BOAR SEMINAL HAEMAGGLUTININ

II. COMBINATION WITH RED CELLS AND SPERMATOZOA

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Summary. The haemagglutinins in boar seminal plasma are absorbed by ejaculated washed motile ram, bull and rabbit spermatozoa. Washed epididymal spermatozoa also absorb the haemagglutinin, but less avidly.

Considerable motility of the heterologous spermatozoa is still present after 15 min contact with boar seminal plasma. Mixed cell agglutination of bull spermatozoa and red cells gives a reticulate agglutination visible microscopically, and tail to tail agglutination occurs.

The haemagglutinins are absorbed at least as well by washed pig red cell ghosts as by the intact washed erythrocytes. The absorptive power of the ghosts is not destroyed by boiling them in n-NaOH or n-HCl for one hour.

It is presumed that a negatively charged 'receptor' group on the surface of the red cell is responsible for the absorption. The receptor does not appear to be connected with erythrocyte sialic acid removable by receptor-destroying enzyme, trypsin or by 0.1 n-HCl.

INTRODUCTION

A powerful haemagglutinin in boar seminal plasma, described by Boursnell & Coombs (1966), has been shown to be caused by the presence of a number of basic proteins by Nelson & Boursnell (1966) who were able partially to separate these proteins and to describe some of their properties. Investigations on the combination of the haemagglutininins with red cells and red cell ghosts and some of their reactions with homologous and heterologous spermatozoa are described in the following paper.

MATERIALS AND METHODS

Collection of semen, epididymal spermatozoa, blood and bakers' yeast cells

Semen was obtained, by the use of artificial vaginae, from animals housed at the Animal Research Station. In all cases the semen was centrifuged, as soon as possible after collection, at about 1000g at room temperature (19° to 21° C) to separate the spermatozoa from the fluid supernatant seminal plasma. Boar

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semen gel was removed before centrifuging by filtering through layers of washed muslin.

Boar epididymal spermatozoa were obtained from freshly excised epididymides obtained from the abattoir, following the method of Lasley & Bogart (1944) as described by Walton (1957). The epididymal spermatozoa were obtained by centrifuging at room temperature at 1000 g.

Blood was collected from slaughtered animals into isotonic acid citrate anti-coagulant. In most cases the red cells were prepared from fresh material, but occasionally after 24 hr storage of the blood at 4° C.

Fresh baker’s yeast cells were obtained commercially and used on the day of harvesting after suspending and washing three times in Ringer–saline.

**Washing of spermatozoa and red cells**

This was normally carried out at about 1000 g with about 10 vol. of the appropriate solution (buffered saline for red cells or Ringer–saline for spermatozoa). Care was taken to obtain a good redispersal of the cells at each wash. The red cells were washed three times and the spermatozoa twice. Microscopical observations of spermatozoal motility were carried out on the twice-washed material.

**Preparation of red cell ghosts**

The red cells, after five washings in buffered saline, were haemolysed by adding about 50 vol. of water and warming for 30 min to 40° C. The ghosts were precipitated at room temperature by adjustment with HCl to pH 5-8, centrifuged and washed with water. The acidification, centrifugation and washing were repeated until the washing fluid was almost colourless. The final washing and suspension was made in buffered saline. Particularly when these ghosts had been chemically treated or extracted as later described, the sometimes rather lumpy product was pulverized in suspension in a Potter Homogenizer before dispensing for the absorption experiments.

**Enzymes**

Pancreatic trypsin (British Drug Houses Ltd) was prepared and used at pH 7-7 according to the method of Morton & Pickles (1951). The proteolytic activity of the preparation was proved by the removal of gelatin emulsion from exposed and developed photographic film in 5 min at 37° C.

Receptor destroying enzyme (Burroughs Wellcome) (RDE) was used in the presence of 0-005 m-Ca and 0-05 m-maleate buffer, pH 5-5.

**Determination of sialic acid and protein**

Sialic acid was determined by the method of Warren (1959) after RDE treatment of red cells in order to prove the activity of the receptor-destroying enzyme. Sialic acid was also determined after trypsin treatment of red cells. Hydrolysis in 0-1 n-H₂SO₄ for 1 hr at 80° C was used to release the sialic acid from the sialomucopside split by the trypsin (Svennerholm, 1958; Warren, 1959) before the sialic acid determination.

