractionation of sera containing antibacterial antibodies with ammonium sulphate has shown that these antibodies are present in the euglobulin fraction, some experiments on refining these sera by the pepsin method were carried out. The results of processing plasma containing antibodies prepared by using as antigen suspensions of the following organisms, *Haemophilus pertussis*, *Proteus vulgaris* OX 19, *Neisseria intracellularis*, and *Erysipelothrix rhusiopathiae* (the organism of swine erysipelas), showed that, as expected, only extremely small antibody yields (less than 20%) could be obtained. Recently a method for concentrating such sera, utilizing the denaturing properties of 'tricrosol' has been worked out (Harms, 1946).

The behaviour of separated fractions of antitoxic horse plasma during pepsin treatment and heat denaturation. It has long been customary to concentrate the antitoxic fractions of plasmas by fractional precipitation with ammonium sulphate or other neutral salt, so that the pseudoglobulin fraction, containing the bulk of the antitoxin is partially separated from the fibrin, euglobulin and albumin fractions. Earlier work relating to the process described here was carried out on this pseudoglobulin fraction (Pope, 1939*a*) which was found to behave in a manner similar to whole plasma when processed by the pepsin method. It seemed therefore of interest to investigate the behaviour of the euglobulin and albumin fractions. Very laborious procedure is necessary to obtain electrophoretically homogeneous fractions, but a simple fractionation with ammonium sulphate can give fractions which contain a preponderance of one component. Plasma diluted with an

equal volume of water was fractionated by precipitation with ammonium sulphate at the concentration indicated: fraction 1 (euglobulin fraction) 0–220 g./l.; fraction 2 (pseudoglobulin fraction) 220–360 g./l.; fraction 3 (albumin fraction) 360–550 g./l. The three precipitates were then dialyzed.

The solution of each fraction was diluted with tap water to contain 5.0% protein and then an equal volume of 0.24% 'tricrosol' in tap water was added to give a concentration of 'tricrosol' suitable for heat denaturation and a protein concentration equal to that obtaining when plasma is used. After treatment with pepsin for 1 hr. at 18.5° , ammonium sulphate (130 g./l.) was added and the pH adjusted to 4.25 with 40.0% (w/v) NaOH. Samples (100 ml.) were then heated for 1 hr. at 55° . Protein and antitoxin were estimated on samples taken at stage B (after addition of ammonium sulphate) and stage C (after heat denaturation); total N and non-protein N estimations (Kjeldahl) were carried out on samples at stage A (after pepsin treatment), in order to determine the amount of true digestion which took place. Electrophoretic analyses, for which I am indebted to Mr P. A. Charlwood, were also carried out on the untreated fractions. The results are summarized in Table 4.

Although the fractions into which the plasma had been separated by the ammonium sulphate fractionation were by no means homogeneous, they showed marked differences in composition as determined by the electrophoretic measurements. The results of pepsin treatment followed by heat denaturation also show differences in behaviour. It is clear (Table 4) that the albumin is almost wholly digested while the other proteins show very little digestion. The impure (in terms of u./g. value) fraction 1 gave a degree of purification almost as high as that obtained with fraction 2, due largely to the fact that a very considerable portion of the protein was precipitated by the addition of ammonium sulphate (stage B). It seems logical to suppose that the protein thus precipitated is largely γ -globulin and fibrin (estimated electrophoretically under β -globulin). Most of the antitoxin would appear to be carried by the β -globulin, which apart from being split by enzyme action was largely unaffected by the processing. The figures for fraction 3 however appear to indicate that a part of the β -globulin had no antitoxin associated with it.

The fractions showed marked differences in rate of filtration. Samples from fraction 1 filtered very rapidly and gave brilliantly clear filtrates, whereas with fractions 2 and 3 the filtrates tended to be opalescent. The precipitates formed from fraction 3 were also very sticky and filtered very badly. The good filtration obtained with fraction 1 is in accordance with other observations in which it has been noted that filtration is always better when plasma

Table 4. *The effect of processing fractions of antitoxic horse plasma*

(Antitoxin and protein remaining in solution after processing are given as % of that present in the original fraction.)

