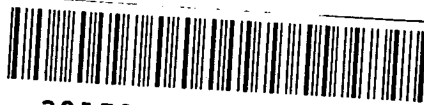
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BOVINE ERYTHROCYTE AGGLUTINABILITY

A thesis submitted by

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Summary

It has been shown that bovine erythrocytescan be divided into 2 classes of antiglobulin agglutinating activity; high agglutinators and low agglutinators, in a test described here using a rabbit anti-bovine red cell sensitiser and a bovine anti-rabbit globulin serum. This is a constant red cell character as has been shown using sera of other species in antiglobulin tests and has been found to be simply inherited. The frequency of each class of agglutinability varies in different breeds but no reason for one breed having a high frequency of one class of agglutinability has been found.

The reaction of antiglobulin agglutination has been investigated by looking at the activity of the IgG and IgM components of the sensitising sera used, both of which fractions are involved although one or other is often more important in specific sera.

The red cell membrane itself was studied using an electron microscope to visualise the receptor sites, After treatment of the red cell ghosts with ferritin labelled sensitising serum it was found that both agglutination classes of bovine erythrocyte had equal numbers of receptor sites and the position of these appeared to be similar. However, the outer, carbohydrate layer of the cell membrane was not studied in the techniques used and it is possible that the inagglutinable cell receptor sites are deeper with respect to this layer than are the agglutinable cell receptor sites.

After an antiglobulin test the inagglutinable cells were never found to approach each other closely whereas agglutinable cells were separated only by

60 nm. This was presumed to be due to some form of steric hindrance probably in the carbohydrate layer surrounding the cell.

The inagglutinable cells could be made to agglutinate by the building up of an antiglobulin-globulin lattice onto the red cell. After three of these treatments the cells agglutinated to the same titre as the agglutinable cells. The cells could also be agglutinated by treatment with proteolytic enzymes but not with neuraminidase, which specifically releases sialic acid, although the red cell agglutinability was found to correlate exactly with the sialic acid content of the membrane protein. Red cells which were highly agglutinable had a low value of sialic acid and inagglutinable cells had a high value of sialic acid. It was shown that neuraminidase treatment reduced all the sialic acid from the ghost and yet the inagglutinable cells remained so. Therefore the mucopeptide chain must also be important in preventing the cells from agglutinating.

Finally a survey of some other bovine red cell characters was undertaken using various European breeds and also some primitive Zambian cattle. The only character studied showing association with the agglutinability was the blood group locus FV in European cattle. It was found that all VV animals were high agglutinators whereas FV animals were of both classes of agglutinability. ~~FF~~ FV negative animals were not found in European cattle and only rarely in the Zambian cattle but these were again of both classes of agglutinability.

Chapter 1 Review of the agglutinability of bovine red cells

Agglutination of red blood cells has been studied for many years, mainly in humans. The reaction is known and used very widely, but it is still incompletely understood. Most of the work on agglutination has been indirect in that workers have been studying cell charges and the change in charge when cells are in media of different concentration and pH. The information gained has some bearing on the study of agglutination. Agglutination of human erythrocytes treated with antibodies has been found to depend partly on the electrophoretic surfaces of shear (Pollock *et al*. 1965), and therefore the many electrophoretic studies on the erythrocyte which give important information on the peripheral zone are relevant to a study of the agglutination reaction.

The electrophoretic mobility of a cell is due to nature of the cell membrane, not to the shape or size of the cell (Abramson, Moyer and Gorin, 1942). Studies have shown that the erythrocyte acts as a large polymer with a negative surface charge (Seaman and Heard, 1960*b*; Cook *et al*. 1961). Since sialic acid and phosphate are components of the red cell present in sufficient amount to cause a surface charge, the charge, presumably, must be due predominantly either to phosphate or to carboxyl groups of the sialic acid molecules present at the surface of the cell. Removal of the carboxyl groups considerably reduces the electrophoretic mobility of the cell (Cook *et al*. 1961). This effect can also be produced by the addition of divalent cations, *e.g.* Cu^{2+}, to the medium (Bangham *et al*. 1958). Addition of a divalent cation increases the ionic strength of the medium and reduces the surface charges on the cell by combining with the charge-determining groups at the cell surface.

Many metallic multivalent cations, <u>e.g.</u> Cr^{3+}, Fe^{3+}, Be^{2+}, Al^{3+}, agglutinate red cells (Jandl and Simmons, 1957), and such agglutinations are morphologically indistinguishable from antibody - red cell agglutination. If polycarboxylic acids are present they act as metal binding agents and inhibit metallic agglutination (Jandl and Simmons, 1957). The agglutination of red cells may depend on the capacity of an agent to lower the surface charge so that cells may approach each other and agglutinate. This would suggest that the red cell agglutination depends on the activity of the free carboxyl group at the red cell surface. However under certain conditions red cells can agglutinate without their surface charge being reduced. For example, if red cells are suspended in a polymer, such as dextran or polyvinyl-pyrollidone, these ionically neutral polymers dilute the ionic double layer (Derjaguin 1954) and agglutination occurs. Antibodies can reduce the electrophoretic mobility of the red cell and may then provide chemical linkages for agglutination (Sachtleben, 1965). There are, however, some exceptional antibody systems, <u>e.g.</u> Rh, M and N, which at higher serum dilutions do not change the electrophoretic mobility although agglutination occurs. Therefore, agglutination cannot be due simply to a decrease in surface charge (Sachtleben, 1965).

The true outer red cell surface is made up of the absorbed serum protein in which it is suspended and the sialic acid residues which contribute the bulk of the cell surface charge. These contribute to the ionic double layer, the outer layer of which is a more diffuse, mobile, positively charged layer, and the inner layer is stable and negatively charged. By electron microscopy the red cell surface has been shown to be composed of an outer layer of plaques on a fibrous network (Hillier and Hoffman, 1953). The outer layer,

as revealed by the scanning electron microscope, shows a smooth surface on normal cells and on abnormal cells surface pits and irregularities similar to those produced in red cell haemolysis (Salsbury and Clarke, 1967).

Agglutination of cells is in general an irreversible process resulting in strong adhesions. It has been suggested that the adhesion involved in red cell agglutination is a result of bridging between them. Easty and Mercer (1962) examined species agglutinability of erythrocytes by an anti-erythrocyte γ globulin and they found gaps of 25 nm. present between agglutinable cells. There was some staining in this gap, possibly indicative of membrane material. Curtis (1967) quoted a gap of 10-20 nm. between cells, but this may have been a fluid-filled gap or even an artefact produced in the preparation of the cells. Palmer and Schmitt (1941) found that repulsive and attractive forces balance when separated by 8.5 nm. and this supports the hypothesis of a gap of approximately 10 nm. between the cell surfaces.

Normally the cells carry negatively charged electrostatic forces which repel other similarly charged cells, but when these forces are balanced by London - Van der Waals forces or by suspending the cells in ionic solutions, agglutination can take place. For identical surfaces the point of balance of these forces will be the stable point of adhesion. According to the Verwey-Overbeek theory there will be a separation of 10-20 nm. between the adhering surfaces (Verwey and Overbeek, 1948; Derjaguin, 1954). Plasma membranes may be separated by 20 nm. and still maintain uniform separation over large areas of plicated contact. The activity of the erythrocyte is directly related to the zeta potential which, in turn, is proportional to the surface charge density and the thickness of the double layer (Pollock *et al*. 1965).

The ability of red cells to agglutinate varies interspecifically and according to the suspension medium. Human red cells are readily agglutinable and are even more readily agglutinable in a medium of high molecular weight, e.g. poly-vinyl-pyrollidone, than in saline. Sheep red blood cells (being poor isoagglutinators) are normally less readily agglutinated than human cells. They can be agglutinated with horse serum containing antibodies produced against sheep red cells by immunisation, but only poorly with rabbit anti-sheep serum (Spooner, private communication). As with human red cells, a higher titre of agglutination is found with sheep red cells when they are suspended in dextran or glucose media. Bovine erythrocytes are recognised as poor agglutinators (Little, 1929); only rarely is iso-agglutination found and then only at a very low titre. These cells resist agglutination by infectious mononucleosis sera (Bailey and Raffel, 1935), but rabbit anti-bovine sera produce agglutination, the extent of the reaction varying with the particular animal used to produce the serum (Spooner et al. 1970).

The ability of red cells to agglutinate can be enhanced by an indirect or antiglobulin test. This test was described originally by Moreschi in 1908, but was rediscovered by Coombs, Mourant and Race (1945) who used it to demonstrate the sensitisation of human red cells by incomplete Rhesus antibodies. Gleeson-White et al. (1950) found a wide variation in the ability of bovine red cells to agglutinate when a dilution of rabbit anti-human globulin serum was added to the cells following their treatment with dilutions of infectious mononucleosis serum. Three broad classes of high, moderate and inagglutinable bovine red cells were found. This was the first report of intraspecific differential agglutinability.

Coombs _et al_. (1951) suggested that the variable agglutinability might be due to variation in the structure of the bovine red cell wall and that the antigen receptors might be at different distances from the effective periphery of the cell, so that although all the cells would be sensitised, the combining molecules could possibly be too short to extend beyond the periphery of the cells and so agglutination of some cells would be impossible. The fact that the inagglutinable cells can be made to agglutinate by building up a lattice of antiglobulin and globulin alternately on the sensitised cells lends support to this idea (Coombs _et al_. 1951).

Uhlenbruck _et al_. (1967) proposed that the inagglutinable cells were subject to greater steric hindrance than were agglutinable cells, so that the effective agglutinating power of the antibody was reduced. Seaman and Uhlenbruck (1963) had previously proposed that the molecular structure of the outer mucoid layer of the red cell consisted of "branched" chains ending in sialic acid residues; treatment with proteolytic enzymes would then render all cells agglutinable by removal of this mucoid layer.

Measurements of the amount of sialic acid released by pronase and neuraminidase showed a definite difference between the bovine cell types, more sialic acid being released from the inagglutinable cells (Uhlenbruck _et al_. 1967). Human erythrocytes, which are readily agglutinable, release less sialic acid than any bovine cells after pronase treatment (Uhlenbruck _et al_. 1967). There is a discrepancy here with the work published by Eylar _et al_. (1962), who, using both mild acid hydrolysis and neuraminidase treatment, showed that calf red cells had less sialic acid than human red cells.

No difference has been shown between the electrophoretic mobilities of the cattle red cells of different agglutinability either before or after enzyme treatment (Uhlenbruck _et al_. 1967), as would be expected if the inagglutinable cells contained more sialic acid. This finding suggests that all cells have the same number of charged terminal sialic acid molecules. This could still be true and yet the inagglutinable cells release more sialic acid if only the outermost of the charged molecules contributed to the electrophoretic charge. The precise distribution of the sialic acid molecules is unknown but under normal conditions (in standard saline) only the region within about 0·8 nm. of the surface of shear is assessed in electrophoretic tests (Uhlenbruck _et al_. 1967). If the more deeply situated mucoid structures were removed they would not affect the electrophoretic charge. Although they would contribute to the amount of sialic acid released. The amount of sialic acid removed by neuraminidase treatment is reflected by the corresponding decrease in electrophoretic mobility of the red cell, except in the horse; in this animal some of the sialic acid is not removed by neuraminidase and is probably lipid bound and so more resistant to enzyme attack (Eylar _et al_. 1962).

The action of neuraminidase on red cells is to release the sialic acid by breaking the α-glycosidic links. Although release of these charged groups reduces the electrophoretic mobility, the inagglutinable cells remain so even after treatment. Treatment with proteolytic enzyme ruptures the peptide links in the cell structure and so the entire sialo-mucopeptide chain is removed from the cell. This rupture creates ionic groups, and unmasks others so that the electrophoretic charge of the cell is not reduced as much as after treatment with neuraminidase (Seaman and Uhlenbruck, 1963). It seems that all the

steric hindrance, in the form of sialo-mucopeptide chains, must first be removed before the inagglutinable bovine cells become capable of agglutinating. These chains are obviously important in maintaining the charge and stability of the cell.

All animal red cells possess sialic acid, but there is a wide variation in type among species. In human cells the neuraminic acid is nearly 100% N-acetyl acid, in cattle it is 40% N-acetyl and 60% N-glycolyl, and in the horse it is almost 100% N-glycolyl (Eylar et al. 1962). Similar values are obtained whether the samples are tested by mild acid hydrolysis or by neuraminidase action.

No correlation has yet been found between the ability of bovine cells to agglutinate and the presence of any blood group antigen (Uhlenbruck and Schmid, 1963). Recently Hatheway et al. (1969) suggested that the blood group factor F, which is destroyed by neuraminidase treatment, is dependent on the cell having a large amount of sialic acid. The V factor, at the same locus as F, is not destroyed by neuraminidase, and so is independent of sialic acid. Both F and V can be released from stroma by papain treatment (Hatheway et al. 1969). It might therefore be expected that cells with V factor would be high agglutinators and those with F factor would be low agglutinators. The correlation between blood-group antigens and agglutinability will be investigated further in this thesis.

The investigations reported in this thesis developed from the following results. The values of the sialic acid content of the protein of the bovine red cell membrane, measured on a number of animals, all lay within 10% of

each other except for one animal, where it was much lower. Repeated tests confirmed the accuracy of this low result, but unfortunately fresh blood samples from the animal were not available. It was first thought that the difference was seasonal, but other animals tested at this time did not show a lowered value of their sialic acid content. Larger numbers of animals from experimental herds were tested and a few animals were found to have this low value. These animals were not ill, or being immunised, nor restricted to one sex or age group; thus the difference appeared to be chemical rather than environmental or physical.

The early work of Gleeson-White et al. (1950) showed that bovine red cells exhibited a variation in agglutinability which in a later paper, (Coombs et al. 1951) they suggested could be due to a variation in the sialic acid layer of the red cell membrane. The bovine red cells mentioned above as showing a variation in their sialic acid values were then tested in an antiglobulin test similar to that used by Coombs et al. (1951). The technique was altered somewhat and the cells were found to fall into two classes of agglutinability (Spooner, Cowpertwait and Maddy, 1970). These two classes showed an exact correlation with the two classes of membrane sialo-protein (Maddy and Spooner, 1970). It was therefore possible to distinguish the two classes of bovine red cell both immunologically and biochemically.

Only cattle have so far shown this distinct intraspecific variation in both agglutinability and membrane sialo-protein. This makes them an interesting species for further studies on the molecular structure and activity of the red cell membrane. In this thesis an attempt has been made to investigate the various aspects of the agglutinability of bovine red cells and to explain as far as possible the reason for this difference in ability to agglutinate.

Chapter 2 The correlation between the antiglobulin agglutinability and the membrane sialo-protein of the bovine red cell

As has been shown in Chapter 1, Uhlenbruck et al. (1967) and other workers previously proposed that cattle showed a continuous range in the agglutinability of their red cells. Uhlenbruck et al. arbitrarily chose animals with high- medium- and low-agglutinable red cells, as shown by their antiglobulin tests, but all the animals were much less sensitive to agglutination by the infectious mononucleosis serum used in their investigations than were sheep cells. In this thesis, the agglutinability of bovine red cells is to be studied by the antiglobulin test reported by Spooner, Cowpertwait and Maddy (1970) and in the work reported in this chapter this antiglobulin test has been used to show that cattle can be divided into two distinct classes whose red cells are of high or low agglutinability.

A. The antiglobulin agglutinability

Materials and Methods

Haemolytic test The whole blood was washed three times with 0.9% saline until the supernatant was clear. A 0.5% suspension of the packed red cells was made up in saline using an automatic diluter. The antiserum to be used was serially diluted in saline in doubling dilutions from 1:4 into round-bottomed microtiter plates. The serial dilutions were made with loops calibrated to hold 0.025 ml. The cell suspensions were then added using a pipette calibrated to deliver 0.025 ml. drops. The complement used was normal rabbit serum diluted 1:3 with saline, 1 drop being added to each well. Two controls were included; the saline control in which 1 drop of the cell suspension was mixed with 2 drops of saline

to detect any unstable cells, lysing in the saline; the complement control in which 1 drop of saline was used instead of the antiserum to detect any haemolytic antibodies present in the complement. The microtiter plates were then covered with sellotape and put on a rotary shaker to shake continuously for 5 hr. The temperature was 18-25°C. The plates were allowed two hours to settle and were then read, the amount of free haemoglobin and the cells remaining in the wells being estimated by eye. Readings were recorded from 0 (Negative) to 5, (Complete lysis).

Agglutination test

A two drop test in which 1 drop of a 2% suspension of three times washed packed red cells in saline was added to 1 drop of a serially diluted antiserum in a conical-bottomed microtiter plate. A saline control was always included. The plates were shaken to mix the cells and serum and were then left to stand overnight at room temperature. The test was read the next day from 0 (Negative) to 4 (Complete agglutination).

Sensitising dose of rabbit antiserum

The concentration of each sensitising serum used to sensitise the red cells in antiglobulin tests was calculated from the haemolytic and agglutination titres of cells tested against that serum. The concentration had to be beyond the titre of the highest direct agglutinated cell so that in the antiglobulin test agglutination was not due to the sensitising serum, and within the haemolytic titre ensuring that all the cells would be sensitised. Thus from Table I the sensitising dose for R4 (18/2/69) was 1:5,000.

Haemolytic test

Red Cell	4	8	16	32	64	128	256	512	1T	2T	4T	8T	16T	32T	64T	128T	Control
	Reciprocal dilutions of R4 (18/2/69)																
HZ127A	5	5	5	5	5	5	5	5	5	5	5	4	4	-	-	-	-
HZ125B	5	5	5	5	5	5	5	5	5	5	5	3	3	-	-	-	-
HZ135A	5	5	5	5	5	5	5	5	5	5	4	3	1	-	-	-	-
HZ141B	5	5	5	5	5	5	5	5	5	5	5	4	3	-	-	-	-
U67A	5	5	5	5	5	5	5	5	5	5	5	5	4	-	-	-	-
U74A	5	5	5	5	5	5	5	5	5	5	5	4	4	-	-	-	-
U85A	5	5	5	5	5	5	5	5	5	5	5	2	-	-	-	-	-
U85B	5	5	5	5	5	5	5	5	5	5	5	5	2	-	-	-	-
U93B	5	5	5	5	5	5	5	5	5	5	5	3	-	-	-	-	-
MZ311A	5	5	5	5	5	5	5	5	5	5	5	2	-	-	-	-	-
FS101A	5	5	5	5	5	5	5	5	5	5	5	3	-	-	-	-	-
TAG967	5	5	5	5	5	5	5	5	5	5	5	4	-	-	-	-	-

Agglutination test

Red Cell	4	8	16	32	64	128	256	512	1T	2T	4T	8T	16T	32T	64T	128T	Control
	Reciprocal dilutions of R4 (18/2/69)																
HZ127A	4	4	4	3	1	-	-	-	-	-	-	-	-	-	-	-	-
HZ125B	4	4	4	4	4	4	4	4	1	-	-	-	-	-	-	-	-
HZ135A	4	4	4	4	4	3	1	-	-	-	-	-	-	-	-	-	-
HZ141B	4	4	4	4	3	-	-	-	-	-	-	-	-	-	-	-	-
U67A	4	4	4	4	4	4	4	4	4	1	-	-	-	-	-	-	-
U74A	4	4	4	4	3	2	-	-	-	-	-	-	-	-	-	-	-
U85A	4	4	4	4	4	4	4	4	4	3	1	-	-	-	-	-	-
U85B	4	4	4	4	4	4	4	4	3	2	-	-	-	-	-	-	-
U93B	4	4	4	4	4	3	1	-	-	-	-	-	-	-	-	-	-
MZ311A	4	4	4	4	3	2	-	-	-	-	-	-	-	-	-	-	-
FS101A	4	4	4	4	4	4	4	4	4	3	1	-	-	-	-	-	-
TAG967	4	4	4	4	2	1	-	-	-	-	-	-	-	-	-	-	-

Table I Typical protocols of haemolytic and agglutination reactions using a rabbit anti-bovine red cell serum R4 (18/2/69)

Results

Whereas Gleeson-White *et al.* (1950) used dilutions of a sensitising serum and a fixed dilution of antiglobulin serum, in the test used here the bovine red cells are sensitised with a fixed concentration of serum and these sensitised cells are added to a titration of antiglobulin serum. (Fig. 1).
Method 1 Red cell + dilutions of sensitiser + fixed concentration of antiglobulin (Gleeson-White *et al.* 1950).
Method 2 Red cell + fixed conc. of sensitiser + titration of antiglobulin. (Spooner *et al.* 1970).

The bovine red cells freshly collected into anticoagulant (acid citrate dextrose), were washed with 0.9% saline and centrifuged at 2000g for 5 min. three times. Then a 2 ml. sample of a 2% red cell suspension was made up from packed cells using an automatic diluter. An equal volume of a fixed concentration of rabbit anti-bovine red cell serum R4 (19/5/66) (see Appendix B) was added and the cells sensitised with this for 1 hr. at room temperature. The sensitised cells were washed in 3 ml. 0.9% saline and centrifuged at 2000g for 5 min. four times and then made up to a final concentration of 2% in saline. One drop of each of these cell suspensions was added to one drop of each dilution of a titration of bovine anti-rabbit globulin K21 (17/5/67) in saline in conical-bottomed microtiter plates. After covering with sellotape the plates were shaken to resuspend the red cells and were then left overnight at room temperature for the cells to settle. The test was read by patterns the following morning. A control of one drop of saline and one drop of sensitised cells was always included to show that agglutination was not

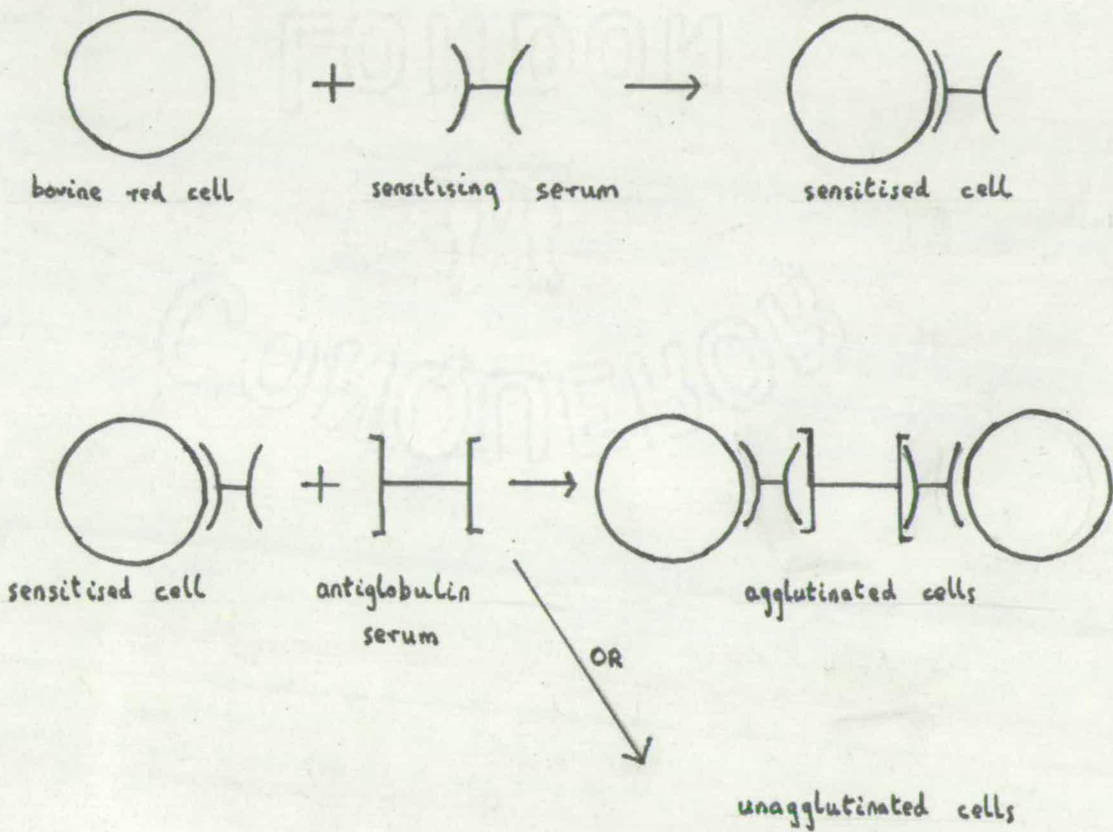
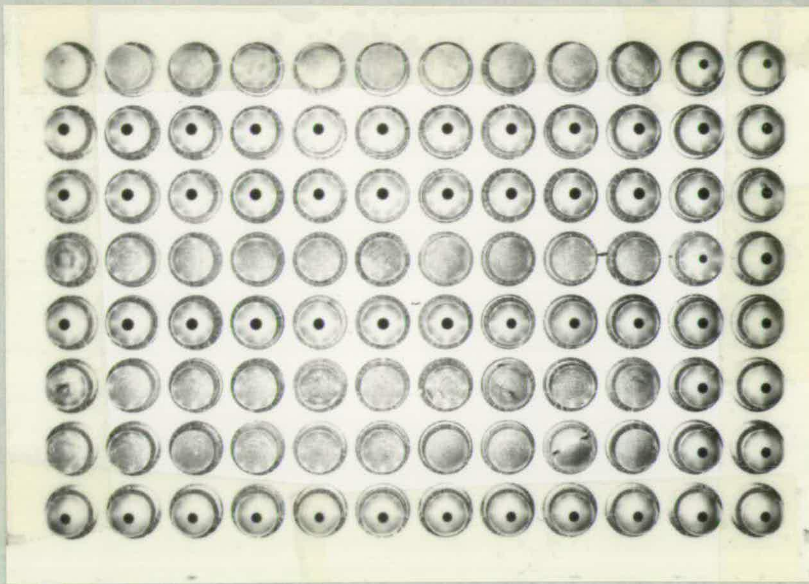


Figure 1. The antiglobulin test

due to the sensitised cell alone. The agglutinability was graded by eye from 0 to 4. (Figs. 2 and 3). When bovine erythrocytes were agglutinated directly by rabbit anti-bovine red blood cell serum a complete gradation of agglutinability e.g. with R4 (see Table I) was produced. (The titres were much lower than the haemolytic titres, but all cells could be agglutinated to some extent). When, however, these same bovine cells were tested in the antiglobulin test described above, using the same rabbit anti-bovine red cell sera as sensitisers, and then added to an appropriate bovine anti-rabbit globulin, K21, there was no longer a complete gradation from low to high agglutinability. Some cells were completely agglutinated at an antiglobulin dilution of 1 in 2000, while others were inagglutinable at an antiglobulin dilution of 1 in 4 (Table II). The tests were repeated using four rabbit antisera to bovine red cells and the same result, two classes of cell, was found; an intermediate group of cells of moderate agglutinability such as was found by previous workers, was not detected.

Discussion

It was found, generally, that cells of high direct-agglutination titre tended to give a high antiglobulin titre and *vice versa*, although one animal, HZ133B, did have a high direct-agglutination titre and a low antiglobulin titre. Only one animal, HZ127A, occasionally showed a partial agglutination reaction, a reading of 2 to an antiglobulin dilution of 1 in 256 but at no antiglobulin dilution with any sensitising serum has the reaction been strong (see Table III). This animal had not been immunised, been visibly ill, nor been treated differently from the other experimental animals.



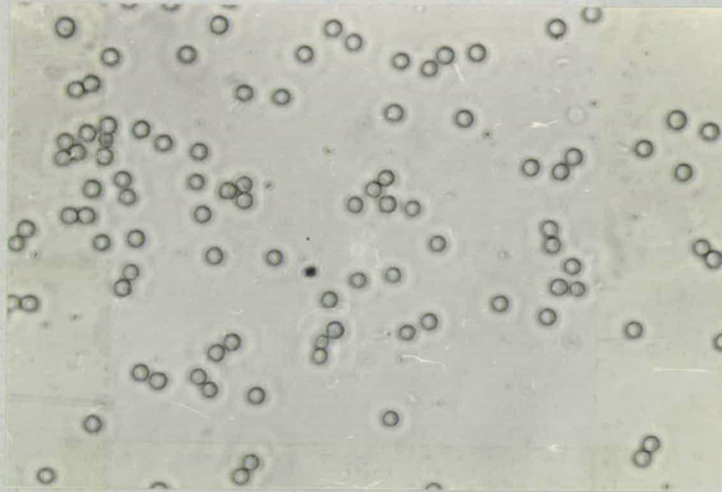
(a)

Reciprocal dilutions of K21 (17/5/67)

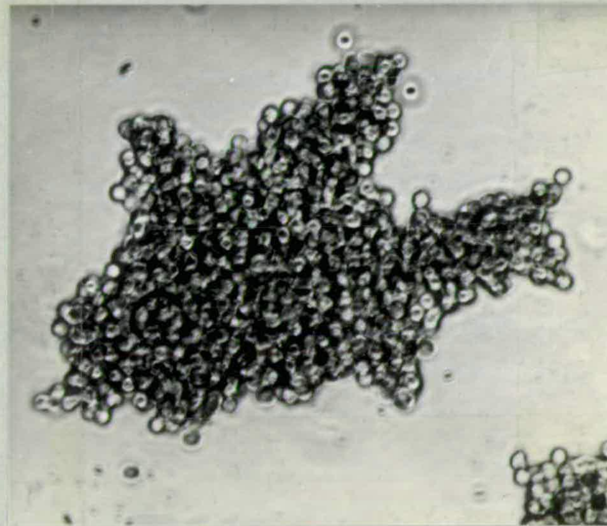
cells sensitised with R4(19/5/66) 1:5000		10	20	40	80	160	320	640	1T	2T	5T	10T	C
	FS101A	4	4	4	4	4	4	4	4	4	3	-	-
	FS106B	-	-	-	-	-	-	-	-	-	-	-	-
	HZ141B	-	-	-	-	-	-	-	-	-	-	-	-
	HZ167B	4	4	4	4	4	4	4	4	4	4	2	-
	MZ308A	-	-	-	-	-	-	-	-	-	-	-	-
	U 85 A	4	4	4	4	4	4	4	4	4	4	-	-
	U 85 B	4	4	4	4	4	4	4	4	4	4	-	-
	U 93 B	-	-	-	-	-	-	-	-	-	-	-	-

(b)

Fig. 2 <u>Antiglobulin test in microtiter plate (a) and the results read from the plate (b)</u>



(a)



(b)

Fig. 3 Inagglutinable red cells (a) and agglutinable red cells (b)

Reciprocal dilutions of K21 (17/5/67)

Red Cell	4	8	16	32	64	128	256	512	1T	2T	4T	8T	16T	32T	64T	128T	Control
HZ127A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HZ125B	4	4	4	4	4	4	4	4	4	4	4	-	-	-	-	-	-
HZ135A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HZ141B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
U67A	4	4	4	4	4	4	4	4	4	4	4	2	-	-	-	-	-
U74A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
U85A	4	4	4	4	4	4	4	4	4	4	4	4	2	-	-	-	-
U85B	4	4	4	4	4	4	4	4	4	4	4	4	-	-	-	-	-
U93B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MZ311A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FS101A	4	4	4	4	4	4	4	4	4	4	4	4	2	-	-	-	-
TAG967	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table II <u>Typical protocol of an antiglobulin test using as sensitiser a rabbit anti-bovine red cell serum R4 (18/2/69) at a dilution of 1:5,000 and a titration of bovine anti-rabbit globulin serum K21 (17/5/67)</u>

Sensitising serum	Cells	4	8	16	32	64	128	256	512	1T	2T	5T	Control
		Reciprocal dilutions of K21 (17/5/67)											
sensitising serum R4 (19/5/66) 1:5,000	U93B	-	-	-	-	-	-	-	-	-	-	-	-
	FS101A	4	4	4	4	4	4	4	4	4	4	4	-
	HZ127A	2	2	2	2	2	2	2	-	-	-	-	-
sensitising serum R2 (30/12/65) 1:2,000	U93B	-	-	-	-	-	-	-	-	-	-	-	-
	FS101A	4	4	4	4	4	4	4	4	4	4	2	-
	HZ127A	2	2	2	-	-	-	-	-	-	-	-	-
sensitising serum R6 (24/5/66) 1:2,000	U93B	-	-	-	-	-	-	-	-	-	-	-	-
	FS101A	4	4	4	4	4	4	4	4	4	4	2	-
	HZ127A	-	-	-	-	-	-	-	-	-	-	-	-

Table III <u>HZ127A in antiglobulin test using various sensitising sera</u>

B. The sialo-protein content of the bovine red cell membrane

Maddy and Spooner (1970) showed that, in the cattle they studied, there were two levels of sialic acid in the red cell membrane. These were shown to correlate exactly with the antiglobulin agglutinability of the red cells as defined in the test described in Section A. An attempt has now been made to repeat and extend this study to show the relationship between the agglutination of cattle red cells and their membrane sialo-protein content.

