Experiments have been reported in Paper I (1) in which the proteins of bovine seminal plasma were subjected to preliminary and electrophoretic characterization. In order to characterize this protein system further, ultracentrifugal and immunological methods have been applied.

A limited amount of work has been reported on the ultracentrifugation of the seminal plasma proteins. Ross (2) has noted a glycoprotein component in human seminal plasma with a sedimentation velocity of 7 (s20,w units), while the proteins of low molecular weight that were present were not observed to sediment under similar conditions.

The proteins of seminal plasma have not been well characterized by immunological reactions, although spermatozoa of different species have been studied by this means (3). Ross (2) has summarized some of the earlier investigations on the human seminal plasma proteins and confirmed with precipitin tests that antisera to blood serum may react more strongly with seminal plasma than does antiserum to the seminal plasma itself. Antisera to seminal plasma also reacted less strongly with blood serum than the antisera to the blood serum did with the seminal plasma.

Several investigators (2, 4–6) have reported a close relationship of the proteins of human seminal plasma to those of blood serum. In comparing the proteins of bovine seminal plasma with those of blood serum (1), it was found that electrophoretically the majority of these proteins resembled the α-globulins. Chemical evidence indicated, however, that they probably were not similar. Subsequently it was noted that there was a striking electrophoretic and ultracentrifugal similarity of the proteins of milk serum (non-casein milk proteins) to the proteins of seminal plasma. Consequently, the proteins of bovine seminal plasma have been further characterized and compared with the proteins of blood and milk serum to determine possible relationships.

**EXPERIMENTAL**

Seminal plasma was prepared by removal of the sperm cells from fresh semen by centrifugation, and blood serum was prepared by removing the
clear serum from clotted blood as described previously (1). The milk
serum proteins were prepared by removing the casein from fresh skim
milk clotted with a minimal amount of rennin at 37°, and were concentrated
by removing part of the water by freezing and exhaustively dialyzing the
concentrated serum against 0.08 M phosphate buffer of pH 6.9.

Electrophoretic analyses were conducted as described previously (1).
Ultracentrifugal analyses were conducted in a Spino ultracentrifuge, model
E, by the double cell technique. All solutions were equilibrated for the
electrophoretic and ultracentrifugal analyses by dialysis against several
changes of 0.12 M Veronal buffer of pH 8.6 at 4°.

For the immunological analyses, blood serum was obtained by pooling
equal amounts of serum from two cows and two bulls. Seminal plasma
was prepared from semen pooled from several bulls of various ages. The
bovine milk serum proteins were prepared from fresh, mixed herd, skim
milk. Penicillin G and dihydrostreptomycin were added to each of the
antigen solutions to give a concentration of 500 units and 100 microgram
equivalents of streptomycin per ml., respectively. The solutions were
dispensed into a number of small tubes and stored at -18° until used as
the antigens for the injections and the precipitin tests.

Antisera to the seminal plasma, blood serum, and the milk serum pro-
teins were prepared by injecting each respective antigen solution into
several rabbits intravenously and intraabdominally until suitable titer had
built up. The antisera obtained from the rabbits were pooled for each
group, stored in a number of small tubes at -18°, and then diluted with
an equal volume of saline (0.9 per cent NaCl) for use in the precipitin tests.

Precipitin tests were carried out according to the procedure of Boyd (7)
in small tubes containing about 0.04 ml. each of antisera and antigen solu-
tion. The antigens were diluted with saline to a protein concentration of
about 1.2 per cent, and serial dilutions of 10 were made of these solutions.
After layering the antigen over the antisera, the tubes were incubated for
2 hours at 37° before being read.

