THE ANTIGENICITY OF SERA OF MAN AND ANIMALS IN RELATION TO THE PREPARATION OF SPECIFIC PRECIPITATING ANTISERA

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(With 4 Figures in the Text)

CONTENTS

INTRODUCTION

The precipitin reaction has been applied to a number of practical problems. Many variations of technique have been described in the literature which is well summarized by Landsteiner (1947). The precipitin test is often used in medico-legal work for the identification of blood stains and the detection of adulterated foodstuffs of protein origin; it is also used extensively for the quantitative and qualitative analysis of proteins (Goettsch & Kendall, 1935; Hewitt, 1937) and for pathological examinations (Chow, 1947). Other investigators have employed the precipitin reaction for the study of animal relationships (Nuttall, 1904; Wolfe, 1939; Boyden, 1942). The reaction has also been used for the identification of blood meals of insects, and the method has proved of value in entomological research, especially

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in ecological studies concerning blood-sucking insects which are capable of transmitting diseases of man and animals (Buxton, 1948). The reliability of many of the results obtained has suffered from a lack of suitable antisera, but some workers have obtained valuable data over relatively limited fields (Bull & King, 1923; Prates, 1928; Rice & Barber, 1934; Rempel, Riddell & McNelly, 1946; Riddell, Rempel & McNelly, 1947; Senior White, 1947; Holstein, 1948).

The identification of blood meals of insects by the precipitin test presents certain difficulties due to the habits of the insects. There is often an unavoidable delay between the feeding of an insect and its capture during which considerable deterioration of the ingested proteins may occur; some insects are very small feeders so that the amount of antigen available for testing is very small. It is, therefore, very important to use antisera which are highly sensitive in that they will react to the presence of very small quantities of the corresponding antigen and, because a very wide range of possible hosts has to be considered, the antisera should also be specific. Specificity is most important when investigating the blood meals of insects which have had multiple feeds on different hosts.

The methods used for the preparation of antisera with the sensitive and specific qualities required for such identification tests are described in this paper. Some experiments designed to determine the nature of the antigen-antibody reactions which take place with such antisera are also recorded.

MATERIALS AND METHODS

(*a*) *Antigens*

The antigens commonly used in this work were the sera of man, ox, sheep, goat, horse, pig, dog, cat and fowl. Other sera were used on occasions.

(i) The sera used as antigens for tests contained no preservatives but were filtered through sterilizing Seitz E.K. filter pads and dried from the frozen state. For the precipitin test the serum was reconstituted to its original volume with sterile distilled water and diluted in normal saline solution (0·9 %) as required.

(ii) The sera used as antigens for inoculating animals were precipitated with a solution of potash alum as described by Proom (1943). The precipitate, after several washings, was resuspended in normal saline to twice the volume of the original serum. Thus 10 ml. of suspension were equivalent to 5 ml. of serum.

(*b*) *Method of immunization*

Groups of healthy mature rabbits were inoculated intramuscularly with a volume of 10 ml. of the alum precipitated serum (A.P.S.) Ten to twelve days after inoculation the rabbits were bled from the ear and their sera tested for sensitivity to the homologous antigen. If the titre of the serum was high enough, about 50 ml. of blood were taken from the rabbit by cardiac puncture. After allowing the blood to clot, the serum was removed and pooled with that of animals similarly immunized. The pools were sterilized by filtration and stored at $-10°$ C. until required. After about 10 days, the rabbits were re-inoculated with 5 ml. of A.P.S.; re-inoculation was repeated at 10-day intervals four or five times. Ten to twelve

days after the last injection the rabbits were bled out by heart puncture under anaesthesia, and the sera from all the bleedings were pooled together to form one large batch of antiserum. In this way a group of five rabbits usually yielded from 500 to 700 ml. of antiserum over a period of approximately 12–14 weeks.

Goats, sheep, calves and a chimpanzee were also used for the preparation of specific antisera. The amount of antigen used is recorded in the experimental section of this paper; in other respects the technique used for inoculating these animals was similar to that used for rabbits.

(*c*) *Storage of antisera*

Antisera must be perfectly clear in order to avoid any confusion which might arise from a cloudy antiserum obscuring or simulating a weak precipitation ring when tested with antigens. Most preservatives tested, including boric acid, phenol and thiomersalate, caused a varying degree of opacity of the antiserum which obscured the accurate observation of positive reactions. Rice & Barber (1934) diluted their antisera with a solution containing phenol and 50% glycerin. This method caused undue dilution of the antiserum with loss in sensitivity and the glycerin content caused a slight separation of the antiserum and antigen solutions at the interface with the consequent formation of rather weak rings. Antisera to which any of these preservatives have been added deteriorate rapidly in quality even when stored in the cold room at $+4°$ C. Samples without preservative stored frozen at $-10°$ C. are preserved more satisfactorily. The ideal method of storage is to dry the antisera from the frozen state in suitable ampoules. No deterioration of quality has been noticed in any dried antisera kept at room temperature for over 2 years. It is necessary, however, to extract the lipoids before drying from the frozen state, as otherwise the serum is opalescent when reconstituted with distilled water. The opacity is due to the breaking up of lipoid complexes at the low temperatures to which they are subjected during the drying process. The lipoids were removed by the method described by MacFarlane (1942); most sera required two or three extractions before the lipoids were sufficiently extracted. No deterioration of the antisera followed the extraction of lipoids by this method; Hartley (1925) has shown that the removal of lipoids with 10 volumes of alcohol-ether at $-10°$ C. inhibits the precipitin reaction. However, after sera have been extracted by the method of MacFarlane using ether at a temperature below $-25°$ C., it is possible to remove an additional 2·5% of lipoids by further extraction with alcohol-ether at $-10°$ C. The lipoids which are extracted with ether below the eutectic temperature seem, therefore, to play no part in the precipitin reaction. Finally, the antisera were filtered through sterilizing asbestos filter pads, ampouled, dried from the frozen state and packed under oxygen-free nitrogen. Antisera thus prepared from rabbits are always crystal clear when reconstituted with distilled water.