Fraction	% of total protein	Protein conc. (%)	Antitoxin				Electrophoretic composition (%)				Stage in process	Antitoxin					Non-protein N/total N (%)
			u./ml.		u./g.		Globulin					Protein (%)	(%)		u./g.		
			<i>In vitro</i> *	<i>In vivo</i> †	<i>In vitro</i> *	<i>In vivo</i> †	γ	β	α	Albumin			<i>In vitro</i> *	<i>In vivo</i> †	<i>In vitro</i> *	<i>In vivo</i> †	
1	33.0	2.52	125	125	4,960	4,960	40.9	38.2	16.3	4.6	A	81.4	—	—	—	—	39.6
											B	36.7	87.0	—	11,600	—	—
											C	23.4	80.8	81.8	17,000	17,200	—
2	47.0	2.55	250	220	9,790	8,620	16.8	43.0	32.9	7.3	A	80.4	—	—	—	—	33.8
											B	70.1	87.1	—	12,000	—	—
											C	37.4	79.7	93.6	20,700	21,400	—
3	20.0	2.54	—	1.5	—	—	0.0	13.5	9.5	77.0	A	31.6†	—	—	—	—	61.3
											B	25.7	—	—	—	—	—
											C	5.5	—	—	—	—	—

* *In vitro* = measured by the flocculation test.† *In vivo* = measured by intracutaneous injection into the skin of guinea pigs (Römer & Sames, 1909).

‡ After 2 min. pepsin treatment the protein concentration had diminished to 75.7% of the original.

is processed rather than serum. There is, therefore, no necessity to convert plasma to serum before processing; in fact it is a disadvantage. It would be an advantage to remove albumin since this would lead to better filtration and obviate the production of proteose and peptone, which tend to contaminate the final product. This, however, would necessitate a laborious initial process involving a difficult filtration.

The treatment of whole blood. An experiment with whole horse blood in comparison with plasma obtained from the same bleeding was carried out. The experiments recorded in Table 5 show that there was a comparatively small increase in total units

recovered, while the degree of purification was slightly less when whole blood was used. Concentrates prepared from the filtrates were very much darker in colour; and there would be a danger that breakdown products of haemoglobin might appear in the concentrates. No advantage would therefore appear to be gained by processing whole blood.

DISCUSSION

The results obtained both in the laboratory and in large-scale processing show that, in general, disaggregation of a major portion of the antitoxic protein molecules, as judged by the resistance of the antitoxic portion to heat denaturation, takes place very rapidly, even in the presence of very small quantities of pepsin. The destruction of antitoxin which may vary from 20 to as much as 50% in some batches (even under optimal conditions) may be explained partly by protein denaturation and partly by the resistance of a portion of the antitoxic protein to disaggregation.

When antitoxic sera (including those obtained by the process described herein) containing phenolic preservatives are stored for a long period a small loss in antitoxic potency occurs; this loss is greatly accelerated by rise in temperature and by alterations of pH from the optimum (pH 5.5–6.0). This loss is caused by denaturation of the antitoxic protein (Harms, 1945) and it is probable that a similar process operates during pepsin treatment at pH 3.2 to account for the 10% loss in antitoxic units which invariably occurs at this stage. Further loss, probably due to the same cause, occurs during the heat treatment, since this loss may be increased by raising the temperature or increasing the time of heating. Whereas the loss of antitoxic units during pepsin treatment is constant, that occurring during the succeeding stages (B and C) of the process is very

Table 5. *The effect of processing whole blood*

(500 ml. of blood were divided into two parts. (A) 250 ml. blood + 750 ml. tap water + 0.12% 'tricrosol', were treated with pepsin (5 g./l.) at pH 3.2 for 2 hr. at 19°. Heat denaturation for 1 hr. at 55°, pH 4.1. (B) 250 ml. of blood were allowed to settle for 24 hr. and the supernatant plasma siphoned off. 184 ml. of plasma were thus obtained and after dilution with 2 vol. of tap water, 0.12% 'tricrosol' was added and the solution treated as (A). The cells and their suspending plasma, after withdrawal of the supernatant plasma, were diluted to 250 ml. with tap water, 0.12% 'tricrosol' was added and the solution was treated as (A). Antitoxin was recovered as recorded below. Original material: blood (diluted): protein 3.15%, antitoxin 204 u./ml.; 6500 u./g. Plasma (diluted): protein 2.64%, antitoxin 320 u./ml.; 12,100 u./g. Total antitoxin units in 250 ml. blood = 204,000.)