Protein determinations were carried out by the Folin–Ciocalteu method as modified by Lowry, Rosenbrough, Farr & Randall (1951).
Absorption by cellular entities of haemagglutinins from boar seminal plasma

Four successive absorptions were performed at room temperature except where indicated, and for this purpose suitable aliquots of the washed packed red cells, ghosts or spermatozoa were transferred to 10 ml polypropylene centrifuge tubes. An aliquot (1·0 or 0·5 ml) of the seminal plasma or vesicular secretion to be absorbed was applied to the first tube and the contents thoroughly mixed. The standard time for any one absorption was 15 min. The tube was then centrifuged (1000 g) for a few minutes until the resulting supernatant could be poured from the cells without unduly disturbing them. The supernatant was transferred as quantitatively as possible with a Pasteur pipette to the second tube in the series. The procedure was then repeated. The four times absorbed supernatant was titrated immediately or stored at −20°C until required for titration.

Microscopical observations of motility were carried out on the first samples of packed spermatozoa after absorption.

'Serological' titrations

Titrations were carried out in 8 × 50 mm tubes in the presence of adequate controls by serial doubling dilutions in 0·2 ml buffered saline, Ringer–saline or other medium as indicated. Then 0·2 ml of 1% suspension of washed red cells or spermatozoa in the appropriate medium were added. After mixing, the titration was allowed to stand at room temperature except in one experiment where the effect of temperature was explored. Washed chicken red cells were normally employed as indicator cells because these gave the result more quickly than any other easily available red cells. In a few cases pig red cells were employed; these took much longer to indicate agglutination, but the end point was little different from that given by the chicken cells. The titre was expressed graphically in terms of the number of tubes (log₂) to the end of the visible reaction.

EXPERIMENTAL

Combination of haemagglutinin with red cells

Attempted reversal of pig red cell agglutination. Attempts to reverse the agglutination by repeated washing with buffered saline did not succeed. Even after the fourth wash, very large lumps of agglutinated red cells were seen under the microscope and small lumps or free cells were almost completely absent. Haemolysis developed which, although not severe, was greater than would normally be seen on washing untreated red cells.

Effect of temperature on agglutination. Identical titrations carried out at 37°, 21° and 4°C gave very similar results. The cold reaction was slow in forming an end point which was ultimately not less definite than at the higher temperatures. An opalescence in the supernatant fluid in the first few tubes of the 4°C titration may well be due to the cold precipitation of the Fraction B noted by Boursnell, Nelson & Cole (1966).

Effect of time and temperature on the absorption by pig red cells. A 5·0 ml volume of packed washed pig red cells was suspended, with stirring, in 5·0 ml of a sample of boar seminal plasma at room temperature. A 2·0 ml aliquot of the stirred
suspension was taken immediately ('time 0') and centrifuged at 1000 g. The supernatant was removed from the deposited cells as quickly as possible and stored until other 2·0 ml samples had been taken at different times and similarly treated. Titrations of the resulting supernatants (Text-fig. 1) showed that the maximum uptake of haemagglutinin was reached at a time between 15 and 30 min.

Two identical absorptions of 1·0 ml seminal plasma with 0·25 ml packed washed red cells were carried out: (1) at 21°, and (2) with all the reagents at 1° C. The reduction from the titre of the unabsorbed seminal plasma (4096) was only slightly greater (to a titre of 8) at room temperature than at 1° C (to a titre of 32).

![Text-fig. 1](image)

**Text-fig. 1.** Haemagglutination titres of samples of boar seminal plasma taken at various times from a 1:1 (v/v) mixture of packed washed pig red cells and boar seminal plasma.

**Effect of pig blood plasma, albumin, globulin and haemoglobin on agglutination.** The results of titrations of boar seminal plasma, carried out with buffered saline and in the presence of 0·1% (v/v), 1% and 10% dilutions of pig blood plasma in the saline (Text-fig. 2) demonstrate a progressive inhibition with increasing concentration of blood plasma. Further titrations in the presence of 1% pig blood albumin and globulin both showed reductions from the titre of the boar seminal plasma similar to that given by pig blood plasma itself. Titration in the presence of 1% pig haemoglobin only reduced the seminal plasma titre by two tubes.