Methods

Preparation of ghosts (by modification of the method of Dodge et al. 1963). Freshly collected whole blood was centrifuged and the serum and white cells removed from the packed red cells. The red cells were washed x 4 with isotonic saline until the supernatant was clear. They were then haemolysed in six volumes of 0.005 M-phosphate buffer pH 6.0. They were spun at 18000g for 15 mins. and the ghosts obtained were washed with this buffer until the supernatant was free of haemoglobin. The ghosts were then pink-red in colour and the remaining haemoglobin was removed by further washing in 0.01 M-phosphate buffer pH 8.0 and then 0.005 M-pH 8.0. The final pad of ghosts was white. It was frozen overnight, then thawed and washed three times with distilled water.

Buffers were made up fresh each time from stock solutions.

stock solutions 0.1 M - Na_2HPO_4

0.1 M - NaH_2PO_4 $2H_2O$

Butanol extraction of protein from ghosts

Ten ml. of freshly prepared ghosts (approximately 100 mg. dry weight) in distilled water were cooled to 0°C in ice water and 7.5 ml. of n-butanol also cooled to 0°C was added, the mixture was shaken vigorously and then allowed to stand at 0°C in ice for 20 min. followed by centrifugation for 5 min. at 18,000g at 0°C. The sample separated out into an upper butanol phase containing the membrane lipid and a lower aqueous phase containing the protein. There was a thin interfacial layer of insoluble protein, but provided the ghost suspension was salt-free then only about 5% of the membrane protein was lost in this layer. The lower aqueous phase containing 95% of the protein was transferred with an ice cold hypodermic syringe and dialysed for 24 hr. against iced water. The dialysis removed any remaining traces of butanol.

The whole extraction process had to be carried out at 0-4°C until all the butanol was removed, as the solubility of butanol decreases with temperature.

Estimation of sialic acid (Aminoff method)

Extracted ghosts, dialysed against water free of any traces of butanol were diluted 5 times with distilled water. Five ml. of the diluted sample was then hydrolysed with 0.5 ml. N-H_2SO_4 at 80°C for 30 min. Four aliquots

of 0.5 ml. of each hydrolysate were treated with 0.25 ml. periodate solution at 37°C for 30 min. The excess periodate was reduced with 0.2 ml. of sodium arsenite. This liberated iodine, showing a bright orange-yellow colouration, which disappeared after about two minutes. Then 2 ml. of 0.1 M-thiobarbituric acid at pH 9.0 was added and the sample heated at 100°C for 7.5 min. A pink colouration was noticed which disappeared as the sample was cooled on ice. Five ml. of acid butanol was added to the sample which was then shaken and spun for 10 min. The butanol extracted the sialic acid, how again pink in colour, and it separated into the top layer over a water phase. The butanol layer containing the sialic acid was separated off and its intensity of colour compared with a control at 549 nm. in a Unicam SP.600 spectrophotometer. 1 $cm.^2$ silica cuvettes were used. The control was distilled water, in place of the protein sample, having had the same treatment as the other samples. The readings were taken within one hour as the pink colour was unstable over larger periods of time. The average of the readings of the four aliquots of each sample was taken as the true value.

Solutions required to estimate sialic acid:-

1. Periodate solution - 25 mM-periodic acid in 0.125 N-H_2SO_4
2. Sodium arsenite - 2% sodium arsenite in 0.5 N-HCl
3. Thiobarbituric acid-0.1M solution of 2-Thiobarbituric acid in water adjusted to pH 9.0 with 2M-NaOH
4. Acid butanol - Butan-1-ol containing 5% (v/v) of 12 N-NCl

All solutions were freshly made up for each experiment.

Determination of Protein (modified Lowry method)

A 1 ml. sample of n-butanol extracted, water-dialysed ghosts was diluted twenty times with sodium deoxycholate solution. To four 0.5 ml. aliquots of this diluted protein 2.5 ml. of copper alkali solution was added. This was shaken and left standing at room temperature for 10 min. Then 0.5 ml. of diluted Folin's reagent was added and mixed with the solution which turned from colourless to yellow, then green and finally blue. The solution was left at room temperature for 1 hr. before the colour intensity was compared with a control at 700 nm. in a spectrophotometer. The control was water, treated in exactly the same way as a protein sample. The average of four readings was taken.

Solutions required for the determination of protein:-

1. Deoxycholate solution - 1 vol. 8% sodium deoxycholate
   3 vols. 0.3 N.NaOH
2. Copper alkali solution - 100 ml. 0.2N-NaOH
   100 ml. 4% (w/v) Na_2CO_3
   2 ml. 2% (w/v) NaK tartrate
   2 ml. 1% $CuSO_4.5H_2O$
3. Folin's reagent diluted x 3 with distilled water.

Results

Ghosts were prepared from the whole red cells and after the protein had been extracted it was analysed quantitatively for sialic acid and protein, and the sialo-protein ratios were calculated. These assays of the sialic acid content of the two types of bovine erythrocyte membrane protein divided

the cells into the same two classes as found previously (Maddy and Spooner 1970), and these classes were identical with the two classes found using the anti-globulin test. Each animal was tested on at least two different occasions and gave identical values (within ± 0.003). Class I animals had a high content of sialic acid, 0.134 (± 0.0078) µmoles/mg. protein, and were low agglutinators; class II animals had a lower sialic acid value, 0.098 (± 0.005) µmoles/mg. protein, and were all high agglutinators (Table IV).

Discussion

The results show clearly that two classes of bovine red cell can be recognised from their ability to agglutinate and from their membrane protein. Maddy (1970) has also found that the pH at which the protein precipitates is correlated with the sialic acid content. He found that at low ionic strength, 0.05 M-citrate-phosphate buffer at pH 3.0 - 4.0, all bovine membrane proteins tested were indistinguishable (Fig. 4) from type I protein. As the ionic strength was increased the type I protein precipitated as two fractions at pH 2.8 and 4.5. As the ionic strength was increased further, the pH 2.8 fraction became progressively more soluble (Fig. 5). In the case of type II protein, as the ionic strength was increased, this protein first separated into two fractions precipitating at pH 3.0 - 3.2 and pH 4.5, and then subsequently the pH 3.0 fraction redissolved but the other was unaffected (Fig. 6). The type II fraction which precipitated at pH 4.5 was dense and of high refractivity, whereas the type I fraction which precipitated at pH 4.5 was fine and of low refractivity.

CLASS I

Animal	Agglutinability	Sialic acid (μmoles/mg. protein)	Mean value (with standard deviation)
U71A	Low	0.125 (2)	
U74A	"	0.134 (4)	
U93B	"	0.128 (4)	
U96A	"	0.130 (2)	
U99A	"	0.134 (2)	
U99B	"	0.134 (2)	
MZ111B	"	0.134 (4)	
MZ245A	"	0.135 (2)	
MZ308A	"	0.134 (2)	0.134
MZ308B	"	0.139 (2)	(± 0.0078)
HZ127A	"	0.110 (2)	
HZ133B	"	0.130 (2)	
HZ135A	"	0.143 (4)	
HZ141B	"	0.141 (4)	
HZ145A	"	0.139 (2)	
HZ155B	"	0.123 (2)	
FS98A	"	0.141 (4)	
FS106A	"	0.135 (2)	
FS108A	"	0.150 (2)	
FS108B	"	0.134 (2)	
FS110A	"	0.143 (2)	
TAG967	"	0.134 (4)	
TZ4D	"	0.134 (2)	
17121	"	0.139	

Table IV <u>Correlation of sialic acid content of bovine erythrocyte ghost protein with agglutinability</u>

Range of values of sialic acid for any one animal was ± 0.003 μM sialic acid/mg. protein.

Figures in brackets indicate the number of assays performed.

Using Arnson's tables the difference between the two groups is statistically significant at the 1% level.

CLASS II

Animal	Agglutinability	Sialic acid (µmoles/mg. protein)	Mean value (with standard deviation)
U85A	High	0.098 (4)	
U85B	"	0.098 (4)	
FS101A	"	0.098 (4)	0.098 (± 0.005)
HZ90B	"	0.090 (s)	
HZ123B	"	0.090 (2)	
HZ125B	"	0.105 (2)	
HZ167B	"	0.098 (4)	
MZ324A	"	0.105 (2)	
MZ324B	"	0.098 (2)	

Table IV <u>Correlation of sialic acid content of bovine erythrocyte ghost protein with agglutinability</u>

Range of values of sialic acid for any one animal was ± 0.003 µM sialic acid/mg. protein.

Figures in brackets indicate the number of assays performed.

Using Arnson's tables the difference between the two groups is statistically significant at the 1% level.

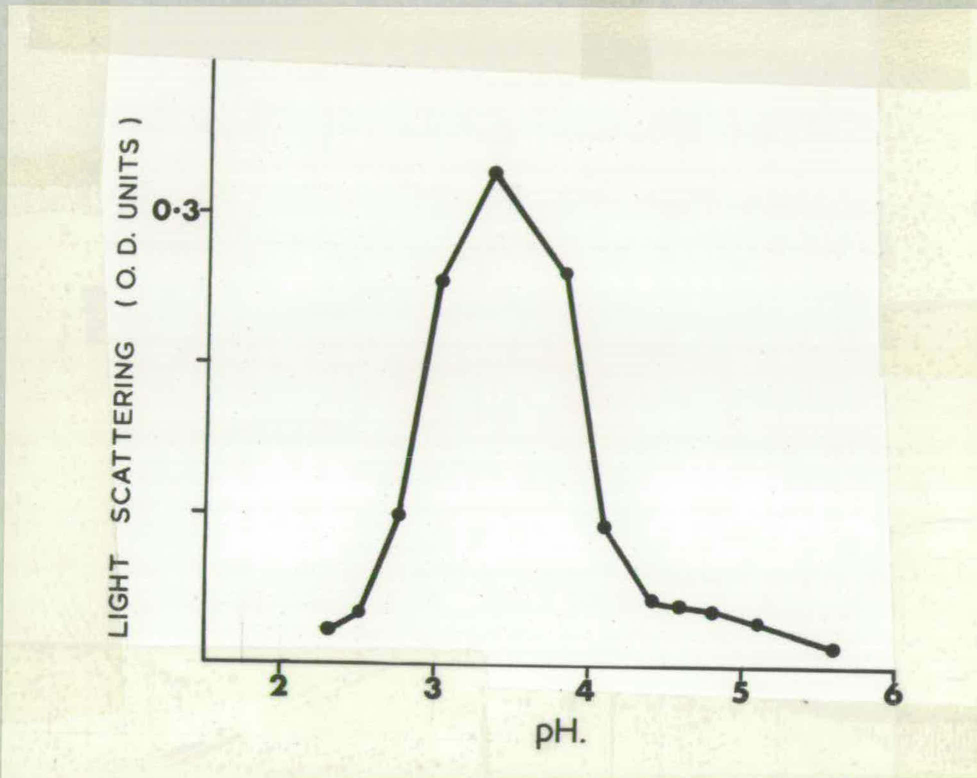
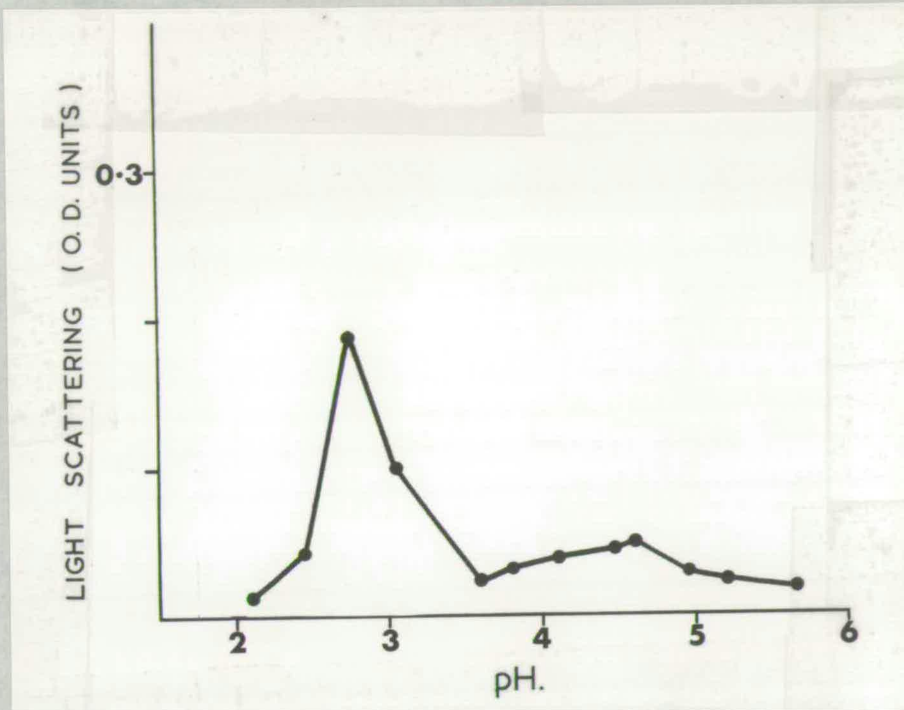
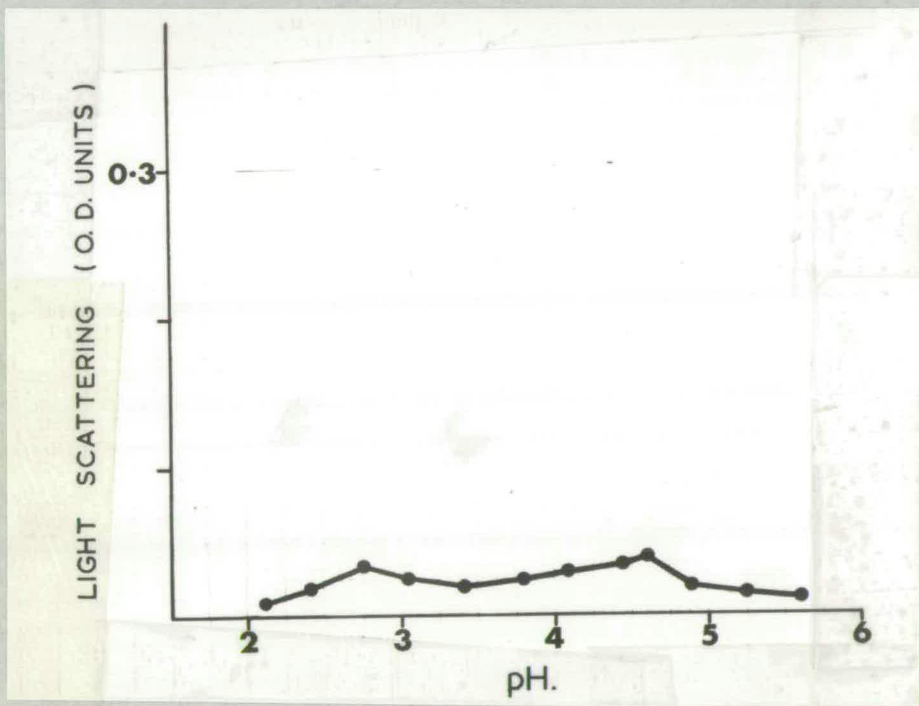


Fig. 4 Precipitation profile of type I and type II red cell membrane protein in 0.05 M-citrate-phosphate, protein solution before addition to buffer 0-sodium chloride.
Precipitation is measured as light scattering at 600nm.

Figs. 4, 5&6 reproduced from Vox Sang 18: 34-41 (1970) by kind permission of Dr. A.H. Maddy.

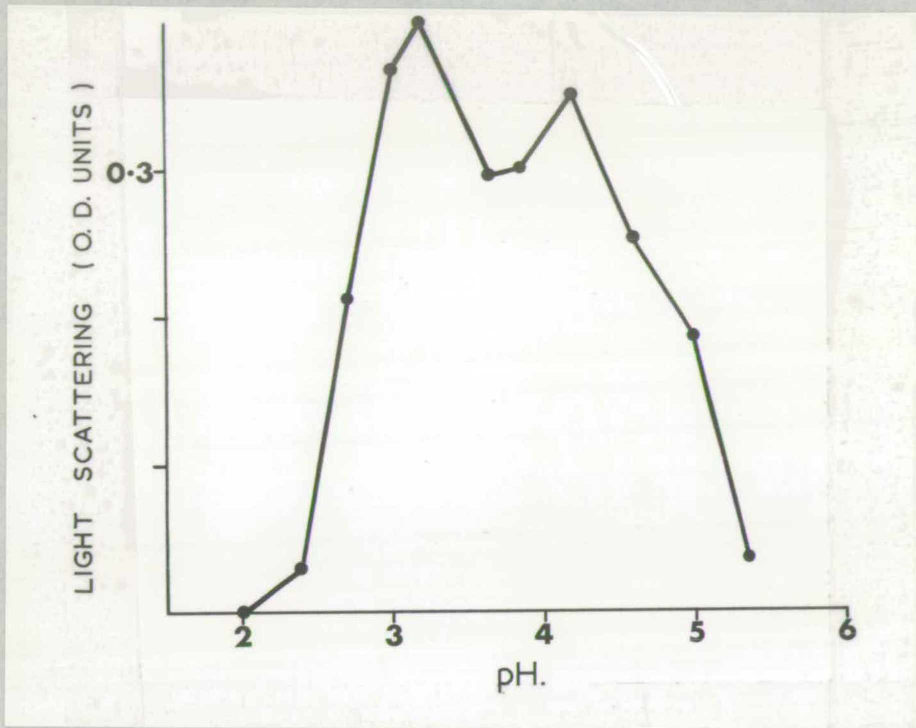


a) Buffer 0.20M citrate-phosphate, 0.10M sodium chloride

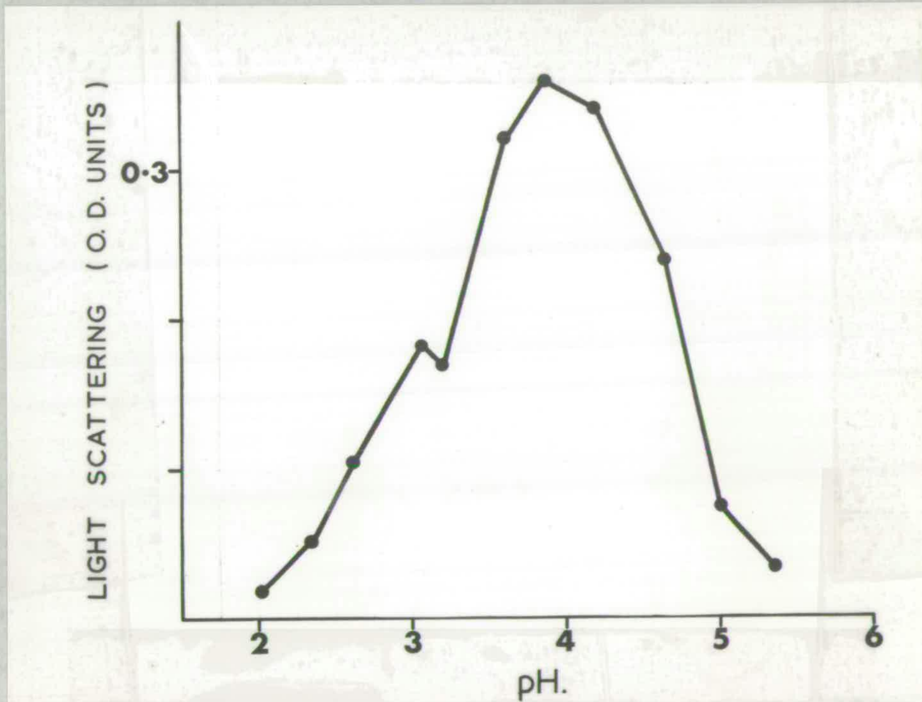


b) Buffer 0.20M citrate-phosphate, 0.30M sodium chloride

Fig. 5 The precipitation of type I protein



a) Buffer 0.20M citrate-phosphate, 0 sodium chloride



b) Buffer 0.20M citrate-phosphate, 0.10M sodium chloride

Fig. 6 The precipitation of type II protein

As proteins are least soluble at their isoelectric point it seems probable that type I had a lower isoelectric point and so a higher net negative charge than type II protein: assays of the sialic acid have shown that the pH at which they precipitate is a function of the sialic acid content. As the results in Table IV show, type I protein has a higher content of sialic acid than has type II.

The partial reaction of HZ127A noted in some antiglobulin tests is interesting as this animal was found to have the lowest sialo-protein ratio of the class I animals tested (mean 0.134 ± 0.0078); in fact, with a value of 0.100 µmoles sialic acid/mg. protein it might be considered to be a class II animal (mean 0.098 ± 0.005) if its protein precipitation were not identical with that of all other class I animals.

Uhlenbruck, Seaman and Coombs (1967) have previously suggested that the variation which they found in bovine erythrocyte agglutinability was correlated with the amount of surface mucoid present, and hence with the surface charge which it provided, thus preventing the cells from agglutinating. As has been shown here the ability of the cells to agglutinate is now correlated with the amount of sialic acid present in each cell, and so this is a possible explanation. If the area of sialic acid surrounding the cell is too dense or too thick then the antigen receptor sites are effectively shielded from the antibody which is trying to combine with them. Those cells with a lower sialic acid value can agglutinate, and although the difference in sialic acid value is ~~may~~ not ~~be~~ chemically large, it seems likely that the inagglutinable cells have enough extra sialic acid to form a completely inhibiting layer to the

cells agglutinating activity. In fact this cannot be the case because the results to be presented ~~as later~~ in this thesis and those of other workers show various ways ~~will be shown~~ in which the low agglutinable cells can be made agglutinable. It is more likely that the sialic acid of low agglutinating cells has a branching structure which makes it more difficult for them than the high cells to agglutinate. All the cattle cells tested were capable of low titre direct agglutination, and so there must be a basic difference between direct and antiglobulin agglutination. An attempt to clarify this will be made in a later chapter.

Chapter 3 Comparison of various systems of antiglobulin test using various sensitising sera with appropriate antiglobulin sera

It has now been shown that two classes of cattle erythrocyte exist in respect of the agglutinability in the modified antiglobulin test described in Chapter 2. As was shown in that chapter this finding appeared to contradict the results of Gleeson-White *et al.* (1950) who reported a variation in agglutinability of the red cells in different cattle. It was clearly necessary to investigate these two apparently contradictory results.

Although it seems unlikely, a possible explanation for the two classes of cattle red cell agglutinability is that the cells with a high agglutinating titre possessed an antigen not present in those with a low titre, and that this difference was detected by the rabbit sera described in Chapter 2 but not by those sera used by Gleeson-White *et al.* Another possibility was that the rabbit sensitising antibody and the bovine antiglobulin sera used were in some way unique. To test this, other species of sensitising antibody and antiglobulin sera were used, including those described by Gleeson-White *et al.*

Materials and Methods

Red Cells - see Appendix A

Antisera production - see Appendix B

Agglutination test }
Haemolytic test } As in Chapter 2

Absorptions

The red cells to be used in the absorption experiments were washed three times in 0.9% saline and centrifuged at 1500 g. for 10 min. The supernatant saline was removed and the antiserum to be absorbed added in the ratio of 2 volumes of serum to 1 volume of cells. This suspension was mixed continuously on a rotator at room temperature for 45 min. The serum was removed after centrifuging at 1500 g. for 20 min.

Results

Comparison of antiglobulin tests

Various systems were tested to compare different types of antiglobulin test, species of sensitiser and antiglobulin sera. Initially the Gleeson-White test was compared with the antiglobulin test described in Chapter 2.

Method 1. Red cells were sensitised with a fixed concentration of rabbit anti-bovine red cell serum R4 (19/5/66) and added to a titration of bovine anti-rabbit globulin K21 (17/5/67).

Method 2. Red cells were sensitised with dilutions of rabbit anti-bovine red cell serum R4 (19/5/66) and added to a fixed concentration of bovine anti-rabbit globulin K21 (17/5/67).

Method 3. Red cells were sensitised with a fixed concentration of infectious mononucleosis serum (see Appendix A) and added to a titration of rabbit-anti-human globulin.

Method 4. Red cells were sensitised with dilutions of infectious mononucleosis serum and added to a fixed concentration of rabbit anti-human globulin.

The results of these four tests on the same cells (Fig. 1) suggest that the previous conflicting results were due to differences in interpretation. When a fixed concentration of sensitiser was used, as in methods 1 and 3, two distinct classes, of agglutinable and inagglutinable cells, were found. Different antisera gave different endpoints, but two classes of cell were always clearly visible. When the sensitiser was diluted, as in methods 2 and 4, the two classes were less obvious, especially on a small panel of animals. However, when a larger number of animals (see Fig. 2) was tested by these methods the division into the same two classes did show more clearly.

Comparison of antiglobulin systems using other species

Differential agglutinability is not confined to the two antiglobulin systems discussed above. Further investigations have shown that red cells from the same animals, when treated with sheep anti-bovine sera 3A146 (17/12/69) and added to titrations of bovine anti-sheep FS99A (22/8/68) or rabbit anti-sheep R51 (16/12/68) globulins, gave the same two agglutination classes, although the titres were lower. Cells which had been sensitised with bovine iso-anti-sera MZ111B (1/8/68) and added to titrations of sheep anti-bovine 7B44 (25/2/69) and rabbit-anti-bovine globulins R17 (24/2/68) also gave the same result. In each case the concentration used for the sensitising dose was determined previously by haemolytic and agglutination tests on the sera, as described in Chapter 2 for the rabbit anti-bovine sensitising sera. Red cells taken from the same cattle always behaved in the same manner no matter which antiglobulin test system was used. Only the agglutination titres varied with the different sera (see Table I). In any particular system there was very little variation

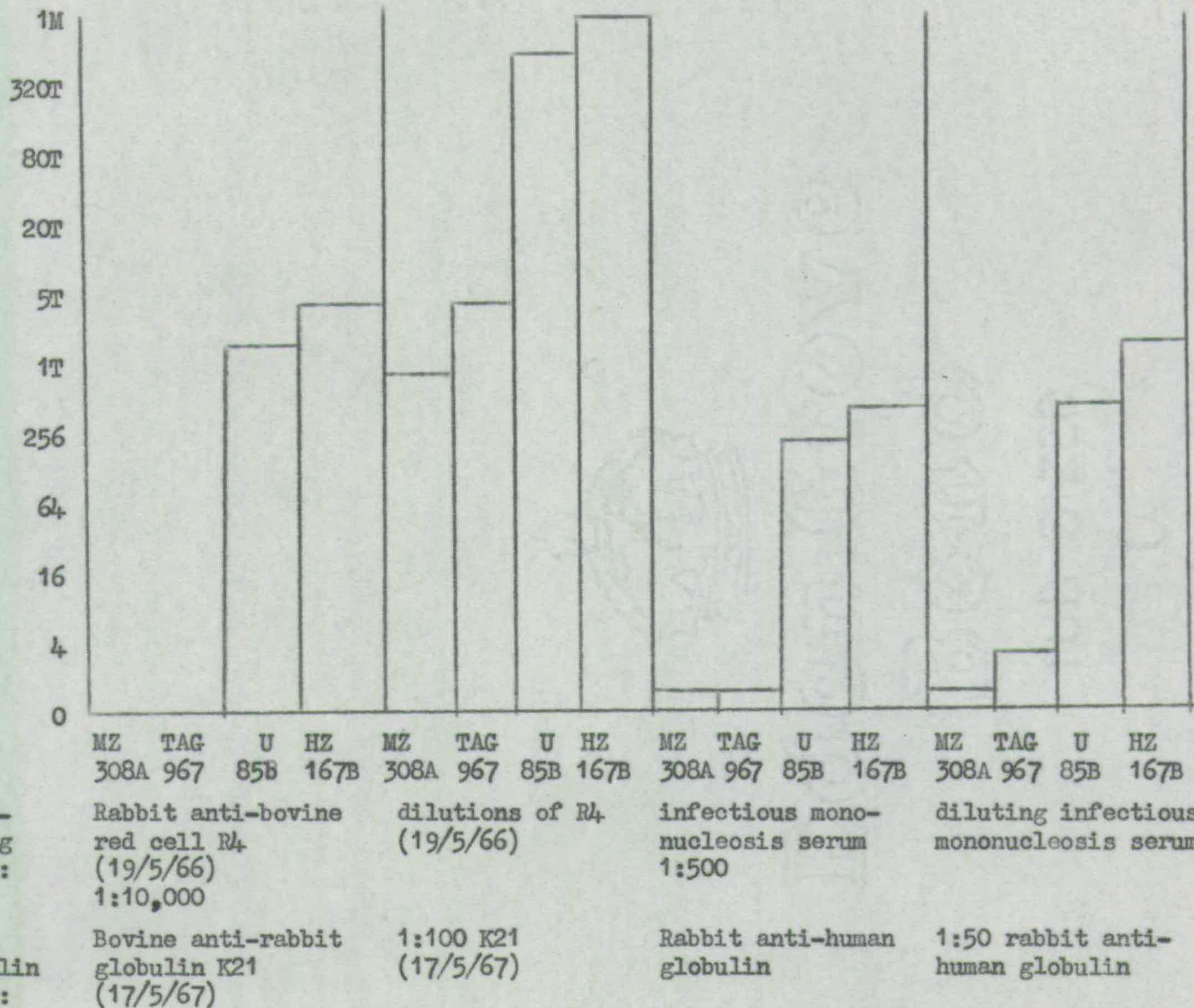
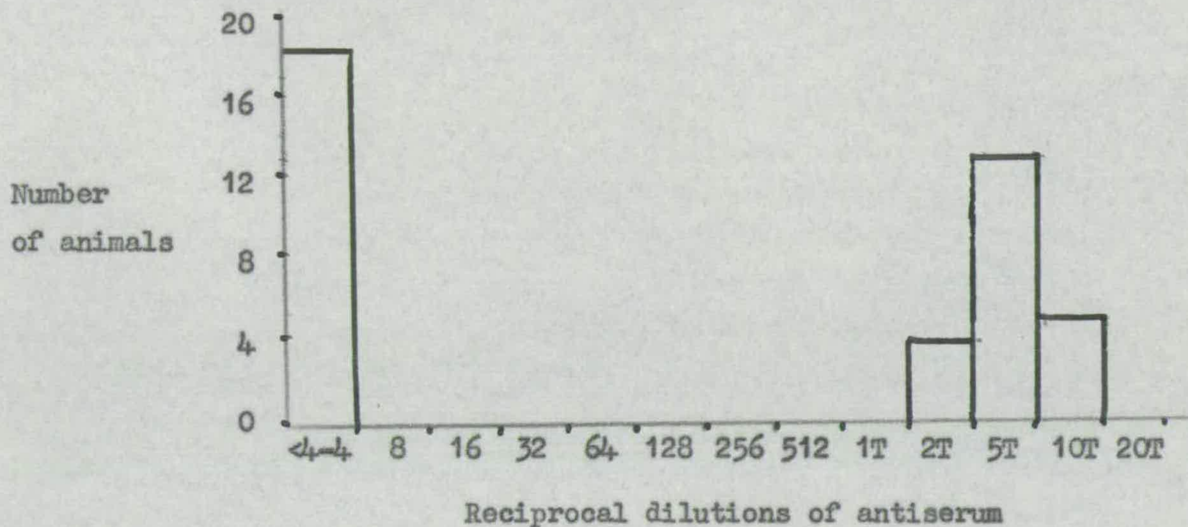
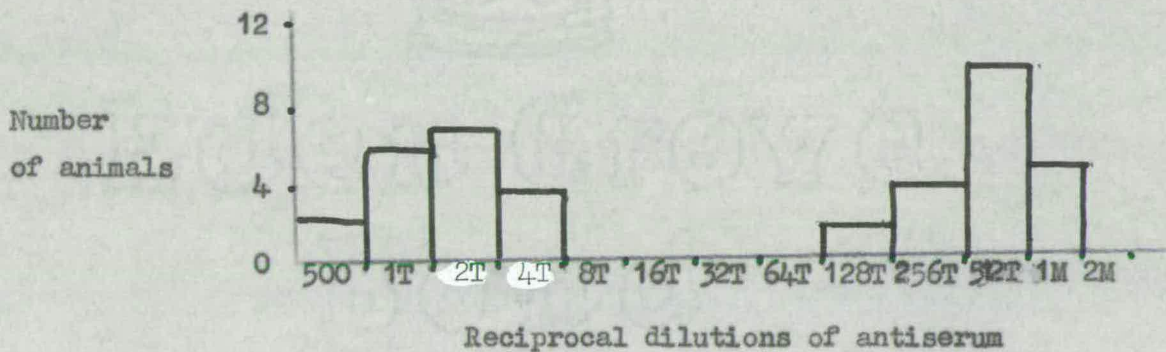


Fig. 1 Comparison of different antiglobulin tests on the same four cells

T = thousand
M = million



a. Antiglobulin test using a fixed concentration of sensitiser and added to a titration of antiglobulin serum.



b. Antiglobulin test using dilutions of sensitiser and a fixed concentration of antiglobulin serum.