(*d*) *The precipitin ring test*

All the determinations in this work were made by the 'ring-test' by overlayering the undiluted antiserum with dilutions of antigen in narrow tubes. In a positive reaction a precipitate appears as a whitish ring at the interface between the

antigen and antiserum. The procedure was carried out by means of a specially constructed apparatus with which twelve tests could be done simultaneously. A row of specially designed glass tubes (2–3 mm. in diameter) is automatically connected to a suction unit, by means of which it is possible to draw up first the antigen dilutions and then antiserum without mixing them, so that a neat interfacial surface is obtained. The tubes are then sealed in a tray containing plasticine and are released from the suction unit. A detailed account of the apparatus will be published.

The tubes are then left at room temperature for 2 hr. before being read under constant indirect illumination against a dark background. In order to obtain the best results with this reaction both the antigen solutions and the antisera should be perfectly clear. To avoid the possibility of contamination with other protein solutions new tubes were used for each test in all these experiments.

(i) *Determination of sensitivity*

The sensitivity of the precipitating antiserum is expressed as the highest dilution of homologous* antigen which shows a positive reaction with the antiserum under test after two hours incubation.

(ii) *Determination of specificity*

The titres of heterologous* antigens were determined by overlayering an antiserum with suitable dilutions of the various sera being tested. An antiserum is described as specific if none of the antigens tested gave a positive reaction at a maximum concentration of 1 in 10 after incubation for 2 hr. Non-specific antisera are those which show little or no difference in titre with heterologous and homologous antigen dilutions.

The results of determinations of the specificity of an antiserum depend on the time allowed for the reaction to take place and also on the concentration of the antigen. These factors should be considered when comparisons are made with results obtained by different workers. Proom (1943) tested the specificity of his antisera using a maximum concentration of antigen of 1 : 50 and reading the test after 30 min. If he had used a maximum concentration of antigen of 1 : 10 and had incubated his tests for 2 hr. his antisera might have appeared far less specific.

In the present work the antigens normally used when testing for specificity were the sera of man, ox, sheep or goat, horse, pig, dog, cat and fowl unless stated otherwise and, if the antiserum gave no reaction with any of these antigens other than the homologous serum, the antiserum was considered specific.

* Throughout this paper the terms *homologous* and *heterologous* are used in the following sense. The homologous antigen is the serum of the animal species used as the antigen for the preparation of the antiserum, while the heterologous antigens are the sera of all other animal species.

EXPERIMENTS

(*a*) *Antibody response in rabbits*

As Proom (1943) and other workers observed, it was found that the specificity of antisera decreases progressively as the course of immunization is repeated, although an increase in titre of homologous antibody may occur. A relatively specific antiserum can be obtained after the first inoculation of antigen but at this stage its homologous titre is fairly low. A highly sensitive antiserum can only be obtained at the expense of specificity.

Table 1. *Precipitin titres of non-specific antisera, prepared by inoculating rabbits with several doses of alum-precipitated serum, when tested with homologous and heterologous antigens*

Antiserum	No. of antiserum	Titre of reaction tested by the ring test with serum of					
		Man	Ox	Sheep	Horse	Pig	Dog
Anti-human	P41/11	**512**	512	256	128	128	128
Anti-ox	P55/13	256	**512**	512	256	128	128
Anti-goat	P67/6	64	64	64	32	32	16
Anti-horse	P50/10	128	128	64	**512**	512	128
Anti-pig	P53/4	256	128	32	256	**512**	256
Anti-dog	P46/3	64	16	16	16	32	**256**

Figures refer to the titre in thousands.
The homologous titre is given in black.

There appear to be no great differences in the antibody response to the injection of the different antigens (see Table 1). Different antisera had titres ranging from 1/64,000 to 1/512,000 when tested with the homologous antigens. Non-specific reactions always occurred at high titres following the second inoculation. Different routes of inoculation gave very similar results although, as was shown by Proom (1943), the antibody response is slower after intravenous injection.

(*b*) *The absorption of non-specific antibodies*

After the titres had been determined with both homologous and heterologous antigens, the antiserum was absorbed with those heterologous antigens which gave positive reactions. A preliminary titration was carried out to determine the amount of antigen required for absorption of the corresponding heterologous antibody before the bulk of antiserum was finally absorbed.

(i) *Preliminary titration*

A series of eight small tubes was used for the preliminary titration of the antiserum with each heterologous antigen. A constant amount of antiserum was introduced into each tube and varying amounts of the serum antigen were added, as shown in Table 2. The tubes were well shaken and were incubated for 2 hr. at 37° C. and then placed overnight in the refrigerator at 4° C. to allow the completion of precipitation. The following morning the tubes were centrifuged for about 15 min.

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at approximately 2000 r.p.m. To determine the presence or absence of the antibody in each tube of a series, the clear supernatants were then tested by the ring method with two dilutions (1/10 and 1/100) of the heterologous antigens used for the absorption of the series. In the preliminary titration this procedure was repeated with each heterologous antigen separately. The proportion of antigen to antiserum required for absorption of each heterologous antibody was indicated by the tube in which the antiserum had been absorbed with the highest dilution of antigen and whose supernatant fluid gave no positive reaction with the corresponding antigen.

Table 2. *Method of setting up a preliminary titration of an antiserum against a heterologous antigen*

No. of tube ...	1	2	3	4	5	6	7	8
Antiserum (undiluted) (ml.)	0·25	0·25	0·25	0·25	0·25	0·25	0·25	0·25
Dilution of heterologous antigen	Neat	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128
Volume of dilution of heterologous antigen added	0·025	0·025	0·025	0·025	0·025	0·025	0·025	0·025
Final antigen/antiserum ratio	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
Amount of precipitate observed during absorption	–	p	p	pp	pp	pp	ppp	ppp
Ring test with heterologous antigen on supernatant fluid	–	–	+	++	+++	+++	+++	+++

+ to +++, intensity of precipitin rings.
p to ppp, amount of precipitate.