**Effect of number of absorptions on agglutination.** Four 1·0 ml samples of boar seminal plasma were subjected to one, two, three or four absorptions, each with 0·5 ml packed pig red cells. The results of this experiment (Text-fig. 3) showed
that a progressive removal of the haemagglutinin occurred with each absorption. Although, in this experiment, there was a trace of haemagglutinin remaining after the fourth absorption, other experiments demonstrated a complete removal under similar conditions.

**Text-fig. 2.** Effect of varying concentrations of pig blood plasma on haemagglutination titre of boar seminal plasma against chicken red cells.

**Text-fig. 3.** Effect of number of absorptions of 1.0 ml aliquots of boar seminal plasma with 0.5 ml quantities of washed pig red cells on the haemagglutinating titre.

*Comparative absorption by pig and ox red cells and washed yeast cells.* Equal packed volumes (0.5 ml) of pig and ox red cells and washed yeast cells were used to absorb the haemagglutinins from 0.5 ml aliquots of a sample of boar seminal plasma (titre, 32,000). Although the red cells of both types absorbed the haemagglutinin completely, the yeast cells were not so effective and the original titre was only reduced to 256.
It is interesting that, although the pig red cells were strongly agglutinated, only small aggregates of three or four ox cells were formed, and no agglutination of the yeast cells could be seen.

**Effect of 'sensitization' of yeast cells by proteins.** Use was made of the comparatively small absorption by yeast cells to observe the effect of proteins, known to reduce the haemagglutinin titre (Nelson & Boursnell, 1966), coating the surface of the yeast cell. Washed packed yeast cells (0.5 ml) were treated for 45 min at room temperature with 0.5 ml of either pig blood plasma or boar epididymal secretion. The yeast cells, 'sensitized' in this way, were then washed either two or five times with buffered saline. They were then used for four absorptions of 0.5 ml quantities of boar seminal plasma. There was no significant difference between the untreated and 'sensitized' yeast cells.

![Text-fig. 4. Comparison of the haemagglutinin titres against chicken red cells of 1.0 ml samples of boar seminal plasma absorbed four times with various volumes of packed pig red cells or ghosts obtained from the same blood source.](image)

**Absorption by equivalent quantities of pig red cells and ghosts.** Early experiments had shown that 1.0 and 0.5 ml quantities of packed red cells or ghosts completely absorbed under the same conditions the haemagglutinins from 1.0 ml boar seminal plasma. Text-fig. 4 shows the results of four successive absorptions of 1.0 ml portions of seminal plasma with lesser quantities of red cells and ghosts which had been prepared from the same blood sample as follows. Two equal volumes of stirred blood were pipetted and the washed red cells either: (1) lysed and washed free from haemoglobin, or (2) retained as a buffered saline suspension. There was very little loss of ghosts during the lysing and washing. The preparations and the absorptions were carried out on the same day. Only a trace of haemolysis occurred during the absorption, even with the 0.25 ml quantity of packed red cells.

The greater apparent absorbing efficiency of the ghosts may be attributed,
in part at least, to the fact that the packed volume of ghosts is about one half that of the red cells from which they were formed. Thus a volume of ghosts represents about twice the number of discrete entities in a similar volume of red cells.

Search for further agglutination inhibitors

The discovery of a number of inhibitors of haemagglutination and of the reaction between the basic proteins and Fraction B (one of the two major protein components of boar seminal plasma) described by Nelson & Boursnell (1966) prompted a search for other substances of known composition which would act similarly. It has been established that the inhibitory action of aqueous extracts from floc cellulose phosphate (Whatman) described by these authors is not affected by boiling in water or saline for 10 min, nor is it apparently dialysable through Visking cellophane tubing.

