FELTON ANTIBODY: ITS DISTRIBUTION AND PURITY AS DETERMINED BY SALTING OUT METHODS.

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(With 2 Graphs.)

The classical division of the protein in serum into two fractions, globulin and albumen, and the subdivision of the globulin fraction into water-insoluble globulin (euglobulin) and water-soluble globulin (pseudo-globulin), are well known. Avery (1915) found that the Pneumococcus antibody is precipitated between 28 and 42 per cent. saturation with ammonium sulphate and that the immune body fraction does not correspond exactly with ordinary euglobulin. Felton (1925) showed that the euglobulin of Pneumococcus immune sera can be further fractionated by "isoelectric" methods to give acid and neutral fractions. The antibody was found to be associated to a large extent with the neutral fraction. Reiner and Reiner (1932) showed that isoelectric fractionation of euglobulin from normal horse serum is possible. Barr (private communication, 1931) obtained a Felton fraction from *B. welchii* antitoxic serum which was non-antitoxic, and I obtained a similar fraction from normal horse serum which exhibited slight protection against Pneumococcus type II in mice. In both cases the amount of Felton protein was very small, and was probably due to natural immunity to Pneumococcus. It is well known that traces of *C. diphtheriae* and *B. welchii* antitoxins can be detected in many sera from normal horses, and recently my colleague Dr Parish has found small amounts of both types I and II Pneumococcus antibody in these sera. On the other hand Felton protein may be a normal constituent of horse serum which increases during immunisation to Pneumococcus as the pseudoglobulin fraction increases during immunisation to diphtheria toxin (Ledingham, 1907 and later workers).

The immunological and chemical properties of this peculiar protein were examined by Felton (1925) and the relations of its solubility at various hydrogen-ion concentrations and phosphorus content to the antibody content were studied. He found that the protein associated with the antibody had an optimal precipitation point at a hydrogen-ion concentration of *p*H 6·8, and that after repeated isoelectric precipitation the ratio of antibody to protein increased. He also studied the effect of repeated precipitation by water dilution, and found that a protective substance free from phosphorus could be obtained. It is therefore apparent that the Pneumococcus antibody

is associated with a very different type of protein from that with which most of the antitoxins are associated. Using solid salts as precipitants, Barr, Glenny and Pope (1931) studied the salting out limits of antitoxins and Barr and Glenny (1931) the purity of fractions from several immune sera, and it was thought that the distribution and purity of Felton antibody in Pneumococcus antisera as determined by similar methods would be of interest.

EXPERIMENTAL.

(1) *Determination of the distribution of antibody and the purity curves for several type I Pneumococcus antisera.*

The sera were fractionated and potency and protein estimations obtained for each fraction so that purity curves could be drawn. In the general method of procedure, a measure volume of serum was diluted with an equal volume of water and 0·5 per cent. of sodium bicarbonate was added. The first fraction was obtained by adding sufficient solid ammonium sulphate to the diluted serum to give a workable precipitate. The precipitate was allowed to remain in contact with the serum for 2 hours, when it was filtered, and washed with a 0·5 per cent. sodium bicarbonate solution containing an equivalent amount of ammonium sulphate. It was then pressed between blotting paper, dialysed against distilled water, and dissolved in 1 per cent. saline to a suitable volume for antibody and nitrogen estimations. The filtrate was measured and its ammonium sulphate content increased by 10 or 20 g. per litre depending on the amount of precipitate produced. The second precipitate was treated in a similar manner to the first, and the concentration of ammonium sulphate in the filtrate from it was again increased to obtain a further precipitate. In this way a series of eight or nine fractions was obtained from each serum.

The protein contents of the fractions were calculated from micro Kjeldahl nitrogen determinations. The factor used for the conversion of the nitrogen results was 7·0; this was determined on specially purified and dried Felton protein obtained from an anti-Pneumococcus type I serum. The potencies were obtained by the mouse protection test investigated by Parish (1930). The test was modified for convenience in that the mice were observed for 4 days instead of 6. Each fraction was compared with the original sample of horse serum from which it was prepared, using about thirty-six mice on each side so that according to Trevan (1930) the estimates of potency are probably not in error by more than about $\pm$ 20 per cent. The purity of each fraction was calculated as the ratio of the percentage of the original antibody present to its protein content. Results for various sera are given in Table I. In fractionation No. 5, to obtain precipitates under as comparable conditions as possible a measured volume of serum was dialysed for a week against distilled water, diluted to twice its original volume on removal from the bath and 0·5 per cent. of sodium chloride added.

Distribution of antibody.