(ii) *Final absorption of antisera*

The bulk of the antiserum was then absorbed by adding the correct amounts of neat antigen which had been determined by the preliminary titration. For the complete absorption of the non-specific antisera a mixture of the antigens was made containing the requisite amounts of each antigen and was then added to the bulk of the antiserum. After 2–4 hr. incubation at 37° C. the antiserum was allowed to precipitate overnight at 4° C. The next morning the antiserum was centrifuged sufficiently to cause most of the precipitate to be deposited. A small sample was filtered through a clarifying asbestos filter pad and tested for specificity. It was sometimes found that one or more of the non-specific reactions had not completely disappeared, in which case the absorption was completed by the addition of further amounts of antigen until the antiserum gave no non-specific reactions.

(*c*) *The results of absorbing non-specific antisera*

The titres of the homologous and heterologous antibodies of an anti-human non-specific antiserum were determined using as antigen the sera of man, ox, sheep, horse, pig and dog. Preliminary absorption tests were made with each antigen in turn to determine the minimum amount of the antigen required for the complete neutralization of its corresponding antibody.

The tests were done in the same manner as the preliminary absorption tests (Table 2), but larger volumes were used, namely 2·0 ml. of antiserum and 0·2 ml. of antigen dilution. The mixtures were incubated at 37° C. for 2 hr. and then left

overnight at 4° C. The following morning the tubes were centrifuged and the supernatants clarified by filtration and tested with dilutions of each antigen ranging from 1 : 10 to 1 : 256,000.

Figs. 1–3 show the results which were obtained when this serum was absorbed with various proportions of man, ox or pig sera, and the supernatant fluids tested

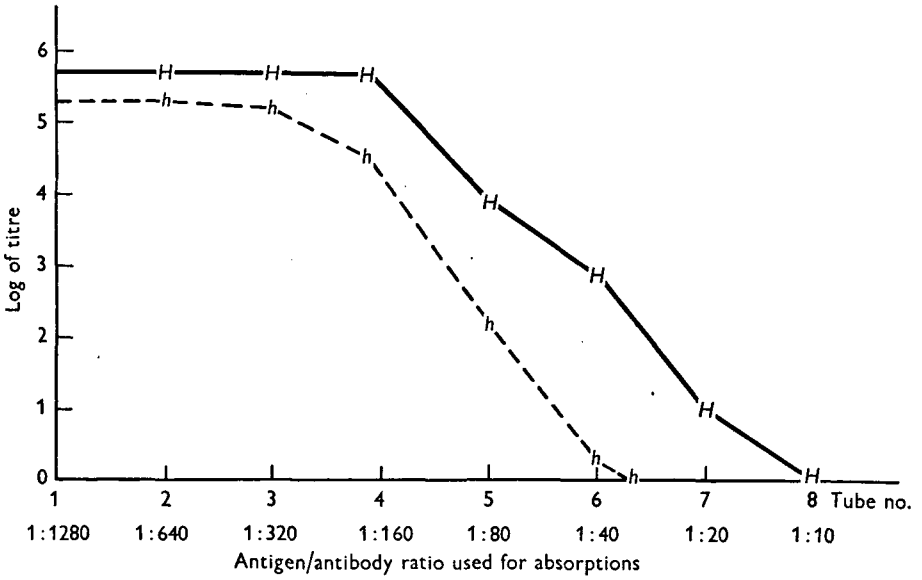

Fig. 1. The absorption of an anti-human non-specific antiserum with various concentrations of homologous antigen (human serum) showing the effect on the homologous and heterologous reactions. *H*——*H*, titre of homologous reaction; *h*– – –*h*, titre of heterologous reaction. Tubes 1–8 are the supernatants from the antiserum after its absorption with increasing amounts of antigen. The antigen/antibody ratio used for absorption is indicated for each tube.

with these antigens at various dilutions. Absorptions were also carried out with sheep, horse, pig, dog and cat antigens, and the results closely resembled those illustrated in the figures.

These results show that the absorption of an anti-human serum with the homologous antigen (human serum) caused the absorption of heterologous antibodies even when small amounts of antigen were added and that heterologous reactions were no longer observed when the point of neutralization of the homologous antibody had been reached. However, the homologous (anti-human) reaction was not affected by the absorption with any of the heterologous antigens, even when an amount of heterologous antigen more than was necessary for the neutralization of the antibody was used.

When this anti-human serum was absorbed with different heterologous antigens, the effect on other heterologous antibodies varied with the species of the antigen used. Ox and sheep heterologous antibodies were more easily removed than the heterologous antibodies of other species. Absorption with ox serum caused the simultaneous disappearance of sheep antibodies (Fig. 2), but absorption with sheep serum did not cause the removal of ox antibody in the same way.