Dextran sulphates (Pharmacia, Great Britain, Ltd) of molecular weight 2 x 10^6 and 1·2 x 10^3 both have powerful inhibitory action even when present in high dilution. At 1/5000 (w/v) saline solution of the lower molecular weight dextran sulphate reduced the titre of a sample of boar seminal plasma from 64,000 to 64. Even at these dilutions the dextran sulphates produced an opalescence in the first few tubes in the titrations, presumably caused by co-precipitation with the free and combined basic proteins. Dextran sulphate, like heparin (Nelson & Boursnell, 1966), produced a considerable precipitate when added to boar seminal plasma; it also precipitated clupeine and salmine from solution.

DNA and RNA (sodium salts, British Drug Houses Ltd) also produced slight inhibition and gave opalescent solutions above the agglutinated red cells in the first few tubes of the titration.

It would seem that all these inhibitors act by combining with the basic haemagglutinins in solution (either free or combined with Fraction B) in competition with the ‘receptor’ groups on the red cell.

Neither the presence of isotonic MgSO_4 7H_2O (Heard, Hinde & Mynors, 1949), 50 mm-cyanide, 30 mm-thiomalate or 25 mm-H_2O_2, nor prior treatment of the cells with tannic acid (Stavitsky, 1954) or chromate (Gray & Sterling, 1950) had any significant effect on the titre.

Treatments of surface of pig red cells or red cell ghosts

An effort was made to identify the receptor group on the surface of the red cell responsible for the absorption of the haemagglutinin.

Short extractions with boiling 80% (v/v) ethanol or boiling ether–ethanol (3:1, v/v) did not reduce significantly the absorptive capacity of pig red cell ghosts.

Treatment of red cell ghosts with N-HCl or N-NaOH in a boiling water bath for 1 hr did not remove the receptor sites. Occasionally, however, some removal or destruction of the sites took place with N-HCl, but the reason for this occurrence could not be determined. Treatment at 100° C for even longer time (2 hr) or with more concentrated acid (6-N-HCl) did not appear to impair the ability of the stroma to absorb the haemagglutinins.
The receptor sites were not removed by heating the red cell ghosts with 0·1 n-HCl for 1 hr in a boiling water bath, a process which would be expected (Svennerholm, 1958; Warren, 1959) to remove N-acetyl neuraminic acid (sialic acid), known to be present on the red cell surface (Klenk & Uhlenbruck, 1958).

In order further to investigate the apparent disconnection between the haemagglutination and the sialomucopeptide (Cook, Heard & Seaman, 1961) on the red cell surface, RDE and trypsin were used to remove the combined sialic acid from the intact red cell surface. It was shown that, after incubation at 37° C for 30 min, sialic acid was present in the supernatant enzyme solutions. It was also present in the supernatant neutralized solution after treatment of ghosts for 1 hr with 0·1 n-HCl. Nevertheless subsequent absorption of boar seminal plasma by the red cells treated with either enzyme showed that the absorptive ability of the red cells had been retained.

Action of heated guinea-pig kidney cells and ammonia

The fact that boiled red cell ghosts were effective in absorbing the haemagglutinin suggested a parallel with the Paul–Bunnell system. However, heated guinea-pig kidney cell suspension (Burroughs Wellcome & Co.), which does not absorb the Paul–Bunnell antibody, did absorb the haemagglutinins completely in four treatments of a sample of seminal plasma with approximately equal volumes of packed kidney cells.

Treatment of boar seminal plasma with 0·2 n-NH₄OH, following the procedure for the inactivation of haemolytic complement (Gordon, Whitehead & Wormald, 1926), had no effect on the haemagglutinin titre of the seminal plasma, tested after neutralization.

Combination of haemagglutinins with spermatozoa

Washing of boar spermatozoa to remove haemagglutinins. Text-fig. 5 shows a typical experiment in which protein determinations and haemagglutinin titres were carried out on the supernatant fluids obtained by repeatedly washing the spermatozoa from a sample of ejaculated semen from which the gel had been removed.

Compared with the initial steep decrease, there is a pronounced change in the slope from the third to the tenth wash in both determinations. Very similar results were obtained with both a greater and a lesser quantity of spermatozoa relative to the volume of the washing fluid. In another experiment a comparison between a 5-min and a 30-min interval between the successive washing (under otherwise identical conditions) showed no significant difference between the results. Nevertheless, prolonged contact of the eleventh washing fluid (18 to 24 hr) at room temperature always produced a slight increase in the haemagglutinin titre above that given by the tenth washing fluid. In the experiment shown in Text-fig. 5, a mean titre of 12 (three or four tubes) was observed when the eleventh washing fluid was titrated.

