The Identification of Natural and Induced Bacterial Antibody with the Serum Components in Sheep

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The association of antibody with γ -globulins was first demonstrated electrophoretically in the serum of rabbits immunized with egg albumin; Tiselius & Kabat (1939) and Enders (1944) found antibodies against a wide range of virus, toxin and bacterial antigens in the γ -globulin prepared from pooled human plasma by ethanol precipitation.

Kekwick & Mackay (1954) used one of these antibodies, the *Salmonella* H agglutinin, as a means of testing the amount of γ -globulin in human globulin mixtures during the development of a method of fractionation of human plasma in aqueous systems containing ether in which the γ -globulins were concentrated in fraction G3. When the method was applied to the sera of other animals, it was found that although, as might be expected from the work of Gibson (1930), an agglutinin against one or more *Salmonella* was present in the pooled sera from most mammalian species, this agglutinin was not necessarily associated with the fraction G3, as in human serum.

In normal sheep serum the agglutinin was in the fraction G2, which was predominantly α - and β -globulins, and the fraction G3, predominantly γ -globulin, had no detectable agglutinin (Mackay, 1955). It was of some interest therefore to know whether the antibody produced by the injection of a bacterial antigen into sheep would be found in the same fraction as the natural agglutinin, and if this was not so, whether the natural agglutinin could be separated from the induced antibody by this method. The term natural agglutinin in this work is used for the agglutinin found *ab initio* in the sheep sera, and induced antibody the agglutinin produced by the injection of a bacterial vaccine. The author appreciates the fact that both agglutinins may have been formed as an immune response to antigens introduced by different routes.

In the course of the work serum and serum fractions from normal sheep, as well as from sheep which had been immunized, were examined electrophoretically. The natural agglutinin in serum and serum fractions of the immunized sheep was also assayed, to find out whether the injection of bacteria with an antigen different from that specific for the natural agglutinin had any effect on the serum titre of the natural agglutinin.

MATERIALS AND METHODS

Mature March Clun sheep taken from the flock at the A.R.C. Institute of Animal Physiology, Babraham, were used in all the experiments. The three animals immunized were housed during and immediately after the injections, and the animal used in the long-term experiment was returned to the field 19 weeks after the last injection of antigen. The *Salmonella* vaccines were injected intravenously, and blood for serum samples was taken into dry bottles. The blood was allowed to clot at room temperature and sent to the Lister Institute, where the serum was separated.

Bacterial vaccines. Strains of *Salmonella* used for testing, and bacterial vaccines, were supplied by Dr B. A. D. Stocker and were from his collection. The vaccines were broth cultures with 0.1% of formalin, and contained approximately 6×10^8 organisms/ml.

Strains SL162 which had the flagellar antigen b, and SL169 with the flagellar antigens 1, 2, 3, were used for immunization. The strain used to test the natural agglutinin had the flagellar antigen c.

Titration of antibody. The flagellar antibody was titrated by the agglutination of a suspension of the relevant organism in 0.85% NaCl containing 0.5% of formalin. The bacteria were grown on nutrient agar at 37° for 16 hr., washed off the agar with formal-saline and the suspension was diluted to an opacity corresponding to 10^8 organisms/ml. A series of twofold dilutions of the fraction or serum to be tested was made in 0.85% NaCl and an equal volume of bacterial suspension was added to each dilution. The mixtures were incubated for 2 hr. at 48°, and the initial end-point was taken as the highest dilution showing flocculation to the naked eye. Results are expressed as the reciprocal of this dilution.

Electrophoretic measurements. Samples were dialysed to equilibrium at 2° against sodium and potassium phosphate buffer, pH 8, *I* 0.2. In the earlier experiments the examinations were carried out in the Tiselius (1937) apparatus for 50 ma hr. In later experiments the duration of the run was extended to 60 ma hr. by the use of the cell with the long centre section (Longworth, 1942). Optical observations by the diagonal-schlieren method (Philpot, 1938) were photographically recorded with a light of 546 m μ wavelength. The electrophoretic picture was analysed as previously described (Mackay, 1955), and the results are expressed as percentages of the total protein in a mixture attributable to any single component. The values quoted are the mean of the values obtained from simultaneous exposures of the ascending and descending limbs.