Fig. 2 <u>Comparison of two methods of antiglobulin test</u>

Number of animals tested	Sensitising serum	Sensitising serum: Haemolytic titre	Agglutination titre	Sensitising dose	Antiglobulin serum	High titres	Low titres
2,000	Rabbit anti-bovine red cell R4 (19/5/66)	8,000	500	1:5000	Bovine anti-rabbit globulin K21 (17/5/67)	> 2000	< 4
100	Rabbit anti-bovine red cell R4 (19/5/66)	8,000	500	1:5000	Sheep anti-rabbit globulin N207 (19/7/64)	> 5000	< 4
30	Sheep anti-bovine red cell 3A146 (17/12/69)	2,000	16	1:1000	Bovine anti-sheep globulin FS99A (22/8/68)	500	0
30	Sheep anti-bovine red cell 3A146 (17/12/69)	2,000	16	1:1000	Rabbit anti-sheep globulin R51 (16/12/68)	500	2
30	Bovine isoantiserum MZ111B (1/8/68)	1:350	4	1:200	Sheep anti-bovine globulin 7B44 (25/2/69)	1000	2
30	Bovine isoantiserum MZ111B (1/8/68)	1:350	4	1:200	Rabbit anti-bovine globulin R17 (24/2/68)	500	2

Table I Antiglobulin systems using other species of animal

in the agglutination titres in the high (H) or low (L) groups tested.

Agglutination using anti-membrane protein

It was suggested by Uhlenbruck et al. (1967) that antibodies to the outer mucoid layer of the red cell should produce agglutination of all bovine erythrocytes. Therefore antibodies to butanol-extracted bovine red cell membrane protein were raised in rabbits, and the antiserum produced, R52 (5/6/69), was used to sensitise the cells in an antiglobulin test. The results were similar to those obtained using antisera to whole red cells, although the titres were slightly lower (Table II and Fig. 3). It seems that the agglutination reaction is slightly inhibited by antibodies to the membrane protein rather than being enhanced by them.

Use of sera produced against high (H) and low (L) agglutinable cells

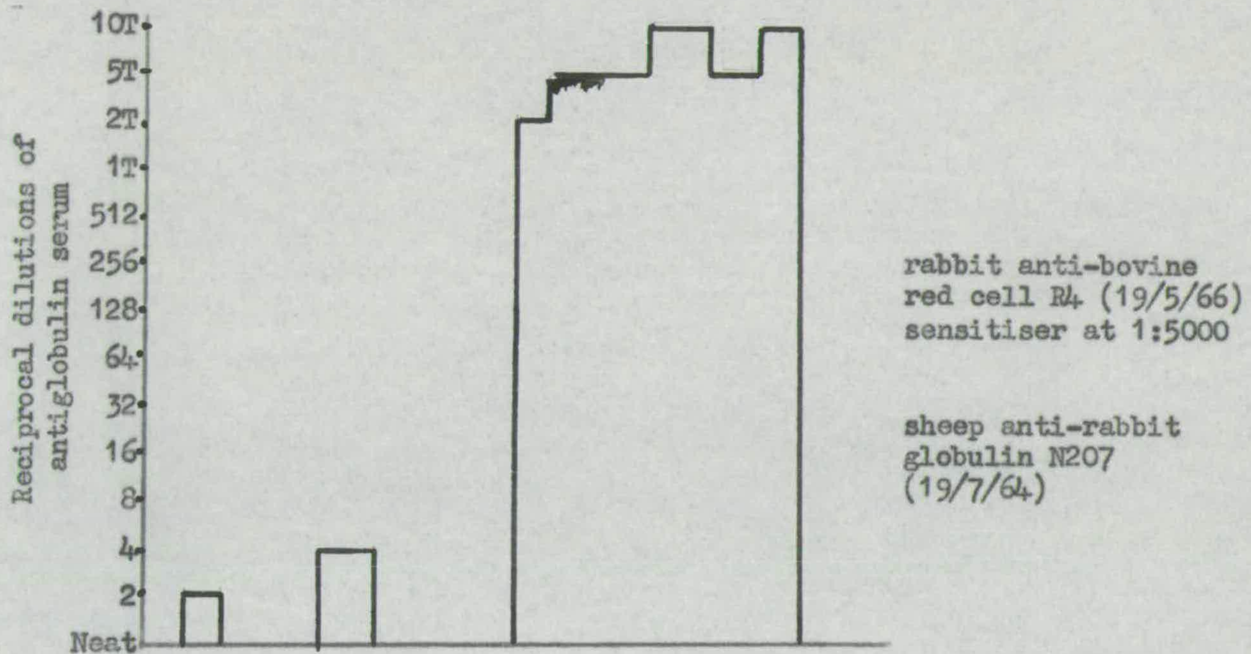
Initially bovine red cells of unknown agglutinability were used to immunise rabbits, but subsequently antibodies were produced which were specific to bovine cells with high or low agglutinability. When the sera containing these anti-high agglutinable R60 (17/6/69) and anti-low agglutinable R62 (17/6/69) antibodies were used as sensitising sera, differential agglutinability was found. On testing with the same anti-globulin serum, a smaller difference was found between the titres of high and low agglutinable cells with rabbit serum against low agglutinable bovine cells than with an anti-high agglutinator sensitising serum (see Table II and Fig. 4).

Absorptions of sensitising sera /

Number of animals tested	Sensitising serum	Sensitising serum			Antiglobulin serum	High titres	Low titres
		Haemolytic titre	Agglutination titre	Sensitising dose			
100	Rabbit anti-bovine red cell membrane protein R52 (5/6/69)	5,000	1,000	1:2,000	Bovine anti-rabbit globulin K21 (17/5/67)	> 1000	2
100	Rabbit anti-bovine red cell membrane protein R53 (5/6/69)	10,000	2,000	1:5,000	Bovine anti-rabbit globulin K21 (17/5/67)	> 1000	0
30	Rabbit anti-high bovine red cell R60 (17/6/69)	12,000	1,000	1:8,000	Sheep anti-rabbit globulin 7B51 (16/1/69)	> 5000	< 10
30	Rabbit anti-low bovine red cell R62 (17/6/69)	12,000	1,000	1:8,000	Sheep anti-rabbit globulin 7B51 (16/1/69)	> 2000	< 20

Table II

Antiglobulin systems using anti-membrane protein serum and anti-high and anti-low bovine red cell sera



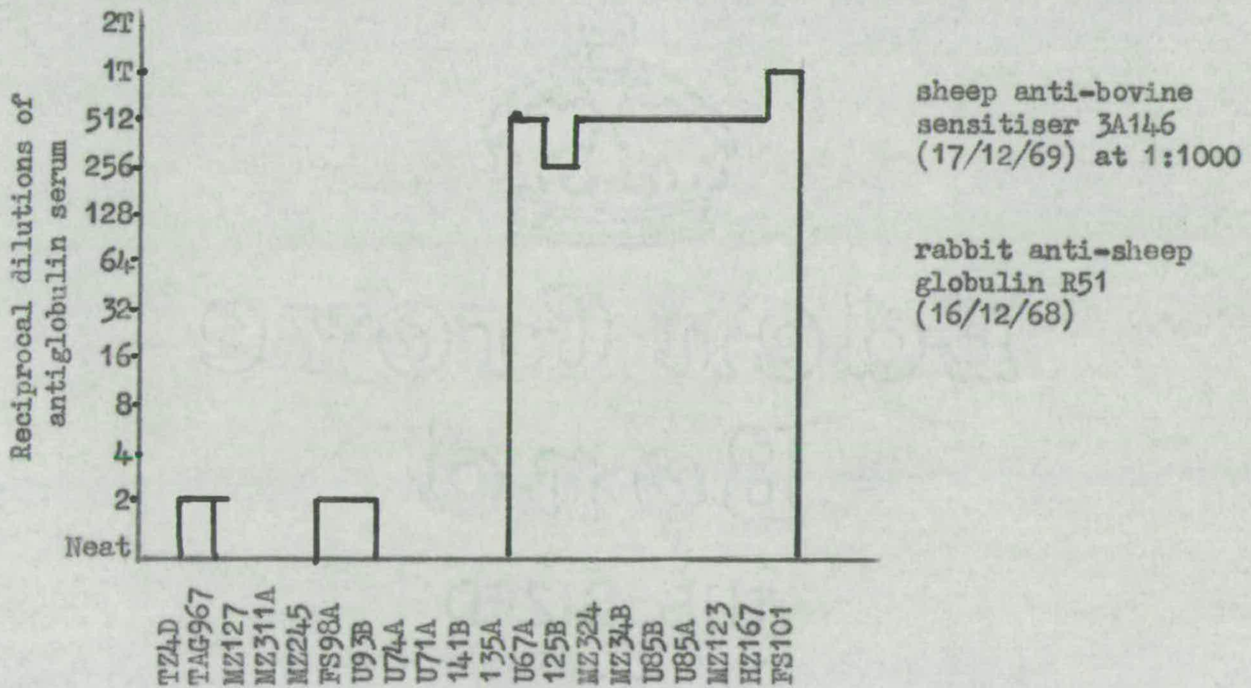


Fig. 3a <u>Comparison of antiglobulin tests using various systems</u>

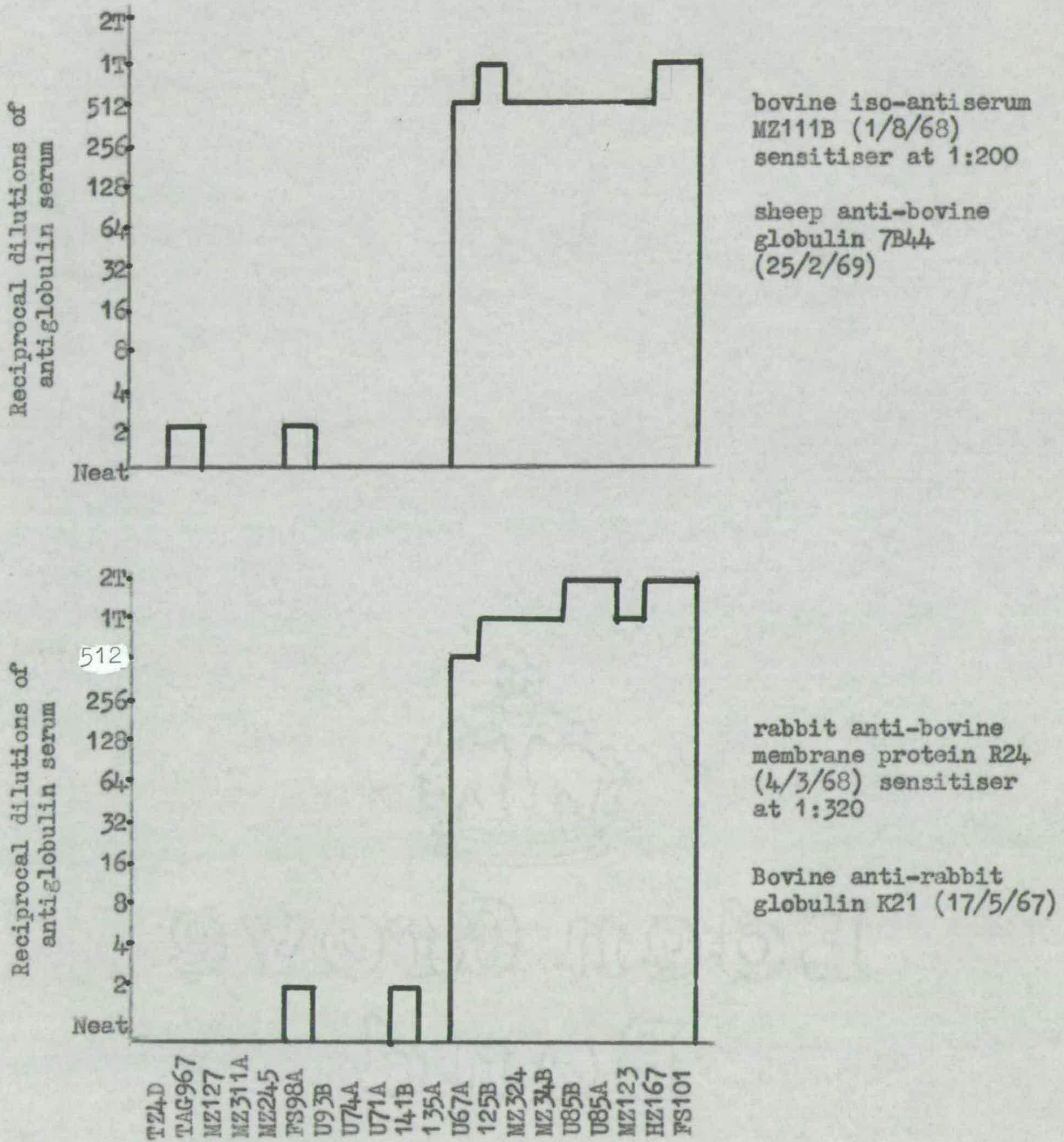


Fig. 3b Comparison of antiglobulin tests using various systems

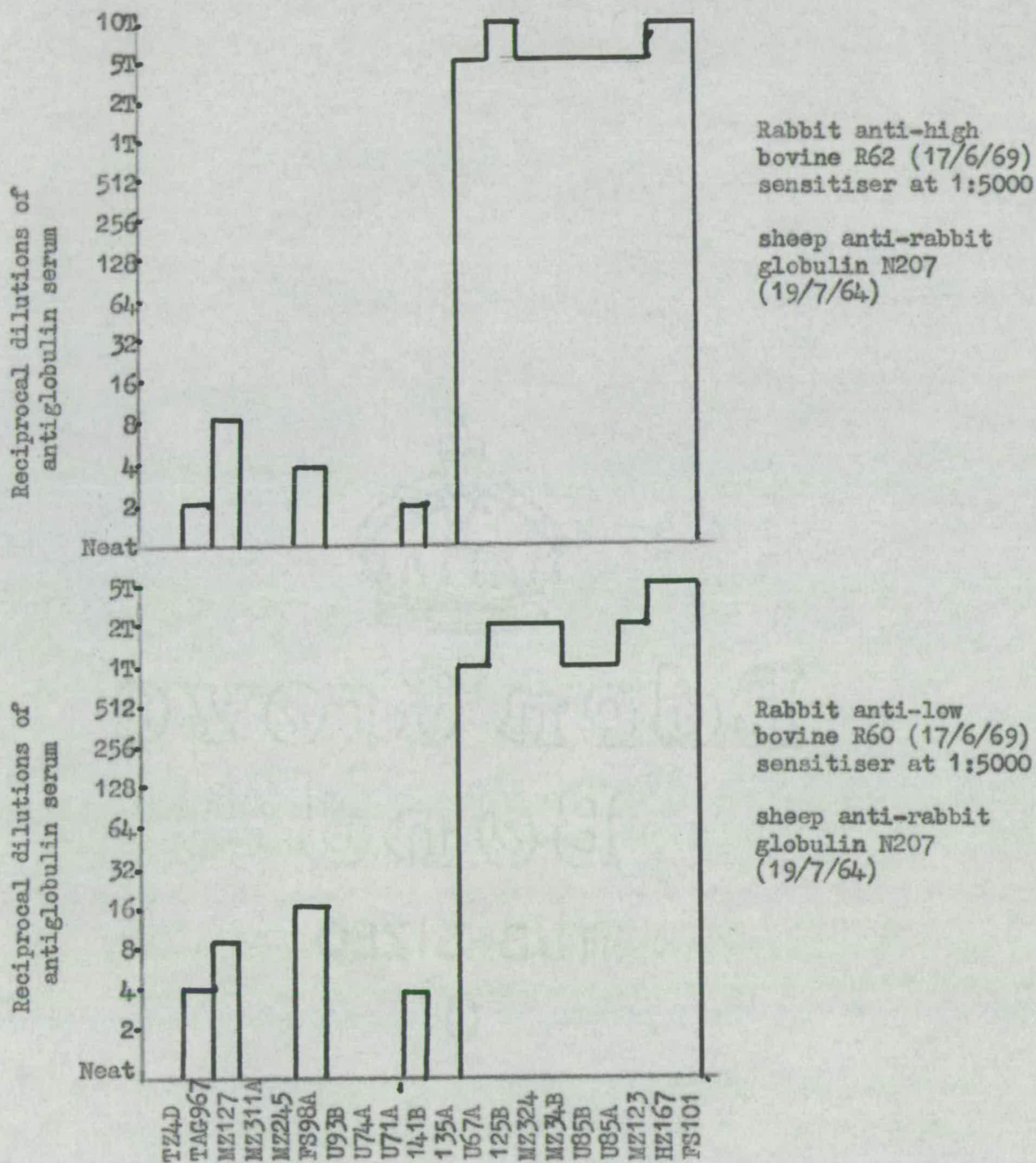


Fig. 3c <u>Comparison of antiglobulin tests using various systems</u>

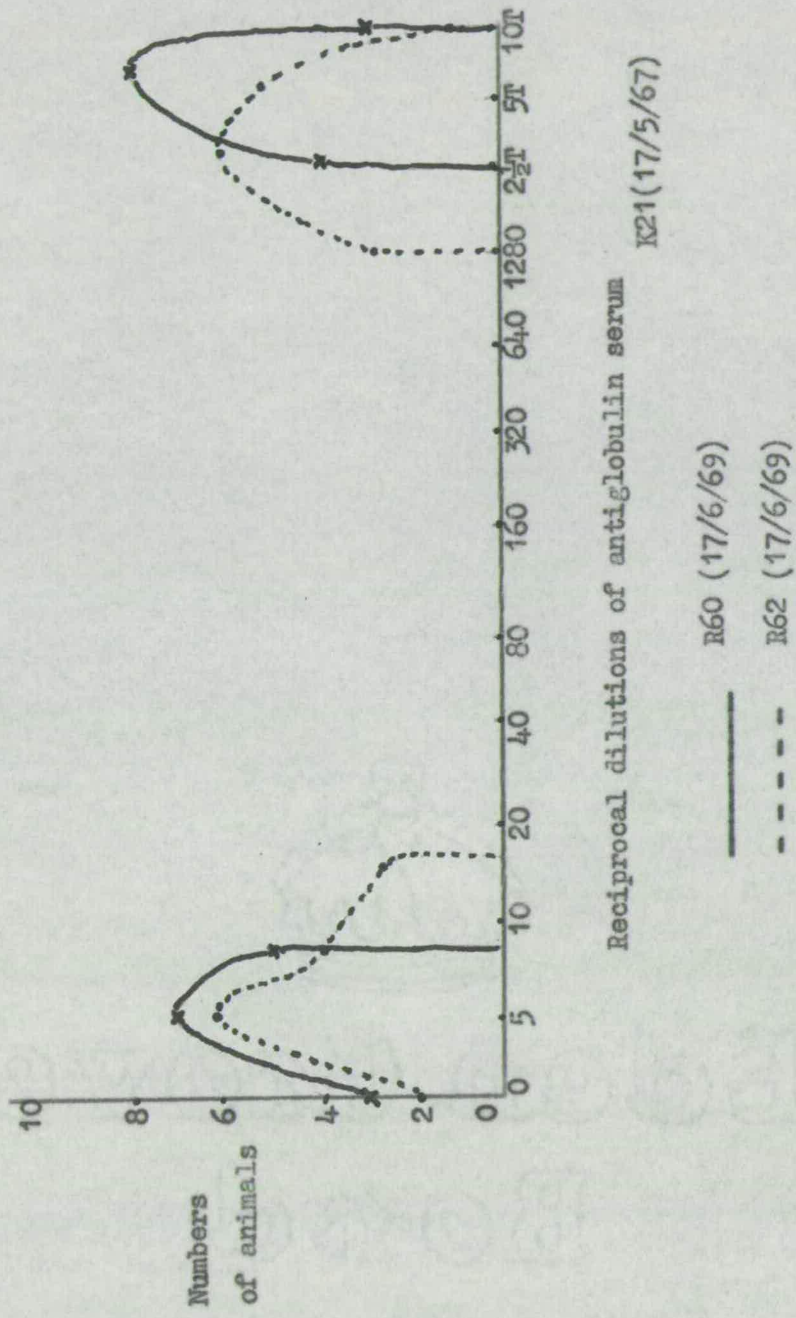


Fig. 4 Antiglobulin test using rabbit anti-high agglutinable R60 (17/6/69) and rabbit anti-low agglutinable R62 (17/6/69) bovine cell sera

Absorptions of sensitising sera

To test the possibility that the antigen causing agglutination was present on the high agglutinable cells but absent from the low agglutinable cells, the sensitising sera were absorbed separately with either high or low agglutinable cells. These absorbed sera were then tested in an antiglobulin test (see Table III). It was found that absorption of the serum by either type of cell neutralised the sensitising activity of the serum and it would no longer sensitise high agglutinable cells. Since the antibodies in the serum were removed completely following absorption by either type of cell, it was concluded that the variability was not due to an antigen which was present on the high agglutinable cells but absent from the low agglutinable cells.

Discussion

All the antiglobulin systems tested with red cells from different cattle have given the same result, viz. that the red cells fall into two distinct classes. Hall (1955) measured the antiglobulin agglutination titres of 60 cattle and found that the titres fell into two distinct classes. One class of animals had a titre of <10 and the other class a titre of >30. He thought that this was an artificial distinction since, as the scale of dilutions was logarithmic, many endpoints were included in the first class. However when these results are compared with the present data, it is obvious that they are in agreement and that he was looking at the same two classes of agglutinability which have been described here. The particular antisera used by Hall give a wider range of titres in each class, but, nevertheless, the division into the two classes is still apparent.

	R4 (unabsorbed)	R4 absorbed U85A	R4 absorbed U93B
High cell U85A	5,000	-	-
Low cell U93B	-	-	-

Table III <u>Antiglobulin titres after absorption of the sensitising serum with high- or low- agglutinable cells</u>

The differential agglutinability shown by R4 (19/5/66) was not due to an antigen which was present only on the high agglutinable cells as the absorptions of this serum have proved. Although the agglutination by the various other anti-red blood cell sera used does not seem to involve antigens which are present only on the high agglutinable cells, the results using antisera to high and low agglutinable cells might suggest that there is some antigenic difference between the two cell types, and that this can be detected using rabbit antisera; this hypothesis requires further investigation, first to confirm the result and then to explain it.

Chapter 4 The mode of inheritance of agglutinability

The various systems used in the antiglobulin test, and discussed in Chapter 3, show that the two classes of agglutinability found in bovine red cells are not due to a freak reaction with one antiserum, but are due to a difference in the structures of the two classes of cell. In the initial studies using the antiglobulin test, a panel of monozygous twin pairs was tested. The results of these tests led to the suggestion that the nature of the bovine red cells' ability to agglutinate may be controlled by a simple genetic mechanism. The suggestion was investigated in the experiments reported in this chapter. During these studies marked differences were noted in the agglutinability of the red cells of different breeds of cattle.

Family studies were carried out, and the hypothesis put forward that the mode of inheritance of high agglutinability is simple, and that high agglutinability is recessive to low agglutinability.

Results

Monozygous twins

In some of the earliest studies using the antiglobulin test a panel of monozygous twin pairs was tested. These animals were identical in every respect including their antiglobulin agglutination class (Table I). When a further study, using a larger number of animals, was made and only the two classes of agglutinator were found, it was discovered that some breeds of cattle were predominantly high agglutinators and others were mainly low

Animal	Agglutinability	Titre	Animal	Agglutinability	Titre
MZ231A	High	1:2 T	MZ329A	Low	0
B	High	1:1 T	B	Low	0
MZ238A	Low	0	MZ339A	Low	0
B	Low	0	B	Low	0
MZ308A	Low	0	MZ341A	Low	0
B	Low	0	B	Low	0
MZ310A	High	1:2 T	MZ342A	Low	0
B	High	1:2 T	B	Low	0
MZ318A	Low	0	MZ343A	Low	0
B	Low	0	B	Low	0
MZ320A	Low	0	MZ344A	Low	0
B	Low	0	B	Low	0
MZ322A	Low	0	MZ345A	Low	0
B	Low	0	B	Low	0
MZ324A	High	1:2 T	MZ346A	Low	0
B	High	1:1 T	B	Low	0
MZ325A	Low	0	MZ347A	Low	0
B	Low	0	B	Low	0
MZ328A	High	1:2 T	MZ351A	Low	0
B	High	1:2 T	B	Low	0
Control Cell			Control Cell		
FS101A	High	1:2 T	U93B	Low	0

Table I <u>Results of an antiglobulin test on monozygous (MZ) twins</u>

T = thousand

agglutinators (or inagglutinable). Therefore, a more intensive breed survey was carried out, an effort being made to test samples from as wide a range of breeds as possible. A great many of these samples were those sent into the Cattle Blood Typing Service for routine parentage testing, others were from the Animal Breeding Research Organisation's farms.

Breed frequencies

The survey showed that there were marked differences among breeds in the frequencies of agglutinability (Table II). For example, of 255 Herefords tested, 84% were high agglutinators. By contrast, of 990 Friesians tested, only 24% were high agglutinators. The predisposition to one or other class was less marked in the Ayrshire and Jersey breeds; 40% of Ayrshires and 58% of Jerseys were high agglutinators. After finding notable breed differences, it was hoped that an association might be found between agglutinability and other blood-group characters. This aspect is dealt with in Chapter 8.

Both from the results of experiments on monozygous twins and from the breed differences in agglutinability it appeared extremely likely that the character was genetically controlled. To test this, blood samples were collected from over 100 families.

Family studies

Antiglobulin tests were carried out on all the samples. Haemolytic tests conducted simultaneously on the first 150 samples proved that all the samples were equally sensitised with antibody.

Breed	Agglutinability High	Low	% High
Aberdeen-Angus	27	8	77.1
Ayrshire	44	64	40.3
Blue Grey	48	56	46.1
Charolais	10	27	27.0
Devon	26	2	92.8
Friesian	230	760	24.1
Guernsey	28	88	23.2
Hereford	213	42	83.6
Jersey	103	73	58
Lincoln Red	11	5	68.7
Longhorn	20	24	45.4
Shorthorn	6	2	75
South Devon	24	37	39.3
Sussex	0	26	0
Welsh Black	1	4	20
Cross breeds			
Friesian x Ayrshire	1	5	
Hereford x Aberdeen-Angus	2	0	
Hereford x Friesian	12	18	
Hereford x Shorthorn	0	2	
Foreign Breeds			
Angoni	544	555	
Brown Swiss	0	7	
Chianina	0	3	
Piemontese	0	6	
Valdostoma	3	4	
Holstein x Brown Swiss	1	10	

Table II <u>An antiglobulin survey of different breeds of cattle</u>

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The results of the family studies (Table III) showed that when both parents gave a high agglutination reaction, all offspring also gave a high agglutination reaction and never showed low agglutinability. If the parents were of different classes of agglutinability, offspring of both classes were produced, irrespective of whether the dam or the sire was the parent with high agglutinability. Matings between two low agglutinating animals also produced offspring of both classes. These results fit the hypothesis that high agglutinability is recessive to low.

	Parents	Offspring
41	High x High	41 High
76	High x Low Low x High	34 High + 42 Low
174	Low x Low	17 High + 157 Low

Table III Family studies

The percentage of animals with high agglutinability in each breed is given in Table II from which it can be seen that there are big differences in the frequency. To test whether the character was in fact simply inherited it was necessary to have a large number of randomly selected families in each breed. The samples were not entirely random, as some were taken from the blood samples sent to the Cattle Blood Typing Service, and several of the bulls involved had been used in two or three different matings. However, the animals were not chosen specially for this experiment and had not been tested previously, so that no animal was included or excluded due to prior knowledge of its class of agglutinability.

Inheritance of agglutinability

The frequencies of the three genotypes, homozygous high and heterozygous and homozygous low, were calculated from the data from Friesians; it was assumed that there was no selection. From this information the probability of a low agglutinator being a homozygous low was calculated, and it was found that 1 in every 3 animals would, in fact, be homozygous low. Then the expected number of high agglutinator offspring of homozygous and heterozygous matings was calculated from the data for Friesian families and compared with the number actually observed. It was calculated that there should be 5 high agglutinator offspring from 44 low x low matings; the observed number was 6. Similarly, from 12 high x low-agglutinator matings there should be 4 high agglutinator offspring; 4 were found. These results indicate that, from the data presented here, the character for high agglutinability is inherited as a simple trait in the Friesian breed. (See Appendix C for calculation).

The Hereford and Ayrshire family data were analysed similarly; the results, shown in Table IV, confirmed that, in the data presented here, the observed values of agglutinability fit with the hypothesis that the character for high agglutinability is inherited and is recessive to the character for low agglutinability.

The volume of family data is too small to test for significance, but the similarity of observed and expected results and the consistency within breeds where there is a wide difference between the frequency of occurrence of high agglutinators and low agglutinators, fit the hypothesis.

Breed	Parents	Offspring	Number of High Agglutinators	
			Expected	Observed
Friesian	2H x H	2H	2	
	12H x L	4H + 8L	4	4
	44L x L	6H + 38L	5	6
Ayrshire	19H x L	8H + 11L	7-8	8
	25L x L	4H + 21L	4	4
Hereford	21H x H	21H	21	
	8H x L	4H + 4L	4	4

H = high agglutinator

L = low agglutinator

Table IV <u>Table to show the number of high agglutinators expected and observed from matings within separate breeds</u>

Chapter 5 Examination of the agglutination process by means of fractionated sera

In Chapter 4 it has been shown that the mode of inheritance of the agglutination character of bovine erythrocytes is simple in the animals studied. The questions of how these cells agglutinate and why one class of cell does and the other does not are considered in the present chapter. Coombs et al. (1951) put forward the hypothesis that the receptor sites in the inagglutinable cells are situated in pits, whereas the receptor sites in agglutinable cells lie on flat areas of the membrane surface. They also proved that all the cells were equally sensitised at these sites by the sensitising antibody. They envisaged a situation in which the sensitising antibody and the anti-globulin molecule were not long enough in two inagglutinable cells for the cells to combine. In the agglutinable cells the receptor sites were exposed and the inter-site distance was short enough for agglutination to occur between cells. Coombs et al. built up antiglobulin - globulin 'lattices' to show that even inagglutinable cells can be made to agglutinate.

With the idea of membrane pits in mind it was decided to investigate the sensitising antibody to see if the $7\overline{S}$ antibody was equally or more important than the $19\overline{S}$ antibody in the antiglobulin agglutination reaction. As the $7\overline{S}$ is smaller (molecular weight 150000) than the $19\overline{S}$ (molecular weight, 900000) it was thought possible that the former might penetrate deeper into the membrane surface, so that the reaction with $7\overline{S}$ would be more intense than with $19\overline{S}$. Alternatively, it might be possible that the $19\overline{S}$ antibody, being the larger, could be the only antibody to react, the $7\overline{S}$ being too short to do so. The $19\overline{S}$ is known to take part in the antiglobulin agglutination, as Paul Bunnell

serum is pure $19\bar{S}$. An attempt has been made to investigate the relative importance of the $7\bar{S}$ and $19\bar{S}$ antibodies by fractionating the sensitising sera and testing the fractions to find out in which fractions antiglobulin activity occurred.

Methods

DEAE-cellulose fractionation

1 ml. of sample was used for every 2 g. dry weight of DE 32 used in packing the column. The serum sample to be fractionated was added to an equal volume of 50% saturated ammonium sulphate solution to precipitate the immunoglobulins. The precipitate was washed twice in 50% saturated ammonium sulphate solution and was then dialysed overnight against the starting buffer. The column was equilibrated with the starting buffer and then the sample applied, and the first elution made with the starting buffer. Subsequent fractions were eluted using either stepwise or gradient buffers, the details of which are shown on the graph of the fractionation of each serum. The eluate was collected in an automatic fraction collector, and the protein concentration estimated at 254 nm in an L.K.B. uvicord. Gradients from the lowest to the highest molarity buffer were effected using a bridge, made of glass tubing, between the two buffers which were stirred continuously. The buffers, which were degassed before use, were made up freshly each time from stock solutions of 0.2M-Na_2HPO_4 and NaH_2PO_4 to pH7.0.

G200 Sephadex fractionation

1 g. of Sephadex was swollen in 100 ml. of distilled water for 1 week. This was fined many times and then degassed before the column was packed. The packed column was equilibrated with buffer, and, after dialysing overnight, the sample was applied. The fractions were collected and the protein concentration estimated as in the DEAE fractionations.

Batch method of preparation of rabbit IgG

An equal volume of whole rabbit serum was added to 50% saturated ammonium sulphate solution. This was left at room temperature for the immunoglobulins to precipitate. The precipitate obtained after centrifuging at 18,000 g for 15 min. was washed twice with 50% saturated ammonium sulphate. The precipitate was then dialysed against 0.01M-phosphate buffer of pH7.4 for 24 hr. The resulting suspension was added to DE52 sludge using 1 ml. of sample to 5 g. wet weight of DE52. This was stirred well and stood at 4°C for 2 hr. when an equal volume of 0.01M-phosphate buffer was added, mixed for 5 min. and then spun at 18,000 g for 15 min. at 4°C. The supernatant then contained the IgG; this was stored and the sludge washed again with the same buffer to obtain the remaining IgG. Both supernatants were filtered to give a pure, dilute IgG fraction, which was concentrated by dialysing against 50% polyethylene glycol in 0.1M-phosphate overnight. This method gives about 70% recovery of IgG.