Effect of the continued presence of boar spermatozoa and boar sperm plus gel. Boar semen, from which the gel had been removed immediately after collection, was kept at room temperature and samples of seminal plasma were obtained by
centrifuging the spermatozoa. There was no difference in the haemagglutinating titre of two samples of the seminal plasma obtained 10 min and 18 hr after collection.

An entire ejaculate of boar semen was allowed to stand with the gel at room temperature. Samples, taken at times from 5 min to 5 hr after collection, were strained through muslin to separate the gel and the spermatozoa were removed by centrifugation. Haemagglutinin titres on the seminal plasma samples showed no loss: a slight increase in the titre observed in the later samples may well be due to concentration caused by imbibition of water into the gel. At 24 hr the

**Text-Fig. 5.** Simultaneous determinations of boar seminal plasma haemagglutinin titre (curve and scale A) and protein (expressed in mg/ml) (curve and scale B) in successive washings of boar spermatozoa from fresh samples (duplicate experiments) of gel-free semen. 1·0 ml packed spermatozoa washed at room temperature ten times with 7·0 ml Ringer-saline with 12 min between each centrifuging. Vertical bars, error of one tube in reading titre.
whole mass was composed of solid gel and it was not possible to obtain a sample of fluid seminal plasma even by filtration under pressure.

No preservative was used in either of these experiments.

Absorptions by rabbit, bull and ram spermatozoa. Packed washed motile rabbit spermatozoa (0·5 ml) were used for absorptions of 0·5 ml boar seminal plasma. No massive agglutination occurred. The presence of the boar seminal plasma appeared to enhance motility. These treatments removed the haemagglutinins completely.

Text-fig. 6. Comparison of the haemagglutinin titres against chicken red cells of 1·0 ml samples of boar seminal plasma thrice absorbed with varying quantities of packed twice-washed bull spermatozoa.

Four absorptions of 1·0 ml boar seminal plasma were carried out with 1·0, 0·5 and 0·25 ml quantities of washed packed bull spermatozoa. Vigorous motility was observed in the washed spermatozoa and at the end of the first 15-min absorption. There was no massive agglutination and complete absorption of the haemagglutinins occurred with all these quantities. Text-fig. 6 shows the results of a further experiment, with lesser quantities of bull spermatozoa and only three absorptions, which was conducted under the same conditions otherwise.

An experiment was carried out to explore the possible effect on the agglutination of traces of ram seminal plasma retained on the surface of the spermatozoa. Samples of spermatozoa from the same semen sample were washed twice or five times. There was no sign of massive agglutination. The results of these experiments are shown in Text-fig. 7.
Absorption by boar epididymal spermatozoa. Epididymal spermatozoa were washed three times in Ringer–saline. Motility, absent before the washing, was very vigorous afterwards. With 1·0 ml boar seminal plasma were used 1·0, 0·5 and 0·25 ml packed spermatozoa. There was no massive agglutination of the spermatozoa, and very vigorous motility was observed after the first absorptions. The result is shown in Text-fig. 8.

Absorptions of seminal plasma haemagglutinins by ejaculated spermatozoa. One volume of packed ejaculated spermatozoa washed between the absorptions. This experiment was undertaken in an effort to determine whether spermatozoa originating in ejaculated semen could absorb more haemagglutinins when the spermatozoa were washed twice between the absorptions.

Sufficient boar semen was centrifuged to give a volume of packed spermatozoa from which two 0·5 ml samples were pipetted. One of these samples was washed twice in Ringer–saline and added to 1·0 ml of the original seminal plasma. After 15-min absorption at room temperature, the spermatozoa were centrifuged, washed twice and returned to the already absorbed sample of seminal plasma. This procedure was repeated until four absorptions had been completed. The other 0·5 ml sample of spermatozoa and another 1·0 ml of the same sample of seminal plasma were subjected simultaneously to exactly the same procedure except that the spermatozoa were not washed between the applications. While this second (control) sample of ‘absorbed’ seminal plasma showed

![Text-fig. 8.](image-url)
Text-fig. 8. Comparison of the haemagglutination titres against chicken red blood cells of 1·0 ml boar seminal plasma absorbed with varying quantities of washed boar epididymal spermatozoa.