Other proteins used in this study were obtained from the following
sources. The bovine blood serum proteins, Fraction II (γ-globulins),
Fraction III-1 (chiefly β-globulins), Fraction IV (chiefly α-globulins), and
crystalline serum albumin were generously supplied by Armour and Com-
pany. The milk proteins, colostrum euglobulin and pseudoglobulin and
crystalline β-lactoglobulin, were prepared as previously described (8).
Twice crystallized α-lactalbumin (9) was kindly furnished by Dr. W. G.
Gordon of the Eastern Regional Research Laboratory, United States De-
partment of Agriculture. These proteins were reconstituted to a 1 per
cent concentration in phosphate buffer, pH 6.9, and ionic strength of 0.1,
and serial dilutions of 10 with saline were used in the precipitin tests.
Results

Electrophoretic Comparison—Electrophoretic patterns of bovine seminal plasma, blood serum, and milk serum are illustrated in Fig. 1. These were the same preparations which were used as the antigens for the immunological analyses. Fig. 1 has been constructed so that the mobilities of the various components in all three systems are directly proportional to their distance from the same adjusted starting position. The mobilities of the various components of seminal plasma and the blood serum proteins have been previously reported under similar conditions (1). It is to be noted that \( \alpha \)-lactalbumin \( (u = -3.8) \) and \( \beta \)-lactoglobulin \( (u = -5.1) \), which compose about 20 and 50 per cent, respectively, of the normal milk serum proteins, possessed mobilities similar to those of the \( \alpha \)-globulins of blood serum and major Components 5 and 8 of the seminal plasma.\(^1\) Previous work has shown that \( \alpha \)-lactalbumin and \( \beta \)-lactoglobulin are apparently not identical with any of the blood serum proteins, whereas albumin and the immune globulins (noted as \( \gamma \) in Fig. 1) are similar to those

\(^1\) Electrophoretic mobilities expressed in \( 10^{-5} \) cm.\(^2\) volt\(^{-1}\) sec\(^{-1}\).
of blood (10–13). Addition of crystalline \( \alpha \)-lactalbumin and \( \beta \)-lactoglobulin to seminal plasma revealed that \( \beta \)-lactoglobulin \( (u = -5.4) \) had greater mobility than Component 8 \( (u = -5.2) \) of the seminal plasma and could be identified as a distinct electrophoretic entity. \( \alpha \)-Lactalbumin,

however, could not be distinguished from Component 5 of the seminal plasma by this means \( (u = -3.9) \).

_Ultracentrifugal Analysis_—A sample of normal blood serum (40 month-old cow), normal milk serum prepared from mixed herd milk, and samples of seminal plasma collected from several young bulls (14 to 15 months), an older bull (120 months), and pooled from several bulls of various ages, were chosen for this purpose. The results (Fig. 2 and Table I) revealed that seminal plasma contains at least five ultracentrifugal components.

![Ultracentrifugal comparison of the proteins of bovine seminal plasma from bulls of various ages with the normal bovine blood and milk serum proteins. All analyses were conducted at 24° in Veronal buffer of pH 8.6 and ionic strength of 0.1. Protein concentrations were 1.8, 1.7, 1.8, 1.7, and 1.2 per cent, respectively. The pictures were taken at the indicated times after the rotor had attained the operating speed of 59,780 r.p.m. (See the text and Table I.)](image)
Three components possessing $s_{20,w}$ values of about 3, 5, and 7 accounted for over 95 per cent of the protein material.

The ultracentrifugal analyses show that, on a quantitative basis, patterns of the seminal plasma proteins do not resemble those of blood. Though the identity of all the ultracentrifugal components of blood serum is not known with certainty, Component D$_0$ represents serum albumin and some of the globulins, and Components D$_2$ and D$_1$ the remainder of the globulins, those of Component D$_1$ having high molecular weights (14). Components 3 and 4 of the seminal plasma possessed sedimentation ve-