Mercaptoethanol and iodoacetamide treatment

Immunoglobulins were precipated from whole serum in the usual manner. The precipitate was then dialysed for 24 hr. against 0.2M-mercaptoethanol in 0.9% saline. The mercaptoethanol broke the disulphide bonds which exist in

IgM. The dialysate was then dialysed against 0.2M-iodoacetamide in 0.9% saline for 6 hr. to remove any remaining mercaptoethanol. A further dialysis against 0.9% saline for 2 hr. was carried out to remove any remaining reagent.

Concentration of protein fractions

All the fractions collected were concentrated by pressure dialysis for 24 hr. before being used in the serological tests.

Immunoelectrophoresis

Preparation of buffer

Oxoid BR II   Barbitone acetate buffer = 5.0g. soluble barbitone
(BAB)
3.25g. sodium acetate
34.2 ml. 0.1N-HCL

16.5g. BAB granules / 1.H_2O pH = 8.2

Preparation of agar

15g. Ionagar No. 2 (Oxoid) were added to 500 ml. distilled water giving the suspension a pH of 8.5. The solution was heated on a water bath, allowed to solidify, and then dialysed against distilled water, containing 2 drops of azide, at 4°C for 24 hr. before being dried and stored in small quantities.

Microscope slides, 3 x 1" or 3 x 3", were cleaned in industrial methylated spirits. They were numbered and put on a level table. 1.5% (w/v) agar in 0.025 M-sodium barbitone, pH 8.2, was boiled and pipetted quickly onto the slides. The slides were allowed to cool in a moist chamber and after 45 min. when the gel was solid, the troughs and appropriate size and number of wells

were cut out. Serum samples were added to the wells by capillary pipettes. The immunoelectrophoresis tank was set up using the prepared barbitone buffer (pH 8.2). The slides were put on the tank; the wicks were in the buffer and also in contact with the gel. A 40V current was passed through each gel. Marker dye was put in the top serum well so that the distance the serum had run would be visible. The current was kept constant until the samples had run fully (about 2 hr.). The slides were removed from the tank and the sera added to the troughs. They were then left in a moist chamber overnight for the sera to diffuse and precipitate. The following day 0.9% saline was added and left for 24 hr. to remove excess unprecipitated protein, and then the slides were put in distilled water for 24 hr. to wash out the saline.

The slides were dried in an oven at 37°C before staining with 10% (w/v) amido black for 1 min. and washing overnight. They were dried again and then read.

Antiglobulin - globulin lattice

2% suspensions of washed, packed red cells were made up to 1 ml. with 0.9% saline. These were treated with 1 ml. of serially diluted rabbit anti-bovine serum R4 (19/5/66) in saline for 1 hr. at 37°C. The cells were centrifuged and washed with saline three times before being resuspended to the original volume in saline. A 2-drop agglutination test was then carried out adding 1 drop of cell suspension to 1 drop of bovine anti-rabbit globulin K21 (17/5/67) at 1:50. The test was shaken and read after standing at room temperature for 30 min. An equal volume of the same antiglobulin serum was added to the remaining cell suspensions and was left at 37°C for 30 min. The cells were

washed in saline three times and then an equal volume of 0.2% normal rabbit serum was added. After sensitising at 37°C for 30 min. the cells were washed and tested again in an agglutination test. The cells were then treated again alternately with the antiglobulin serum and then the normal rabbit serum until the agglutination test showed the cells to be agglutinated. Controls of saline and normal bovine serum instead of rabbit anti-bovine serum were used.

Results

The original serum used in the antiglobulin tests R4 (19/5/66) was fractionated (Fig. 1a and b) on a DEAE-cellulose column. Using a phosphate buffer and a gradient of increasing molarity, the first protein peak was eluted at 0.0175 M-phosphate. This peak was found by immunoelectrophoresis to contain a pure IgG (or 7S̄ immunoglobulin). The fourth peak which was eluted at 0.4M-phosphate, contained IgM (or 19S̄ immunoglobulin). The first peak was pure but subsequent ones were contaminated with other proteins. Therefore, to obtain a pure 19S̄ fraction the same serum was fractionated on a G200 Sephadex column (Fig. 2a and b). When this gel is used the smaller protein molecules penetrate into it and are retarded, so that the larger protein molecules are eluted first. The first pure peak in this case was the 19S̄, the second one containing the 7S̄. The fractions containing the protein peaks were collected and concentrated by ultrafiltration before being tested for antiglobulin activity. The 19S̄ was present in very small amounts as it was not readily detected in immunoelectrophoresis. Therefore, to ascertain that the peak did contain pure 19S̄, the whole serum was treated with mercaptoethanol and iodoacetamide and then re-

FIG. 1a. DEAE II CELLULOSE FRACTIONATION of R4 (19/5/66)

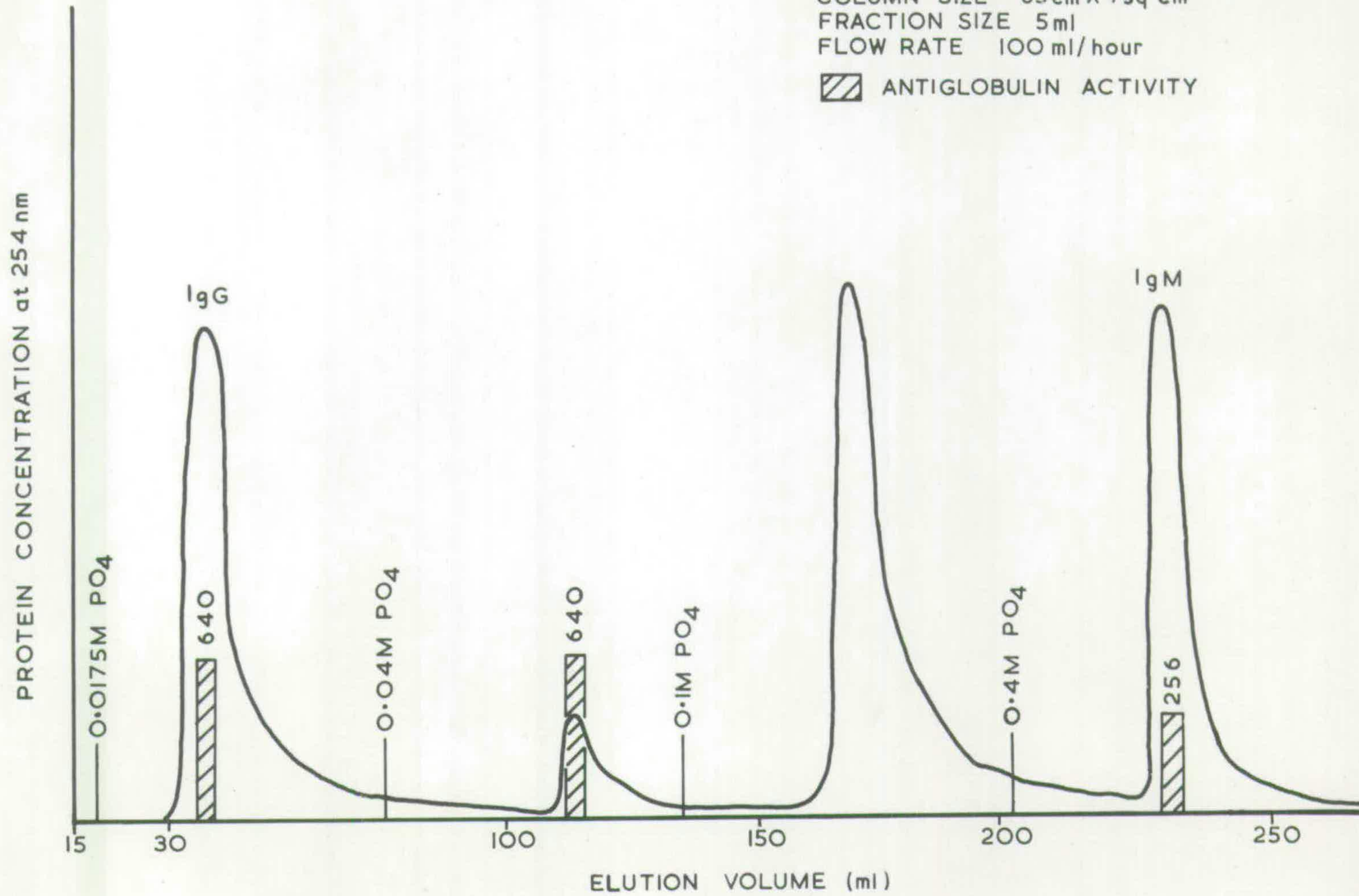
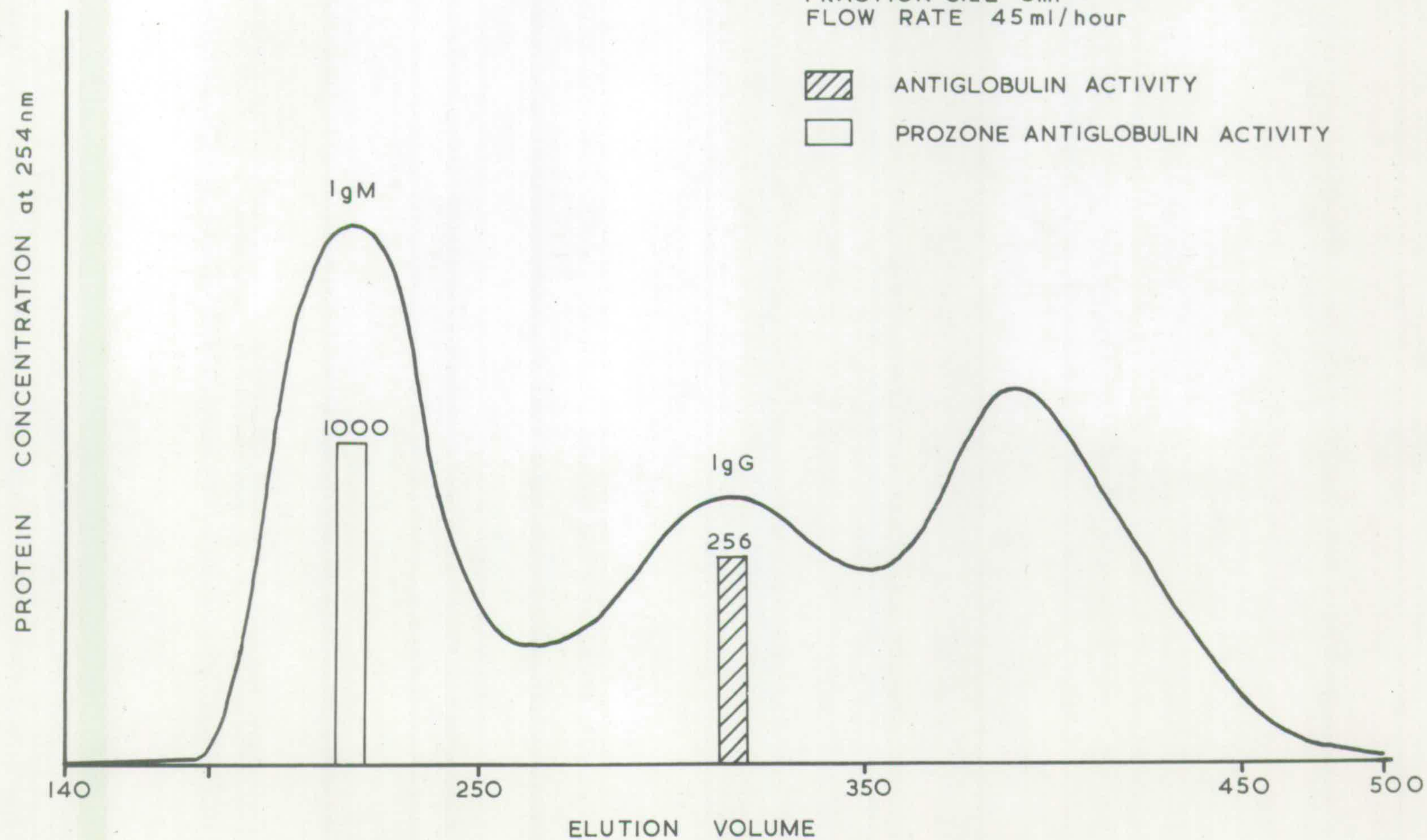
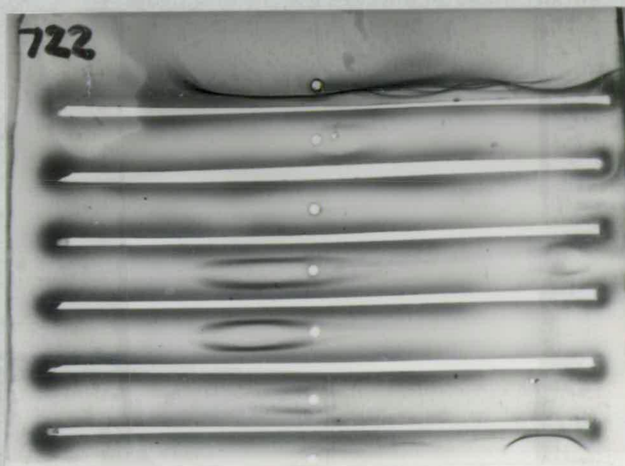


FIG. 2 a. G200 SEPHADEX FRACTIONATION of R4 (19/5/66)

DATE 3/12/69
SAMPLE 3ml SERUM
BUFFER 0·5M TRIS/HCl pH 7·3
COLUMN SIZE 68 cm X 7sq cm
FRACTION SIZE 5ml
FLOW RATE 45 ml/hour





whole serum R4

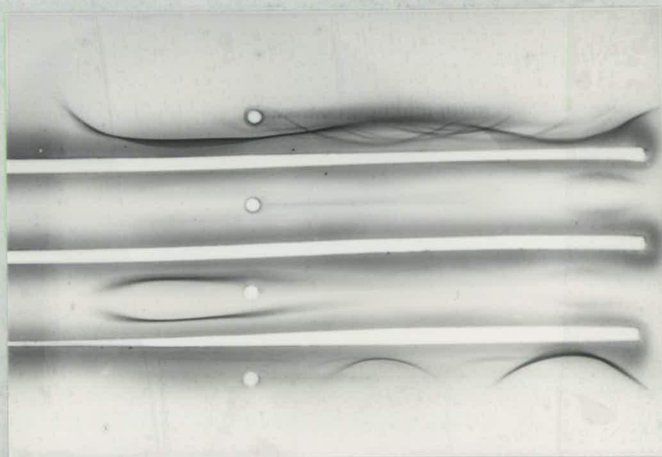
peak 1 IgG

peak 2 IgG

peak 4 IgM

peak 4 albumin and transferrin.

Fig. 1b <u>DEAE fractions of R4(19/5/66) using a sheep anti-rabbit serum in the troughs</u>



whole serum R4

peak 1 IgM

peak 2 IgG

peak 3 albumin and transferrin

Fig. 2b <u>G200 fractions of R4(19/5/66) using a sheep anti-rabbit serum in the troughs</u>

fractionated under exactly the same conditions (Fig. 3). This treatment, which specifically destroys disulphide bonds (Deutsch and Morton 1957), reduced the size of the first protein peak drastically. It is known that these double bonds occur in the structure of $19\bar{S}$ and when they are destroyed the $19\bar{S}$ loses its activity.

The pure $7\bar{S}$ and $19\bar{S}$ from all three fractionations were studied in haemolytic, agglutination and antiglobulin tests (Table I). Haemolytic activity was found in all the fractions tested, although it was much reduced from the whole serum titre of 1:5000. The $7\bar{S}$ generally gave a stronger reaction by one doubling dilution than the $19\bar{S}$ fraction, but there was no difference between the cells with high and low agglutination when tested with any fraction. In the direct agglutination tests activity was again found in all fractions. The whole serum had a complete reaction to a titre of 1:320 with cells with high agglutination, but this value was reduced to 1:80 after treatment of the serum with mercaptoethanol and iodoacetamide. This shows that the $19\bar{S}$ was active in the agglutination reaction and its titres were similar to that of the $7\bar{S}$. The cells with low agglutination were only agglutinable to a titre of 1:40 when whole serum was used and the titre was reduced to 1:20 after inactivation of the $19\bar{S}$ component. In the antiglobulin agglutination, the whole serum reaction with an agglutinable cell was complete to an antiglobulin titre of 1:2000. After the $19\bar{S}$ activity had been destroyed, the whole serum titre was reduced to 1:640. This finding, when considered together with the $7\bar{S}$ titre of 1:640 and the much lower $19\bar{S}$ titre, implies that in this serum the $19\bar{S}$ activity is not as important as the $7\bar{S}$ activity.

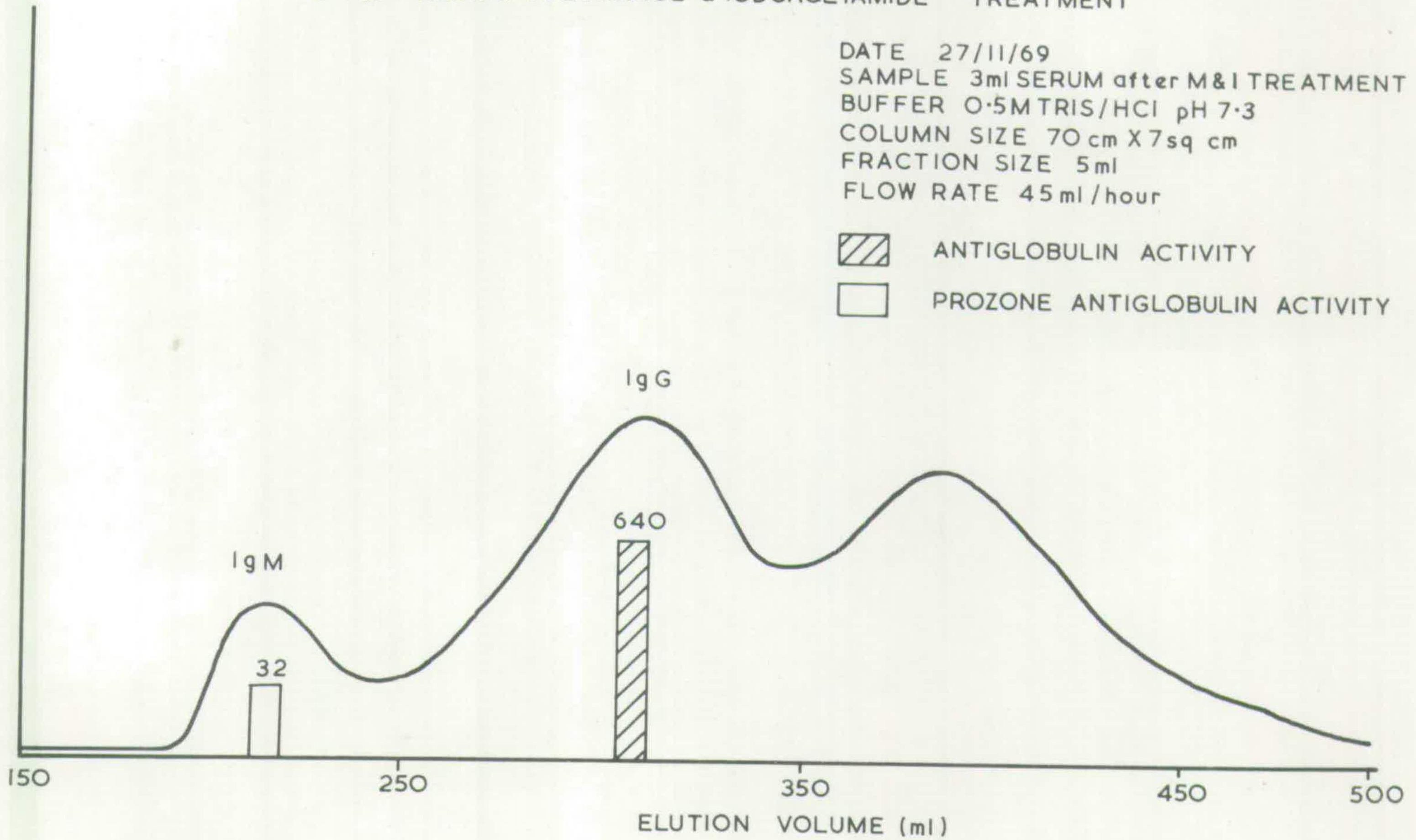


FIG. 3
G200 SEPHADEX FRACTIONATION of R4(19/5/66)
AFTER MERCAPTOETHANOL & IODOACETAMIDE TREATMENT
DATE 27/11/69
SAMPLE 3ml SERUM after M&I TREATMENT
BUFFER 0·5M TRIS/HCl pH 7·3
COLUMN SIZE 70cm X 7sq cm
FRACTION SIZE 5ml
FLOW RATE 45ml/hour
ANTIGLOBULIN ACTIVITY
PROZONE ANTIGLOBULIN ACTIVITY
PROTEIN CONCENTRATION at 254nm
IgM
32
IgG
640
150
250
350
450
500
ELUTION VOLUME (ml)

Table I **Titres obtained using fractions of R4 (19/5/66)**

Test	Agglutinability	whole serum	DEAE 7S	DEAE 19S	G200 7S	G200 19S	M&I treated whole serum	M&I treated 7S	M&I treated 19S
Haemolytic	High	1:5,000	1:64	1:32	1:128	1:64	1:320	1:32	1:16
	Low	1:5,000	1:64	1:32	1:128	1:64	1:320	1:32	1:16
Agglutination	High	1:320	1:16	1:8	1:32	1:32	1:80	1:8	1:6
	Low	1:40	1:4	1:2	1:16	1:8	1:20	1:2	0
Antiglobulin	High	1:2,000	1:640	1:256	1:1,000	P	1:640	1:640	P
	Low	0	<2	<2	<5	0	<5	<10	0

M&I - mercaptoethanol and iodoacetamide

P - prozone (see Table IV)

A sheep anti-bovine red blood cell serum 3A146 (17/12/69) (Fig. 4a and b) and a bovine isoantiserum MZ111B (1/8/68) (Fig. 5a and b) (see appendix B for sera production), were also fractionated to investigate the activity of their components in haemolytic and agglutination tests (Table II). In the sheep anti-bovine serum (3A146) the $19\overline{S}$ fraction showed very little haemolytic or direct agglutination activity. The whole serum and $7\overline{S}$ fraction both exhibited haemolytic activity but virtually no direct agglutination activity. In an antiglobulin test the whole serum (1:640) and both fractions (1:320) gave titres with cells of high agglutination but were negative with cells of low agglutination. The results with the bovine isoantiserum (MZ111B) were similar.

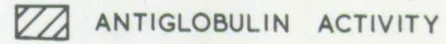
The antiglobulin serum used most frequently in this study was K21 (17/5/67). This was fractionated (Fig. 6a and b) and showed no activity with bovine cells, other than as the antiglobulin serum; the reaction with whole serum was much higher than with either the $7\overline{S}$ or $19\overline{S}$ components. (Table III).

Discussion

By fractionating the sera used as sensitisers in the antiglobulin test it has been shown that, while rabbit sera show haemolytic and agglutinating activity in all $7\overline{S}$ and $19\overline{S}$ fractions, sheep and bovine sera show activity only in the IgG ($7\overline{S}$) fraction, apart from antiglobulin activity which is again found in both fractions. Therefore both IgG ($7\overline{S}$) and IgM ($19\overline{S}$) are important in antiglobulin agglutinability. From the results of the sera tested here it would seem that IgM was less important than IgG, but these results are probably due to the sera having very little IgM. As has been shown previously by Gleeson-

FIG. 4a. G 200 SEPHADEX FRACTIONATION of 3A 146 (17/12/69)

DATE 16/12/70
SAMPLE 3ml SERUM
BUFFER 0·5M TRIS/HCl pH 7·3
COLUMN SIZE 68cm X 7sq cm
FRACTION SIZE 5ml
FLOW RATE 45ml/hour

 ANTIGLOBULIN ACTIVITY

whole serum 3A 146

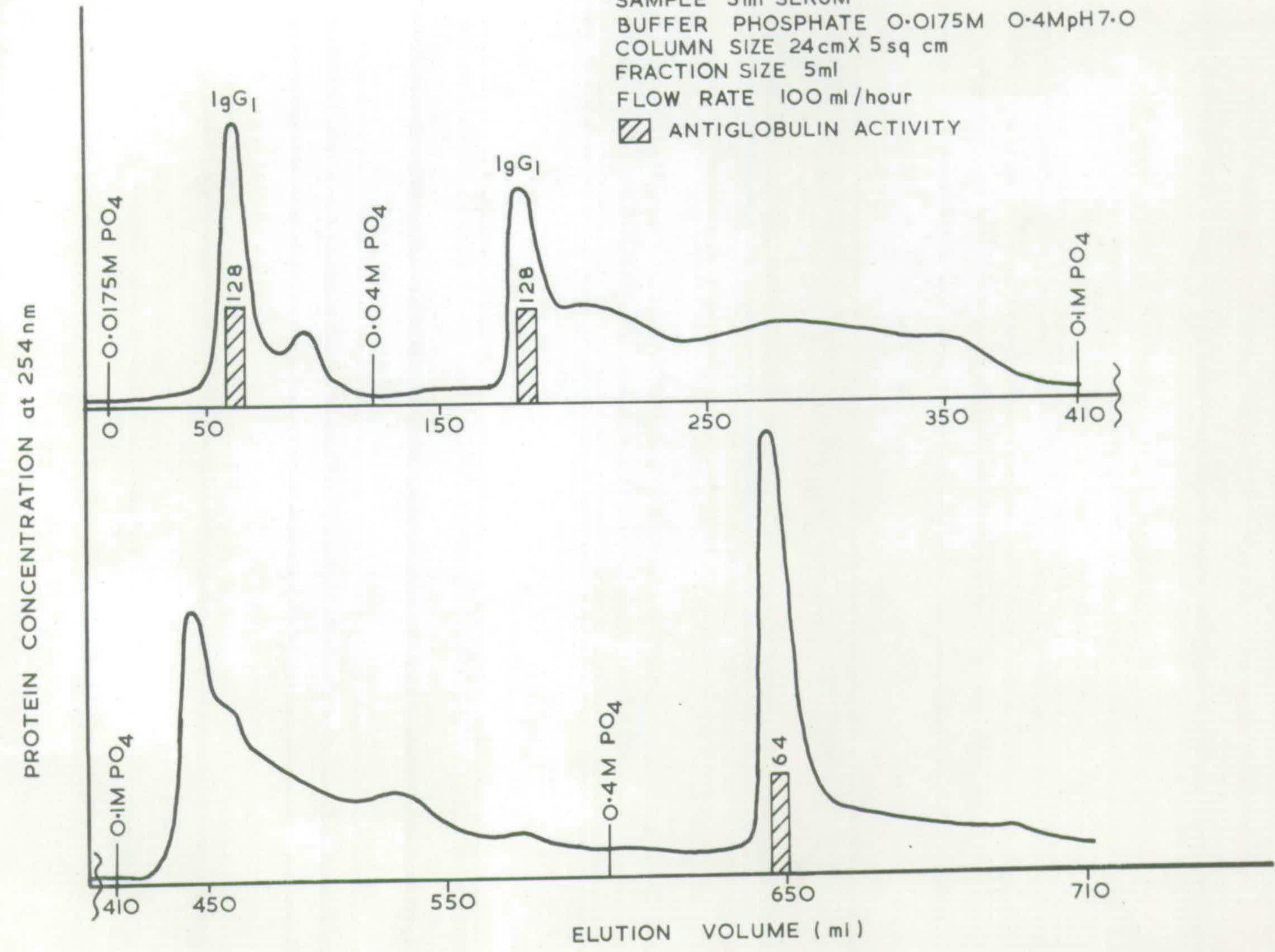
peak 1 IgM

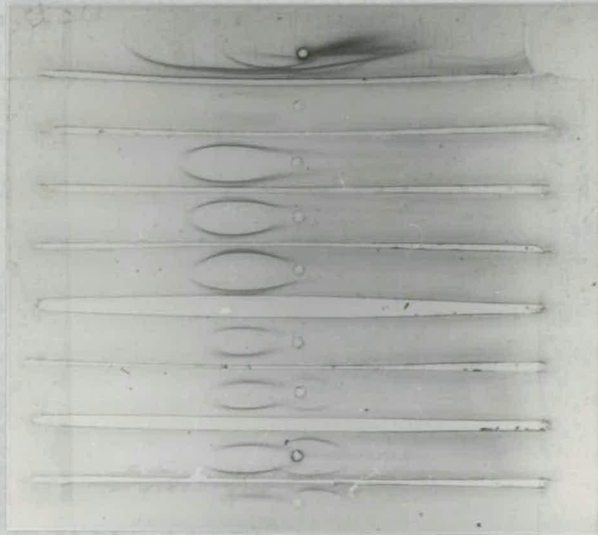
peak 2 IgG

peak 3 albumin and
transferrin

Fig. 4b <u>G200 fractions of 3A 146 (17/12/69) using a rabbit anti-sheep serum in the troughs</u>

FIG. 5a. DE 52 FRACTIONATION of MZ111B (1/8/68)





whole serum MZ 111 B

peak 1 IgG

peak 2 IgG

whole serum MZ 111 B

peak 4 IgM

peak 4 transferrin

Fig. 5b DEAE fractions of MZ 111 B (1/8/68) using a rabbit anti-bovine serum in the troughs

Test	Agglutinability	whole serum	7$\bar{S}$	19$\bar{S}$
Haemolytic	High	1:320	1:64	-
	Low	1:320	1:64	-
Agglutination	High	-	<1:5	<1:5
	Low	-	<1:5	-
Antiglobulin	High	1:640	1:320	1:320
	Low	<1:10	-	-

(a) Fractions of 3A146 (17/12/69) after G200 fractionation

Test	Agglutinability	whole serum	7$\bar{S}$	19$\bar{S}$
Haemolytic	High	1:320	1:16	-
	Low	1:320	1:16	-
Agglutination	High	-	1:4	-
	Low	-	1:4	-
Antiglobulin	High	1:1,000	1:128	1:64
	Low	<1:10	-	-

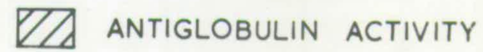
(b) Fractions of MZ111B (1/8/68) after DEAE fractionation

Table II

Titres obtained using fractions of (a) sheep anti-bovine serum 3A146 (17/12/69) and (b) bovine isoantiserum MZ111B (1/8/68)

FIG. 6a. G200 SEPHADEX FRACTIONATION of K21(17/5/67)

DATE 20/1/69
SAMPLE 3ml SERUM
BUFFER 0·5M TRIS/HCl pH 7·3
COLUMN SIZE 75cm X 7sq cm
FRACTION SIZE 5ml
FLOW RATE 45ml/hour



PROTEIN CONCENTRATION at 254nm

IgG

IgM

128

16

140 200 250 300 350

ELUTION VOLUME (ml)

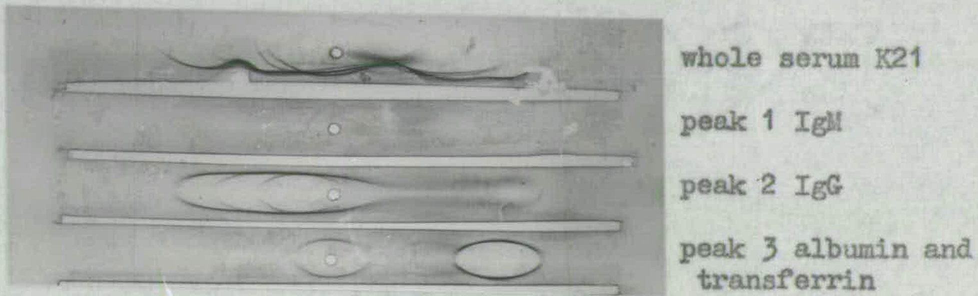


Fig. 6b <u>G200 fractions of K21 (17/5/67) using a sheep anti-bovine globulin in the troughs</u>

Test	Agglutinability	whole serum	$7\bar{S}$	$19\bar{S}$
Haemolytic	High	-	-	-
	Low	-	-	-
Agglutination	High	-	-	-
	Low	-	-	-
Antiglobulin	High	1:5,000	1:128	1:16
	Low	-	-	-

Table III

Titres obtained using fractions of K21 (17/5/67) after G200 fractionation

White *et al.* (1950) using infectious mononucleosis sera as sensitisers in antiglobulin tests, pure IgM alone can cause antiglobulin agglutination. It is concluded, therefore, that both IgG and IgM can be effective antibodies in the antiglobulin test, although one or other may be of greater importance depending on the particular serum and its species. A prozone effect was shown by the $19\bar{S}$ fraction of the serum R4 (19/5/66) (see Table IV). This took the form of a partial reaction (a reading of 3) at antiglobulin titres from 1:16 to 1:256. This was a true prozone reaction and not a low-titre reaction, as the results were not affected by varying the concentration of rabbit sensitising serum. There are two possible explanations for a prozone reaction (Van Loghem *et al.*, 1950). In the first case, if antiglobulin is present in excess, then by increasing the number of combining sites available, agglutinations could occur. This is unlikely to be the explanation, as equal volumes of sensitised cells and antiglobulin serum were used throughout the tests and only in this one case was prozoning found. When the sensitised red-cell concentration was increased by a further 0.5% the result was the same as before.