Text-fig. 9. Comparative absorption of 0·5 ml of: (i) boar vesicular secretion (vs) and (ii) boar seminal plasma (sp) by 0·5 ml packed unwashed boar spermatozoa obtained from the seminal plasma sp. U, Unabsorbed; A, absorbed.
no reduction from the titre of the untreated seminal plasma, there was a slight reduction (three tubes) in the titre caused by the absorption on the spermatozoa washed twice between the absorptions.

Absorption of seminal plasma and vesicular secretion by ejaculated spermatozoa. The spermatozoa from a gel-free sample of boar semen were centrifuged and dispensed without washing into eight tubes each containing 0·5 ml packed spermatozoa. Four of these were used to absorb 0·5 ml of the same seminal plasma and four to absorb 0·5 ml boar vesicular secretion. Text-fig. 9 shows that there was a considerable reduction in the titre (nine tubes) of the vesicular secretion due to further absorption by the spermatozoa already partly saturated with haemagglutinins. In this experiment there was a reduction by three tubes in the titre of the seminal plasma caused by further absorption of the haemagglutinins, but in another comparable experiment there was no reduction in the titre of the seminal plasma at all.

However, not all the reduction in titre in the absorption can be attributed entirely to absorption on the surface of the spermatozoa. Investigation revealed that mere dilution of the vesicular secretion reduced the haemagglutinin titre by an amount slightly greater than could be accounted for by arithmetic dilution alone. The dilution of the vesicular secretion by the Ringer solution entrained in the packed spermatozoa applied in the absorptions is small and certainly does not account for the reduction of the titre of the vesicular secretion by nine tubes.

Aggregation of heterologous spermatozoa by boar seminal plasma haemagglutinin

Although, as mentioned above, massive agglutination of heterologous spermatozoa on the scale of that observed with pig red cells does not occur, an aggregation phenomenon nevertheless can be demonstrated.

When a sample of boar seminal plasma (0·2 ml) is titrated against 0·2 ml of 1% suspension of washed motile bull spermatozoa in Ringer solution, a moving granulation rapidly appears in the first tubes, and more slowly develops in those with lesser seminal plasma concentrations. Settlement of the spermatozoa under these conditions begins after a few minutes, considerably more rapidly than in a corresponding suspension in Ringer solution alone.

In the absence of boar seminal plasma, the spermatozoa settled as a fluffy deposit at the bottom of the tube. In the titration with boar seminal plasma present (haemagglutinin titre about thirteen tubes), the spermatozoa appear as a fluffy deposit occupying a considerably greater volume in the first eight or nine tubes.

The volume of the fluffy deposit does not completely correspond with the concentration of the seminal plasma. The maximum volume occurs at 16-to 64-fold dilutions of the seminal plasma, when the deposit occupies as much as 75% of the total volume of the fluid in the tube. After 12 hr or more, further settlement occurs to about 40% of the total volume with a corresponding settlement on the other tubes of the titration. In the tubes without seminal plasma the spermatozoa only occupy at this time about 5% of the volume of the fluid.

Mixed cell agglutination, using 0·2 ml of a mixed suspension of 1% bull
spermatozoa and 1% red cells, shows, to a lesser extent, this same characteristic increase in the volume of the settled cells in the presence of boar seminal plasma. The fluffiness of the spermatozoa gives way to a more granular appearance of the mixture of cells which remains uniformly pink. When boar seminal plasma is absent, the red cells finally settle out and form a small red button underneath the fluffy white deposit of spermatozoa.

Microscopic examination showed that the early stage was associated with a star-like appearance of the motile spermatozoa and that there was little occurrence of head to head or head to tail agglutination. As aggregation became more pronounced the tails tended to become entwined and the number of spermatozoa in each group increased, obscuring the original star-like pattern. No agglutination took place in Ringer solution alone or in the presence of boar seminal plasma from which the haemagglutinin had been removed by absorption with red cells.