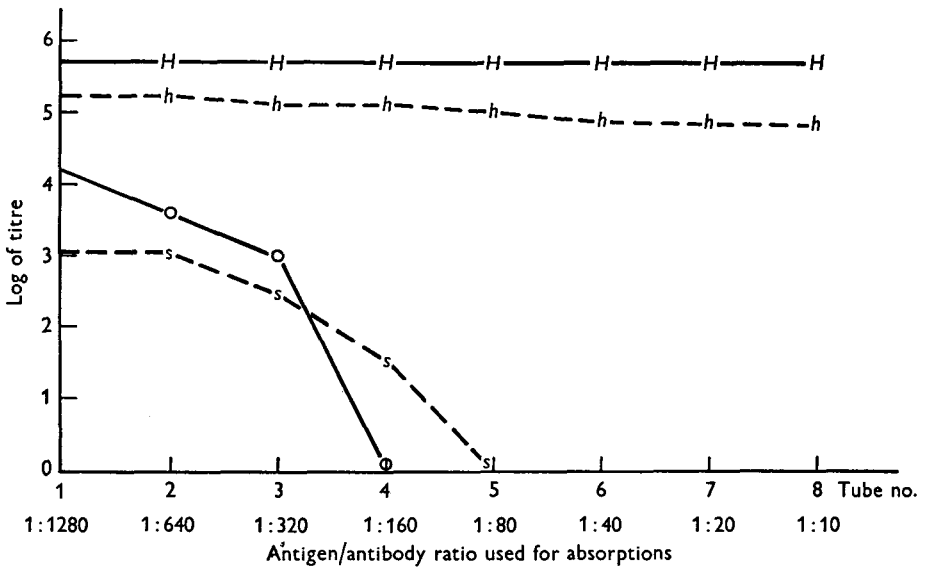

Fig. 2. The absorption of anti-human non-specific antiserum with various concentrations of ox serum (heterologous antigen) showing the effect on the homologous and other heterologous reactions. *H*——*H*, titre of homologous reaction; O——O, titre of ox serum reaction; *s*- - - -*s*, titre of sheep heterologous reaction; *h*- - - -*h*, titre of other heterologous reactions.

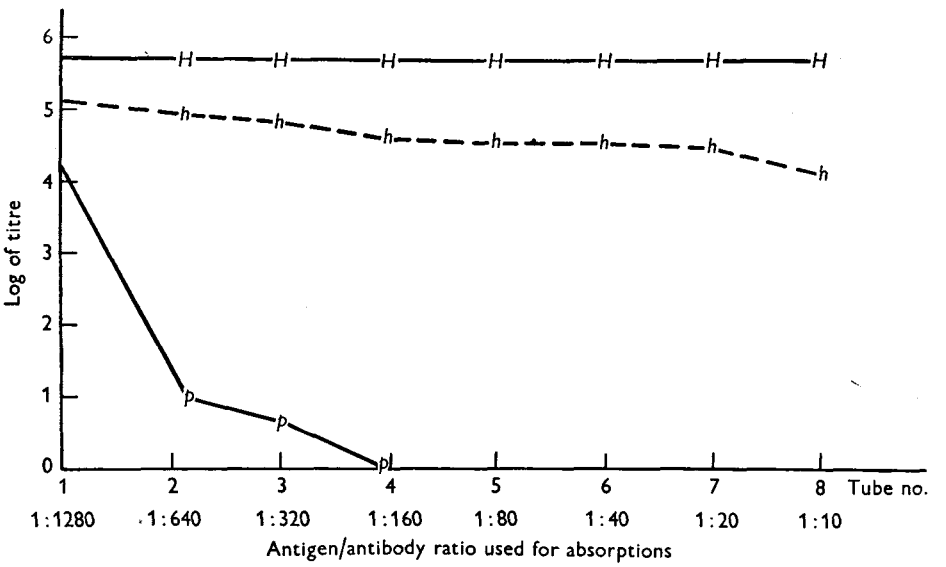

Fig. 3. The absorption of anti-human non-specific antiserum with various concentrations of pig serum (heterologous antigen) showing the effect on the homologous and other heterologous reactions. *H*——*H*, titre of homologous reaction; *p*——*p*, titre of pig serum reaction; *h*- - - -*h*, titre of other heterologous reactions.

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These experiments show that the reactions of the homologous and heterologous antigen-antibody systems are different and distinct reactions.

A. The Homologous reaction

In the strictest sense, the homologous antigen-antibody reaction should refer only to the reaction which occurs when an antiserum is tested with the antigen which had been used to inoculate the antibody-producing animal. In fact, a number of antigens closely related to the homologous serum were also found to react with the antiserum after the heterologous antibodies had been absorbed. This point is illustrated by the homologous reaction of an anti-goat serum (prepared by the inoculation of rabbits with goat A.P.S.), which can be made specific in relation to the sera of other domestic animals and man, by absorbing it with the sera of man, ox, horse, pig, dog and cat. However, the absorption of this antiserum with sheep serum causes the disappearance of both the homologous and the heterologous reactions. Similarly, an anti-sheep serum absorbed with goat serum, or an anti-human serum absorbed with sera from some of the higher apes will loose their power to cause homologous reactions. Thus, the antigen-antibody reactions which can still be observed after the absorption of antisera with heterologous antigens, indicate the range of specificity of the homologous antibody.

(*a*) *The specificity of the homologous reaction*

In these experiments four different antisera were used; they were an anti-man, anti-ox, anti-goat, and an anti-horse serum. These antisera had been prepared in rabbits by multiple inoculations and, when tested with a large number of different antigens, were found to be very non-specific. The species of animals, the sera of which were used for testing these antisera, represent a fairly wide distribution throughout the mammals and include a few birds. For convenience the order and family of each animal species is indicated in col. 1 of Table 3; most of the important mammalian orders are represented by at least one animal species.

Because only small quantities of sera were available, it was not possible to determine the titre of the antisera for each species before absorption. In a number of tests, however, when similar non-specific sera were tested, it was invariably found that the sensitivity titre with all heterologous mammalian sera was very similar to that obtained with the homologous serum, and a few tests confirmed these findings on the antisera used for these experiments. When these antisera were tested with the sera of a small number of species of birds, including the domestic fowl, shoebill, penguin, seagull, crane and ostrich, none of the antisera reacted with any of these avian sera, which suggests that the heterologous reaction is confined to mammalian sera. Unfortunately no reptilian sera were available for these tests.