The second possibility is that the antiglobulin serum may contain a second incomplete form of antibody, of lower titre, than the actual agglutinating antibody. The combination of this second incomplete antibody with the sensitised cells could produce a partial reaction at some dilutions. In the presence of the actual agglutinating antibody, for example when the whole serum is used, the minor reaction of the low titre antibody is masked. This seems to be the probable cause of the prozone reaction.

Reciprocal dilutions of K21 (17/5/67)

Animal	4	8	16	32	64	128	256	512	1T	2T	4T	8T	16T	C
FS101A	-	-	3	3	3	3	3	2	1	-	-	-	-	-
HZ135A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MZ308A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
U85A	-	2	3	3	3	3	3	1	-	-	-	-	-	-
U85B	-	-	3	3	3	3	3	1	-	-	-	-	-	-
U93B	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table IV <u>Protocol of a prozone in an antiglobulin test using the 19S fraction of a G200 fractionation of R4 (19/5/66) as sensitiser</u>

The two classes of bovine red cell could be explained by suggesting that, in the case of the inagglutinable cells, the antibody was not long enough to span the distance between the two cells. This does not mean that the length of the antibody varied but that the distance between receptor sites of the two classes of cell did. As stated earlier Coombs <u>et al</u>. (1951), suggested that the receptor sites in inagglutinable cells may be in pits and that the length of the antibody-antiglobulin chain is less than the distance between the sites (see Fig. 7), so that the antibody cannot join the two cells. In the case of agglutinable cells, the distance between the sites, which are exposed and not in pits, is shorter than the length of the antibody-antiglobulin chain, so that the two cells can be joined.

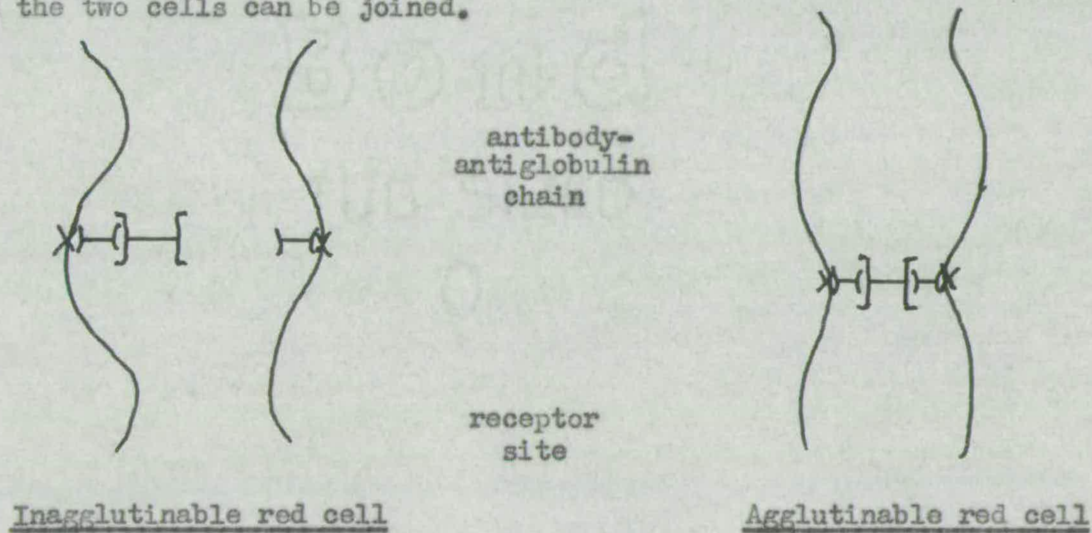


Fig. 7
<u>Positions of receptor sites on inagglutinable and agglutinable red cells</u>

These pits need not be large, as the $7\bar{S}$ antibody is only about 10 nm in length. The positioning of the sites on each class of cell is most important. If the suggested pits are the explanation of the differential agglutinability,

it is possible that observation of the position of the cell sites would immediately ascertain the class of cell being studied.

Another possible explanation of the differential agglutinability of bovine erythrocytes is that the sialic acid content which is correlated with the cell agglutinability, could, in the case of the inagglutinable cells, be hindering agglutination. If insufficient sialic acid is present to form a continuous dense band, it may occur as a branched chain structure. The low agglutinable cells which contain larger amounts of sialic acid may have a multi-branched structured which does not prevent the sensitisation of the cells with antibody, but prevents two cells from getting close enough to agglutinate, even with the antiglobulin antibody (Fig. 8). The high agglutinable cells have small amounts of sialic acid and therefore a simple sialic acid structure, so that the antibody-antiglobulin-antibody can extend easily between two such cells. Therefore the shortest distance between two high agglutinable cells is much less than that between two inagglutinable cells.

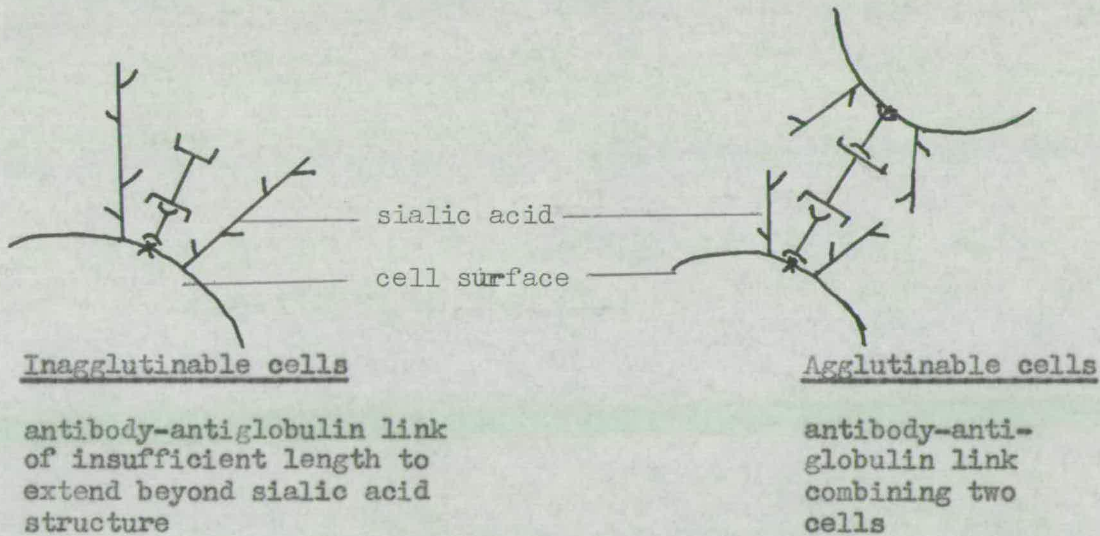
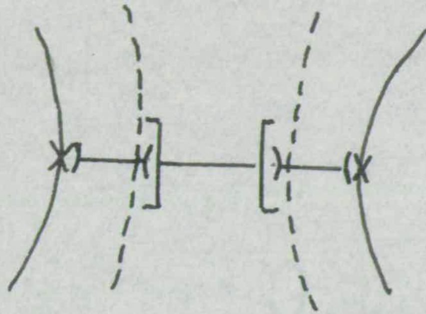


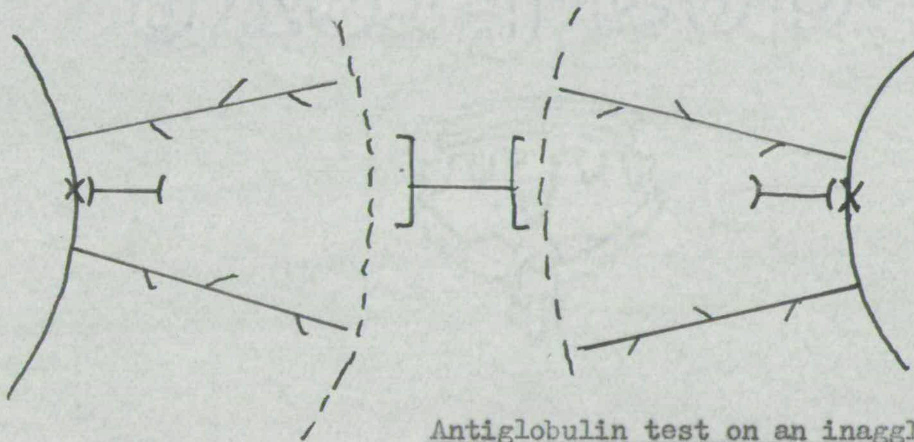
Fig. 8 Steric hindrance around inagglutinable and agglutinable red cells

The cause of inagglutination could be a combination of these two possibilities. The receptor sites of the inagglutinable cells may be in pits in which the sialic acid chains are also situated.

If either of these possibilities is correct then it should be possible to convert low agglutinable cells into high agglutinable cells. Coombs et al. (1951) succeeded in agglutinating inagglutinable cells; they built up a lattice structure of alternate antiglobulin serum and globulin on to the inagglutinable cells. By this device they built up the receptor sites (Fig. 9) until they were outside the effective cell surface and protruded from the pits or the branched chains of the sialic acid. The free antibodies combined in turn with more antiglobulin serum and the cells were agglutinated. The lattice of Coombs et al. consisted of bovine red cells, sensitised with Paul Bunnell antibody, which were treated alternatively with rabbit anti-human globulin serum and human γ-globulin. A similar lattice has now been built up here using red cells sensitised with rabbit anti-bovine antibody and then alternate treatments of bovine anti-rabbit globulin and normal rabbit serum. After two double treatments the inagglutinable cells were agglutinated to the same titre of antiglobulin serum as the high agglutinable cells, which did not increase in titre of agglutination (Table V). The agglutination obtained by building up a lattice was not due to the sensitised cells absorbing the antiglobulin-globulin complex, as no agglutination was found when other species of antiglobulin and globulin were used. Therefore there must be specific combination between the sensitised cells and the appropriate antiglobulin serum before agglutination occurs.



Antiglobulin test on an agglutinable cell



Antiglobulin test on an inagglutinable cell

Fig. 9 (i)

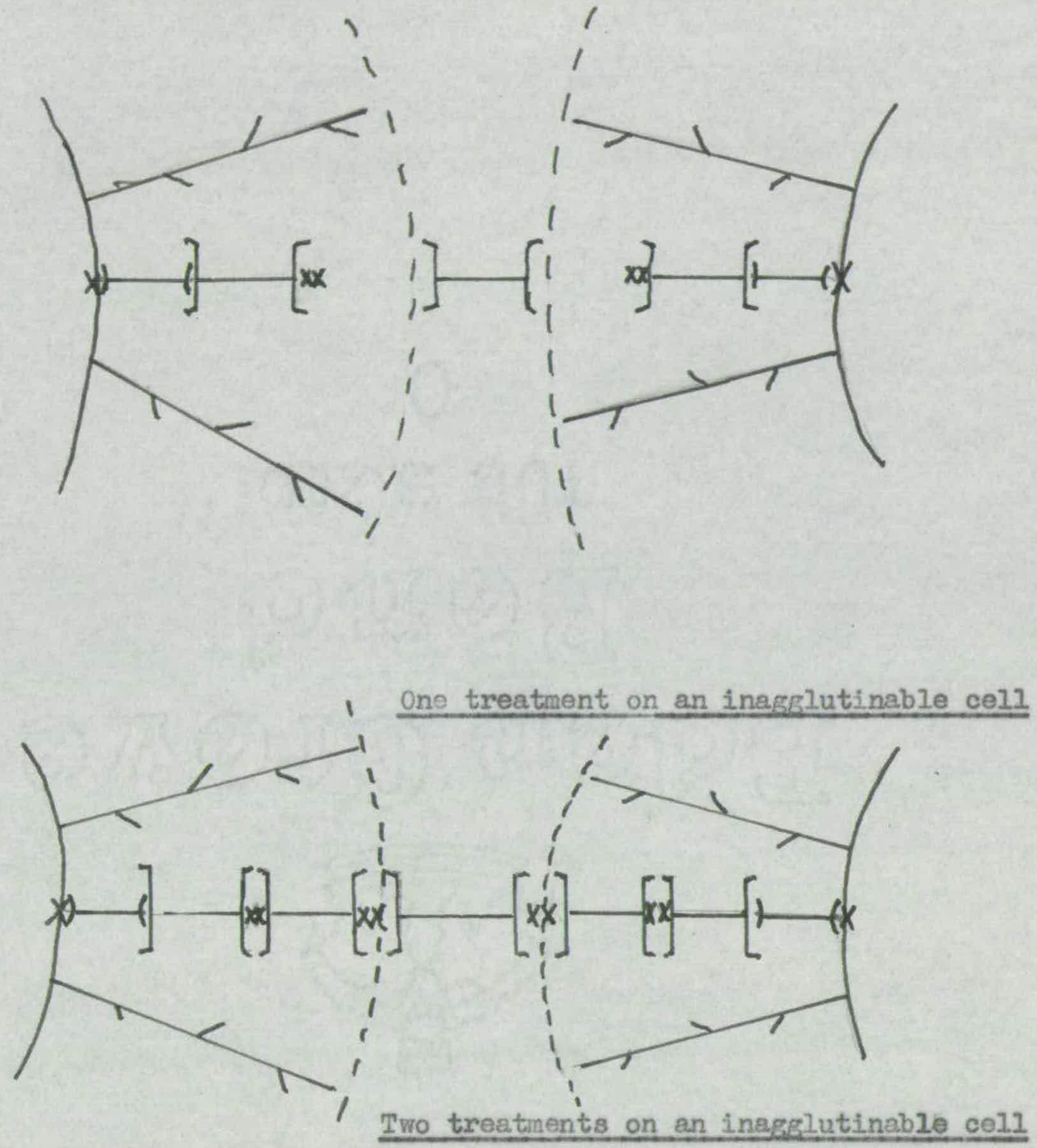


Fig. 9(ii) Theoretical structure of antiglobulin-globulin lattice

Key:-

- X receptor site on red cell membrane
- sensitising antibody
- antiglobulin
- XX globulin
- steric hindrance
- limit of cell beyond which no hindrance

Treatment	Agglutinable cell - U85B													Inagglutinable cell - MZ308A												
Reciprocal dilutions of R4 (19/5/66)	10	20	40	80	160	320	640	1T	2T	5T	10T	SC	NBS	10	20	40	80	160	320	640	1T	2T	5T	10T	SC	NBS
Haemolytic test	5	5	5	5	5	5	5	5	5	5	5	-	-	5	5	5	5	5	5	5	4	2	-	-	-	-
Direct agglutination	4	4	4	4	4	4	4	4	3	3	-	-	-	4	4	4	3	3	2	-	-	-	-	-	-	-
Reciprocal dilutions of K21 (17/5/67)																										
Antiglobulin test sensitised cells + antiglobulin	4	4	4	4	4	4	4	4	3	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sensitised cells + 1 treatment of antiglobulin and antibody	4	4	4	4	4	4	4	4	4	4	-	-	-	3	3	-	-	-	-	-	-	-	-	-	-	-
Sensitised cells + 2 treatments of antiglobulin and antibody	4	4	4	4	4	4	4	4	4	4	-	-	-	4	4	4	4	4	4	4	4	3	3	-	-	-

SC - saline control

NBS - normal bovine serum

Table V <u>Reactions during the building up of an antiglobulin - globulin lattice</u>

It has been proved that all bovine erythrocytes can be made agglutinable and therefore the cells must all possess the particular antigen necessary, although normally it is not apparent in the inagglutinable cells. Therefore there must be some differences in the site of the antigen receptors in the two classes of cell, i.e. they must be at effectively different levels in the cell membrane.

Chapter 6 Electron microscopic studies on the bovine red cell membrane

High resolution microscopy has been employed here to detect any morphological differences which may exist between the membranes of the two classes of bovine red cell. From these studies it was hoped to show the presence or absence of pits in the membrane as hypothesised by Coombs et al. (1951) and reported in Chapter 5. The possibility that other differences might exist between the inagglutinable and agglutinable cells was also examined. Easty and Mercer (1962), in a survey of electron micrographs of sections of agglutinable rat erythrocytes, demonstrated two extreme forms of agglutination, which were distinguished by the closeness of contact of the cells. As described in Chapter 2, two extremes of bovine red cell agglutinability have been found using the antiglobulin test; the electron microscope studies reported here were undertaken to show the nature and extent of the adhesion between the cells of each type. Specific antibodies to erythrocytes have been identified and located using ferritin-labelled globulins (Harris, 1964; Lee, 1963); it is known that the sensitising serum antibody attaches itself to the receptor sites of the red cell, and therefore it was decided to try and count the number of these sites by labelling the serum with ferritin and then treating the cells with the labelled serum. The red cells were too dense for much structure to be visible under the electron microscope, and so it was necessary to use ghosts. Initially, the ghosts were negatively stained, but in later studies thin sectioning techniques were employed. Pure $7\bar{s}$ and $19\bar{s}$ immunoglobulins of human and rabbit have been demonstrated by electron microscopy (Feinstein and Rowe 1965; Feinstein and Munn 1969) and it was hoped to gain more information about the antigen-antibody link by labelling these fractions of the sensitising serum.

Materials and Methods

Ghosts in antiglobulin and direct agglutination tests

A 2% suspension of packed ghosts was tested by the direct agglutination and antiglobulin test in exactly the same way as were the whole red cells. Drops of the test samples were placed on microscope slides and were read under the light microscope.

Preparation of ghosts for the electron microscope

The ghosts were prepared by the method described in Chapter 2 for whole red cells, 1 ml. im M calcium chloride solution per 50 ml. ghosts being added to prevent the ghosts from fragmenting and producing stromolytic forms. After preparation in the usual phosphate buffers (0.01 M pH 8.0) the ghosts were centrifuged at 18,000g for 15 min. and resuspended (1:200) in 0.01 M acetate buffer (pH 7.0). Acetate buffer was used as ammonium acetate being highly volatile, does not crystallise out as the phosphate would have done under electron microscopic examination.

Electron Microscope

Most of the microscopy was performed with an AE 1 6B electron microscope using a 60kV accelerating voltage with a 50µ objective aperture. Later microscopy was performed using a Siemens Elmiskop 1A microscope at 80kV with a 50µ objective aperture. In both cases Ilford Special Contrasty Lantern plates were used.

Negative staining

2% (w/v) ammonium molybdate, pH 7.0 was used as a negative stain.

One drop of the ghost sample was placed on a copper specimen grid carrying a carbon support film and two drops of stain were added to it. Excess solution was drawn off with filter paper and the grid was then ready for microscopic examination.

Ferritin labelling

The sensitising antibodies were first precipitated from R60 (17/6/69) with 50% saturated ammonium sulphate, (1 vol. serum : 1 vol. $(NH_4)_2SO_4$), and the precipitate was washed twice with 50% saturated ammonium sulphate. The serum was found to contain 3% antibody. Twice-crystallised ferritin (Koch-Light) was diluted with 0.1M-borate buffer to obtain 20mg. ferritin/ml. and was complexed with toluene - 2,4-di-isocyanate (0.1 ml./15 ml. diluted ferritin) and this was conjugated with the antibodies (4 parts ferritin : 1 part antibody), as described by Hsu (1967) after Singer and Schick (1961). The conjugate was washed four times with 0.01 M-acetate buffer at pH 7.0 to remove excess unbound ferritin. The active complex was added to a 2% suspension of packed ghosts (1:1) and the samples were examined.

Thin-sectioning

After a 2% suspension of ghosts in 0.01M ammonium acetate pH 7.0 had been prepared the ghosts were centrifuged out as a pellet at 18,000g for 15 min. and fixed overnight with 1 ml. 2% (w/v) buffered osmium tetroxide solution (pH 7.2). They were then dehydrated in alcohol of increasing concentrations and embedded in araldite, (Glauert and Glauert 1958). Thin sections were cut on a Porter-Blum microtome and were stained with a drop of lead hydroxide solution before examination under the electron microscope.

Results

Antiglobulin test on ghosts

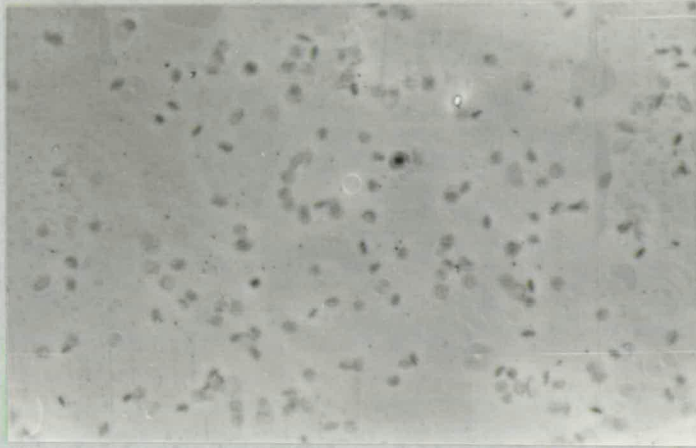
Red cell ghosts were tested to show that the agglutinable character of the cell lay in the membrane. Antiglobulin tests using ghosts were read under the light microscope and readings of 0 (negative) to 4 (complete agglutination) were recorded as for whole red cells (Fig. 1 and Table I). In the antiglobulin tests the ghosts gave the same results and titres as red cells from the same animals.

Negative staining

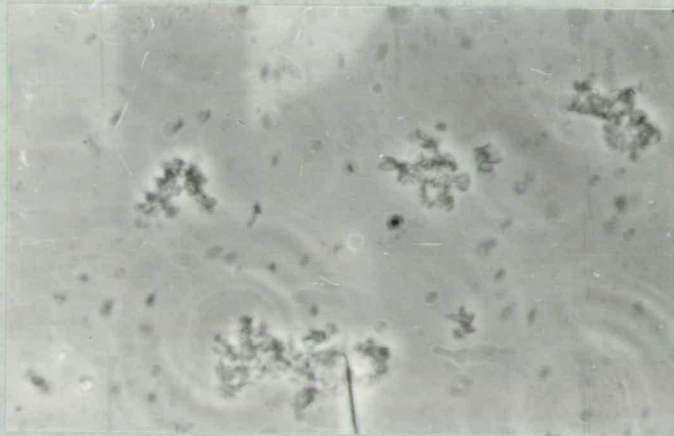
Using negative staining of the two classes of ghost with 2% (w/v) ammonium molybdate pH 7.0 no difference could be discerned in the membrane at x 60,000 magnification. Inagglutinable and agglutinable ghosts respectively, after an antiglobulin test shown in Figs 2 and 3. It can be seen that the agglutinable cells are much closer to each other than are the inagglutinable ones. The inagglutinable cells remain rounded, often separated by considerable gaps. In many instances the ghosts had dried out on top of each other and so their agglutinability could not be estimated. The agglutinated cells were largely in close contact with each other, and this led to some distortion of the cell shape from the normal rounded form. The average gap found between the agglutinated ghosts was in the range of 58-135nm. (Table II), as measured on the micrograph (Fig. 3).

Ferritin conjugates

After the ferritin was conjugated with the antibodies in the



(a)



(b)

Fig. 1 <u>Inagglutinable red cell ghosts (a)</u> and <u>agglutinable red cell ghosts (b)</u>

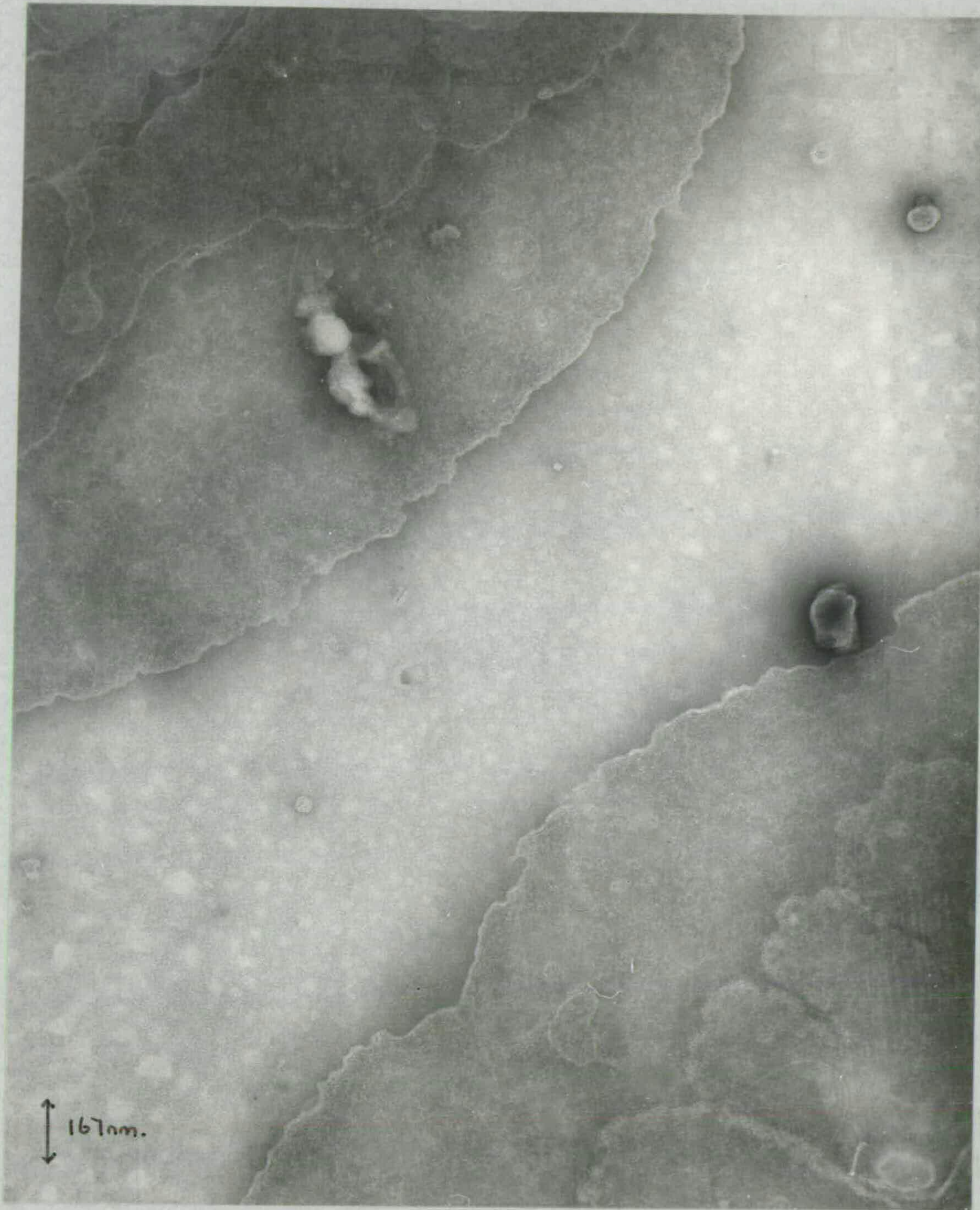


Fig. 2 Inagglutinable red cell ghosts negatively stained with 2% ammonium molybdate after an antiglobulin test, x 60,000

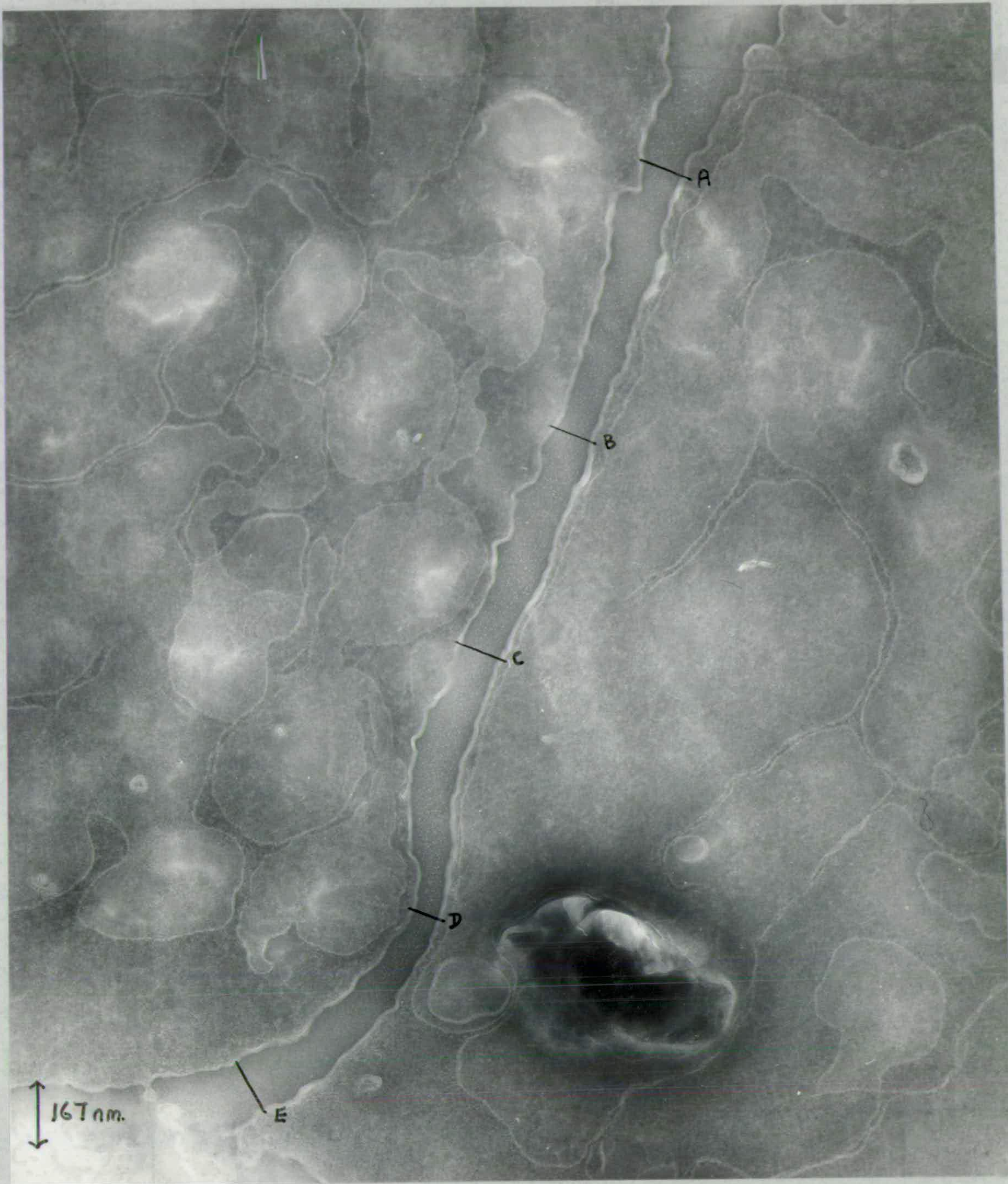


Fig. 3 Agglutinable red cell ghosts negatively stained with 2% ammonium molybdate after an antiglobulin test x 60,000

Position	Distance
A	83 nm.
B	83 nm.
C	100 nm.
D	58 nm.
E	134 nm.

Table II Distances separating two high-agglutinable red cell ghosts (see Fig. 3)

sensitising serum, the serum was subjected to agglutination and haemolytic tests (Table III): it was shown that ferritin conjugation did not interfere with the reaction, and therefore that ferritin could be used satisfactorily as a labelling agent. The specificity and purity of the antibody conjugated to ferritin was checked by immunoelectrophoresis (Fig. 4). Ferritin has a greater electrophoretic mobility than the immunoglobulin, which it confers to the immunoglobulin when they are conjugated. The intermediate ferritin-toluene complex has an even greater mobility than the ferritin, therefore any unconjugated ferritin or immunoglobulin can be detected, (Howe et al. 1969). Also, any contamination with the intermediate complex can be demonstrated. Prior treatment of ghosts with unconjugated serum completely inhibited binding of subsequent conjugated serum.