In fractions containing several components, the identity of these was established by comparing mobilities with those of the components of the serum from which they were

Table 1. *Agglutination of Salmonella strains by normal sheep serum*

Agglutinin titres: values given are the reciprocal of the highest serum dilution showing agglutination.

Flagellar antigen	Serum from sheep no.										
	F70	F21	F20	F22	F57	F67	F6	F18	E108	E81	F37
a	—	—	—	—	5	—	—	5	—	—	40
b	—	5	5	—	10	—	5	10	—	—	—
c	40	40	80	40	80	40	160	80	80	40	40
d	—	—	—	—	5	10	—	10	—	—	—
1, 2, 3	—	—	10	—	5	—	—	5	—	—	—
eh	—	—	5	—	10	5	5	—	—	—	—
ehx	5	10	20	20	20	5	20	40	5	5	—
gm	—	5	—	10	20	20	10	10	—	—	40
gp	—	5	10	5	10	20	5	10	5	—	10
i	20	5	40	20	40	20	20	40	5	40	40

derived. The mobilities were calculated from the positions of the maxima of the peaks of the individual components obtained in the geometrical analysis.

Determination of pH. The pH values were determined with a MacInnes-type glass electrode, calibrated against a solution containing 0.09M-KCl and 0.01M-HCl, which was assumed to be pH 2.1. Measurements were made at room temperature with a valve potentiometer, the accuracy being 0.01 pH unit.

Calculations of ionic strength. These were made as described by Kekwick & Mackay (1954). Volumes of water to be added were calculated from the known salt concentrations, the ionic strength effects of the protein being disregarded.

Nitrogen determinations. The protein nitrogen was determined by the micro-Kjeldahl technique; the results are expressed as mg. of N/ml.

Cholesterol determinations. These were made by the method of Sperry & Brand (1943). Results are expressed as mg. of cholesterol/g. of N.

Fractionation of sheep serum proteins. This was carried out by a modification of the method of Kekwick & Mackay (1954). Serum was roughly separated into globulins and albumin by the precipitation of the globulin (G1) at pH 5.5 ± 0.02 , I 0.037, with ether (18.5 vol. %) at -3.5° . The precipitate was dissolved in 40% of the serum volume of water and 60–70% of the serum volume of buffer, pH 4. The dissolved G1 was brought to a pH between 4.50 and 4.60 to ensure complete solution. The pH of the G1 solution was then adjusted to 5.00 with buffer, pH 6, and the ionic strength to 0.01. This was done by adding a volume of water to a total volume equal to 2.5 times the volume of pH 4 buffer added, plus 4.84 times the volume of pH 6 buffer added. The calculation of the total volume includes the volume of the G1 solution, the volume of the pH 6 buffer and the amount of ether to be added to bring the ether concentration to 10 vol. % (v/v). The fraction G2 was precipitated at pH 5.00 ± 0.02 , I 0.01, with 10 vol. % of ether at 0° . The pH of the supernatant was brought to 6.70 ± 0.02 with 0.5M-NaHCO₃ and concentration of ether to 18.5 vol. % at -3.5° (fraction G3).

The fractions G1s/p and AP were prepared as described in a previous paper (Mackay, 1955). All the precipitations were carried out in vessels suspended in a refrigerated bath, thermostatically controlled to $\pm 0.5^\circ$. The mixtures were allowed to come to equilibrium at the temperature of the precipitation, and the precipitates were removed by centrifuging at the temperature of the precipitation in a

Table 2. *Composition of normal sheep serum measured by electrophoresis*

First technique: duration of expt., 50 mA.hr.; second technique: duration of expt., 60 mA.hr. (see Methods).