The four non-specific antisera were then absorbed with a mixture of the sera of man, ox, goat, horse, pig and dog, using the amounts determined by preliminary titration and omitting in each absorption the corresponding homologous serum. The absorbed antisera were then retested with the mammalian sera, and the titres of the reactions determined are recorded in Table 3. Such absorptions removed the

Table 3. *Reactions occurring with antisera after their absorption with the sera of domestic animals and man*

	Sera tested		Antisera			
Order	Family	Common name	Anti-human	Anti-horse	Anti-ox	Anti-goat
Primates	Hominidae	**Man**	**1 : 512000**	0	0	0
	Cercopithecidae	Vervet Monkey	1 : 8000	0	0	0
		Rhesus Monkey	1 : 8000	0	0	0
		Baboon	1 : 32000	0	0	0
Rodentia	Thryonomyidae	Cane Rat	0	0	0	0
	Hystricidae	Porcupine	0	0	0	0
Insectivora	Erinaceidae	Hedgehog	0	0	0	0
Carnivora	Canidae	Dog	0	0	0	0
	Felidae	Cat	0	0	0	0
		Lion	0	0	0	0
	Ursidae	Polar bear	0	0	0	0
Marsupialia	Macropodidae	Wallaby	0	0	0	0
Tubulidentata	Orycteropodidae	Aardvark	0	0	0	0
Hyracoidea	Procaviidae	Hyrax	0	0	0	0
Perissodactyla	Equidae	**Horse**	0	**1 : 32000**	0	0
		Zebra	0	1 : 16000	0	0
	Rhinocerotidae	Rhinoceros	0	1 : 1000	0	0
Artiodactyla:						
1. Suiformes	Suidae	Pig	0	0	0	0
		Warthog	0	0	0	0
2. Ruminantia	Giraffidae	Giraffe	0	0	0	0
	Bovidae					
	Bovinae	**Ox**	0	0	**1 : 64000**	0
		Eland	0	0	0	1 : 10
		Kudu	0	0	1 : 4000	1 : 10
		Buffalo	0	0	1 : 8000	0
		Bushbuck	0	0	1 : 100	0
	Cephalophinae	Duiker	0	0	0	1 : 8000
	Hippotraginae	Hartebeest	0	0	1 : 10	1 : 16000
		Wildebeest	0	0	0	1 : 100
		Waterbuck	0	0	1 : 4000	1 : 8000
		Reedbuck	0	0	0	1 : 8000
	Antilopinae	Thomson's gazelle	0	0	0	1 : 16000
		Grant's gazelle	0	0	0	1 : 100
		Impala	0	0	0	1 : 10
		Dikdik	0	0	0	1 : 2000
	Caprinae	Sheep	0	0	0	1 : 64000
		Goat	0	0	0	**1 : 64000**

reaction which occurred previously with all mammalian sera, except that a positive reaction remained when tested with the sera of animals closely related to the species of the homologous serum. Thus it appears that the homologous reaction takes place in varying degrees with the sera of zoologically related species whilst the heterologous reaction occurs with all mammalian sera from zoologically related or unrelated species. These distinctions were clearly noticed in the anti-human serum which, after its absorption with the sera of domestic animals, reacted only with human and monkey sera. Similarly, the anti-horse serum reacted with zebra serum and to a less extent with the serum of the rhinoceros which is classified in the same order as the horse. With the anti-ox and anti-goat sera the range of the homologous reactions appeared to be much wider, but in fact only included sera from the family Bovidae. The goat antiserum reacted with a wider group of sera than any of the other antisera, which may indicate that goat serum contains a more complex type of antigen.

The homologous reaction therefore appears to be group-specific, and this specificity broadly matches the accepted zoological classification.

(*b*) *Cross-immunization experiments*

The findings recorded in Table 3 show the limitations of the ordinary methods for the preparation of specific precipitating antisera by absorption. The identification of sera of closely related animal species is not possible by the use of such absorbed sera. There are undoubtedly degrees of relationship, and consequently specific sera can be prepared by the absorption of antisera which show cross-reactions with antigens which are not too closely related to the homologous antigen. A relationship of this kind is illustrated in Table 3. The cross-reactions which occurred with the anti-horse serum when tested with rhinoceros antigen was eliminated by another absorption of the antiserum with a small amount of rhinoceros antigen. It was not possible to absorb the serum with zebra antigen without removing the anti-horse antibodies at the same time.

Qualitative differences in the antigenic behaviour of serum proteins derived from very closely related animal species have never been successfully established by means of rabbit precipitating sera. Nuttall (1904) prepared a number of anti-human sera in rabbits and found them to react identically with the sera of man, chimpanzee and other apes. Subsequent workers have observed differences in the titre of rabbit anti-human antisera (Wolfe, 1939) when tested with the sera of man and apes, and differences have also been observed when antisera prepared against the sera of various members of the family Bovidae were tested with sera from different members of this family. These observations cannot be accepted as proving differences of identity of serum protein between the related species.

Uhlenhuth (1905) devised a method which was suitable for the differentiation of the sera of hares and rabbits by inoculating the serum of one species into the other. In the experiments recorded in this paper, this method of 'cross-immunization' was applied for the differentiation of serum proteins from the ox, sheep and goat and also from man and the chimpanzee.

(i) *Experiments with sheep and goats*

On the basis of the experiments of Uhlenhuth it was not expected that the sheep would produce antibodies against serum proteins antigenically similar to its own proteins. Sheep were, therefore, inoculated with the serum proteins of the goat in the hope that specific antisera would result. Conversely, goats injected with sheep serum should give a specific anti-sheep serum. Another group of goats and sheep was inoculated with ox serum to observe whether heterologous antibodies for goat or sheep would appear as a result of the injections.

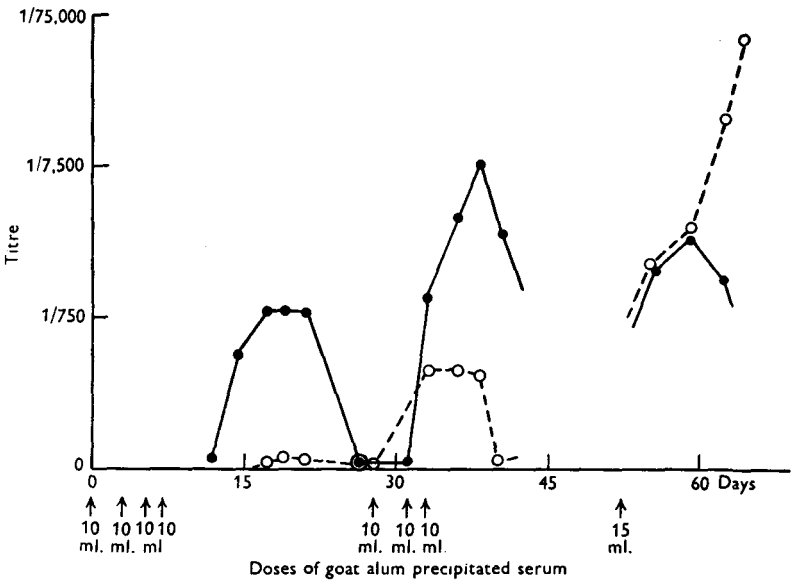

Fig. 4. Antibody response to the injection of goat alum-precipitated serum in a sheep. ●——●, titre of anti-goat reaction (homologous); O- - - -O, titre of anti-ox reaction (heterologous).