When an early ferritin-antibody conjugate was negatively stained it was found to be impure containing an unidentified protein molecule; it was very similar in appearance to ferritin but was slightly larger and had a smaller central core (Fig. 5). This protein also tended to clump forming cylindrical structures. In later pure preparations of conjugate ferritin molecules were sometimes found to be bound singly to the membrane, but more often they were found in small clumps apparently attached to various points on the cell membrane. These molecules were presumed to be bound to the membrane as repeated washing did not reduce the number of molecules which appeared to be in contact with the membrane, but it did reduce the number found in the background. As a control, cells were treated with unconjugated antibody and then ferritin. These samples, when examined, showed an insignificant number of ferritin molecules and so it was assumed that every

Reciprocal dilutions of K21 (17/5/67)

	4	8	16	32	64	128	256	512	1T	2T	5T	C
U85A	4	4	44	4	4	4	4	4	4	4	-	-
U93B	-	-	-	-	-	-	-	-	-	-	-	-
FS101A	4	4	4	4	4	4	4	4	4	4	4	-
HZ141B	-	-	-	-	-	-	-	-	-	-	-	-
HZ123B	4	4	4	4	4	4	4	4	4	4	2	-
HZ90B	4	4	4	4	4	4	4	4	4	4	2	-
MZ111B	-	-	-	-	-	-	-	-	-	-	-	-
MZ308A	-	-	-	-	-	-	-	-	-	-	-	-

Table III (a) Antiglobulin test using ferritin-conjugated sensitising antibody R60 (17/6/69) and red cell ghosts

	4	8	16	32	64	128	256	512	1T	2T	5T	C
U85A	5	5	5	5	5	5	5	5	5	5	5	-
FS101A	5	5	5	5	5	5	5	5	5	4	2	-
U93B	5	5	5	5	5	5	5	5	5	5	3	-
HZ141B	5	5	5	5	5	5	5	5	5	4	2	-

Table III (b) Haemolytic test using ferritin-conjugated serum and ghosts

	4	8	16	32	64	128	256	512	1T	2T	5T	C
U85A	4	4	4	4	4	4	4	4	4	3	-	-
FS101A	4	4	4	4	4	4	4	4	4	4	1	-
U93B	4	4	4	4	4	4	3	1	-	-	-	-
HZ141B	4	4	4	4	4	4	1	1	-	-	-	-

Table III (c) Agglutination test using ferritin-conjugated serum and ghosts

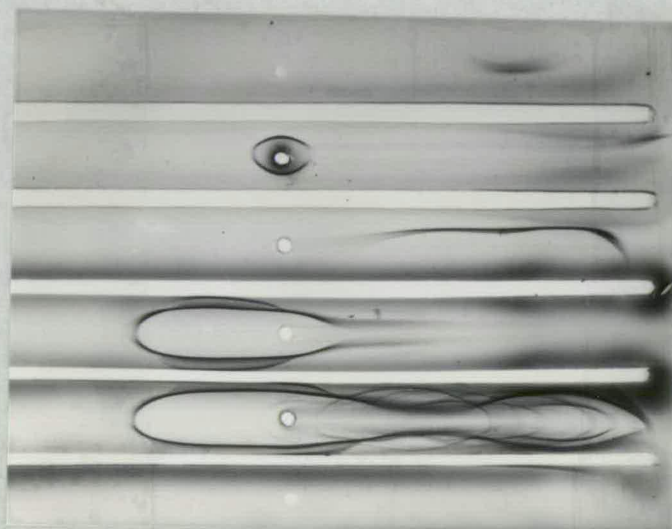


Fig. 4 An immunoelectrophoretic test showing the stages of conjugation of ferritin and the purity of the conjugate

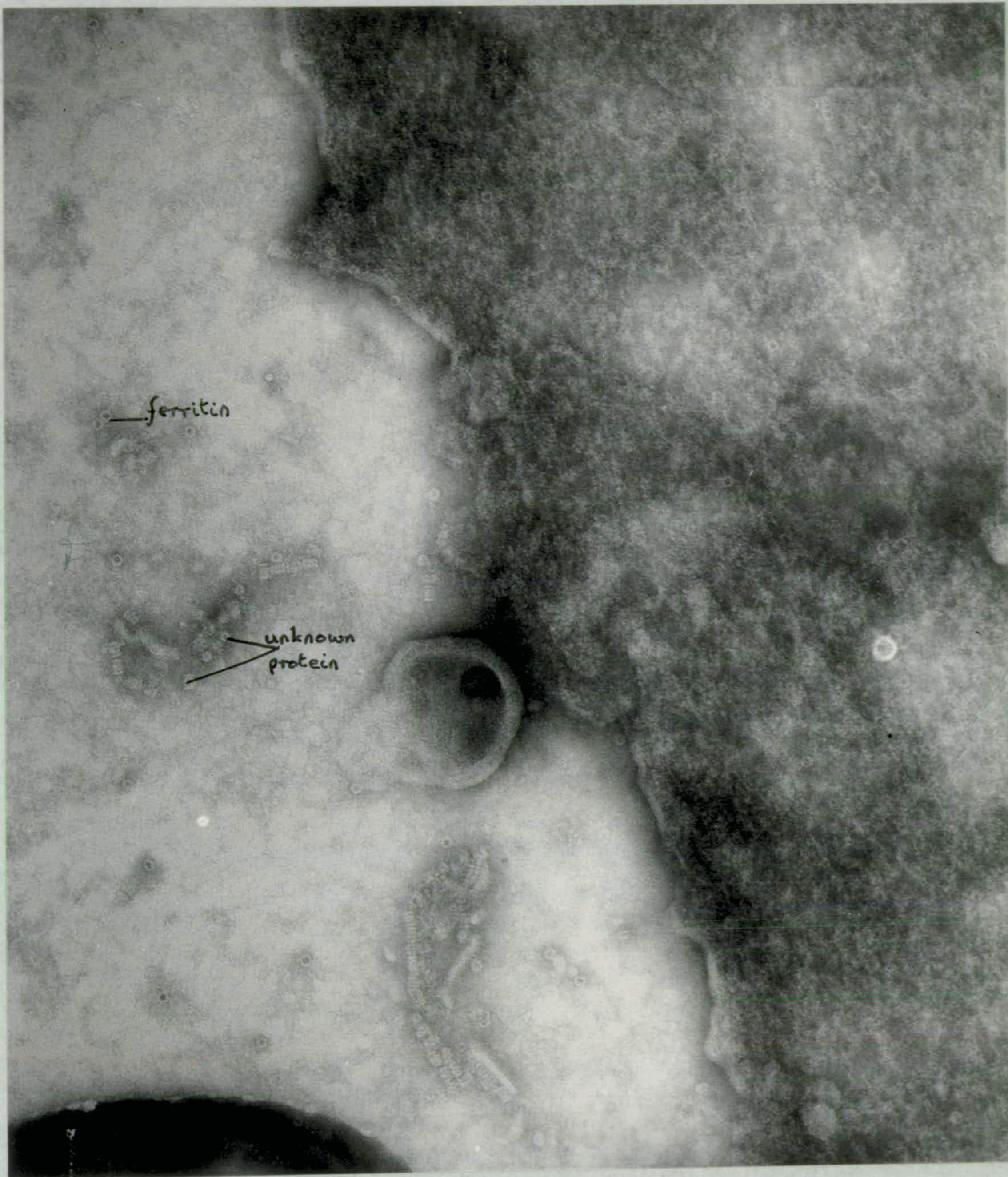


Fig. 5 Agglutinable red cell ghosts stained with 2% ammonium molybdate after treatment with ferritin conjugated rabbit IgG. x 200,000.

molecule or clump of molecules found bound to the membrane represented a receptor site.

Negative staining of the ghosts was unsuitable for counting the number of receptor sites, and so thin-sectioning techniques were employed which had the advantage of showing if the ferritin molecules entered the membrane, thus preventing misleading results.

Thin-sectioning

Samples prepared as thin sections showed ferritin only on the outside of the membrane (Fig. 6), thereby proving that the ghosts were still intact and that no molecules had passed through the membrane. The number of molecules found on a measured length of membrane was counted for each of the two cell classes and was found to be similar (Figs. 7a and b and 8 and Table IV).

Discussion

The distance of 30-60 nm. between agglutinable ghosts may not be the true distance as the exact periphery of the membrane is uncertain, the outer carbohydrate layer not being visible under these conditions (Glauert 1970, private communication), and the cells may in fact have been closer together than is shown. Recently the existence has been reported of stains which probably stain the carbohydrate layer around the cell, but these have not yet been fully investigated and were not used here.

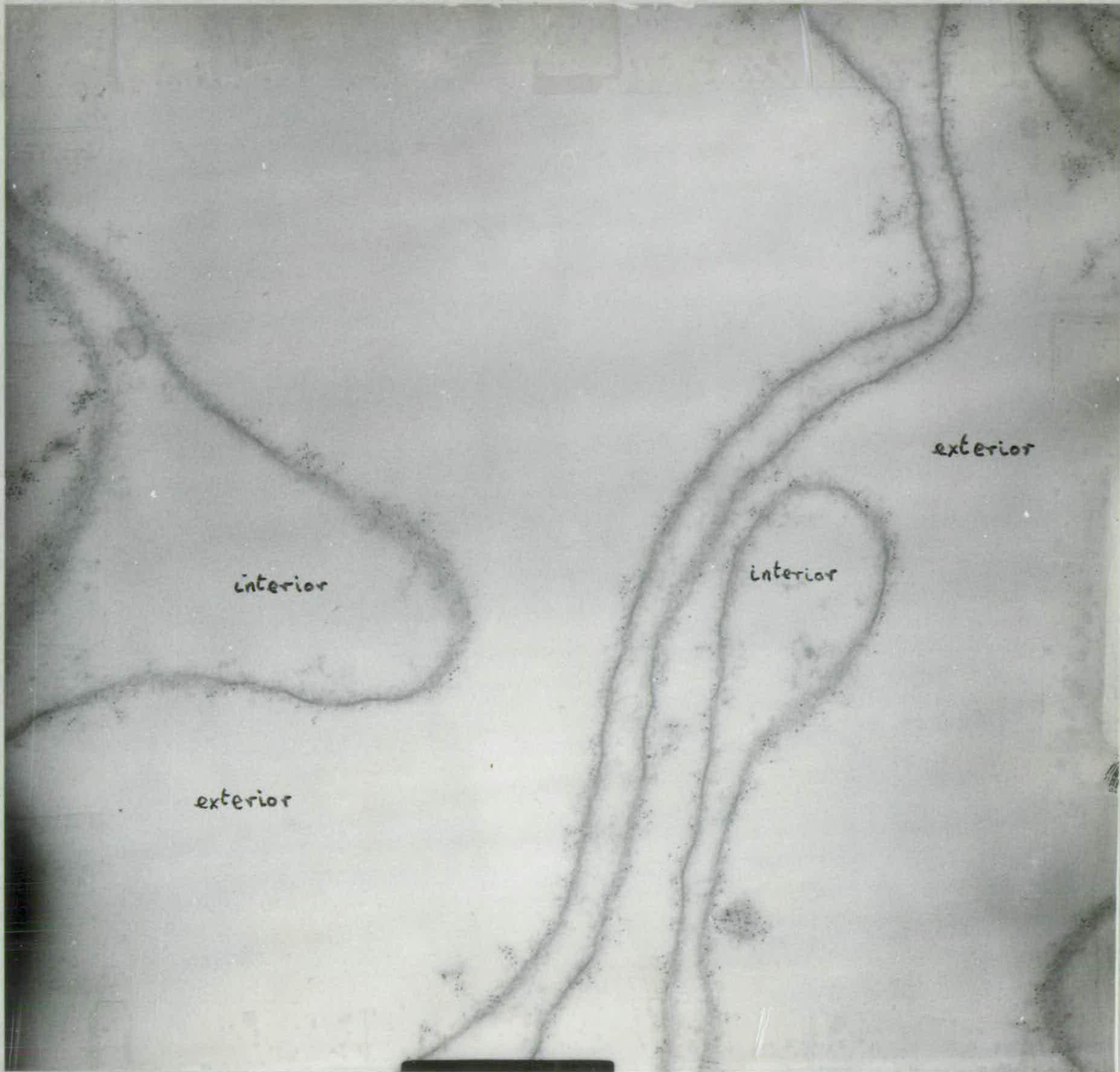


Fig. 6 Thin section of agglutinable red cell ghost, after treatment with ferritin conjugated rabbit IgG, showing ferritin molecules only on the exterior of the membrane. x 50,000

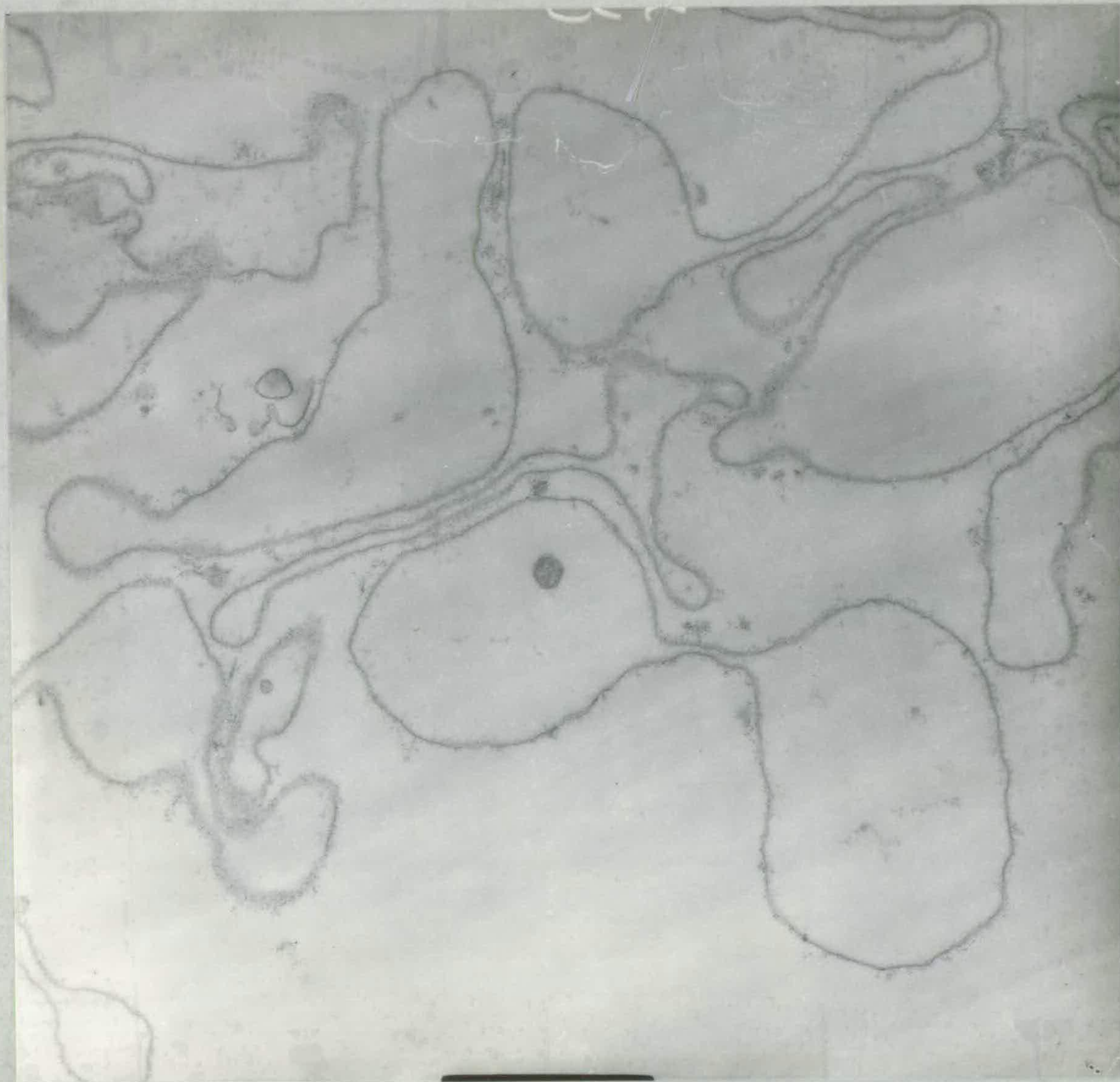


Fig. 7a Thin section (low power) of inagglutinable red cell ghosts after treatment with ferritin conjugated rabbit IgG, showing bound ferritin molecules. x 20,000

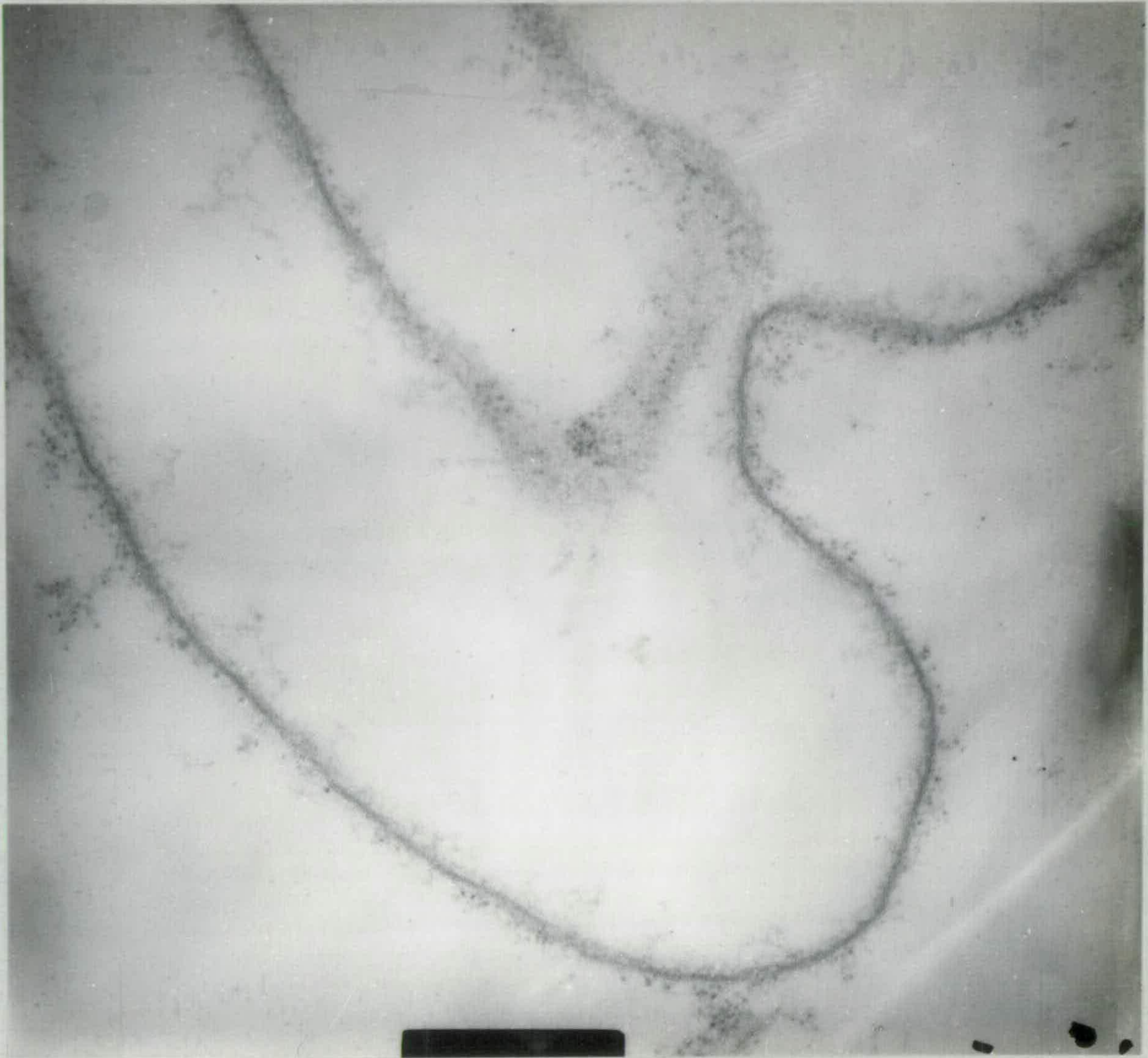


Fig. 7b Thin section (high power) of inagglutinable red cell ghosts after treatment with ferritin conjugated rabbit IgG, showing bound ferritin molecules. x 100,000

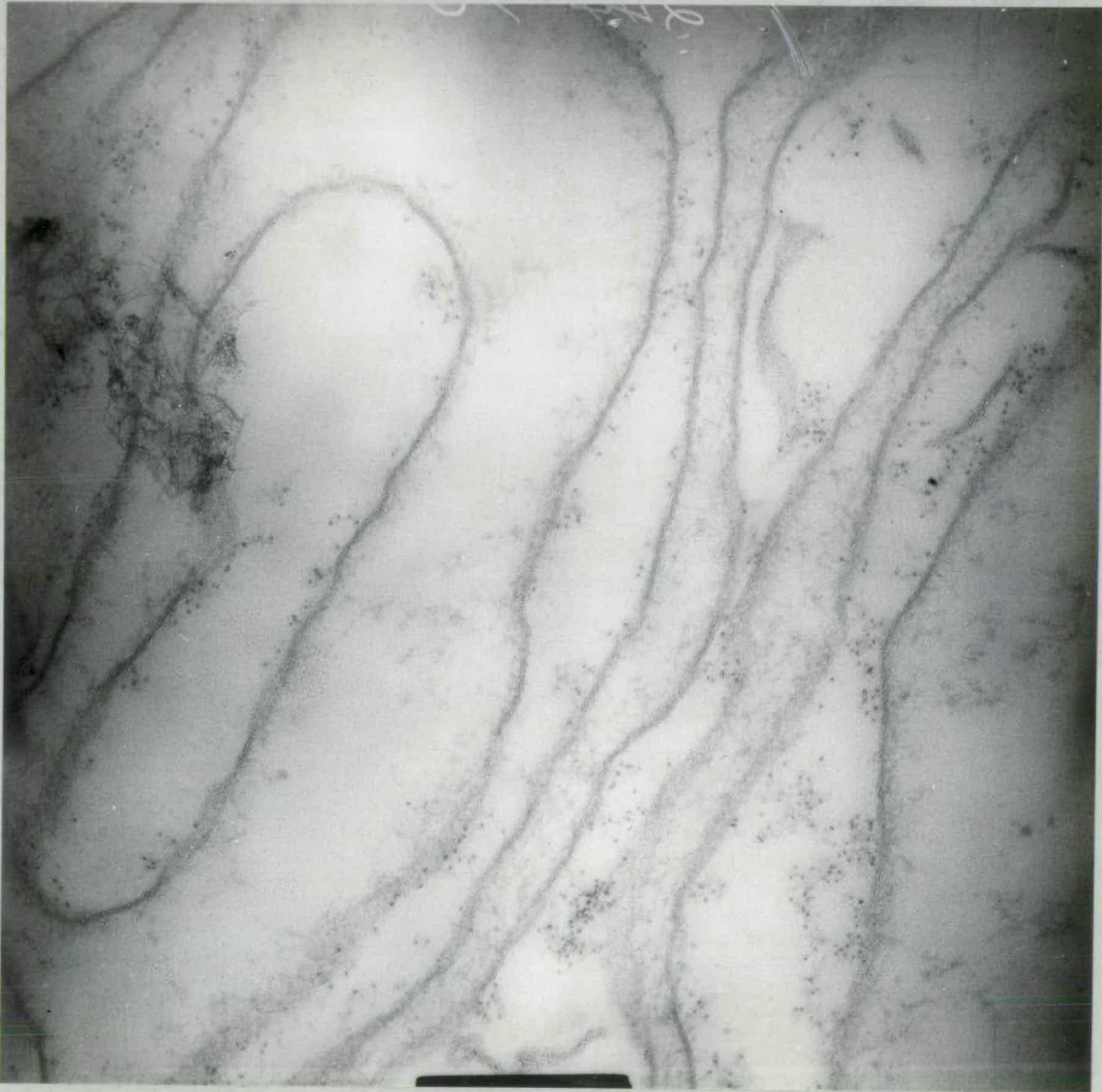


Fig. 8 Thin section of agglutinable red cell ghosts after treatment with ferritin conjugated rabbit IgG, showing bound ferritin molecules. x 100,000

Number of ferritin molecules on 7cm.

High-agglutinable cell membrane	Low-agglutinable cell membrane
13	14
17	13
16	18
14	15
16	18

Table IV Comparison of the number of ferritin molecules found bound to 7cm. lengths of high- and low-agglutinable ghost cell membranes

Easty and Mercer (1962) concluded from work involving rat and human red cells, that the agglutination process began with similar changes in the surface properties of both the agglutinable and inagglutinable cells and that this led to a change in the physico-chemical conditions. The cell membranes of inagglutinable cells appeared to be inadhesive, whereas the highly agglutinable cells were obviously adhesive and the adhesive force was bringing these cells into close contact and in so doing distorting the membrane shape. A similar result has been obtained in the present work and it is shown in Figs. 1 and 3; there is a fairly uniform, less dense layer between the adhering membranes. This layer can be stained with lead salts, but even under these conditions it was found by Easty and Mercer (1962) to be structureless. The presence of such a uniform layer implies that the cells already have an outer component external to the phospholipid-protein complex. Under microscopic examination the cells were seen to have a diffuse layer external to the individual cells and so it was assumed that the layer was always present. If these layers were always present, but the cells were not normally adhesive, then it is probable that the layer was in fact the layer containing the sialic acid and that the rabbit antibodies cross-link between these layers on separate cells thereby causing agglutination.

Examination of thin sections of the ferritin-labelled cells showed that the ferritin molecules lay a short distance from the visible membrane. This distance was not always equal, and was probably due to the combining antibody. There was no difference in the number of ferritin molecules on the two classes of cell indicating that there was an equal number of receptor sites on each class. If it had been possible to prepare specific antisera

reflecting the difference between the two classes of cell then a difference in the number of receptor sites if it exists might have been demonstrated. There are many antigens common to both types of cell and so a quantitative difference in this one antigen could well have been masked by the other common antigens. In this study no ferritin molecules were found within the cell membrane. Of all the samples examined in the electron microscope none showed evidence of pits in the membrane surface. It is however still possible that when more of the external erythrocyte structure is revealed pits may be found.

In preliminary studies using whole bovine red cells treated with rabbit antisera, projections of the membrane from the main cell body were seen. These were always indistinct and their origin in the cell body obscure (Fig. 9). They were dissimilar to the stromolytic forms described by Furchgott, (1940) and found in some early preparations of ghosts, and the control cells were normal, with no projections. At first these projections were thought to be artefacts caused by the buffer concentration or the preparative techniques for microscopy, but they were only found in agglutinated samples. Salsbury and Clarke (1967) who examined human red cells under the stereoscan electron microscope described similar cellular protrusions which they considered to be due to agglutination, as their control red cells were normal in structure, with biconcave discs of 7µ diameter and smooth regular surfaces. It seemed therefore that the cell wall protrusions were not artefacts. It had been hoped to examine agglutinated bovine red cell ghosts with the scanning microscope to enable a three-dimensional view of detailed surface structure to be obtained which was not possible with the transmission electron microscope

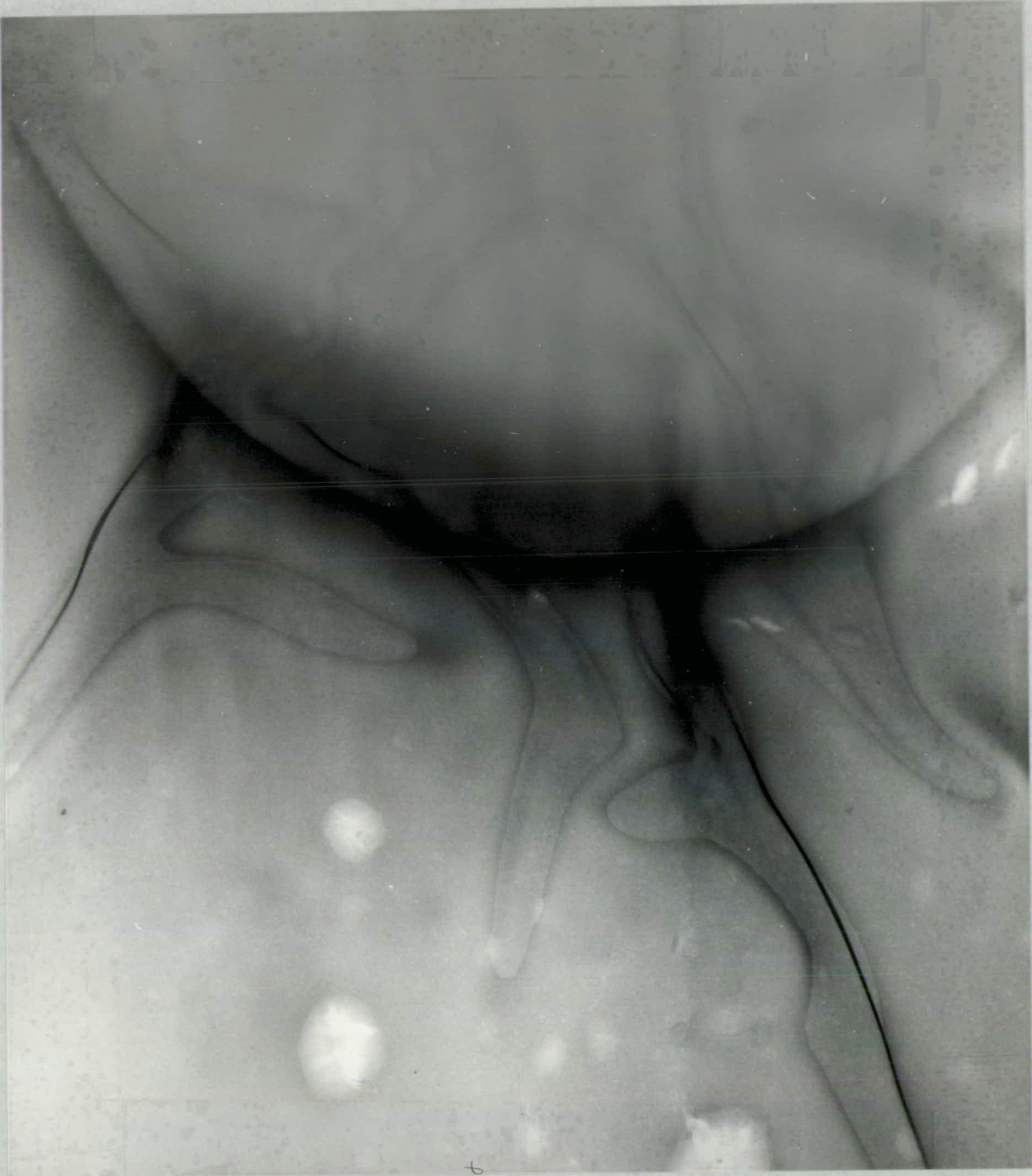


Fig. 9 Agglutinable red cell, negatively stained with 2% ammonium molybdate, showing "stromolysis". x 50,000.

which shows little surface structure. Unfortunately the only scanning microscope available was in constant use and it was impossible to carry out this study.

Assuming that Salsbury and Clarke's findings apply to bovine red cells, it is possible to correlate this structural change in the cell membrane with the agglutinability. The removal of the charges necessary for cells to agglutinate would result in changes in the electrostatic field around the cell and cell protrusions might well develop as a direct result of these changes. The changes would occur prior to agglutination. It may be that the agglutination process itself alters the electrokinetic charge sufficiently for cell protrusions to develop. If the charged surfaces of the two cells are able to approach each other sufficiently closely, then there is attraction between charges and agglutination of the cells occurs. This is only a suggestion, as the bovine red cell projections found (Fig. 9) were not studied fully and the results may not be comparable with those for human red cells. It is also possible that the previous results of Salsbury and Clarke were due to artefacts in the prepared material, as the cells were air-dried before examination. The use of freeze-dried preparations should overcome the possible formation of artefacts.

Salsbury _et al._ (1968) also found that agglutination of human cells at 4°C could be reversed with separation of the red cells and retraction of the projections to give apparently normal red cells by incubating them at 37°C. Control red cells at 4°C and cells incubated with complement at 4°C did not show any abnormal structures.

Chapter 7 The effect of various enzymes on bovine erythrocytes

The two types of cattle red cell have been shown to be microscopically similar with no discernible differences in either the number or position of the receptor sites. It seems therefore that the charged area surrounding the cell must be of importance to the cell's agglutinating activity. It has been shown (Chapter 5) that inagglutinable cells can be built up with a lattice of sensitiser and globulin to become agglutinable. This suggests that it is a matter of the effective distance between the cells which prevents them from agglutinating: only if the antibody can bridge the intercellular distance will the cells be able to agglutinate. It seems likely that the inagglutinable cells have more steric hindrance than the agglutinable cells. In this chapter the effect is investigated of altering the cell surface charge, by removing the steric hindrance by enzyme treatment, and then comparing the amount of sialic acid removed and the agglutinability of the cells after the specific enzyme treatment. The results are discussed in the light of previous reports on the effect of enzyme treatment on the electrophoretic mobility of red cells.

Materials and Methods

Neuraminidase treatment.

BDH neuraminidase from *Vibrio cholerae* 500 units/ml. was diluted 1:24 (v/v) with standard saline and 0.005M calcium chloride buffered to pH 7.0 with 0.5M aqueous sodium bicarbonate.

2ml. of diluted neuraminidase solution was added to 0.5ml. of washed packed erythrocytes and these were mixed and incubated for 1 hr. at $37^{o}C$. The suspensions were centrifuged at 1500g for 2 min. and the supernatants decanted and tested by the Aminoff method for sialic acid. The treated cells were then resuspended in saline and antiglobulin tests carried out in the normal way. When ghosts were being tested, 0.2ml. of washed packed ghosts were used in place of 0.5ml. red cells. These were spun at 18000g for 2 min.