Inspection of the results reveals that traces of antibody are precipitated with the first appreciable precipitate, and that less than 2 per cent. remains in the filtrate after the addition of 260 g. of ammonium sulphate. The actual amount remaining varies with each serum. In every fractionation only about 50 per cent. of the total antibody originally present was recovered owing to loss by washing; each precipitate was washed in a similar way. It may be noted that the distribution of the antibody as given by the percentage precipitated in each fraction varies in the two sera investigated. With serum No. 1 there is less antibody in the first fractions than in the corresponding fractions of serum No. 2 and more antibody in the later fractions. Also it can be seen that addition of purified Felton protein to an immune serum lessens the percentage of antibody precipitated by small concentrations of ammonium sulphate and appears to increase the percentage of antibody precipitated between 180 and 220 g. of ammonium sulphate per litre. This may mean that there is a redistribution of all the antibody present or that all the purified antibody added is precipitated in the fractions obtained by increasing from 180 to 220 g. of ammonium sulphate per litre. Dialysis of serum containing tricresol affects the distribution of antibody as determined by this method. In the presence of this preservative a measurable amount of antibody is precipitated by 160 g. of ammonium sulphate per litre of serum, while it requires 10–20 g. more per litre to precipitate a measurable quantity after dialysis of the serum.

Purity of fractions.

The purity of fractions, as measured by the percentage of antibody precipitated divided by the percentage of protein, shown in Table I, appear in Graph I. It is apparent that the purity of the first fractions of serum No. 2 is greater than that of the corresponding fractions of serum No. 1, while that of the fractions of serum No. 2 prepared by salting out with larger quantities of ammonium sulphate is practically identical with that of corresponding serum No. 1 fractions. Blending serum No. 2 with purified Felton antibody causes a flattening of the purity curve. This may be due to redistribution of the protective antibody and protein in the presence of the added antibody. The serum precipitated in the presence of 0·4 per cent. tricresol shows a distinct peak in the purity curve whereas fractionation after dialysis reveals practically no peak. As there is a possibility of change in protein precipitability after dialysis it would not be permissible to assume that the peak is due only to the presence of tricresol, but the curves tend to point to this conclusion. Thus it has been shown that:

(*a*) Serum from different horses may give fractions, each produced at definite ammonium sulphate concentrations which differ in potency and purity.

(*b*) The addition of Pneumococcus antibody, purified by the Felton process

Table I. *Showing the relative amounts of Felton antibody and protein precipitated from several Type I Pneumococcus antisera by various quantities of solid ammonium sulphate.*

I. Fractionation of serum 1 containing no tricresol.

A	0–140	140–160	160–180	180–190	190–200	200–220	220–240	240–260	260–280
B	0·3	0·7	7·0	5·0	7·0	23·0	6·0	4·0	0·5
C	0·03	0·16	0·64	0·19	0·17	0·53	0·19	0·20	0·10
D	10·0	4·4	11·0	26·3	41·2	43·4	31·6	20·0	5·0

II. Fractionation of serum 2 containing no tricresol.

A	0–140	140–160	160–180	180–200	200–220	220–240	240–260	260–280	280–300
B	0·5	1·5	10·0	23·0	12·0	5·0	1·0	0·3	0·2
C	0·13	0·14	0·42	0·56	0·27	0·13	0·046	0·04	0·10
D	4·6	10·7	23·8	41·2	44·4	38·4	21·7	7·5	2·0

III. Fractionation of serum 2 blended with 10 per cent. of purified Felton antibody.

A	0–140	140–160	160–180	180–200	200–220	220–240	240–260	260–280	—
B	0·15	0·6	8·0	25·0	15·0	3·0	0·8	0·1	—
C	0·043	0·10	0·57	0·92	0·50	0·15	0·066	0·013	—
D	3·5	6·0	14·0	27·2	30·0	20·0	12·1	7·7	—

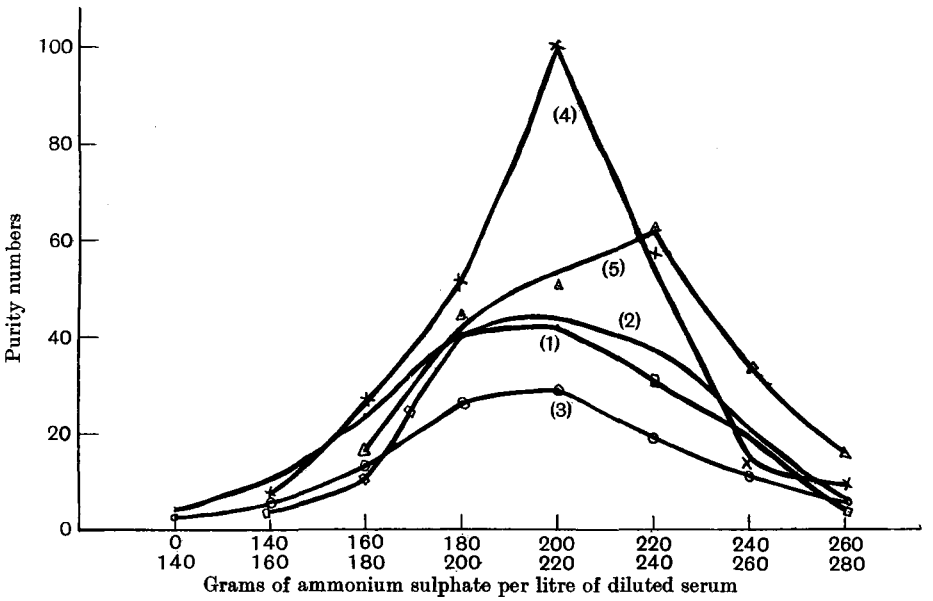
IV. Fractionation of serum 3 containing 0·4 per cent. tricresol.