	Percentage total protein				
	Globulin				Albumin
	α_1	α_2	β	γ	
First technique					
E108	49	18	8	25	
E81	56	18	8	18	
F44	44	13	7	36	
F22	40	14	7	39	
F18	48	15	5	32	
F70	41	16	7	36	
Second technique					
F70	49	7	10	8	26
F67	47	7	10	6	30
F21	51	6	8	7	28

refrigerated bucket centrifuge, thermostatically controlled to $\pm 0.5^\circ$. The precipitates were dissolved in sodium-potassium phosphate buffer, pH 8.0, or in 0.85% sodium chloride solution, so that the total volumes were one-fifth to one-third of the original volume of serum.

Buffers. (i) pH 4; 0.043M-acetic acid, 0.007M-Na₂HPO₄; (ii) pH 6; 0.023M-acetic acid, 0.027M-Na₂HPO₄; (iii) pH 8, I 1.0; Na₂HPO₄·2H₂O (58.054 g./l.), KH₂PO₄ (2.981 g./l.), diluted to the ionic strength required.

Ether. Anaesthetic-grade ether free from peroxide was used and stored in the dark at 4° .

EXPERIMENTAL AND RESULTS

Serum of normal sheep all had low titre agglutinins against a number of *Salmonella* flagellar antigens (Table 1). There were considerable differences in the electrophoretic compositions of the sera from individual animals (Table 2). Protein fractions prepared from the pooled serum of normal sheep were examined electrophoretically (Fig. 1) and the proportion of total nitrogen precipitated in the main fractions was estimated. From the electrophoretic analysis of the fractions, and their nitrogen

Table 3. *Electrophoretic analysis of sera taken during immunization of sheep E 108 with a Salmonella vaccine (flagellar antigen 1, 2, 3)*

Agglutinin titres given are the reciprocals of the highest dilution showing agglutination. c, Natural agglutinin; 1, 2, 3, induced agglutinin.

Time after first injection (days)	Vol. injected (ml.)	Percentage total protein				Agglutinin titre	
		Albumin	Globulin			c	1, 2, 3
			α -	β -	γ -		
0	1	49	18	8	25	64	< 2
12	5	57	16	7	20	80	320
16	7	57	17	6	20	80	10 240
25	8	48	20	7	25	80	3 200
33	—	55	18	6	21	80	3 200
37	—	56	18	6	20	80	3 200

Fig. 1. Electrophoresis of fractions from normal sheep serum in the Tiselius (1937) apparatus; 50 mA.hr. at 0° in sodium and potassium phosphate buffer, pH 8, I 0.2.

contents, the recovery of protein at different stages was estimated. A typical fractionation is shown in Table 9, and similar values were obtained with a normal serum as with the antiserum shown. Agglutinin titres of the fractions indicated that all the agglutinin was in the fraction G1, and subsequently in G2. There was none in G3.

Examination of antisera. Two sheep, E81 and E108, whose serum had a natural agglutinin titre of 40 and 80 respectively against the flagellar antigen c, but less than 2 against antigen b or antigen 1, 2, 3, were immunized, sheep E81 with

antigen b and E108 with antigen 1, 2 and 3. Four doses of 1, 5, 7 and 8 ml. were injected intravenously at approx. 7-day intervals, and then 10 ml. was given intramuscularly 28 days after the last intravenous injection. A sample of 200 ml. of blood was taken before the first injection, a similar quantity 5 days after the last intravenous injection, and another 8 days after the intramuscular injection. In addition, 20 ml. samples were taken intermediately for electrophoretic examination and for antibody titration. Table 3 shows the electrophoretic analyses of serum samples from E108 and the development of the antibody. Similar results were obtained with E81 immunized with the b antigen. In neither animal was the serum titre of the natural agglutinin altered.