Material	Units recovered		
	Total	% of original*	u./g.
Blood	89,225	43.7	40,000
Plasma	82,325	40.4	42,500
Cells	5,719†	2.8	45,400

* Low yields due to mechanical loss in processing small quantities.

† Presumably from the plasma left with the cells.

variable and cannot be accounted for by heat denaturation of disaggregated antitoxic protein. Thus, when ammonium sulphate is added at stage B a quantity of antitoxic protein, which varies considerably from batch to batch, is precipitated. Should the quantity of ammonium sulphate added be lowered in order to decrease this precipitation it is found that an extra loss equivalent to this increase occurs during stage C. The most probable explanation of this phenomenon is that the globulin which can be precipitated by a low concentration of ammonium sulphate carries an antitoxic protein resistant to disaggregation by pepsin treatment. This is confirmed by the observation that when antitoxic globulin solutions prepared by the ammonium sulphate fractionation of immune plasma (during which a fraction of the globulin, insoluble in low concentrations of ammonium sulphate, is removed) are processed, yields are generally higher than when immune plasmas are processed.

Rosenheim (1937) found that whereas *Eberthella typhosa* O agglutinins were destroyed by pepsin treatment, H agglutinins were more resistant and the resistance depended on the degree of immunization of the horse from which they were obtained. Also it has been found that antibacterial antibodies are almost completely destroyed when processed by the method described in this paper. The work of Cohn, Oncley, Strong, Hughes & Armstrong (1944) on the ethanol fractionation of human plasma indicates that many of the antibodies, including antitoxins, are associated with the γ -globulin fraction. It is possible that the method of immunization of horses affects the distribution of antitoxin between the β -globulin and γ -globulin fractions. If, as evidence would seem to indicate, the γ -globulin of antitoxic horse plasma is more resistant to disaggregation by pepsin than the β -globulin, this may account for the destruction of antibacterial antibodies because these are largely confined to the γ -fraction and it might account for the variable yields obtained with different batches of antitoxin. The experiment (Table 4) in which three fractions of antitoxic horse plasma were processed shows that very much more protein was precipitated and heat-denatured in fraction 1, containing the high proportion of γ -globulin, than in fraction 2 which contained a much smaller proportion. Although the yield of antitoxin from both fractions was substantially the same, this can be explained by the fact that fraction 1 still contained a high percentage of β -globulin, with which the majority of the antitoxin was associated.

Further work on pure globulin fractions is indicated in order to clarify this point, for it has been suggested that the loss of certain portions of the antitoxic protein during purification may have led to the failure of antitoxin therapy in some types of diphtheria infection (O'Meara, 1939).

The purity of antitoxin (u./g.) which can be obtained depends mainly on the purity of the original plasma, although the presence of antitoxins other than the specific one, formed either during the course of immunization or during a previous immunization to produce another type of antitoxin, will contribute protein to the filtrate after heat denaturation and so lower the final purity.

Should it be possible to produce an immunization in a horse in which only one specific antitoxin was formed and the horse possessed no significant quantity of other antitoxin, it seems possible that 'pure' antitoxin could be produced by the pepsin process. Using diphtheria antitoxic plasma containing 3600 units of antitoxin/ml. it was possible to obtain an antitoxin solution of 100,000 u./g., which is of the same order as that produced from refined antitoxin by the method of Pope & Healey (1939) using toxin-antitoxin floccules. Experimental evidence indicates that, even under optimal conditions, the degree of purification and yield of antitoxin which can be obtained by pepsin treatment followed by heat denaturation are limited by the quality of the original plasma. Improvement may therefore come from better methods of immunization designed to produce only the specific antitoxin in a form in which it is easily disaggregated by pepsin.