### Table I

<table>
<thead>
<tr>
<th>Protein system</th>
<th>Component No.*</th>
<th>Per cent component*</th>
<th>Sedimentation velocity†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A  B  C  D  E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seminal plasma</td>
<td>1†  1  1  2</td>
<td>11.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2†  1  1  3</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3  41  41  10</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4  24  23  21</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5  33  31  34</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>Blood serum</td>
<td>1  8</td>
<td>16.4</td>
<td>16-20 (14)</td>
</tr>
<tr>
<td></td>
<td>2  23</td>
<td>7.2</td>
<td>7 (14)</td>
</tr>
<tr>
<td></td>
<td>3  69</td>
<td>4.2</td>
<td>4 (14)</td>
</tr>
<tr>
<td>Milk serum</td>
<td>1†  3</td>
<td>19.9</td>
<td>20 (15)</td>
</tr>
<tr>
<td></td>
<td>2  8</td>
<td>6.8</td>
<td>6-7.4 (12, 16)</td>
</tr>
<tr>
<td></td>
<td>3†  89</td>
<td>2.6</td>
<td>2.7-3.2 (12, 16)</td>
</tr>
<tr>
<td></td>
<td>4†</td>
<td>1.8-2 (12, 16)</td>
<td></td>
</tr>
</tbody>
</table>

* See Fig. 2 for identification of solutions and components.
† Sedimentation velocities ($s_{20,w}$) in $1 \times 10^{-13}$ cm. sec.$^{-1}$ unit field$^{-1}$.
‡ Estimated.

velocities similar to those of Components D$_2$ and D$_3$ of the blood serum, whereas Components 1, 2, and 5 appeared to be absent from blood. Thus, there is a strong possibility that at least 30 to 40 per cent of the bovine seminal plasma proteins (principally Component 5) are unlike anything present in blood.

On a quantitative basis, the ultracentrifugal patterns of seminal plasma more closely resemble those of milk serum than those of blood serum. Component E$_4$ of the milk serum proteins has been identified as $\alpha$-lactalbumin, Component E$_3$ as $\beta$-lactoglobulin, and Component E$_2$ as immune globulins (named, respectively, the $\alpha$, $\beta$, and $\gamma$ components by Pedersen (16)). Smith (15) has noted the fast immune globulin component, E$_1$. Serum albumin, identical with that in blood (10), was apparently not
present in sufficient concentration to be seen in the ultracentrifugal patterns or formed complexes with the other proteins. Components 3 and 5 of the seminal plasma possessed sedimentation velocities similar to those of Components E₂ and E₅ of the milk serum, whereas Components 1, 2, and 4 appeared to be absent in the milk serum. Only 20 to 25 per cent of the bovine seminal plasma proteins (principally Component 4) are, on an ultracentrifugal basis, unlike anything present in the milk serum.

It is to be noted that, because of similar protein concentrations and viscosities, the ultracentrifugal pictures of the seminal plasma proteins and the blood serum proteins are almost directly comparable. Viscosity analyses of the solutions which were subjected to ultracentrifugation indicated that the Veronal buffer alone possessed a relative viscosity at 24° of 1.06 and the 1.7 per cent blood serum proteins in the Veronal buffer of 1.19. The 1.8 per cent solution of seminal plasma protein possessed a relative viscosity of 1.22 under similar conditions. The viscous nature of bovine seminal plasma is apparently due to the high protein concentration and the dialyzable constituents, such as fructose present. Dilution and dialysis yield solutions which are comparable in viscosity to other protein systems of similar concentration.

**Immunological Analysis**—The results of the precipitin tests, data for which are shown in Table II, indicate that the seminal plasma proteins elicited a marked antibody response in the injected rabbits. Although some cross-reaction existed between the respective antisera to the seminal plasma and the blood and milk serum, it was in each case considerably less than the response of the antiserum towards its injected antigen system.