All these animals were given large doses of A.P.S. and were tested daily to check the rise in their antibody titres. Fig. 4 shows the response which was observed in the serum of a sheep when inoculated with goat serum, and was characteristic of the reaction which occurred in sheep or goats when injected with each other's serum. The response to the first series of injections of doses of 10 ml. of alum-precipitated goat serum given at 2-day intervals was very slow and of low titre. Further inoculations produced a sudden rise in titre of the homologous reaction which, however, rapidly returned to normal. Of the five other sheep in this group given similar doses of antigen one gave practically no antibody response but the responses in the remaining four followed that recorded in Fig. 4. Three goats which were injected with sheep serum produced antibody responses similar to those obtained after injecting sheep with goat serum.

As shown in Fig. 4, a marked heterologous antibody response, but confined to the ox, was produced in sheep inoculated with goat serum. When tested with the sera of other domestic animals and man, the general heterologous reaction which

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occurs in rabbits was not found. The fact that antibodies to the ox persisted even when the homologous titre had dropped suggests that goat serum contains an antigen common to the ox.

At the peak of homologous antibody production the goats and sheep were bled and the sera were separated. The heterologous anti-ox antibody was absorbed out with ox serum and resulted in antisera completely specific for sheep and goat respectively.

Six calves which were injected with goat and sheep serum responded very rapidly, and yielded high-titred sera. In these animals, however, the goat and sheep antibodies were present together and could not be absorbed out separately; in this respect the preparation of antisera in calves yielded no better antisera than those made in rabbits. These findings confirm the observations made in rabbits that the ox is not sufficiently closely related to the sheep or goat to inhibit the stimulation of antibody production to their proteins.

(ii) *Experiments with human and chimpanzee sera*

Rabbit anti-human serum gives homologous reactions with the sera of the chimpanzee and other higher apes. The differentiation of the proteins of the higher apes was investigated by preparing an anti-human serum in a chimpanzee.

An adult chimpanzee was inoculated intramuscularly with human A.P.S. in doses of 20 ml. (= 10 ml. whole serum) on the 1st day, 10 ml. (= 7·5 ml. serum) on the 11th day, 20 ml. (= 15 ml. serum) on the 18th day and 20 ml. (= 10 ml. serum) on the 25th day. Before each inoculation a sample of blood was withdrawn and the serum tested for the presence of antibodies to human serum. No response was observed until the 33rd day when a positive reaction occurred with a dilution of human serum of 1 : 2000. On this day a final inoculation of 10 ml. of whole serum was given intraperitoneally and the animal was bled out under anaesthesia on the 43rd day. The serum obtained on the 43rd day was then tested for its sensitivity to human antigen and for the presence of non-specific antibodies. Its reactions are shown in Table 4.

Although the titre with human antigen was low, the antiserum was very specific.

These findings are similar to those obtained when sheep and goats were inoculated with closely related antigens. It was necessary to inoculate comparatively large amounts of antigen in these animals to stimulate antibody production.

Specific antisera obtained by these methods should prove useful for critical identification tests of blood meals of blood-sucking arthropods or for medico-legal purposes if results from 'group-specific' antisera would not be sufficiently accurate.

B. The Heterologous reaction

The experiments already described have shown that the heterologous antibodies can be absorbed from an antiserum without reducing the titre of the homologous antibody. The heterologous antigen-antibody reaction, however, appears far more complex than the homologous, since the reaction occurs with the serum of all mammals (p. 283). The experiment described on pp. 283–5 shows that con-

Table 4. *Precipitin reactions which occurred with an anti-human serum prepared in a chimpanzee*

Antigens (whole serum)	Dilution of antigens 1/10	1/100	1/500	1/1000
1. Man (*Homo sapiens*)	+ +	+ +	+ + +	+
2. Man (*H. sapiens*)	+ + +	+ +	+ +	+
3. Man (*H. sapiens*)	+ + +	+ + +	±	±
4. Chimpanzee (*Pan troglodytes*)	−	−	−	−
5. Chimpanzee (*P. troglodytes*)	−	−	−	−
6. Baboon (*Papio cynocephalus*)	−	−	−	−
7. Baboon (*P. cynocephalus*)	−	−	−	−
8. Olive Baboon (*P. doguera*)	−	−	−	−
9. Rhesus monkey (*Macaca mulatta*)	−	−	−	−
10. Rhesus monkey (*M. mulatta*)	−	−	−	−
11. Common monkey (*Cercopithecus* species)	−	−	−	−
12. Common monkey (*Cercopithecus* species)	−	−	−	−
13. Ox	−	−	−	−
14. Sheep	−	−	−	−
15. Goat	−	−	−	−
16. Horse	−	−	−	−
17. Pig	−	−	−	−
18. Dog	−	−	−	−
19. Cat	−	−	−	−
20. Fowl	−	−	−	−

+ + + to ±, intensity of ring precipitate. −, no reaction visible after 2 hr. or more. Precipitin reactions which occurred with an anti-human serum made in chimpanzee.

siderable overlapping occurs amongst the heterologous antigens, for it was possible to eliminate the heterologous reaction completely by the addition to the antisera of antigens from the domestic animals only (Table 3). Antisera which had been successfully absorbed by the methods described contained excess of the absorbing antigen, which was revealed by testing with unabsorbed antiserum. Some preliminary experiments indicate that there is no neutral zone in which neither antigen nor antibody are present in the supernatants of an antiserum which has been absorbed with varying amounts of the antigen. In fact, it was found that over a small range both antigen and antibody could be demonstrated in the same supernatants. This phenomenon may be explained by the presence of multiple antigenic components which are present in the serum used for absorption of the antisera, the latter being capable of combining with only some of the components and thus leaving some uncombined antigens in solution (see discussion).