Samples of the serum from E108 before the injection of antigen and 33 days after the first intravenous injection were fractionated. The agglutinin titrations and the electrophoretic composition of the fractions from E108 are shown in Table 4. and similar results were obtained with E81. The fractions were relatively constant in composition in the same animal, and were unaffected by immunization. In some sheep the γ -globulins precipitated in fraction G3 were complex and showed varying amounts of a component (γ_3) with a lower mobility than the main γ -globulin component (γ_2); see Table 5 and Fig. 1. The amount of the slower component bore no relation to the agglutinin titre of the serum.

The natural agglutinin was found in fraction G2 only, as in previous experiments, but the induced antibody was also in fraction G3, although the greater proportion of this antibody was in G2. Fraction G3 contained α -globulin which could be separated electrophoretically from γ -globulin, and tests showed that the induced antibody was associated with the γ -globulins. Because resolution of the components of G2 was not possible by electrophoresis, attempts were made to separate the two types of agglutinin by solvent fractionation.

Fractionation of G2. Previous experiments had indicated that the natural agglutinin was associated with α - and β -globulins (Mackay, 1955). Surgenor, Strong, Taylor, Gordon & Gibson (1949) had described a method for the precipitation of α - and β -lipoprotein at low pH and ionic strength, and it was possible that the proteins containing the natural agglutinin could be precipitated by these means.

Fraction G1 from serum taken 50 days after the first injection of vaccine was brought to pH 5.00 and 1.0.01 at 0°. The precipitate was collected by centrifuging at 0° (fraction G2/1). The ether concentration was brought to 10 vol. % and after equilibration at 0° a second fraction was collected (G2/2) and G3 was made from the supernatant. The protein content of the subfractions G2/1 and

G2/2 was found by nitrogen assay and the cholesterol content and agglutinin titre were determined; the fractions were also examined electrophoretically (Table 6).

Approximately half the protein nitrogen and most of the cholesterol were precipitated in G2/1; the concentration of natural agglutinin in G2/1 was double that in G2/2, whereas the immune antibody was more concentrated in G2/2. The fractions were similar electrophoretically in respect to α - and β -globulin, but the fraction G2/2 had a greater amount of a less-mobile component identified as γ_1 -globulin. The greater concentration of both γ_1 -globulin and induced antibody in G2/2 suggested that the induced antibody was associated with this component.

Table 4. Comparison of fractions from serum from sheep E 108 before and 33 days after the initial antigen injection (flagellar antigen 1, 2, 3)

Agglutinin titres given are the reciprocals of the highest dilution showing agglutination. c, Natural agglutinin; 1, 2, 3, induced agglutinin. Concentration factor: the concentration of the fraction by volume relative to the initial volume of serum fractionated.

Serum fractionated.		Percentage total protein				Concn. factor	Agglutinin titre	
Fraction	Percentage initial N in fraction	Albumin	Globulins				c	1, 2, 3
			α -	β -	γ -			
Before immunization								
Serum	—	49	18	8	25	—	64	<2
G 2	12	4	59	22	15	4	320	—
G 3	11	2	10	—	90	3	<2	—
After immunization								
Serum	—	55	18	6	21	—	80	3 200
G 2	12	2	53	29	16	5	320	25 600
G 3	11	1	17	—	83	5	<5	400

Table 5. Electrophoretic distribution of sheep serum γ -globulins precipitated in fraction G3

Sheep	No. of estimations	Percentage total γ -globulin in G3	
		γ_2	γ_3
F70	8	86 $\pm$ 2	14 $\pm$ 5
E81	2	74	26
E108	2	100	0
B	2	75	25
C	1	77	23

Table 6. Subfractionation of G2 from sheep serum by preliminary precipitation of lipoprotein. (Sheep E108, immunized with flagellar antigen 1, 2, 3.)

Agglutinin titre given is the reciprocal of the highest dilution showing agglutination. c, Natural antibody; 1, 2, 3, induced antibody.