The various preparations of immune globulins used (blood Fraction II-γ-globulins, blood Fraction III-1-β-globulins, colostrum euglobulin and pseudoglobulin) reacted strongly with the antiserum to the bovine blood serum and the milk serum proteins in the precipitin tests. This is understandable, since these proteins were isolated from blood and colostrum, and considerable evidence has shown (10, 11, 15) that immune globulins are present not only in the colostrum, but also in the normal milk, passing there from the blood. Little reaction of these proteins with the antiserum to the bovine seminal plasma proteins was found. The data in Table II also show there was little reaction between the seminal plasma proteins and the antiserum to the milk serum proteins. The large reaction of the antiserum to the milk serum proteins with the various immune globulin preparations and the small reaction with the milk proteins, α-lactalbumin, β-lactoglobulin, and serum albumin, indicated that the precipitins in the immune sera from the rabbits injected with milk serum were essentially against the immune globulins. Thus, if immune globulins were present in the seminal plasma, a reaction of these components with the antiseras
### Table II

**Precipitin Reactions of Bovine Blood Serum, Seminal Plasma, and Milk Serum Proteins**

<table>
<thead>
<tr>
<th>Antisera against</th>
<th>Test antigen and dilution†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood serum</td>
</tr>
<tr>
<td>Blood serum</td>
<td>7+</td>
</tr>
<tr>
<td>Seminal plasma</td>
<td>3+</td>
</tr>
<tr>
<td>Milk serum</td>
<td>3+</td>
</tr>
<tr>
<td>Immune globulins‡</td>
<td>6+</td>
</tr>
<tr>
<td></td>
<td>Milk serum</td>
</tr>
<tr>
<td>Blood serum</td>
<td>5+</td>
</tr>
<tr>
<td>Seminal plasma</td>
<td>2+</td>
</tr>
<tr>
<td>Milk serum</td>
<td>5+</td>
</tr>
<tr>
<td></td>
<td>Blood serum albumin</td>
</tr>
<tr>
<td>Blood serum</td>
<td>6+</td>
</tr>
<tr>
<td>Seminal plasma</td>
<td>Tr.</td>
</tr>
<tr>
<td>Milk serum</td>
<td>“</td>
</tr>
<tr>
<td></td>
<td>Milk α-lactalbumin</td>
</tr>
<tr>
<td>Blood serum</td>
<td>2+</td>
</tr>
<tr>
<td>Seminal plasma</td>
<td>1+</td>
</tr>
<tr>
<td>Milk serum</td>
<td>2+</td>
</tr>
</tbody>
</table>

* All analyses run at least in triplicate.
† Readings for 2 hour tube. 5+ to 7+, precipitate plus heavy ring; 1+ to 4+, ring present; Tr., trace of ring present; —, no observable reaction.
‡ Mixture of blood γ-, β-, and Colostrum globulins.
to the milk serum proteins would be expected. Such was not the case, the small reaction observed indicating that only a slight amount of the immune globulins could be present in the seminal plasma. This was further tested by immunizing several rabbits against a mixture of the immune globulins isolated from blood and colostrum. The antisera obtained demonstrated a large reaction with the blood serum, but only a slight reaction with the seminal plasma; the amount of reaction suggests that the concentration of immune globulins in the seminal plasma was less than 1 per cent of the total protein.

The results indicate that the crystalline milk proteins, α-lactalbumin, β-lactoglobulin, and serum albumin, reacted little with the antisera to the seminal plasma proteins. Since the antibody response to these milk proteins was small even in the rabbits injected with milk serum, owing to their low antigenicity or concentration, the absence of a pronounced reaction with the antisera to the seminal plasma suggests only that they were not present in great concentration.

None of the purified proteins tested accounted for the high antigenicity of the seminal plasma proteins. Attempts to dissolve preparations of the blood α-globulins prepared by solvent and low temperature fractionation were not successful, a characteristic of lipoproteins (14, 17). Thus, direct evidence for the immunological relationship of the seminal plasma proteins to the blood α-globulins was not obtained. However, previous evidence (1) showing that the bovine seminal plasma proteins are physically stable and possess low lipide, nitrogen, and carbohydrate contents indicates that proteins identical with the labile blood α-globulins are not present or are in low concentration in the seminal plasma.