A	0–140	140–160	160–180	180–200	200–220	220–240	240–260	260–280	—
B	Trace	1·0	10·0	10·0	14·0	7·0	0·8	0·1	—
C	0·015	0·12	0·37	0·19	0·14	0·12	0·053	0·010	—
D	—	8·3	27·0	52·6	100·0	58·3	15·1	10·0	—

V. Fractionation of serum 3 after dialysis.

A	0–140	140–160	160–180	180–200	200–220	220–240	240–260	260–280	—
B	—	—	4·0	10·0	15·0	10·0	2·5	0·8	—
C	—	0·002	0·24	0·22	0·30	0·16	0·073	0·45	—
D	—	—	16·7	45·5	50·0	62·5	34·2	17·7	—

A = grams of ammonium sulphate to a litre of diluted serum.
B = percentage of original antibody present.
C = percentage of protein precipitated.
D = purity of precipitate = B/C.



Graph I. Purity curves for fractionations given in Table I. (1) Fractionation I; (2) Fractionation II; (3) Fractionation III; (4) Fractionation IV; (5) Fractionation V.

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to an unconcentrated Pneumococcus antiserum redistributes the antibody content and reduces the purity of all fractions.

(*c*) The presence of tricresol in the serum appears to increase the purity of certain fractions.

(2) *The investigation of the distribution of Felton antibody and Pneumococcus I agglutinins in type I serum obtained from a horse bled at intervals during immunisation.*

Similar methods were used in this investigation. The serum from each small bleeding was diluted with an equal volume of water and divided into four suitable volumes which were precipitated respectively with the equivalent of 160, 180, 200 and 220 g. of ammonium sulphate per litre. The four precipitates were filtered, washed, pressed and dialysed as previously described and dissolved in 1 per cent. saline for test purposes. The antibody contents were determined as before, and the agglutinins were determined against a standard suspension of living type I culture at 100 per cent. differences. The actual values of protective power and agglutinin titre are given in terms of the standard serum used in these laboratories, and are shown in Table II.

Table II. *Showing the amounts of Felton antibody and agglutinin precipitated by ammonium sulphate from serum bled from a horse at intervals during immunisation.*

			Fraction I		Fraction II		Fraction III		Fraction IV	
			Ammonium sulphate							
Date of bleeding of horse	Protective power	Agglutinin titre	0–160 g.		0–180 g.		0–200 g.		0–220 g.	
			A	B	A	B	A	B	A	B
9. iii. 31	$\frac{1}{2}S$	None	6·4–9·6	—	18–27	—	36–54	—	52– 78	—
4. v. 31	$S-$	$2S$	2·6–3·9	2	8–12	12·5	40–60	50–100	76–114	50
1. vi. 31	$1\frac{1}{2}S$	S	3·0–4·5	3	12–18	12	31–45	50	64– 96	50
5. xii. 31	$2S+$	$S/6$	—	—	—	—	—	—	—	—

A column = per cent. of original antibody in precipitates allowing a 20 per cent. testing error.
B column = per cent. of agglutinins in precipitates.
S represents the titre of the standard serum used in these laboratories with which all protective and agglutinin titres were compared.
$S-$ indicates that the titre of the serum is slightly less than standard.
$2S+$ that the titre is slightly greater than twice standard.
First immunisation of horse commenced 18. ii. 31.