Fraction	Percentage initial serum protein in fraction	Cholesterol mg./g. of N	Electrophoretic analysis (% total protein)				Agglutinin titre	
			Albumin	Globulin			c	1, 2, 3
				α -	β -	γ -		
G2/1	5.6	455	—	57	32	11	160	3 200
G2/2	5.8	47.6	3	50	27	20	80	12 800
G3	12.1	60.2	1	21	—	78	<2	400

Further efforts were made to obtain a better separation of agglutinin by varying the pH of the G2/1 precipitation. Samples of G1 were titrated to pH 5.1, 5.2 and 5.5, and fractions G2/1, G2/2 and G3 prepared. There was not sufficient material for electrophoresis, but the assay of agglutinin suggested that the pH of minimum solubility of the natural agglutinin was 5.1–5.2, and of the induced antibody 5.5.

The titre of specific agglutinin caused by the immunization of sheep E108 and E81 was maximal in the serum taken 16 days after the first injection of antigen, and then declined in later samples in spite of the continued injections. There was no change in the titre of natural agglutinin at any stage. In the immune serum, the natural antibody was all in the G2, but approximately 20% of the induced antibody was in G3, the remainder being in G2. Further fractionation of G2 suggested that the natural agglutinin had a lower isoelectric point and, like the lipoproteins, was insoluble at low ionic strength and ether concentrations. In the experiments on E108 and E81, the immune serum for fractionation was taken after the peak of the antibody level had been passed. There remained the possibility that at an earlier stage of immunity the distribution of agglutinin among the serum proteins might be different. Accordingly, antisera taken at an earlier stage and over a longer period were examined in greater detail.

Location of antibody in serum at different stages of immunity

One sheep (F70) was given six intravenous injections of vaccine SL162, as shown in Table 7. Serum was collected for electrophoretic examination and for fractionation 10, 19 and 28 days after the first injection of antigen, and thereafter at intervals up to 350 days. As in previous experiments, no change was observed in the electrophoretic pattern or in the total protein nitrogen during the course of the immunization. Each serum sample was then fractionated. G1 was prepared at pH 5.5, *I* 0.037, with ether (18.5 vol. %) at –3.5°; G2/1 at pH 5.2, *I* 0.01, at 0°; G2/2 at pH 5.5, *I* 0.01, and ether (10 vol. %) at 0°; G3 at pH 6.7, ether (18.5 vol. %) at –3.5°. The fractions were dissolved in phosphate buffer, pH 8, and were three to four times as concentrated as the serum by volume. The percentage of the total protein precipitated in the various fractions was estimated, and they were examined electrophoretically. Table 8 shows the mean values of eight such examinations, and Table 9 the recovery as nitrogen of the electrophoretically characterized protein components of serum in the different fractions. The results of the agglutinin titrations are shown in Table 10. The distribution of antibody among the fractions varies significantly during immunization. As in the previous experiments, in most sera the

Table 7. *Electrophoresis of serum from sheep F70 during immunization with Salmonella vaccine SL162*

Agglutinin titre given is the reciprocal of the highest dilution showing agglutination. c, Natural agglutinin; b, induced agglutinin. Intravenous injections of vaccine were given on 24, 27, 30 June, 4, 7, 11 July 1956.

Serum sample (days after first injection)	Total serum protein (mg. of N/ml.)	Electrophoretic analysis (% total protein)						Agglutinin titre	
		Albumin	Globulin					c	b
			α_1 -	α_2 -	β -	γ -			
0	10.6	49	7	10	8	26		80	<5
10	10.3	47	6	11	9	27		80	1600
19	10.0	46	8	12	6	28		80	3200
28	10.1	46	8	12	9	25		80	3200
45	10.6	51	6	12	7	24		80	3200
110	12.3	41	5	10	10	34		80	640
195	11.6	47	5	10	6	32		80	320
350	12.6	50	6	10	5	29		80	160

Table 8. *Electrophoresis of sheep serum fractions after immunization*

Sheep F70. Mean values from 8 samples. For details of immunization see Table 7.

