Isoimmunization of the female with semen results in reduced fertility in a number of species, notably guinea-pigs (Katsh, 1959; Isojima, Graham & Graham, 1959; Otani, Behrman, Porter & Nakayama, 1963), mice (McLaren, 1964; Edwards, 1964), rabbits (Menge, 1968, 1970; Bell, 1969) and cattle (Menge, 1967, 1969). Attempts to define the site and mechanism of such immunological inhibition of fertility using univalent (Fab, non-aglutinating, non-immobilizing) antispem antibody fragments showed that cervical passage of spermatozoa (Metz & Anika, 1970) and one or more additional processes are vulnerable to antibody inhibition (Metz, 1972, 1973). The latter evidently includes an event(s) at the normal time and site of sperm-egg interaction because antibody-pretreated capacitated spermatozoa fail to produce conception following intratubal insemination (Metz, 1973). The intratubal insemination procedure is inherently deficient in quantitative precision, as, for example, when attempting to assess the sperm concentration and composition of the medium in the vicinity of the egg. Fertilization in vitro provides a more satisfactory degree of precision with the added advantage that the material can be directly observed as in the classical studies on sea urchin fertilization (Metz, 1967). We report here the inhibition of fertilization of rabbit eggs in vitro by univalent antispem isoantibodies.

Isoantisera were prepared in virgin female New Zealand rabbits against rabbit semen and epididymal spermatozoa (Menge, 1968). Preinjection sera served as controls. Univalent (Fab) antibody and control globulin, prepared by papain digestion (Porter, 1959; Metz & Anika, 1970), were used in all experiments.

The synthetic medium of Brackett (1969) supplemented with 20% heated (56°C, 30 min) rabbit serum, 3 mg crystalline bovine albumin (Sigma)/ml, 100 Units penicillin (Lillie)/ml and 160 µg streptomycin/ml and equilibrated with 5% CO₂-air mixture (280 mosmol, pH 7.8, 38°C) was used in all procedures. Rabbit ova were obtained after superovulation (Brackett, 1969). Rabbit spermatozoa were capacitated (1·2×10^6 injected per capacitor uterus; spermatozoa recovered 18 hr later), washed in supplemented synthetic medium and finally suspended to 9×10^6/ml in this medium.
Of this suspension, 0.3 ml was mixed with 0.9 ml univalent rabbit globulin (6 mg protein/ml). After incubation (38°C for 30 min), excess univalent globulin was removed by centrifuging (395 g for 5 min) and the capacitated, univalent globulin-treated spermatozoa were resuspended to 4 ml in synthetic medium.

These samples of univalent globulin-treated capacitated spermatozoa were added to ten to fifteen ova (surrounded by cumulus envelopes) in 0.5 ml culture medium (the total volume after the addition of spermatozoa was 4.5 ml and the final sperm concentration was $2 \times 10^6$/ml). All cultures were then covered with sterile mineral oil, incubated for 24 hr or more in 5% CO$_2$–air atmosphere at 38°C and were then examined for cleavage. Eggs were regarded as fertilized if they consisted of two or more cells of normal shape and size.

Ova were inseminated with univalent antibody and univalent control globulin-treated spermatozoa. Additional controls included ova incubated in the absence of spermatozoa as a standard for egg fragmentation and parthenogenesis, and ova inseminated with untreated capacitated spermatozoa. Ova from capacitation females were cultured through cleavage as controls for the success of capacitation.

Experiments were performed using spermatozoa pretreated with univalent anti-whole semen (Table 1) and anti-epididymal sperm isoantibodies (Table 2). Little (3%) if any parthenogenesis occurred since only one of the thirty-two ova in the two experimental series apparently cleaved. Ova inseminated with saline and with control univalent globulin-pretreated spermatozoa gave acceptable cleavage levels, namely twenty-four out of sixty-eight (35%) and twenty-two out of fifty-nine (37%) ova, respectively (Tables 1 and 2). Ova inseminated with univalent antibody globulin pretreated spermatozoa did not give significant levels of cleavage in either the univalent anti-whole semen or the anti-epididymal sperm globulin experiments (e.g. only three of fifty-

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**EXPLANATION OF PLATE 1**

**Fig. 1.** Uninseminated rabbit egg after 24 hr in synthetic medium. The cumulus and corona (c) are intact. z: Zona pellucida. Nomarski optics, $\times$ 400.

**Fig. 2.** Uncleaved rabbit egg incubated with capacitated spermatozoa for 19 hr. The corona and cumulus cells are completely removed showing the denuded zona (z) and clear perivitelline space (ps). Nomarski optics, $\times$ 400.