Experiments with heterologous antigens

The anti-human serum used was the same as in previous tests and was non-specific. Preliminary titration tests were made with the sera of ox, sheep, horse, pig, dog and cat to determine the minimal amount of each of these sera which was required for the complete absorption of the corresponding antibody when these antigens were added singly. Thus it was found that the proportion of ox serum to anti-human antiserum which was required to absorb the anti-ox heterologous antibodies was 1 : 3000. Similarly, the proportions with other sera were sheep 1 : 60, horse 1 : 500, pig 1 : 250, dog 1 : 250 and cat 1 : 250.

A mixture of these heterologous sera (antigen mixture) was then made so that it contained the correct proportions of each of the antigens to absorb a given volume of antiserum, and was added to the antiserum and allowed to precipitate. Ring tests on the clear supernatant fluid showed that, as expected, absorption of the heterologous antibodies was complete (Table 5). Aliquots of the antiserum were

Table 5. *The titre of heterologous reactions, after absorbing anti-human serum with various proportions of an antigen mixture*

ıtiserum (ml.)	Antigen mixture* used for absorption	Volume (ml.)	Titres in thousands when absorbed antiserum was tested with sera of						
			Man	Ox	Sheep	Horse	Pig	Dog	Cat
1·0	Neat	0·32	512	0	0	0	0	0	0
1·0	1 : 2	0·32	512	0	0	0	0	0	0
1·0	1 : 3	0·32	512	0	0	0	0·01	0	0·1
1·0	1 : 4	0·32	512	0·1	0	0	0·01	0	0·1
1·0	1 : 6	0·32	512	1	0·1	0·01	0·01	2	2
1·0	1 : 8	0·32	512	2	2	1	2	4	8
1·0	1 : 10	0·32	512	2	2	4	64	32	32
1·0	Normal rabbit serum	0·32	512	256	512	128	512	512	128

* The antigen mixture was composed of sera mixed in such amounts that the final antigen/antibody io was ox 1/300, sheep 1/60, horse 1/500, pig 1/250, dog 1/250 and cat 1/500 when 0·32 ml. of the mixture s added to 1·0 ml. of the antiserum.

then absorbed with similar volumes of the antigen mixture which had been diluted 1 : 2, 1 : 3, 1 : 4, 1 : 6, 1 : 8 and 1 : 10 respectively, and the supernatant fluids were tested in order to determine the degree of absorption of each of the heterologous antibodies. A control sample was treated with an identical volume of normal rabbit serum. Even when the antigen mixture was diluted three times almost complete absorption of all the heterologous antibodies had occurred, although single antigens would have caused only incomplete antibody absorption at this dilution (Figs. 2 and 3). This indicates that these sera must contain common antigens which act on several heterologous antibodies at the same time.

A number of tests were made to establish whether a single heterologous antibody could be absorbed out of an antiserum without using the corresponding species antigen. Table 6 shows the results of absorbing the anti-human serum with the antigen mixture from which a single heterologous serum had been omitted. This antigen mixture was used neat and diluted 1 : 2, 1 : 4, 1 : 8 and 1 : 10. After absorption the resulting supernatants were tested by the ring test with the heterologous serum which had been omitted (Table 6). It was found that this procedure resulted in the complete absorption of the antibody concerned. As the antigen mixture was diluted, the degree of absorption became less, although even when the antigen mixture (less ox) was diluted by half or by a third complete absorption of the ox antibody occurred. With the dilutions of antigen mixture which were too weak to complete the absorption of the antibody corresponding to the serum which was omitted from the mixture, it was possible to complete the absorption of the antibody by the addition of very small quantities of the antigen

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previously omitted. In every case the total amount of antigen necessary for complete absorption was far less than that determined by the preliminary absorption tests with the single antigen. For example, the amount of ox antigen required to be added to the antigen mixture from which ox serum was previously omitted, and which was diluted 1 : 10 (Table 6, col. 5), was such that the proportion of ox serum to antiserum was less than 1 : 500, as compared with 1 : 300 required for the absorption of the anti-ox reaction when the antiserum was absorbed with ox serum alone.

Table 6. *Absorption of anti-human serum with antigen mixture**

Material used for absorption	Dilution of antigen mixtures: Neat (Titre of heterologous reaction)	1 : 2	1 : 4	1 : 8	1 : 10	Antigen used for test of supernatant fluids after absorption
Antigen mixture* less ox serum	0	0	8	32	128	Ox serum
Antigen mixture less sheep serum	<1	1	1	2	4	Sheep serum
Antigen mixture less pig serum	0	0·1	8	256	512	Pig serum
Antigen mixture less horse serum	0	N.T.	N.T.	N.T.	N.T.	Horse serum
Antigen mixture less dog serum	0	N.T.	N.T.	N.T.	N.T.	Dog serum
Antigen mixture less cat serum	0	N.T.	N.T.	N.T.	N.T.	Cat serum

Titres in thousands. N.T., not tested. 0, no reaction.

* The antigen mixture was composed of sera mixed in such amounts that the final antigen/antibody ratio was ox 1/300, sheep 1/60, horse 1/500, pig 1/250, dog 1/250 and cat 1/500 when 0·32 ml. of the mixture was added to 1·0 ml. of the antiserum.

It follows from these observations that the heterologous antigens contain varying proportions of common antigens which have an additive effect on the absorption of the heterologous antibodies when a mixture of heterologous sera are used together for the absorption of an antiserum.