Fraction	Percentage initial serum protein	Electrophoretic analysis (% total protein)				
		Albumin	Globulin			
			α_1 -	α_2 -	β -	γ -
Serum	—	47.1±3.4	6.2±1.8	10.9±0.8	7.5±1.3	28.3±2.3
G2/1	7.7±1.4	—	—	45.4±9.7	41.1±4.7	13.5±2.8
G2/2	8.0±1.8	—	—	—	44±5.8	55.5±9.2
G3	13.1±1.7	—	—	10.9±3.8	—	89.1±10

Table 9. *Recovery of protein of the electrophoretic components in fractions from the serum of an immunized sheep (F70)*

Values were estimated from electrophoretic analyses and nitrogen assays of fractions. For details of immunization see Table 7.

Electro-phoretic component	Fraction				Percentage initial serum N	Fraction			Percentage initial G1 N
	Serum	G1 (mg. of N)	G1s/p	AP		G2/1	G2/2 (mg. of N)	G3	
Albumin	420	43	33	211	74	—	—	—	—
α -Globulin	162	138	21	27	84	31	—	11	75
β -Globulin	66		6	2	—	29	32	—	—
γ -Globulin	235	215	—	17	98	9	43	108	74
Total N	886	384	60	286	—	66	75	119	—
% serum N	—	43.4	6.8	32	82.3	17	19.5	31	67.5

Table 10. *Agglutinin titres of serum fractions from sheep F70 after immunization*

Agglutinin titre given is the reciprocal of the highest dilution showing agglutination. c, Natural agglutinin; b, induced agglutinin. Concn. factor: this refers to fractions G2/1, G2/2 and G3 and is the volume concentration of the fraction relative to the initial serum volume. The fractions G1 were all diluted to a volume equal to that of the initial serum before the agglutinin titration, i.e. concn. factor = 1.

Fraction ... Time after first injection (days)	Agglutinin titre										Concn. factor
	Serum		G1		G2/1		G2/2		G3		
	c	b	c	b	c	b	c	b	c	b	
0	80	<5	80	<5	160	<5	40	<5	<5	<5	3.1
10	80	1600	80	1600	160	800*	80	3 200	<5	200	3.6
19	80	3200	80	3200	160	1600	80	12 800	<5	800	3.2
28	80	3200	80	3200	160	800	80	3 200	<5	800	3.2
111	80	640	80	640	320	640	80	320	<8	160	3.2
195	80	320	80	320	160	320	40	640	<5	40	4.0
350	80	160	80	160	160	160	80	320	<5	20	3.0

natural agglutinin content of the G2/1 was double that of G2/2. As the induced agglutinin titre of the serum rose, the ratio of the induced agglutinin titre of fraction G2/2 to that of G2/1 rose to a value of four at the height of the immunity, and was paralleled by the change in the ratio of the γ_1 -globulin content of these fractions. As the serum titre of the induced agglutinin fell, the ratio of the titre of fraction G2/2 to that of G2/1 fell to two, but the ratio of the γ -globulin content of these fractions remained unaltered. The proportion of induced antibody precipitated in G3 was maximal in the samples taken at 19 and 28 days after the first injection of antigen. During fractionation 25 % of the α - and β -globulins and 26 % of the γ -globulins of G1 were lost, presumably in the G3 supernatant which was discarded (Table 9).

DISCUSSION

Experiments with the three sheep show that the flagellar agglutinin stimulated by the intravenous injection of a bacterial vaccine reached its maximum titre in the serum about 20 days after the initial injection of antigen. In two animals (E108 and

E81) the antibody titre declined in spite of continued immunization. The titre of the natural agglutinin was not enhanced by the injection of an organism with a different flagellar antigen.

In normal sheep all the natural agglutinin was in fraction G2; there was none in fraction G3 (Mackay, 1955). In serum fractions from immunized sheep, induced antibody, but never natural antibody, was found in G3.