In the mixed cell agglutination, when a deep preparation was viewed under relatively low power, it was possible to see a three dimensional reticulum in which red cells and spermatozoa were aggregated together (Plate 1). This was particularly striking, viewed against a dark ground, when the still motile spermatozoa caused the whole network to vibrate.

**DISCUSSION**

These investigations have shown clearly that the ability to absorb the basic haemagglutinins in boar seminal plasma is not confined to red cells alone but is a property also of some heterologous spermatozoa, bull and ram in particular.

In order to throw some light on the possibly relevant chemical structure of the spermatozoal surface, attempts were made to investigate the nature of the ‘receptor’ group presumed to be present on the surface of the red cell. Treatment of the red cell by papain (Nelson & Boursnell, 1966) and trypsin suggested that blood plasma proteins, still absorbed after prolonged washing, were not involved. This suggestion was strengthened by the failure of the treatments with tannate and chromate and the efforts to influence the absorption by yeast cells previously ‘sensitized’ with proteins known to combine with haemagglutinins.

The binding of the haemagglutinin factors to red cells and the irreversibility of the agglutination by saline-washing reaction suggests a strong similarity with the reaction between polylsine and the red cell surface, investigated electrokinetically by Katchalsky, Danon, Nevo & de Vries (1959).

Cook et al. (1961) have shown that a sialomucoprotein accounts for a considerable proportion of the negative electrokinetic charge on the intact red cell. Although RDE and trypsin were shown to have removed much of the sialic acid or sialomucoprotein, there was no apparent decrease in the ability of the enzyme-treated red cells to absorb haemagglutinins. It would seem that the sialomucopolymers, if they are involved, are not the sole ‘receptors’ on the cell surface. The observation of Nelson & Boursnell (1966) that sialic acid does not inhibit the precipitation of Fraction B Seph by the haemagglutinin, also suggests that the sialomucopolymers may not be directly involved.

The nature of the ‘receptor’ on the surface of the red cell is puzzling. Cook
Mixed agglutination of washed bull spermatozoa and washed pig red blood cells (1% and 0.2%, suspension respectively in Ringer-saline) produced by 1:10 dilution of boar seminal plasma in Ringer-saline. Late stage of agglutination showing lattice formation. An identical lattice also forms when bull spermatozoa are treated with a 1:10 dilution of boar seminal plasma in the absence of red cells. × 320.
et al. (1961) have shown that the sialomucoprotein is not the only group on the red cell which confers a negative electrophoretic charge. Combination of such a residual acid group on the surface of the red cells with the basic proteins of the haemagglutinins is suspected because apparently only strongly acid substances markedly compete with the agglutination reaction.

Whatever groups are present appear to be normally resistant to destruction by N-NaOH or N-HCl for 1 hr at about 100°C.

Although red cells from the pig are strongly agglutinated, ox red cells appear to be much less so. Nevertheless ox cells absorb haemagglutinin apparently as well as pig cells. The occurrence of a gradation of agglutinability in ox red cells from individual cattle towards some antisera has been demonstrated by Gleeson-White, Heard, Mynors & Coombs (1950). The difference in agglutinability between pig and ox cells could be due to the position of the 'receptor' sites on the cell surface (cf. Coombs, Gleeson-White & Hall, 1951).

The results (Text-fig. 5) obtained by repeatedly washing the seminal plasma from boar spermatozoa suggest that a proportion of the seminal plasma proteins is firmly absorbed on the spermatozoal surface and is only partly removed by prolonged washing. This is indicated by the marked change in the rate of removal which occurs after the second wash when about 1% of the proteins (and apparently about 0.1% of the haemagglutinins) remain. Protein determinations on the washing fluids from comparable experiments with either ram or bull spermatozoa demonstrate a similar effect after the second wash, also when approximately 99% of the seminal plasma proteins have been removed. A kindred change of slope, occurring when pig red cells are being washed free of blood plasma, can certainly be attributed to the release of the plasma proteins from a graded absorption on the surface of the red cell (Boursnell, Coombs & Rizk, 1953).