DISCUSSION AND CONCLUSIONS

The induction of antibodies in rabbits by the injection of animal sera demonstrates that these sera must contain at least two distinct groups of antigenic components: the homologous and the heterologous group. That these main groups of antigenic components are distinct is clear from the experiments described on pp. 280–85, where absorption of the homologous antibody causes the complete loss of the heterologous antibodies, whereas the absorption of the heterologous antibodies does not influence the titre of the homologous antibody. Dr R. I. N. Greaves (personal communication) has also demonstrated differences between the homologous and the heterologous reactions by means of the anaphylactic reaction in guinea-pigs. He found that when plain muscle of guinea-pigs which had been passively sensitized with a non-specific antiserum was exposed to the action of the homologous antigen,

a reaction occurred, whereas no reaction took place when the muscle was exposed to heterologous antigens.

(1) *The homologous antigenic component* is specific for the species of the animal from which the serum used as the antigen is derived. This major specific component has associated with it minor components which are group-specific for antigens in the sera of species of closely related mammals.

(2) *The heterologous antigen* is a more complex group of components and is responsible for the production of antibodies which react with antigens present in most mammalian sera. This antigen must contain several components because it is possible to remove part only of its reacting antibodies by absorption of an anti-serum with the serum of another animal species.

A schematic representation of these antigenic complexes is suggested. The homologous specific and group-specific components could be represented by letters, while a combination of figures might represent the several components of the heterologous antigen. Thus, horse-serum antigens would contain the homologous specific antigen H and several group-specific antigens such as Z and A to represent the cross-reaction with zebra and ass sera. The heterologous group of antigens could be represented by the digits 1236. This notation is chosen at random, but nevertheless represents the pattern of the antigenic components as determined in this work.

Examples can be used to illustrate the pattern of antigen-antibody reactions which have been described. For this purpose the following antigenic formulae are suggested:

	Homologous antigen			
Antigen	Specific	Group-specific	Heterologous antigen	Formula
Horse serum	H	Z A	1236	(HZA) 1236
Zebra serum	Z	H A	1236	(ZHA) 1236
Ox serum	O	X Y	1247	(OXY) 1247
Pig serum	P	V W	2356	(PVW) 2356

If a rabbit is immunized with horse serum (HZA 1236) the antiserum produced will react with (i) horse serum because of the identical components both homologous and heterologous, (ii) with zebra or ass sera because of the group specific anti-Z and anti-A components, (iii) any mammalian serum which has in its heterologous antigenic formula the numbers 1, 2, 3 or 6.

The absorption of the homologous reaction, i.e. (HZA) 1236 + anti-(HZA) 1236 or of the group-specific reaction (ZHA) 1236 + anti-(HZA) 1236, etc., would inevitably cause the absorption of the heterologous components because of the identical numbers in both antigen and antibody.

The heterologous antibody (i.e. anti-1236) can be partially removed by any single serum which contains some of the antigens 1, 2, 3 or 6, and will be completely removed by a combination of two or more sera which together contain all the components 1, 2, 3 and 6. Thus, the absorption of anti-horse antisera with the heterologous sera of the ox can be represented as:

Anti-horse serum (non-specific)		Absorbed with ox antigen		Absorbed antiserum (non-specific)
anti-(HZA) 1236	+	(OXY) 1247	→	anti-(HZA) 36

Such an absorption yields an antiserum which is still capable of reacting with any heterologous sera which contain the components 3 or 6 in their antigenic make up. The antiserum can be further absorbed with another heterologous serum which contains these components, e.g. pig serum. This can be represented as:

$$\text{anti-(Hza) } 36 + \text{(Pvw) } 2356 \rightarrow \text{anti-(Hza)}$$

which results in a group-specific antiserum, reacting only with the homologous sera of the horse, donkey and zebra. This double absorption can of course be carried out in a single step by the simultaneous addition of ox and pig sera to the antiserum. This can be represented as:

Anti-horse serum		Absorbed with ox and pig antigen		Group-specific anti-horse
anti-(Hza) 1236	+	(Oxy) 1247 + (Pvw) 2356	→	anti-H (Hza)

When the supernatant is tested with ox or pig antiserum (see experiments, p. 288) a positive reaction is obtained because of the uncombined antigens (Oxy) 47 and (Pvw) 5.

This hypothesis would explain the results which have been obtained in this work. It is based on qualitative differences of the antigenic components as there is no evidence for a system based entirely on quantitative differences in the composition of a multiple antigen. In fact, quantitative differences occur among the antigenic components, and these determine the amounts of the various antigens required for the absorption of various antibodies.

The extent to which the anti-ox antibody was absorbed when the anti-human non-specific serum was absorbed with sheep antigen (p. 281, Fig. 2) compared with the extent to which the anti-sheep titre was reduced by absorption with ox antigen indicates such a quantitative difference in the various antigenic components.

In the cross-immunization experiments it was found that very large quantities of antigen had to be inoculated to elicit a relatively low titre antiserum. This can be explained by the similarity of the 'group-specific' and 'species-specific' antigens. Thus, when a goat (Gsx) is inoculated with sheep antigen (Sgx) it is natural that the goat will react only and with difficulty to the S component of sheep antigen since it has some relationship to the group-specific s component of its own antigenic composition.

As the relationships of group-specific antigens broadly follow the accepted zoological relationships of the animals from which they are derived the reaction has been used for the determination of the classification of animals. Such determinations should be based exclusively on the homologous antigen-antibody reactions.

SUMMARY

Specific precipitating antisera can be prepared in rabbits by the repeated inoculation of alum-precipitated serum.

High titre of sensitivity to the homologous serum is obtained only with a complete loss of specificity.

By absorption of the non-specific antiserum with heterologous sera the specificity can be restored.

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Two types of reactions can be observed in such non-specific antisera: (1) the homologous reaction which occurs with the sera of closely related animals, and (2) the heterologous reaction which is attributed to the presence of antigens which are widespread among mammalian sera.

Cross-immunization experiments have demonstrated the presence of specific antigenic material in the serum of indvidual species.

A schematic representation of the antigenic arrangement of mammalian sera is suggested.

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