A modification of the fractionation technique, and the use of the Tiselius apparatus with the long centre-section cell allowed a more critical appraisal of the composition of the fractions, and revealed a substantial amount of γ -globulin in G2. Although there was a considerable coprecipitation of β - and γ -globulins at low ionic strength and low solvent concentration, the natural agglutinin appeared to have a minimum solubility at pH 5.1–5.2, and the induced agglutinin at pH 5.5. In the fractions G2/1 and G2/2 the induced agglutinin showed some correlation with the amount of γ -globulin. It was not possible definitely to associate natural agglutinin with any component. As the fractions G1s/p and G3 both contained α -globulin, but no natural agglutinin, and as G3, which was substantially

γ -globulin, contained only induced agglutinin, it was reasonable to assume that the natural agglutinin was associated with β -globulin. The complete separation of natural from induced agglutinin was not achieved, although in G3 a part of the induced antibody, free from natural antibody, could be isolated.

The fractionation experiments suggest that in the serum from immunized sheep agglutinins are present in three electrophoretically distinct protein components. Immune agglutinin was found in γ_1 -globulin precipitated at pH 5.5, and in γ_2 -globulin precipitated at pH 6.7, whereas the natural agglutinin was in the β -globulin. The separation of human and ox γ -globulins into two components with different antibody properties has also been described by Deutsch, Alberty, Gosting & Williams (1947) and Hess & Deutsch (1949).

There are various explanations for the association of the two types of antibody with different electrophoretic components, but there is no real proof. Kekwick & Record (1941) observed that the early reaction in horses to the intramuscular injection of diphtheria toxoid was the production of antibody in γ -globulin and after prolonged immunization in β -globulin. The antibodies which were separated electrophoretically showed qualitative differences, and electrophoresis of the whole serum showed an increase in the amount of β_2 - (or γ_1 -) globulin. Sheep may behave in a similar manner, the immune antibody appearing first in γ -globulin, and later in β -globulin. There was some evidence for this in the experiments on sheep F70. The method of assaying agglutinin was not sufficiently accurate to allow a firm conclusion to be drawn with respect to the G2 fractions, but in sheep, as in horses, the first response was the appearance of agglutinin in γ -globulin. As the titre of induced antibody waned the proportion of agglutinin in G3 also decreased, as well as the ratio of agglutinin in G2/2 to G2/1. The ratio of natural agglutinin in G2/1 and G2/2 did not alter during the experimental period. It should be noted, however, that there was still some antibody in G3 12 months after the injection of antigen, when the serum titre had fallen almost to the level expected in a normal animal.

An alternative explanation is suggested by the work of Treffers, Heidelberger & Freund (1947). The natural *Salmonella* agglutinin is presumably acquired as the result of an infection, and certainly not by intravenous injection, and the location of antibody may depend on the route by which the antigen is introduced. Treffers *et al.* studied the effects of subcutaneous and intravenous injection of protein antigens into horses and found that when the antiserum was fractionated with ammonium sulphate a small amount of the antibody produced by intravenous injection was in the fraction pre-

cipitated by 30 % saturation, and some in the water-insoluble part of the fraction precipitated between 30 and 50 % saturation. The antibody produced by the subcutaneous injection was in the water-soluble part of the 30–50 % saturation fraction. Treffers *et al.* did not examine their fractions electrophoretically, but Cohn, McMeekin, Oncley, Newell & Hughes (1940), who examined the fractions precipitated from horse serum by increasing concentrations of ammonium sulphate, showed that the fraction precipitated at 30 % saturation was mainly γ -globulin, the water-insoluble part of the 30–50 % saturation fraction was β - and γ -globulin and the water-soluble part was α - and β -globulin. If a similar series of events takes place in sheep immunized with a bacterial vaccine, the agglutinin from the intravenous injection would be in the γ_2 -globulin (the 30 % saturation fraction) and in γ_1 -globulin (water-insoluble part of the 30–50 % saturation fraction) and the natural agglutinin in β -globulin (the water-soluble part of the fraction precipitated at 30–50 % saturation).