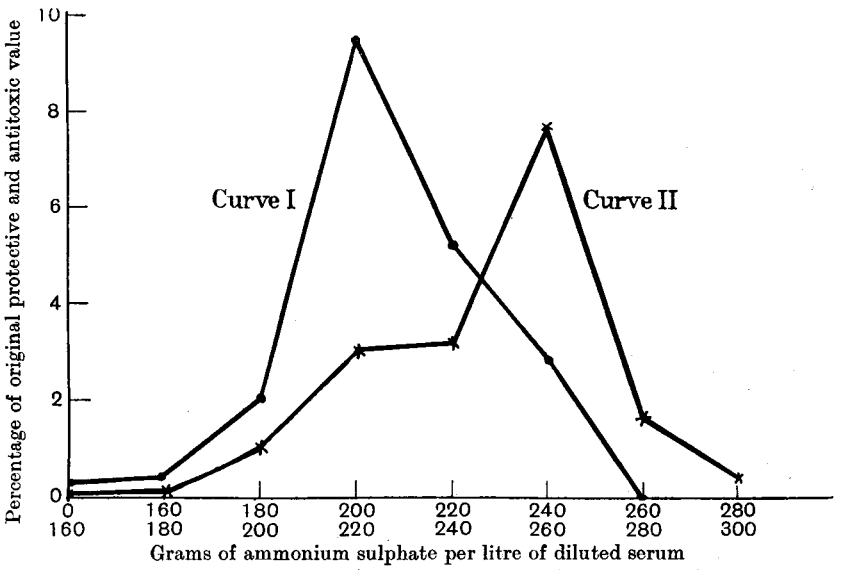
The results recorded in Table II show that fractions from the first bleeding prepared by salting out at low ammonium sulphate concentrations contain a higher percentage of the total Felton antibody than the corresponding fractions of later bleedings. This may be due to alterations in the proportions of proteins present. It has been shown by Barr, Glenny and Pope (1931) that addition of euglobulin and of albumen to antitoxic sera affects the distribution of the antitoxin as determined by salting out methods. It is therefore highly probable that any increase in the total amount of Felton protein present as immunisation proceeds would cause similar disturbances in the precipitability of proteins by ammonium sulphate. The effect rather resembles that which was produced by the addition of purified Felton antibody to whole immune

serum (see p. 254). When agglutinins are present they are distributed with the antibody, *i.e.* similar percentages of the total protective power and agglutinating titre appear in each fraction. This indicates that there is a close chemical resemblance between the proteins to which these properties are due.

The titres of protection and agglutination determined for each bleeding show no constant relationship. It is possible that the correlation of immunological properties for sera from various horses given by Felton (1931) may be due to the sera examined, having been obtained from horses all at the same stage of immunisation.

(3) *Fractionation of serum from a horse immunised to Pneumococcus type I and to diphtheria toxin.*

The first series of fractions was prepared in the same way as those for the purity curve determinations and each fraction was tested for its Pneumococcus antibody and diphtheria antitoxin content. The usual mouse test was used for the Pneumococcus antibody determination and the intracutaneous method of titration for the diphtheria antitoxin determination. The percentage yield of both antibodies in each fraction was calculated and the results are given in Graph II.



Graph II. The precipitation of Felton antibody and diphtheria antitoxin from the same serum with solid ammonium sulphate. Curve I, Felton antibody; Curve II, diphtheria antitoxin.

The fraction containing the maximum amount of Felton antibody is that precipitated by increasing from 200 to 220 g. of ammonium sulphate per litre, whereas the maximum diphtheria antitoxin precipitation occurs from 240 to 260 g. It is therefore clear that a partial separation of these two antibodies can be obtained by salting out methods. A second series of fractions was prepared in a manner similar to that described on p. 259. Each fraction

was tested as previously described for Pneumococcus antibody and for diphtheria antitoxin and the ratios of the percentage of diphtheria antitoxin to Pneumococcus antibody present in the fractions were calculated. They are given in Table III, and show quite definitely that the Felton antibody is more precipitated by lower concentrations of ammonium sulphate than is diphtheria antitoxin, when both antibodies are present in the same serum. This experiment therefore confirms the results given in Graph II.

Table III. *Showing the relative amounts of Felton antibody and diphtheria antitoxin precipitated from a serum by various quantities of ammonium sulphate.*

Gram of $(NH_4)SO_4$ added/litre of diluted serum	Percentage of antibody present in the precipitates		Ratio Felton antibody / Diphtheria antitoxin
	Diphtheria	Pneumococcus	
0–200	4·2*	11*	2·62 : 1
0–220	18·3	30	1·64 : 1
0–240	36·3	50	1·38 : 1
0–260	52·5	45	0·86 : 1

* For the diphtheria antitoxin results are correct to ±10 per cent. of the determined values and for Pneumococcus antibody to ±20 per cent.

Summary.

The results obtained show:

1. The limits of precipitation of Felton antibody with solid ammonium sulphate in several sera; the effect on these limits of the addition of purified Felton antibody and the removal of tricresol from the serum.

2. That within these limits of precipitation small fractions can be obtained which differ from each other in their ratio of antibody to protein.

3. That the purity of such fractions throughout a given serum may be reduced by the addition of purified Pneumococcus antibody to the serum, and apparently increased by the addition of tricresol.

4. That the distribution of antibody in serum taken at intervals from a horse during immunisation changes progressively.

5. Differences in the distribution of the two antibodies in serum from a horse immunised to both Pneumococcus type I and diphtheria toxin.

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