The cross-reaction between the blood serum and the seminal plasma and their respective antisera shows that these systems immunologically have some material in common. The reaction with the antiserum to the purified immune globulin preparation suggests that there is a small amount of immune globulins present in the seminal plasma. Trace amounts of these immune globulins and possibly serum albumin could account for the reaction with the respective antisera to the blood and milk serum. The cross-reaction of the antisera to the seminal plasma with the blood and milk serum is probably due to other components which are either antigenically weak, showing a non-specific cross-reaction, or more likely present in low concentration. The identification of such components must await the fractionation and characterization of the individual proteins of seminal plasma.

**DISCUSSION**

Although previous work is lacking on the characterization of the bovine seminal plasma proteins, various investigators have reported conflicting
similarities between the human blood serum proteins and the human seminal plasma proteins (2, 4–6). Apparently an analogy cannot be extended from this work on the human seminal plasma proteins to the bovine seminal plasma proteins. Wide differences among many of the constituents of seminal plasma from different species have been reported (18–21).

Bovine seminal plasma is composed of proteins which are highly antigenic. In fact, one rabbit receiving injections of the seminal plasma proteins died of anaphylactic shock, and two others usually went into partial shock after each injection. The rabbits receiving the blood serum and milk serum proteins maintained their weight and were healthy in all respects. Those receiving the seminal plasma generally lost weight for several days after the injections and were in a poorer state of health. At the site of the seminal plasma injection, an erythematous edema followed by severe necrosis resulted. In some cases, large sections of the ear were completely sloughed away after several days, and at the site of the intra-abdominal injections secondary infections occurred, with intestinal adhesions. The primary causative factor, which was heat-labile, non-dialyzable, and still effective at a 1:50 dilution, is being investigated further.

The results of this investigation, together with previous evidence (1) on the relationship of the proteins of seminal plasma to those of blood serum, have been summarized in Table III. It should be noted that the electrophoretic mobility and sedimentation velocity of Component 5 of the seminal plasma are similar to those of α-lactalbumin, which contains a high tryptophan content and is similar to the seminal plasma proteins in this respect (22). Certainly, with this possible exception, the evidence

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Evidence for presence in seminal plasma*</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood serum, γ globulins</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>&quot; β-globulins</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>&quot; α-globulins</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>&quot; albumin</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Milk serum, immune globulins</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>&quot; α-lactalbumin</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>&quot; β-lactoglobulin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>&quot; albumin</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

* +, possible presence (implies that the presence in seminal plasma of proteins with similar electrophoretic or ultracentrifugal properties does not eliminate the possibility of their being present; however, it does not prove their presence); 0, inconclusive; -, absent (or present in low concentration).
suggests that the major protein components of bovine seminal plasma are
definite chemical entities which are not similar to any of the major protein
components of blood or milk serum.

SUMMARY

Ultracentrifugal analyses of the bovine seminal plasma proteins revealed
at least five components with the majority of the proteins in three com-
ponents having $s_{20,w}$ values of about 3, 5, and 7. A great similarity was
shown in the ultracentrifugal patterns of the seminal plasma proteins ob-
tained from several bulls.

Immunological analyses indicated that the bovine seminal plasma pro-
teins are highly antigenic. Chemical, electrophoretic, ultracentrifugal, and
immunological comparisons suggest that the major proteins of seminal
plasma are distinct entities which are either absent from or are only minor
constituents of blood and milk serum.

The authors are grateful to Dr. N. L. VanDemark of the Department
of Dairy Science for his helpful suggestions and aid in procuring the samples
of semen and also wish to extend appreciation to Dr. B. R. Ray of the
Department of Chemistry for conducting the ultracentrifugal analyses.

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THE PROTEINS OF BOVINE SEMINAL PLASMA: II. ULTRACENTRIFUGAL AND IMMUNOLOGICAL STUDIES AND COMPARISON WITH BLOOD AND MILK SERUM
Bruce L. Larson, Ralph S. Gray and G. W. Salisbury


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