Papain treatment

(Sigma 2 x recrystallised)

One volume of 0.05% papain in saline was added to 1 volume washed packed red cells and these were mixed and incubated for 40 min. at $37^{o}C$. The cells haemolysed in the enzyme solution and were washed in 0.9% saline (x 3) until the supernatant was clear and were tested in the antiglobulin test. Papain solution:- 1% papain diluted to 0.05% with L-cysteine hydrochloride and adjusted to pH 7.0 with 0.5M-$NaHCO_3$.

Pronase treatment

(B grade Calbiochem)

A 0.1% solution of pronase in saline was prepared. 2ml. of pronase solution was added to 1 ml. of washed packed cells. They were mixed and incubated for 1 hr. at $37^{o}C$. The haemolysed cells were washed in saline (x 3) until the supernatant was clear. The cells were then tested in the antiglobulin test.

Trypsin

(crude BDH powder)

A 2% (w/v) solution of trypsin was prepared in 0.067 M-phosphate to give a pH of 7.2. 1 ml. of 1% trypsin solution in saline was added to 1 ml. of washed packed red cells and incubated for 1 hr. at 37°C. The cells were washed in saline and tested in the antiglobulin test.

Results

Red cells from three high agglutinable and three low agglutinable animals were treated separately with neuraminidase to remove sialic acid. The treatment produced lysis but when the washed cells were tested in an antiglobulin test the same differential was found, showing that neuraminidase treatment does not affect the agglutinability of the cells (Table 1). When red cells from the same animals were treated with the proteolytic enzymes; pronase, papain and trypsin, which remove not only the sialic acid but also other charged groups connected to the acid and extending further into the cell, both classes of cell were found to be fully agglutinable (Table II), and the two cell classes were indistinguishable. Red cell haemolysis occurred during all enzyme treatments of red cells, but this was ignored and the cells were washed with saline until a clear supernatant liquid was obtained. The washed cells were then used in antiglobulin tests.

Whole red cells were not entirely satisfactory due to their haemolysing during enzyme treatment. Therefore the further tests were carried out on ghosts as it has already been shown (Chapter 6) that these give the same antiglobulin reaction as their red cells.

Reciprocal dilutions of K21(17/5/67)

Animal	4	8	16	32	64	128	256	512	1T	2T	4T	8T	16T	C
FS101A	4	4	4	4	4	4	4	4	4	4	1	-	-	-
HZ135A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MZ2308A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
U85A	4	4	4	4	4	4	4	4	4	2	-	-	-	-
U85B	4	4	4	4	4	4	4	4	3	2	-	-	-	-
U93B	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Red cells sensitised with R4(19/5/66) 1:5,000 after treatment with neuraminidase for 30 min. at 37°C

Reciprocal dilutions of K21(17/5/67)

Animal	4	8	16	32	64	128	256	512	1T	2T	4T	8T	16T	C
FS101A	4	4	4	4	4	4	4	4	4	3	-	-	-	-
HZ135A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MZ308A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
U85A	4	4	4	4	4	4	4	4	4	4	1	-	-	-
U85B	4	4	4	4	4	4	4	4	4	2	-	-	-	-
U93B	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Red cell ghosts sensitised with R4(19/5/66) 1:5,000 after treatment with neuraminidase for 30 min. at 37°C

Reciprocal dilutions of K21(17/5/67)

Animal	4	8	16	32	64	128	256	512	1T	2T	4T	8T	16T	C
FS101A	4	4	4	4	4	4	4	4	4	3	2	-	-	-
HZ135A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MZ308A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
U85A	4	4	4	4	4	4	4	4	4	2	-	-	-	-
U85B	4	4	4	4	4	4	4	4	3	1	-	-	-	-
U93B	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Control red cells sensitised with R4 (19/5/66) 1:5,000

Table I Neuraminidase treatment of high- and low-agglutinable bovine red cells

Reciprocal dilutions of K21 (17/5/67)

Animal	4	8	16	32	64	128	256	512	1T	2T	4T	8T	16T	C
FS101A	4	4	4	4	4	4	4	4	4	4	4	4	4	3
HZ135A	4	4	4	4	4	4	4	4	4	4	4	4	4	-
MZ308A	4	4	4	4	4	4	4	4	4	4	4	4	4	-
U85A	4	4	4	4	4	4	4	4	4	4	4	4	4	2
U85B	4	4	4	4	4	4	4	4	4	4	4	4	4	2
U93B	4	4	4	4	4	4	4	4	4	4	4	4	4	-

Red cells sensitised with R4 (19/5/66) 1:5,000 after treatment with pronase for 1hr. at 37°C

Reciprocal dilutions of K21 (17/5/67)

Animal	4	8	16	32	64	128	256	512	1T	2T	4T	8T	16T	C
FS101A	4	4	4	4	4	4	4	4	4	4	4	4	4	2
HZ135A	4	4	4	4	4	4	4	4	3	3	2	2	2	-
MZ308A	4	4	4	4	4	4	4	4	3	2	2	2	2	-
U85A	4	4	4	4	4	4	4	4	4	4	4	4	4	2
U85B	4	4	4	4	4	4	4	4	4	4	4	4	4	2
U93B	4	4	4	4	4	4	4	4	3	2	1	-	-	-

Red cells sensitised with R4 (19/5/66) 1:5,000 after treatment with papain for 40 min. at 37°C

Reciprocal dilutions of K21 (17/5/67)

Animal	4	8	16	32	64	128	256	512	1T	2T	4T	8T	16T	C
FS101A	4	4	4	4	4	4	4	4	4	4	4	4	3	2
HZ135A	4	4	4	4	4	4	4	4	4	4	4	4	2	-
MZ308A	4	4	4	4	4	4	4	4	4	4	4	4	2	-
U85A	4	4	4	4	4	4	4	4	4	4	4	4	2	-
U85B	4	4	4	4	4	4	4	4	4	4	4	4	2	-
U93B	4	4	4	4	4	4	4	4	4	4	4	4	1	1

Red cells sensitised with R4 (19/5/66) 1:5,000 after treatment with trypsin for 1 hr. at 37°C

Table II Enzyme treatment of high- and low-agglutinable bovine red cells

When the two classes of red-cell ghost were subjected to mild acid hydrolysis they were found (Chapter 2) to release different amounts of sialic acid. If the ghost protein had been extracted with butanol prior to treatment then slightly less sialic acid was released than if the protein had not been extracted previously (Table III). When the ghosts were extracted before hydrolysis they probably lost this small amount of sialic acid in the interfacial layer of insoluble protein.

When the red-cell ghosts were treated with neuraminidase and the resulting suspension was centrifuged at 18000g for 15 min. to separate the ghosts from the supernatant liquid, and the ghosts were then subjected to mild acid hydrolysis, virtually all the sialic acid was recovered in the supernatant liquid, none in the ghosts (see Table IV). This shows that all the sialoprotein of the cell must be on the outer surface of the membrane. When these ghosts shown to be without sialic acid were tested in the antiglobulin test they still showed the two classes of agglutinability. The ghosts tested gave identical reactions to those cells which had had no neuraminidase treatment and were taken from the same animals at the same time. The differing amounts of sialic acid on the two classes of cell cannot be the direct cause of the different reactions. The sialic acid content and the agglutinability of bovine red cells are correlated but the first is not the direct cause of the second, although obviously related to it.

Discussion

Neuraminidase treatment releases sialic acid specifically (Gottschalk,

Animal	Agglutinability	Acid treated ghosts	Butanol extracted acid treated ghosts
FS101A	High	0.102	0.098
HZ123B	High	0.100	0.098
U85A	High	0.095	0.090
HZ141B	Low	0.131	0.128
MZ308A	Low	0.142	0.141
U93B	Low	0.137	0.134

Table III Release of sialic acid (uM/mg. protein) from red cell ghosts

Animal	Agglutinability	Ghosts before neuraminidase treatment	Ghosts after neuraminidase treatment	Supernatant after neuraminidase treatment
HZ123B	High	0.046	0.000	0.044
U85A	High	0.045	0.000	0.041
HZ141B	Low	0.079	0.000	0.074
MZ308A	Low	0.054	0.000	0.049

Table lV Release of sialic acid (extinction at 549 nm.) from supernatant and ghosts after treatment of red cell ghosts with neuraminidose

1957) but even although it has been shown that the enzyme removes all the sialic acid from the ghosts, these still show their normal antiglobulin character. Thus it is not entirely the presence of larger quantities of sialic acid on the inagglutinable cells which makes them inagglutinable, unless when the neuraminidase treatment releases these charged groups which prevent agglutination. In the agglutinable class of cell too, which has a smaller amount of sialic acid, the sialic acid charges may be replaced by others during neuraminidase treatment, but the new charges, as before, do not prevent agglutination.

The action of proteolytic enzymes differs from that of neuraminidase in that the former break down the molecular structure of the cell to a deeper level within the membrane surface. The enzymes release sialomucopeptide chains. Other charged groups are presumably formed or unmasked but these apparently do not prevent either class of red cell from agglutinating.

The larger quantities of sialic acid released from the inagglutinable cells suggests either that these have more complex multibranched chains of mucopeptide or else that there are more short chains positioned deeper into the cell structure than in the agglutinable cells. As the amount of sialic acid liberated from the red-cell ghost by mild acid hydrolysis corresponds to that found in the supernatant liquid after neuraminidase treatment the total sialic acid content of both classes of cell must be situated at the outer surface of the membrane. Also, neuraminidase is a large molecule (Heard and Seaman, 1960) and because of its size, it could not enter the red cell to remove sialic acid from the interior of the cell. Therefore all the sialic acid of the cell must be vulnerable to neuraminidase.

Work on human red cells has shown that their agglutinability varies with age, the older cells showing increased agglutinability (Buchholz and Bove, 1967). Also, the increase in agglutinability of human red cells is directly proportional to the amount of sialic acid removed by neuraminidase treatment. In the cattle red cells studied here, no change was found in the agglutinability of the cells with increasing age of the animal. Neuraminidase treatment released sialic acid from human red cells and their agglutinability was enhanced (Uhlenbruck et al. 1967), in contrast to the results found for cattle red cells.

Eylar et al. (1962) measured electrophoretic mobilities of red cells of various animals and compared the alteration in charge after neuraminidase treatment with the amount of sialic acid removed. They found in each case that the number of sialic acid molecules removed exceeded the number of charges lost; for human and calf cells, they found that 100% more sialic acid molecules were released than the number of charges lost. This can be explained if one assumes that of the total number of sialic acid molecules lost, only some, equal in number to the number of charges lost, were situated at the surface of shear. Only these surface molecules would affect the mobility of the cell although other more deeply situated sialic acid molecules might also be released by the neuraminidase treatment.

Treatment with proteolytic enzyme resulted in a more complex reaction, and more charged groups were produced than by neuraminidase treatment (Seaman and Uhlenbruck, 1963). Treatment with neuraminidase after proteolytic enzyme treatment had no effect on the electrophoretic mobility, from which it can be concluded that no neuraminidase-susceptible groups have been uncovered by the

enzyme treatment (Seaman and Uhlenbruck, 1963). Measurements of electrophoretic mobility showed no difference between the inagglutinable and agglutinable bovine cells either before or after enzyme treatment, Uhlenbruck _et al_. (1967).

The negative surface charge is not due solely to sialic acid (Eylar _et al_, 1962); it is thought that α-carboxyl groups also contribute to it (Maddy, 1970). However a roughly linear relationship has been found between the surface density of the sialic acid and the electrophoretic mobility of the bovine red cells (Eylar _et al_, 1962).

The inagglutinable bovine erythrocyte has been found to remain inagglutinable even after removal of all of its sialic acid component. It seems therefore that removal of the sialic mucopeptide chains also is necessary for agglutination. This suggests that it is the arrangement of these charged groups which hinders the cells' agglutinating potential. The precise distribution of the sialic acid molecules is unknown, but it is probable that they are situated at different depths in relation to the surface of shear, and, with the mucopeptide chains, form a barrier so that the cells cannot get close enough together to agglutinate.

Chapter 8 Correlations between bovine red cell agglutinability and other blood characters

Up to 1962, 11 loci had been identified as controlling blood groups in cattle (Stormont 1962). Since then others have been identified but not fully studied. Other loci control other blood characters, e.g. haemoglobins, transferrins, amylases and carbonic anhydrases. Many of these characters are independent of each other and, therefore, are probably controlled by loci on different chromosomes. These loci can be used to clarify the mode of inheritance of characters controlled by other loci on the same chromosome. Two characters can be genetically related either by pleiotropy (where one gene controls both characters) or by linkage (where the genes are situated on the same chromosome) with or without recombination through crossing over.

The existence of correlations between blood groups and production characters such as milk yield, would be of great practical importance, but it is considered (Neimann-Sorensen and Robertson, 1961) unlikely that such correlations will be found as the production characters are controlled by genes at a large number of loci and therefore each locus will have only a limited influence. Production characters are also largely influenced by environmental factors and therefore close genetic relationships between the production and blood characters are unlikely. Subdivision of populations into heterogenous groups has been known to change blood group frequencies (Kraay and Bouw, 1964, Nasrat et al., 1964). When the two heterogenous groups are bred in separate selected environments differences in blood group, frequencies can arise due to inbreeding, and an association between blood groups and other characters can be induced in the whole population (Kraay, 1964).

It has already been shown in this thesis that bovine red cell agglutinability is genetically controlled (Chapter 4), and in this chapter, investigations are reported which were undertaken to identify any character that might be correlated with red cell agglutinability.

Materials

The animals from European breeds were chosen as randomly as possible. As it was not possible to get sufficient family data for testing, none has been included. Separate groups of animals were used for the different investigations as stated in each section.

The material relating to Zambian cattle was collected and studied with the permission of the Zambian Government and the Ministry of Overseas Development during a blood group survey in Zambia. The blood samples were all from Angoni cattle and were collected as described in the Appendix. The animals came from four separate crushes; two of the crushes were in an area with a high incidence of trypanosomiasis, and were separated by 50 miles from the other two crushes which were in an area with a low incidence of such disease, i.e. free of tsetse flies. The animals within each crush belonged to many individual farmers. The cattle wandered freely and were not herded. There were no breeding programmes and it was impossible to recognise families, although in each crush there must have been many related animals. To minimise the effects which the family material would have on the data, only animals under an estimated 12 months of age have been included in the studies presented here. Amongst these there are certain to be paternal half-sibs, but these could not be identified and the results have been analysed bearing this in mind.

Methods

Separation of blood into young and old cells

Red cell samples taken from each animal were separated into old and young cells by centrifuging them in their own plasma at 2000g for 20 min. The specific gravity of red cells increases with age (Danon and Marikovsky, 1964); as a result the old cells separated out and packed into the bottom of the tube, while the top fraction contained the younger cells. Plastic tubes of 80mm. with a diameter of 7mm. were used.

Measurement of cell volume

The mean cell volume of several red-call samples was measured in a model B Coulter counter. The red cells were collected into anticoagulant, washed four times in 0.9% phosphate buffered saline and then one drop of packed red cells was diluted 40,000 times. The lower threshold of the counter was set at 20 for all counts; the amplitude and aperture current were set at 0.5. The initial window for cell counting was 2 and the difference between windows set at 6µ. The number of cells in 0.5 ml. of each diluted sample was counted using a 200µ orifice tube, and the cell volume plotted. The mean cell volume ($\bar{v}$) was calculated using the formula $\bar{v} = \frac{\Sigma hv}{\Sigma h}$ where v is the volume of the cell represented in each window and h is the pulse as measured on the graph. The pulse is a measure of the number of cells in that window. ~~(see plot in Appendix C).~~

Measurement of potassium levels in the red cell

Blood samples were collected in heparin and used within two days. Red cells were centrifuged out; red cell and serum lysates were made up

using 0.25ml./50ml. distilled water. The potassium values of the whole blood were measured using a flame photometer with a potassium filter. A stock solution of 0.477 g/l. KC1 diluted x50 was used as a standard. The standard and distilled water controls were checked after every other reading.

The packed cell volume of each sample was measured by spinning a capillary tube of whole blood in a haematocrit centrifuge for 10 min. and reading the value on a cell volume counter. The potassium concentrations per litre were then calculated for each sample of plasma and whole blood.

Haemoglobin gels

A continuous buffer system at pH 8.9 was used in the gels; it was stored at 4°C until required. The buffer consisted of 40.4g. tris 4.0g. disodium E.D.T.A. and 3.0g. boric acid dissolved in distilled water and made up to 2l.

50ml. of buffer was added to 25g. of starch for each gel. This was left at 56°C and shaken at intervals so that the starch stayed in solution. Another 200ml. of buffer was boiled and added to the starch solution to give a final concentration of 10% starch, the mixture being shaken continuously. The gel solution was degassed, poured into a glass plate 25cm. x 12.5cm. x 0.75cm. then covered and allowed to cool. Small inserts (0.5cm. x 1.0cm.) of Whatman No. 54 filter paper were dipped into water-lysed blood samples and put into a slit made in the gel 1-2cm. from the cathode wick. The distance between wicks was about 19cm. The inserts were left in the gel for 1 min. at 300V and 55mA, and then removed, and the samples run until the haemoglobin separated out into bands (0.5 - 1 hr.). The unstained gels were then read.

Transferrin gels

The gel consisted of 0.016M tris cacodylic acid at pH 7.45. 2l. of 0.1M-boric acid brought to pH 8.7 with 0.1M sodium hydroxide was used as the tank buffer. This buffer was stored at 4°C. The nigrosin stain used, consisted of 5.0g. nigrosin, 37.0ml. glacial acetic acid, 278.0ml. methyl alcohol and 185.0ml. distilled water. The washing solution was 10% glacial acetic acid in 50% methyl alcohol.

The gels were prepared exactly as for the haemoglobin gels using 14.5% (w/v) starch. The serum samples were applied on 0.5cm. x 1.0cm. strips of Whatman No. 54 filter paper inserted 1-2cm. from the cathode wick. The inserts were left for 5 min. at 300V and 55mA and then removed and the samples run until the buffer boundary was 7-8cm. from the insert line. The gels were then cut and stained with 1% nigrosin for 10 min., rinsed in cold water and excess stain removed overnight with washing solution.

Carbonic anhydrase and esterase gels

The gel buffer consisted of 0.144M tris citric acid at pH 7.3. This was made up from 0.05M citric acid monohydrate pH 2.4 and 0.2M tris phosphate of pH 9.9. The tank buffer was 0.1M boric acid broughtoto pH 8.6 with 0.1M sodium hydoxide. This was stored at 4°C until required. The α-naphthyl acetate stain used consisted of 2ml. 1% α-naphthyl acetate in acetone, 4ml. phosphate buffer pH 6.8, 0.1g. fast blue BB salt (Sigma), made up to 1l. with distilled water. The washing solution used was the same as for transferrins.

The gels were prepared exactly as for haemoglobin gels using 12% starch (w/v). Inserts (0.5cm. x 1.0cm.) were put in the gel 2cm. from the cathode.

They were run for 3 min. at 300V and 40mA. The inserts were then removed and the gels run for a further 2 hr., the plates being kept cool with ice packs. The gels were stained with α-naphthyl acetate for 10 min. and were then left in washing solution overnight. Both carbonic anhydrases and esterases were read the following day.

Blood typing of red cells

The red cells were blood-typed using a three drop haemolytic test. A 1 ml. drop of each sample of whole blood was washed four times with 0.9% saline and then made up to an 0.5% suspension using an automatic diluter. The antisera used were specific reagents which detected specific antigenic blood-group factors. These were diluted in saline and the lowest concentration to give a complete reaction was used. One drop (0.025 ml.) of antiserum was put into a U-bottomed microtitre plate and one drop of cell suspension added followed by one drop of complement (normal rabbit serum diluted 1:3 in saline), after which the plate was covered with sellotape and put onto a mechanical shaker for 5 hr. at 18-25°C., to keep the cells suspended. The cells were then allowed 2 hr. to settle and the free haemoglobin, ~~left as a button at the bottom of the tube~~, was estimated by eye. The haemolysis was scored from 0 to 5.

Results

Cell age

As there was a possibility of the bovine red cell agglutinability being associated with the age of the cells, two calves, one of each class of

agglutinability, were tested from the age of 6 months until they were 2 years old. No change was found in the agglutinability of the red blood cells with age. Samples were also taken from adult animals and the cell population separated into young and old cells by centrifugation. The two fractions were tested separately in the antiglobulin test, but in no case did the young and old cells from one animal differ in the class of agglutinability (see Table I).

Cell volume

Samples were taken from 10 Ayrshire cattle from an Animal Breeding Research Organisation experimental farm, and their red cell volume was measured to detect any difference in cell size which might exist between the high and low agglutinable cells. The results showed an unexpectedly wide range of values (see Table II). The results were analysed using the t-test and a value of 1.18 obtained, thus proving that there was no significant difference between the volumes of the two classes of cell.

Potassium levels in the red cell

The levels of potassium in the whole blood and plasma of 20 Ayrshire cattle were determined, using flame photometry, to see if these were associated with agglutinability. The animals were found to have very similar levels of potassium (see Table III); the values were low in comparison with the values found in other animals, e.g. sheep. The results were analysed using a t-test, and a value of 2.14 obtained, thus proving that there was no significant difference between the potassium levels of the two classes of cell.

Carbonic anhydrases and esterases of the red cell /

		Reciprocal dilutions of K21 (17/5/67)											
		5	10	20	40	80	160	320	640	1T	2T	4T	C
U67A	Young	4	4	4	4	4	4	4	4	4	4	-	-
	Old	4	4	4	4	4	4	4	4	4	3	-	-
U93B	Young	-	-	-	-	-	-	-	-	-	-	-	-
	Old	-	-	-	-	-	-	-	-	-	-	-	-
HZ123B	Young	4	4	4	4	4	4	4	4	4	4	2	-
	Old	4	4	4	4	4	4	4	4	4	4	1	-
HZ141B	Young	-	-	-	-	-	-	-	-	-	-	-	-
	Old	-	-	-	-	-	-	-	-	-	-	-	-
HZ167B	3 mths.	4	4	4	4	4	4	4	4	4	4	-	-
	6 mths.	4	4	4	4	4	4	4	4	4	3	1	-
	12 mths.	4	4	4	4	4	4	4	4	4	4	-	-
	18 mths.	4	4	4	4	4	4	4	4	4	3	-	-
	24 mths.	4	4	4	4	4	4	4	4	4	4	1	-
FS106A	3 mths.	-	-	-	-	-	-	-	-	-	-	-	-
	6 mths.	2	-	-	-	-	-	-	-	-	-	-	-
	12 mths.	-	-	-	-	-	-	-	-	-	-	-	-
	24 mths.	-	-	-	-	-	-	-	-	-	-	-	-

Table I <u>The agglutinability of young and old populations of red cells from the same animals</u>

Animal	Mean cell volume μ^3	Mean	Agglutinability
FS101A	66		High
HZ125B	66		"
HZ135A	66	64.2	"
HZ145A	63		"
HZ167B	60		"
U85A	66		"
HZ141B	60		Low
MZ111B	72		"
U74A	66	67.2	"
U93B	72		"
TAG967	66		"

Table II <u>Comparison of mean cell volume with agglutinability</u>

High agglutinable		Low agglutinable	
Animal	K (m. eq. /l)	Animal	K (m.eq./l.)
FS101A	18.6	FS98A	20.0
HZ90B	15.3	HZ133B	19.6
HZ125B	17.6	HZ141B	17.2
MZ324A	14.3	MZ308A	14.1
MZ324B	15.9	MZ308B	15.2
MZ328A	17.2	MZ311A	15.5
MZ328B	18.1	MZ346B	20.9
U67A	15.2	TAG967	19.1
U85A	17.4	U74A	18.2
U85B	20.0	U93B	15.6
Mean	16.96	Mean	17.54

Table III <u>Potassium levels of high and low agglutinable red cells</u>

Carbonic anhydrases and esterases of the red cell

The carbonic anhydrase and esterase activity of the red cells of 27 Ayrshire cattle from the ABRO experimental farm were tested to see if any association existed between the red cell agglutinability and these characters. Six monozygous and 1 dizygous twin pairs were tested and in every case the phenotype of both members of each twin pair was the same. In the samples tested only animals homozygous for the slow component and heterozygous animals were identified, no animals homozygous for the fast component were found (see Table IV). A X^2-test ($X^2 = 0.14$; 1 degree of freedom) was used to show that there was no significant association between the class of agglutinability and the carbonic anhydrase.

The same panel of animals was tested for esterases, and here it was found that although the low-agglutinable cells had both types of esterase, the high-agglutinable cells always had only one type. Analysis of these results ($X^2 = 0.75$; 1 degree of freedom) showed that the difference was non-significant.

Haemoglobins and transferrins of red cells

A survey was carried out of the haemoglobin and transferrin types in blood samples from 300 Hereford and Friesian cattle (see Tables V and VI). The samples were chosen randomly from those sent to the Cattle Blood Typing Service for typing and were from unrelated animals. The results were considered in relation to the agglutination type of the animals concerned. The majority of animals in both breeds were of haemoglobin type AA. 90% had blood of high agglutinability and the most frequently occurring transferrin types were the AD_1, D_1D_2 and AA, with AD_1 predominating. Among animals

	Carbonic anhydrase	
	SS	FS
High agglutinator	5	1
Low agglutinator	16	5

$X^2 = 0.14$ for 1 degree of freedom

	Esterase	
	+	-
High agglutinator	6	0
Low agglutinator	15	6

$X^2 = 0.75$ for 1 degree of freedom

Table IV Distribution of numbers of animals by agglutinability and carbonic anhydrase and esterase

Breed	Agglutination type	% Haemoglobin type AA	AB	AF	BB
Hereford	High	86	1	3	-
	Low	10	-	-	-
Friesian	High	38	-	2	-
	Low	55	1.5	3.5	-

Table V[illegible] Comparison of agglutinability and haemoglobin type in Hereford and Friesian cattle

Breed	Agglutination type	% Transferrin type									
		AA	AD_1	AD_2	AE	D_1D_1	D_1D_2	D_1E	D_2D_2	D_2E	EE
Hereford	High	16	29	11	-	9	18	1	5	1	-
	Low	5	1	2	-	-	-	-	2	-	-
Friesian	High	7	10	7	2	0.5	6.5	-	4	3	-
	Low	14	7	20	1	0.5	9	-	5.5	2.5	0.5

Table VI <u>Comparison of agglutinability and transferrin type in Hereford and Friesian cattle.</u>

having blood of low agglutinability, the most frequently found transferrin type was the AA. Thus it looked as if there might be some correlation between high agglutinability and the presence of the AD_1 transferrin. The expected values were too low for an accurate X^2 test to be carried out. However, from the expected values calculated from the data given in Table V it was obvious the correlation was not significant.

The transferrin and haemoglobin type of 88 Angoni cattle were recorded (see Table VII and VIII). There was little difference in the frequencies of the transferrin types of the genotypes in animals with high and low agglutinability. It was noticeable that the transferrin D genotypes occurred less frequently in the data for Zambian cattle than in that for European cattle, and that D_2E which was commonly found in the Zambian data hardly ever occurred in the European data. The haemoglobin which occurred most frequently in both high- and low-agglutinable cells was the AC type which was not found in the European data. The values in these results also were too low for an accurate X^2-analysis, but from the observed and expected values it was clear that there was no significant relationship between the class of agglutinability and the transferrin or haemoglobin type.

Blood-group antigens

Samples from 125 unrelated European cattle selected at random from different breeds (samples sent in to the Cattle Blood Typing Service) were tested for the agglutinability of their red cells and also for 40 blood-group antigens. The results were kindly analysed by Mr. R.G. Carpenter. The only associations found between the agglutinability and the red cell antigens were

Agglutination type	% Transferrin type									
	AA	AD_1	AD_2	AE	D_1D_1	D_1D_2	D_1E	D_2D_2	D_2E	EE
High	6	3	8	8	1	-	1	5	10	4
Low	8	2	8	1	-	2	2	6	9	4

Table VII <u>Comparison of agglutinability and transferrin type in Zambian (Angoni) cattle</u>

Agglutination type	% Haemoglobin type						
	AA	AB	AC	BB	BC	BD	CC
High	8	9	13	3	4	1	8
Low	9	4	18	-	5	-	6

Table VIII <u>Comparison of agglutinability and haemoglobin type in Zambian (Angoni) cattle</u>

negative correlations with V and ϵ_1; (see Table IX). These show that there is a slight negative correlation between agglutinability and V, significant at the 1% level and also a negative correlation between agglutinability and ϵ_1 significant at the 1% level.

The agglutinability and blood group antigens of blood samples from 1,000 Zambian cattle were tested and the results were analysed by computer, for associations using X^2 tests (with the assistance of Mr. D. Nicholson). About 50% of the animals had cells which were highly agglutinable and all the cattle belonged to one of the two classes of agglutinability being studied here. Most of the family material was omitted by including only the results from the 88 animals estimated to be under 12 months old. The results were found as in Table X.

From these results it appears that there is a negative correlation of agglutinability with ϵ_1, and with F. In fact, because of the low number of animals negative for F and ϵ_1, the result is misleading. When the results were analysed using X^2 tests, it was found that agglutinability and the antigens V, ϵ_1 and F were all independently assorted and there was no association between these characters.

Discussion

The results of experiments to detect any association between red-cell agglutinability and other blood-group characters have revealed little positive information. The number of samples tested for each character was small and restricted to a few breeds, and it may be that further tests using larger

	Antigen V	
	present	absent
High agglutinator	14	53
Low agglutinator	28	30

$$x^2 = 10.44$$

Correlation between agglutinability and V is -0.289

	Antigen ε_1	
	present	absent
High agglutinator	48	19
Low agglutinator	58	0

$$x^2 = 19.39$$

Correlation between agglutinability and ε_1 is -0.3939

Table IX <u>Distribution of numbers of unrelated European cattle by agglutinability and antigens V, and ε_1</u>

	Antigen V	
	present	absent
High agglutinator	8	39
Low agglutinator	4	37

	Antigen ε_1	
	present	absent
High agglutinator	38	9
Low agglutinator	33	8

	Antigen F	
	present	absent
High agglutinator	45	2
Low agglutinator	41	0

Table X Distribution of numbers of Zambian cattle by agglutinability and antigens V, ε_1 and F

numbers of cattle and breeds might show more positive results.

There was no significant association between cell age and its agglutinability, although in human red cells a variation in agglutinability with age has been found by Buchholz and Bove, (1967). These workers also found that the agglutinability of human cells was directly proportional to the amount of sialic acid removed following neuraminidase treatment. There must, therefore, be a difference in the ageing process in bovine and human red cells.

The measurements of the mean cell volume of cattle red cells showed a wide range of values as compared with those of other animals (Wintrobe, 1933). When animals were separated into two classes according to their red cell agglutinability, the animals showed an overlap in their values, and this indicates that there is a complete range of cell volume, rather than two discrete classes, and that cell volume has no association with the agglutinability of the cell.

Investigations into the potassium concentration of the red cells were prompted by results published by other workers. Rasmusen and Hall (1966) showed that there is an association between the potassium concentration in sheep red cells and the occurrence of the blood group M. There were two levels of potassium concentration, and all sheep in which M was absent had a low cell potassium content. They also showed that the M system in sheep was homologous to the S system in cattle. Ellory and Tucker (1970) found that red cell potassium levels in a Jersey herd varied. Of 91 animals, 13 had a high potassium

content (> 78m$\underline{M}$/1.) and 78 had a low potassium content (< 62m$\underline{M}$/1.). Previous to this, all cattle were considered to have a low potassium level (Evans and Phillipson, 1957). In the study of 20 Ayrshire cattle reported here, the potassium concentrations were all found to be very low compared with the values found by Ellory and Tucker (1970) for Jerseys. It seems that in the red cells of some breeds of cattle two levels of potassium are found, whereas in others only one level is found. It has been shown that 40% of Ayrshires have cells of high agglutinability; possibly if Herefords, in which 84% have high agglutinability , had been tested, a difference would have been detected between the potassium levels in these two breeds.

The results of the tests involving the carbonic anhydrases did not show any association between them and agglutinability, but no animals homozygous for F were found. It is possible that no Ayrshires are homozygous for the F carbonic anhydrase, but if the frequency of the gene was low, then it is possible that the phenotype would not be expressed in a small number of animals. However, analysis of the data presented has shown no such association of either carbonic anhydrase or esterase with agglutinability.

The transferrin results, although not showing an association of transferrin type with agglutinability did show that a high percentage of the Herefords and Friesians were of the AD_1, D_1D_2, AA, and AD_2 transferrin types. The frequency of the gene $\underline{Tf}^{E}$ in the data for European cattle was very low, but it was much higher in that for Zambian cattle. The transferrins with the highest frequency in the Zambian data were D_2E, AD_2 and AA.

The two European breeds were predominantly of haemoglobin type AA, whereas the range of types in Zambian cattle was much wider, although 35% of these tested were of type AC which is not found in European cattle.