SUMMARY

1. A description is given of a large scale process suitable for the purification of true antitoxins, but not for antibacterial antibodies, by treatment of immune plasmas with pepsin followed by critical heat denaturation in the presence of a protein denaturant. The preparation of the purified antitoxin for therapeutic use is described.
2. It has been established that the optimal conditions on the large scale are pepsin treatment (0.5 hr. at 30° at pH 3.2 with plasma diluted 1 : 3) and heat denaturation (1 hr. at 55° at pH 4.3 in the presence of 130 g. ammonium sulphate/l. and 0.12 % 'tricrosol').
3. It has been shown that it is preferable to process plasma rather than whole blood, serum, or salted-out fractions of plasma.
4. The limitations of the process and the bearing of methods of immunization on yields and purity (u./g.) are discussed.

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Fig. 3. Photograph showing the ultrafiltration in progress.

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Fructose and Glucose in the Blood of the Foetal Sheep

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Little is known of the distribution of D-fructose in animal tissues, apart from its demonstration in certain embryonic fluids (see, e.g., Needham, 1931, 1942). Recently it has been positively identified by Mann (1946) as the reducing sugar in the seminal plasma; the fact that hitherto it had been taken for granted that this was glucose suggests that fructose may have escaped detection in other instances.

There is no excuse for this, for a characteristic, though not entirely specific, colour reaction, due to Seliwanoff (1887), has been available, and in the methylphenylosazone Neuberg (1902; Neuberg & Strauss, 1902) found a derivative by which it could be distinguished from accompanying glucose, provided always that a sufficiently concentrated solution was available. Our own attention was drawn to the problem of identifying fructose by the late Sir Joseph Barcroft and Mr S. W. Cole, who in the course of recent work on the foetus of the sheep had confirmed an observation of Paton, Watson & Kerr (1907). The latter authors detected by the Seliwanoff test a fructose-like substance in the amniotic and allantoic fluids of sheep, cow, pig, cat, dog, rabbit, ferret and guinea pig, and in foetal blood. They investigated the blood more closely: 'by extracting large quantities of foetal blood [of the sheep?] with alcohol, evaporating with charcoal, and taking up in a small quantity of water, we were able to extract a sugar which was laevorotatory and which gave Seliwanoff's reaction distinctly...'. Orr (1924) obtained similar results using lead acetate instead of charcoal, with blood from a newly born kid, and blood taken from the human placenta after birth.

The observations of Cole & Hitchcock (1946) indicated that appreciable quantities of fructose (up to 100 mg./100 ml.) were present in the blood of the

foetal sheep, and at Sir Joseph Barcroft's request we attempted (a) to prove that the substance responsible for the colour reaction was in fact fructose, and (b) to isolate a derivative of the sugar.

EXPERIMENTAL

Methods

Standard fructose and glucose solutions. These were made in saturated benzoic acid solution (Folin, 1929); a concentrated solution (c. 2%) was made for polarimetric measurement of the concentration, and this was diluted with more saturated benzoic acid solution to give a final sugar concentration of 0.5 mg./ml. In our experience a solution of either sugar at this, or a lower concentration, does not deteriorate when kept for several years at room temperature in a well-stoppered bottle.

Total reducing substances (t.r.s.). (More strictly, reducing substances estimated by the ferricyanide-cerium method.) The procedure used was that of Miller & Van Slyke (1936), the solutions being heated in boiling tubes without stoppers, and titrated to the nearest drop (0.02 ml.) after cooling and acidification, using xylene cyanole FF as an internal indicator. Instead of standardizing the ceric sulphate solution and using a fixed time of heating, a standard fructose, and occasionally a glucose solution, containing 100 µg. of sugar, was included in each set of estimations, and heating was carried out for 10–12 min. in a boiling water bath. The ceric sulphate solution was of such a concentration that 1 ml. was equivalent to about 100 µg. of reducing substance, calculated as hexose. Contrary to the findings of Miller & Van Slyke (1936) we found no discrepancy greater than the experimental error, between the reducing values of glucose and fructose, nor did we find any appreciable variability in the reducing value of fructose.

Fructose. For the estimation of those substances giving the Seliwanoff reaction, called here 'fructose' for the sake of brevity, a modification of Roe's method (1934) was used. This was devised by Mr S. W. Cole, who very kindly gave