SUMMARY

1. The electrophoretic pattern and natural agglutinin titre against a range of H antigens of *Salmonella* was determined in sera from one flock of sheep.
2. The effects of the injection of a bacterial vaccine on the electrophoretic pattern of sheep serum during, and for 12 months after, immunization were studied and showed no characteristic change in pattern.
3. An attempt was made to isolate the natural and induced antibodies by solvent fractionation. Natural antibody to certain *Salmonella* flagellar antigens were found in fraction G2, which was shown electrophoretically to consist of α -, β - and γ_1 -globulins. They did not increase in amount during immunization with an unrelated *Salmonella* antigen.
4. Induced antibody reached a maximum titre in serum 16–21 days after the first injection of antigen. Antibody, free from natural antibody, was present in the fraction G3, which was substantially γ -globulin. In fraction G2 it was mixed with natural antibody. The isoelectric points of the two antibodies appear to be different.

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The Formation of Complexes between Haemoglobins and Plasma Proteins in a Variety of Animals

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Heilmeyer (1933), who investigated by spectrophotometry the effect of adding alkaline haematin to serum, showed that there was a definite combination of the haematin and some protein constituents. Later Fairley & Bromfield (1934) and Fairley (1941*a, b*) identified methaemalbumin formed from the combination of haemoglobin released by the intravascular breakdown of erythrocytes and the albumin fraction of the plasma in man and monkey. This complex was not found in other animals. Clark, Harris & Rosenberg (1949) investigated the combination of mesohaem with a number of proteins. They found that a variety of proteins formed haemochromogens, including human serum albumin, and showed that methaemalbumin is not a haemochromogen. Rosenfield & Surgenor (1949) have shown that haematin and serum albumin combine in the molar ratio 1:1 to form methaemalbumin, but found no spectrophotometric evidence for the combination of haematin with other plasma proteins. Haem combines with denatured proteins in the molar ratio 1:2 to form haemochromogens, but does not combine with native proteins (Anson & Mirsky, 1928, 1934; Holden & Freeman, 1929). Haem combines with native globin to form a complex which resembles native haemoglobin in many respects (Anson & Mirsky, 1930, 1934; Joep, 1949). It differs from native haemoglobin in its spectral absorption in the near-ultraviolet region (Joep, 1949; Joep & O'Brien, 1949). Very little is known of complex formation between haemoglobins and other fractions of serum proteins in other vertebrate animals.

Changes in electrophoretic mobility of proteins have been used widely to investigate their interactions with a number of large molecules, including other proteins. This paper describes the use of paper electrophoresis, supplemented by spectrophotometry, to reveal the formation of a family of complexes between haemoglobin and its derivatives and plasma proteins in a variety of animals: man, horse, ox, dog, pig, goat, rabbit, guinea pig, duck, turtle and frog.

METHODS

Blood was collected by venepuncture or heart puncture into an oxalated tube and centrifuged at 2500 rev./min. for 20 min. to separate the plasma. Haemoglobin was prepared from the sedimented erythrocytes by the method of Drabkin (1946) and diluted to a concentration of 40 mg./ml. with barbitone buffer (*I* 0.075; pH 8.6). From this solution, the following were prepared: (*a*) oxyhaemoglobin, by saturation with air; (*b*) reduced haemoglobin, by addition of a trace of sodium dithionite and subsequent storage under CO₂; (*c*) carbon monoxy haemoglobin, by saturation with coal gas; (*d*) methaemoglobin, by addition of a minimal amount of potassium ferricyanide and subsequent dialysis; (*e*) alkaline haematin, from purified ox haemin by the method of Fischer (1941).

Paper electrophoresis was conducted under kerosene (b.p. 200–300°), with broad sheets of Whatman no. 1 filter paper and barbitone buffer (*I* 0.075; pH 8.6). The potential gradient was 5–7 v/cm. A graded series of buffered mixtures of plasma and haemoglobin was prepared as shown in Table 1. Immediately after mixing each sample was examined spectrophotometrically to confirm the exclusive presence of the appropriate haem–protein. Spots (0.005 ml.) of a series of dilutions of the mixture were applied at 15 mm.