In the boar the similarity in the shape of the curves showing the contents of the proteins and the haemagglutinins in the washings suggests that some, at least, of these proteins may be associated on the spermatozoal surface. The only protein in boar seminal plasma definitely known to combine with the basic haemagglutinins is the relatively acidic Fraction B (Nelson & Boursnell, 1966) but these are of course not necessarily the only proteins absorbed. It is possible, but perhaps less likely, that the protein determined by the Folin–Ciocalteu method is solely the haemagglutinating basic protein.

It is not possible to say from these results at what stage in the repetitive washing the spermatozoal membranes become permeable to the intracellular proteins (Mann, 1964). Throughout this work efforts have been made to minimize damage to the spermatozoa caused by centrifugation and washing. The number of washings before the absorptions was therefore confined to two which removed about 99% of the free seminal plasma proteins in the semen.

In assessing the results of the spermatozoal absorption experiments reported in this paper the following four facts have to be borne in mind: (1) during ejaculation when boar epididymal spermatozoa come in contact with the seminal plasma, they only partially absorb the basic proteins contained in the vesicular secretion, (2) boar spermatozoa are not naturally agglutinated by the basic proteins, (3) boar epididymal spermatozoa are not so effective as bull or
ram spermatozoa in absorbing the basic proteins from seminal plasma, and
(4) when ejaculated seminal spermatozoa, apparently in equilibrium with the
basic proteins in seminal plasma, are separated and exposed without washing
to the greater concentration of basic proteins in vesicular secretion, they absorb
more of the basic proteins.

The most likely explanation of these facts is that there is a lesser number of
receptor sites available on the boar spermatozoal surface than on either ram
or bull spermatozoa.

It is not to be expected that full saturation of the receptor sites could be
achieved with only one ‘application’ of the basic proteins. Nevo, De Vries &
Katchalsky (1955) have shown that the absorption of polylysine on to red cells
or ghosts follows a parabola of the Freundlich isotherm type and Boursnell,
Coombs & Rizk (1953) have demonstrated that it is far from easy to saturate
antigenic receptors on the red cell surface with the homologous antibody and
many successive applications of fresh material were required even in cases
where the number of antigenic sites was known to be small (e.g. 5500 Rh (D)
antigenic sites on the human red cell). These considerations may help to answer
the question why all the haemagglutinin present in the seminal plasma is not
absorbed naturally by the spermatozoa in the semen. If a figure of about
3 \times 10^6 spermatozoa/\mu l represents the right order of magnitude for packed
spermatozoa and the normal spermatozoal density in boar semen is taken as
10^5/\mu l (Mann, 1964) then, with four absorptions of a given amount of seminal
plasma with equal volumes of packed washed epididymal spermatozoa, the
experiment involves the use of about 100 times the number of spermatozoa
normally present in the same volume of semen. Even then the absorption is
incomplete, in marked contrast to the absorptive capacities of ram and bull
spermatozoa.

Although there is no evidence for the presence of an inhibitory substance which
prevents the absorption of the basic proteins on to boar spermatozoa, the
possibility of its occurrence cannot be ruled out at present.

No massive agglutination comparable to that of the pig red cell has been
shown to occur with any species of spermatozoa, in spite of the fact that some
species (e.g. bull) absorb the haemagglutinins better than pig red cells. It has
been shown, however, that the haemagglutinins cause a tail to tail agglutina-
tion of bull spermatozoa. There appears to be little or no head involvement;
this could be related to the observations of Nevo, Michaeli & Schindler (1961)
that the negative charge on the bull spermatozoa tail is greater than that on the
head.

The appearance of the progressive tail to tail agglutination is obviously
similar to that described by Smith (1949) who was investigating the effect of
goat antiserum to rabbit seminal spermatozoa upon the washed suspension of the
homologous cellular antigen.

The increased volume of the fluffy deposit occurring when boar seminal
plasma is titrated against washed bull spermatozoa is undoubtedly due to the
formation of the lattice-like tail agglutination of the spermatozoa. The shrink-
age observed after a period of time must be due to the subsequent contraction
of the lattice after the spermatozoa have become immotile.
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REFERENCES