The association, found between the red cell agglutinability and the blood group antigen V in European cattle was expected because a direct association has been proved to exist between the agglutinability and sialic acid content of the bovine red cell membrane. The work of Hatheway _et al_. (1969) suggested that the red cell membrane of animals in which the antigen V was present contained less sialic acid than that of animals in which F was present. This is not in complete agreement with the results found here, as all the VV animals were of low sialic acid and high agglutinability, whereas the FV and FF animals were of both types of sialic acid and agglutinability. There was no significant difference between the sialic acid values of VV, FV and FF animals.

The F and V antigens can be recovered in soluble form from the sialic acid-rich fraction of a butanol extract of the membrane (Spooner and Maddy, in press). Also, the F antigen is destroyed by neuraminidase treatment while the V is unaffected (Hatheway _et al_. 1969). This, together with the computer analysis, proves that there is an association between agglutinability and the _FV_ locus in European cattle. The Zambian cattle did not show this association; however some of these animals were probably related, several cows having been mated by the same bull, and, as it was not possible to eliminate these related animals this may be a reason for no apparent association between the red cell agglutinability and _FV_ locus as found in the European data.

Amongst the European cattle tested, FF, FV and VV phenotypes were found and no FV negatives were found whereas 13 of the 1000 Zambian cattle tested were FV negative. These animals showed both classes of agglutinability. This finding is in agreement with the work of Osterhoff and Politzer (1968) who recorded the absence of both F and V antigens in indigenous South African cattle (Zebu). The sialic acid content of the red cell membrane of the African cattle has not been measured, but since the Zambian animals show the same two classes of agglutinable red cells it is probable that they also have two levels of sialic acid. If this is true then it is surprising not to find the same associations as were found in the European cattle.

Chapter 9

Conclusion

The results of the antiglobulin test described in this thesis show that in all the systems tested there are only two classes of bovine erythrocyte - those with high agglutinability and those with low agglutinability. The previous reports by Gleeson-White et al. (1950) of a gradation of agglutinability and the work of Hall (1955) have been reconciled with the two classes of agglutinability found here. The division of the agglutinable cells into two classes was more obvious when antiglobulin serum rather than sensitising serum was titrated, and when a large number of animals was tested. From the results obtained using different antiglobulin systems and by titrating both types of sera used, it is clear that it is the same character of agglutinability which is being studied, and that the results given here are in agreement with those of Gleeson-White et al. and Hall. It is also clear that the direct agglutination test involves red-cell receptor sites which differ from the sites involved in antiglobulin agglutination since in the direct test there is a continual gradation of agglutinability of the red cells.

It is shown that the agglutinability observed in an antiglobulin test is a character of the red cell which can be demonstrated in sera from many different species and which is constant throughout the life of an animal. The character has been shown to be inherited simply with the character for high agglutinability recessive to that for low. This is the first case reported of an inherited intraspecific variation in red cell agglutinability. It is interesting that the frequencies of cells of high and low agglutinability vary widely among breeds, especially as the beef breeds are predominantly high agglutinators and the dairy breeds mainly low agglutinators. Although this

fact has been recorded here, it was not investigated in detail. There may be some association between agglutinability and production characters and it would be worthwhile to extend the survey to larger numbers of animals and breeds to see if these results are confirmed.

It had previously been shown by Maddy and Spooner (1970) that there are two classes of bovine red cell and that these possess two different levels of sialic acid in the membrane protein. The present investigation has shown that the same classes are found when bovine red cells are classified according to their agglutinability. It was found that highly agglutinable red cells had a low sialic acid content and low agglutinators had a high sialic acid content. However, Eylar et al. (1962) have shown that human red cells, which are far more readily agglutinated than bovine cells with even the highest agglutinability have a higher sialic acid content in their membrane than either of the two classes of bovine red cell. The chains in which sialic acid is terminal are important in the agglutination of bovine red cells, clearly the difference between the two types of red cell must be in the carbohydrate and proteins to which the sialic acid is attached, as after removal of all the sialic acid the cells can still be divided into the same two classes of agglutinability; however, in the present investigation it has been shown that after removal of all the sialo-mucopeptide chains from the cell by proteolytic enzyme treatment the cells can agglutinate. It is therefore probable that the proteolytic enzymes completely break down the cell surface structure. However, if individual bonds in the mucopeptide chains can be broken selectively, it should be possible to see how many and which charged groups need to be removed before the cells become agglutinable.

The electron microscope studies reported in this thesis show that the agglutinable cells approach each other more closely than the inagglutinable cells, and are in fact distorted over the area of contact. The distance between the cells may be less than has been stated here, as the outer carbohydrate layer of the membrane is not visible. Electron microscope techniques will yield more valuable information when the techniques for visualising the carbohydrate layers have been perfected. The information reported here is that all bovine erythrocytes appear to possess the same number of receptor sites. When more of the membrane structure has been rendered visible, the receptor sites in the inagglutinable cell will probably be found to lie deeper in the membrane than those of the agglutinable cells. If this is discovered to be the case, then it is possible to envisage a situation in which the antibody, be it IgG or IgM is able to sensitise all the cells, but is not sufficiently long to protrude beyond the outer layers of the membrane of the inagglutinable cells. This is the present state of knowledge of the two classes of bovine erythrocyte agglutinability as found in the antiglobulin test.

The association of agglutination of bovine red cells with other blood-group characters has been studied. From work involving very limited numbers of animals, mainly of the Ayrshire, Friesian and Hereford breeds, it was found that the only association with agglutinability was a correlation between the agglutinability and the FV locus of the red cell. In the data on European breeds studied, it was found that all VV animals had highly agglutinable red cells and were of low sialic acid content. There were no FV negative animals, but of the FF and FV animals which were low agglutinators, their sialic acid

values were almost identical. This does not entirely agree with the work of Hatheway et al. (1969), who suggested that cells of VV type animals had less sialic acid than those of FV type and that FF had the highest value. This was probably because by chance they picked low agglutinating FV and FF and high agglutinating VV animals in the few they tested. It is evident that the agglutinability, the FV locus and the sialic acid content of the red cell membrane are associated. The fact that F and V can be recovered in soluble form from the sialic acid-rich fraction of the butanol extracted membrane protein, and also the F antigen can be destroyed by neuraminidase, lend further support to this idea.

Blood samples from Zambian cattle were tested similarly for agglutinability and FV status, but were not analysed for sialic acid content. No correlation between agglutinability and the FV locus was found as in the case of the European breeds, possibly due to the inter-relationship of the animals tested, or it may be that the animals were of a primitive type which had not been bred for desired characters as had the European cattle.

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Appendix A

Materials

Cattle samples - The panel of cattle used regularly in the work in this thesis came from animals on the Animal Breeding Research Organisation (ABRO) farms at Blythbank and Stanhope, Peeblesshire. The majority of the animals were Ayrshires and were numbered according to family relationships.

MZ - monozygous twins

DZ - dizygous twins

FS - full sisters

HZ - half sisters

U - unrelated animals of the same age.

Other blood samples were taken from those sent in to the Cattle Blood Typing Service. The Hereford family material was from the herd of Mr. F.K. Retson, Whitmuir Farm, Peeblesshire.

The Angoni cattle samples were from the animals of Zambian native farmers.

Sheep samples - The sheep blood samples were mostly from animals at the ABRO field station at Dryden. The sheep serum N207 (19/7/64) was kindly donated by Dr. E.M. Tucker.

Rabbit samples - The rabbit serum samples were from rabbits kept at A.B.R.O.

Collection and storage of red cells

Blood samples were taken from cattle and sheep using hypodermic needles inserted into the jugular vein. The blood was collected into sterilised evacuated tubes containing 1/7 of their volume of anticoagulant. The blood was immediately mixed with the anticoagulant and then stored at 4°C until required; it was always used within one week of collection.

Collection and storage of serum samples

Serum samples were collected from cattle and sheep in the same way as red cells but into dry tubes without anticoagulant. Rabbits were bled from the ear vein. The blood was allowed to clot at room temperature and the serum pipetted off. The serum was stored at -25°C until required.

Anticoagulant

One volume of acid citrate dextrose (A.C.D.) to 7 volumes of blood.

sodium citrate	13.2g.
dextrose	14.2g.
citric acid	4.8g.
distilled water	16.

Complement

Normal rabbit serum was used as a source of complement. Blood was obtained from a rabbit farm and the serum taken off and stored in 1ml. quantities at -196°C in liquid nitrogen. It was thawed and diluted 1:3 with 0.9% saline as required. Thawed unused complement was discarded, never frozen and reused.

Antisera

The antisera were prepared according to the immunisation schedule in Appendix B. The sera were stored at -25°C until required and when necessary heated to 56°C to inactivate the complement before use.

The infectious mononucleosis serum was kindly donated by Professor R.R.A. Coombs.

Appendix B

Immunisation Schedules

Rabbit Immunisation Schedule

Animal	Course	Immunisation material	Immunisation method	Immunisation dates	Dates of bleeds used
R2		1cc. 50% washed bovine red cells	I/V	2/12/65 - 23/12/65 7 times, twice weekly	30/12/65
R4	1.	1cc. 50% washed bovine red cells	I/V	2/12/65 - 23/12/65 7 times, twice weekly	19/ 5/66
	2.	" " "	"	28/ 4/66 - 12/ 5/66 4 times, twice weekly	18/ 2/69
	3.	" " "	"	9/ 1/69 - 7/ 2/69 4 times, once weekly	
R6	1.	1cc. 50% washed bovine red cells	I/V	2/12/65 - 23/12/65 6 times, thrice weekly	24/ 5/66
	2.	" " "	"	28/ 4/66 - 12/ 5/66 4 times, twice weekly	
R17	1.	1cc. whole bovine blood.	1/Ml. hind leg	26/ 4/66 - 23/ 9/66 6 times, once monthly	24/ 2/68
	2.	2cc. whole bovine blood	"	8/ 5/67 - 10/ 6/67 2 times, once monthly	
R24	1.	2mg. bovine red cell membrane protein in multiple emultion (2cc.)	1/Ml. hind leg	21/11/66 - 12/12/66 2 times, once monthly	4/ 3/68
	2.		"	16/ 1/68 - 12/ 2/68 2 times, once monthly	

Rabbit Immunisation Schedule (cont'd)

Animal	Course	Immunisation Material	Immunisation method	Immunisation dates	Dates of bleeds used
R51		1cc. sheep serum	1/Ml. hind leg	30/ 7/68 - 25/12/68 6 times, once monthly	16/12/68
R52		½cc. bovine red cell membrane protein in multiple emulsion (2cc.)	1/Ml. hind leg	3/ 4/69 - 4/ 6/69 6 times, twice monthly	5/ 6/69
R53		½cc. bovine red cell membrane protein in multiple emulsion (2cc.)	1/Ml. hind leg	3/ 4/69 - 4/ 6/69 6 times, twice monthly	5/ 6/69
R60	1. 2.	1cc. whole bovine blood from low agglutinator	I/V	5/ 6/69 - 16/ 6/69 6 times, thrice weekly 16/ 7/69 - 10/ 8/69 8 times, twice weekly	17/ 6/69 22/ 8/69
R62		1cc. whole bovine blood from high agglutinator	I/V	5/ 6/69 - 16/ 6/69 6 times, thrice weekly 16/ 7/69 - 10/ 8/69 8 times, twice weekly	17/ 6/69

Cattle Immunisation Schedule

Animal	Course	Immunisation material	Immunisation method	Immunisation dates	Dates of bleeds used
FS99A	1.	5ml. sheep serum in multiple emulsion	1/M	14/ 8/68 - 18/ 9/68 5 times, once weekly	22/ 8/68
K21		5ml. rabbit serum in multiple emulsion	1/M	6/ 1/67 - 10/ 5/67 5 times, once monthly	17/ 5/67
MZ111B	1.	10ml. of 50% washed bovine red cells	1/M	14/12/65 - 20/ 4/66 7 times, twice monthly	1/ 8/68
	2.	" " "	"	17/ 8/66 - 31/ 8/66 2 times, once weekly	
	3.	" " "	"	20/ 6/68 - 23/ 7/68 5 times, once weekly	

Sheep Immunisation Schedule

Animal	Course	Immunisation material	Immunisation method	Immunisation dates	Dates of bleeds used
3A146	1.	5ml. whole bovine blood	1/M	29/ 7/69 - 12/ 8/69 2 times, once weekly	17/12/69
	2.	" "	"	4/11/69 - 3/12/69 4 times, once weekly	
7B44	1.	5ml. whole bovine blood in multiple emulsion	1/M	28/ 5/68 - 5/ 7/68 3 times, twice monthly	
	2.	" " "	"	1/ 2/69 - 30/ 2/69 2 times, twice monthly	25/ 2/69
7B51		5ml. rabbit serum in multiple emulsion	1/M	9/ 1/68 - 14/ 1/69 7 times, once bimonthly	16/ 1/69

Appendix C

1. Calculations of sialic acid values (Chapter 2)

Type I - Low agglutinator e.g. FS.108A

sialic acid values read at 549 nm. total protein read at 700 nm.

0.450	0.434
0.462 mean 0.455	0.433 mean 0.438
0.450	0.442
0.450	0.432

$$\therefore \frac{S}{P} = \frac{0.455 \times 5.5}{0.438 \times 20} = 0.28$$

From calibration curves 0.36 OD units = 0.06µM sialic acid
1 OD unit = 310 µg/ml protein

$$\therefore \frac{\text{amount of sialic acid}}{\text{amount of protein}} \text{ of } 0.112 = 0.06\mu M \text{ sialic acid/mg protein}$$

$$\therefore \frac{0.28}{0.112} \times 0.06 = \text{number of } \mu M \text{ sialic acid/mg protein}$$

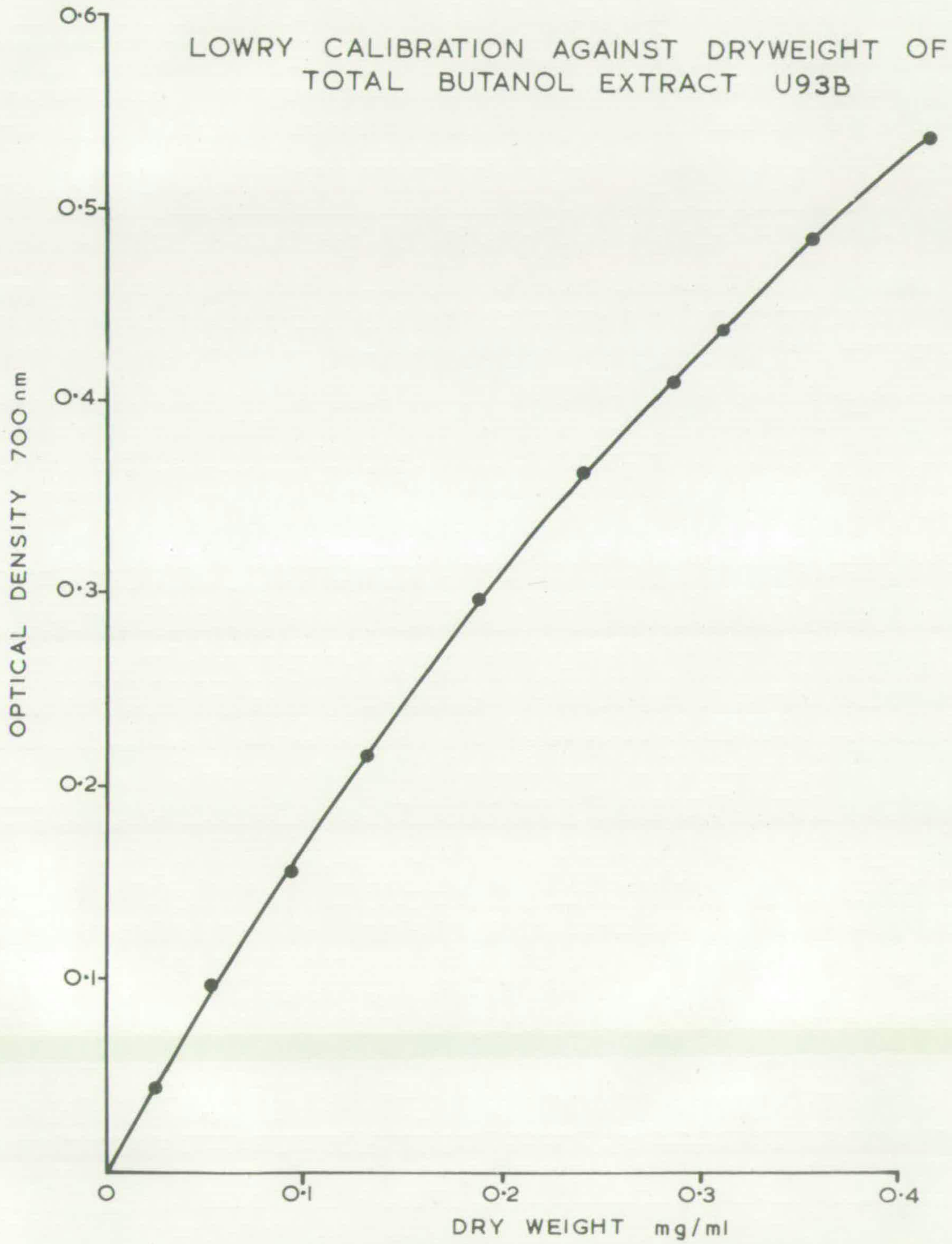
$$= 0.15$$

∴ FS.108A has 0.15µM sialic acid/mg. protein

Type II - high agglutinator e.g. U85B

sialic acid values read at 549 nm. total protein read at 700 nm.

0.261	0.412
0.265 mean 0.262	0.406 mean 0.407
0.261	0.405
0.263	0.404


LOWRY CALIBRATION AGAINST DRYWEIGHT OF
TOTAL BUTANOL EXTRACT U93B
OPTICAL DENSITY 700 nm
0·6
0·5
0·4
0·3
0·2
0·1
0
0·1
0·2
0·3
0·4
DRY WEIGHT mg/ml

$\therefore \frac{S}{P} = \frac{0.262 \times 5.5}{0.407 \times 20} = 0.18$

$\therefore \frac{0.18}{0.112} \times 0.06$ = number of uM sialic acid/mg. protein

= 0.096

$\therefore$ U85B has 0.096 uM sialic acid/mg. protein

2. Calculation of the inheritance of agglutinability (Chapter 4)

Ayrshire data

There are 108 Ayrshire animals of which

44 are high agglutinators (H)
64 are low agglutinators (L)

Consider frequency of A_L in population to be p

" " a_H " " " " q

$\therefore$ Frequency of genotypes assuming no selection

$A_L A_L$	$A_L a_H$	$a_H a_H$
p^2	$2pq$	q^2

$q^2 = \frac{44}{108}$

$q^2 = \frac{11}{27}$

$= 0.41$

$\therefore q = 0.64$

$\therefore p = 0.36$

Probability that an animal with low agglutination will be $A_L A_L$ is

$$\frac{p^2}{p^2 + 2pq} = \frac{0.13}{0.13 + 0.46}$$

$$= \frac{0.13}{0.59}$$

$$\doteqdot \frac{2}{10}$$

$\therefore$ On average 2 out of every 10 L animals will be homozygous for L

From Ayrshire family data

25 L x L $\longrightarrow$ 4H + 21L

Of these 25 (L x L) matings we expect

AA x AA	AA x Aa	AaAa
$\frac{4}{100}$	$\frac{32}{100}$	$\frac{64}{100}$

Now Aa x Aa $\longrightarrow$ 1 in 4 aa

and $\frac{64}{100}$ of the matings are AaAa

$\therefore$ $\left(\frac{64}{100} \times \frac{1}{4}\right)$ of the progeny expected to be aa.

i.e. $\frac{64}{100} \times \frac{1}{4} \times$ 25 animals

$= 4$

No. highs expected = 4

$\therefore$ No. highs observed = 4

$\therefore$ Hypothesis fits

$$19 \begin{pmatrix} H \times L \\ L \times H \end{pmatrix} \longrightarrow 8H + 11L$$

If $\frac{2}{10}$ of the matings are expected to be aa x AA

and $\frac{8}{10}$ of the matings to be aa x Aa

and aa x Aa $\longrightarrow$ 1 in 2aa

Then $\left(\frac{8}{10} \times \frac{1}{2}\right)$ of progeny will be aa - high

$\therefore$ $\left(\frac{8}{10} \times \frac{1}{2} \times 19\right)$ of progeny will be high

$$= \frac{76}{10}$$

$$\simeq 8$$

$\therefore$ No. highs expected = 8
No. highs observed = 8

Hereford Data

There are 255 animals of which

213 highs

42 lows

Consider frequency of A_L in population to be p

" " a_H " " q

Frequency of genotypes, assuming no selection

A_LA_L $\quad$ A_La_H $\quad$ a_Ha_H

is p^2 $2pq$ q^2

$$q^2 = \frac{213}{255}$$

$$= 0.83$$

$\therefore$ $q = 0.9$

$\therefore$ $p = 0.1$

Probability that a low animal will be $A_L A_L$ is

$$\frac{p^2}{p^2 + 2pq} = \frac{0.01}{0.01 + 0.18}$$

$$= \frac{0.01}{0.19}$$

$\therefore$ On average 1 in every 19 low animals will be homozygous low

For the Hereford family data

$$21 \quad H \times H \longrightarrow 21\ H$$

$$8 \begin{pmatrix} H \times L \\ L \times H \end{pmatrix} \longrightarrow 4\ H + 4\ L$$

$\therefore \frac{18}{19}$ of matings expected to be aa x Aa

aa x Aa $\longrightarrow$ 1 in 2 aa

$\therefore$ $8 \times \frac{1}{2} \times \frac{18}{19}$ of progeny will be high

$$= \frac{76}{19}$$

$$\simeq 4$$

$\therefore$ No. highs expected = 4
No. highs observed = 4

Vox Sang. *18:* 251–256 (1970)

Ox Erythrocyte Agglutinability

2. Differential Agglutinability

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Abstract. This paper describes a form of antiglobulin test which detects two types of cattle red cells – high and low agglutinators. There are no intermediates. The type of agglutinability correlates with the sialic acid content and precipitation profile of their red cell membrane protein.

Introduction

In our first paper [4] we referred to the work on the differential agglutinability of ox red cells by Gleeson White, Heard, Mynors and Coombs [2], and Coombs, Gleeson White and Hall [1]. These workers used two systems for detecting the agglutinability of ox red cells, one, the direct agglutination of ox cells with rabbit and guinea pig antisera, and the other, antiglobulin tests using Paul-Bunnell antibody or rabbit or guinea pig antisera to sensitise the cells and then adding an appropriate antiglobulin serum. This latter technique involves sensitising the cells with a titration of the sensitising antibody followed by a fixed concentration of the antiglobulin serum. Cattle were divided into three classes of agglutinable, moderately agglutinable and inagglutinable, but these were only arbitrary divisions in a continual gradation from agglutinable to inagglutinable. Moreover, cells that were inagglutinable with one antigen-antibody system were inagglutinable with other antigen-antibody systems. In their latest paper Uhlenbruck, Seaman and Coombs [5] showed that more sialic acid was released from inagglutinable cells than from agglutinable cells. They found that neuraminidase did not make the cells more agglutinable although treatment with pronase did have this effect.

This paper shows that using an antiglobulin test where the antiglobulin serum and not the sensitising antibody is titrated, cattle red cells can be divided into two groups – high and low agglutinators. The low agglutinators have a high level of sialic acid and the high agglutinators a lower level.

Materials and Methods

Antisera. (1) Bovine anti-rabbit globulin K21 (17/5/67) prepared by multiple intramuscular injections of whole rabbit serum in a double emulsion [3] of Freund's complete adjuvant. The serum was collected after five monthly immunisations.

(2) Rabbit anti-bovine red cell R2 (30/12/65), R3 (8/5/66) R4 (19/5/66) (21/1/69), and R6 (24/5/66). The rabbits were injected intravenously three times per week for four weeks with washed bovine red cells. After a delay of two months the course of injections was repeated. Serum was collected one week after the last injection and was then stored at –25°C. The rabbit serum was heated at 56°C for 30 min to inactivate complement.

(3) R24 (4/3/68) Rabbit anti-bovine red cell membrane protein was prepared by bimonthly injections of membrane protein (2 mg) in double emulsion adjuvant. The animal was bled after three immunisations.

Agglutination tests. Serial dilutions of the rabbit sera were distributed to the wells of conical bottomed microtiter plates (Cooke Engineering) in complement fixation diluent (c.f.t., Oxoid), followed by the addition of one drop of a 2% suspension of thrice washed red cells. The test was read by patterns after being allowed to settle overnight.

Antiglobulin test. Red cells were sensitised for 1 h at room temperature with an appropriate dilution of the different rabbit sera. They were then washed three times in saline and made up to a final concentration of 2%. These cells were then added to a titration of the antiglobulin serum (K21). Again the test was read by patterns after being allowed to settle overnight.

Haemolytic test. Sera were diluted in c.f.t. diluent in round bottomed plates and a 1% suspension of red cells followed by a 1:4 dilution of rabbit complement were added. The tests were incubated at 26°C overnight with constant shaking for 6 h.

Red cells. Blood was collected in Acid Citrate Dextrose from ABRO farms and used within one week of collection.

Results

When bovine red cells were agglutinated by a rabbit antiserum to ox red cells (R2, R3, R4, R6) or with an antiserum to red cell membrane protein (R24) there was a complete gradation in agglutinability from low to high (fig. 1), which is in agreement with earlier work [2].

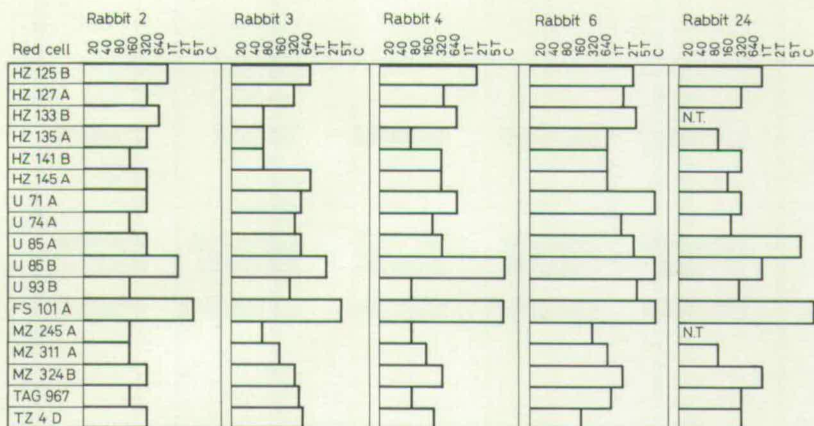

Fig. 1. Direct agglutination titres of 5 rabbit antisera to ox red cells.

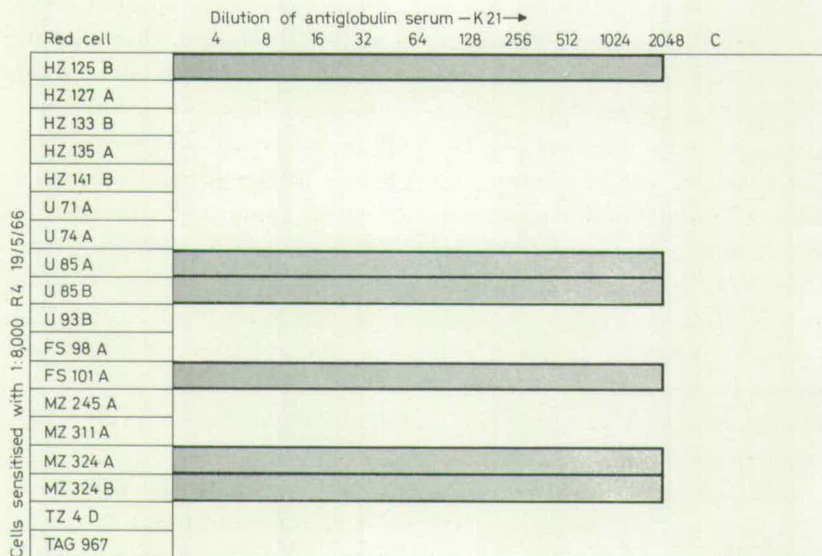

Fig. 2. Typical protocol of an antiglobulin test using a fixed dilution of sensitising rabbit serum R4 19/5/66 and a titration of ox anti rabbit globulin serum. In this test the antiglobulin serum was not titrated beyond 1:2048.

Sensitised R 2 1:10,000 | Sensitised R3 1:16,000 | Sensitised R4 (21/1/69) 1:16,000 | Sensitised R6 1:16,000 | Sensitised R24 1:3,000

Dilutions of K21: 4 16 64 256 1T 5T 20T C

Red cell: HZ 125 B, HZ 135 A, HZ 141 B, HZ 145 A, U 74 A, U 85 A, U 85 B, U 93 B, FS 101 A, MZ 311 A, TZ 4 D, TAG 967

Fig. 3. Antiglobulin titres using 4 other rabbit antisera to ox red cells and one to ox red cell protein.

However, if these same ox cells were sensitised with a sub-agglutinating dose of R4 (beyond the agglutination end point of the most highly agglutinable cell), washed three times in saline, and then added to a titration of a bovine anti-rabbit globulin serum (K21), the picture was quite different. Some cells were highly agglutinable, others not agglutinated at all (fig. 2). The antiglobulin test was repeated using the other four sera and a later bleed of R4. To confirm that all cells were in fact sensitised with rabbit antibody at the dilutions used for sensitisation a haemolytic test was performed. The sensitisation titres used were in the region of the haemolytic end points. The haemolytic end point for any one serum showed very little variability between cells. The antiglobulin results are shown in figure 3. Unfortunately not all animals were available on all occasions hence producing some differences between the three figures in the animals tested, but it can be seen that the five sera give essentially the same result.

It was then found that the difference between red cells, in their ability to agglutinate, corresponded with the difference in the sialo protein ratio and precipitation characteristics of their red cell membrane protein [4], (table I).

Although most cells giving a high direct agglutination titre have a Type II protein, a few, e.g. HZ 133B figure 1 and figure 2 have a high direct titre but a low antiglobulin titre and have Type I protein.

Although it is naturally not feasible to extract the red cell membrane protein from large numbers of animals it is possible to perform extensive antiglobulin tests and so far of several hundred animals

Table I. Correlation between the agglutinability and sialo protein ratio of ox red cell membranes

Cell	Antiglobulin agglutinability	Sialo protein μM/mg	
HZ 127 A	Inagglutinable	0.110	Precipitation profile Type I (4)
HZ 133 B	Inagglutinable	0.130	
HZ 135 A	Inagglutinable	0.143	
HZ 141 B	Inagglutinable	0.139	
U 71 A	Inagglutinable	0.125	
U 74 A	Inagglutinable	0.146	
U 93 B	Inagglutinable	0.148	
FS 98 A	Inagglutinable	0.141	
MZ 245 A	Inagglutinable	0.135	
MZ 311 A	Inagglutinable	0.120	
TZ 4 D	Inagglutinable	0.134	
TAG 967	Inagglutinable	0.150	
HZ 125 B	Agglutinable	0.099	Precipitation profile Type II (4)
U 85 B	Agglutinable	0.100	
FS 101 A	Agglutinable	0.098	
MZ 324 A	Agglutinable	0.098	
MZ 324 B	Agglutinable	0.105	

tested we have only found two types of reaction, high or low agglutinability. Amongst the animals we have looked at so far we have found marked breed differences in the frequency of high and low agglutinability.

Discussion

The characteristic of agglutinability was compared in 18 animals with the sialo protein ratio and precipitation profile of the red cell membrane protein [4]. It was found that all animals with Type I protein and high sialic acid levels were of the low agglutinable type and all animals with Type II protein and low sialic acid were of the highly agglutinable type. On occasions we have, however, seen partial agglutination with HZ 127A. This has taken the form of slight agglutination to an antiglobulin titre of 1:256 but at no dilution of the antiglobulin serum is the reaction strong. This trace reaction is more marked with some sera than others. It may be significant that it has the lowest sialo protein ratio of the Type I animals tested. There has been no obvious illness in the animal and so far no explana-

tion can be found for the variability. It does not appear to be a seasonal variation. Other cattle, notably Tag 967, have been sampled as very young calves and later as adults, and have given the same result on each occasion, thus showing no evidence that age affects the protein type.

It was postulated by Coombs *et al.* [1] that the red cell had an irregular surface and that in some cells the antigen was more inaccessible to the antibody. Such deeply sited antibody could not be agglutinated by an antiglobulin serum. In their recent paper Uhlenbruck, Seaman and Coombs [5] suggested that the variation in agglutinability depended on the amount of mucoid outside the protein backbone of the cell membrane. It is not clear, as no test of significance was applied, whether these workers have been looking at three levels of sialic acid release or whether in fact they have only two types of cell as we find here. Our results show that the variation of agglutinability is a discontinuous variable rather than a continuum of high through intermediate to low agglutinability. The variation of the three characters of agglutinability, protein precipitation and sialic acid content is bimodal.

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