**Fig. 3.** Pronuclear stage of a rabbit ovum incubated with capacitated spermatozoa for 14 hr. P: Pronuclei. Phase contrast, $\times$ 900.

**Fig. 4.** Two-cell rabbit egg 24 hr after insemination with control globulin-treated spermatozoa. Corona and cumulus cells are completely removed and spermatozoa adhere to the zona (z) (arrows). The perivitelline space is shown (ps). Nomarski optics, $\times$ 400.

**Fig. 5.** Four-cell rabbit egg 32 hr after insemination with control globulin treated spermatozoa. Nomarski optics, $\times$ 400.

**Fig. 6.** Four-cell rabbit egg recovered from the oviduct of a capacitor doe 36 hr after artificial insemination and after 24 hr of incubation in vitro. Nomarski optics, $\times$ 400.

**Fig. 7.** Uncleaved rabbit egg incubated for 24 hr with immune globulin-treated spermatozoa. The cumulus has been removed by mechanical means. The corona cells (c) are strongly attached to the zona. No spermatozoa were observed in either the corona or zona pellucida. Nomarski optics, $\times$ 400.

**Fig. 8.** Higher magnification of the egg inseminated with immune globulin-treated spermatozoa (Fig. 7). The corona cells (c) are observed closely attached to the zona pellucida (z). No spermatozoa are observed in the corona, zona or perivitelline space. Nomarski optics, $\times$ 1000.
Antibody inhibition of fertilization in vitro

Table 1. Cleavage of normal rabbit eggs in vitro following exposure to capacitated spermatozoa pretreated with univalent (Fab) anti-whole semen isoantibody globulin fragments

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>(1) Immune globulin</th>
<th>(2) Control globulin</th>
<th>(3) Saline</th>
<th>(4) Uninseminated ova</th>
<th>(5) Capacitator ova</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>0/2*</td>
<td>2/3</td>
<td>1/4</td>
<td>0/4</td>
<td>5/5</td>
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</tr>
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<td>0/5</td>
<td>2/4</td>
<td>2/6</td>
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<td>10/28</td>
<td>10/38</td>
<td>0/16</td>
<td>31/37</td>
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<tr>
<td>% Cleaved</td>
<td>7</td>
<td>35</td>
<td>26</td>
<td>0</td>
<td>83</td>
</tr>
</tbody>
</table>

Column (1). Eggs inseminated with capacitated spermatozoa pretreated with univalent (Fab) anti-sperm (whole semen) antibody fragments.
Column (2). Eggs inseminated with capacitated spermatozoa pretreated with univalent (Fab) control globulin fragments.
Column (3). Eggs inseminated with capacitated spermatozoa in saline.
Column (4). Uninseminated ova cultured in synthetic medium as controls for parthenogenetic cleavage and egg fragmentation.
Column (5). Eggs from the female providing the capacitated spermatozoa. Cleavage, e.g. fertilization of these eggs, confirms capacitation of spermatozoa in the capacitator female.
* Cleaved/uncleaved eggs.

seven (5%) ova cleaved). Such antibody treatment evidently blocks one or more sperm antigens, not egg antigens, that function in sperm–egg interaction because the antibody-treated spermatozoa were washed to remove excess antibody before insemination.

Apart from the cleavage rates, the most striking effect of antibody treatment was on the dispersion of the cumulus and corona coats of the ova. The cumulus and corona remained intact for over 19 hr (Pl. 1, Fig. 1) in the absence of spermatozoa, but dispersed in less than 5 hr (Pl. 1, Figs 2 to 6) in the presence of control (saline or univalent globulin-pretreated) spermatozoa. In the latter, cumulus dispersion probably occurred within a few minutes (Metz, Seiguer &

Table 2. Cleavage of normal rabbit eggs in vitro following exposure to capacitated spermatozoa pretreated with univalent (Fab) anti-epididymal sperm antibody globulin fragments

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>(1)* Immune globulin</th>
<th>(2) Control globulin</th>
<th>(3) Saline</th>
<th>(4) Uninseminated ova</th>
<th>(5) Capacitator ova</th>
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<td>3/6</td>
<td>5/8</td>
<td>0/1</td>
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<tr>
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<td>12/31</td>
<td>14/30</td>
<td>1/16</td>
<td>22/27</td>
</tr>
<tr>
<td>% Cleaved</td>
<td>3.3</td>
<td>39</td>
<td>47</td>
<td>6</td>
<td>81</td>
</tr>
</tbody>
</table>

* The experiments were performed as in Table 1 except that univalent (Fab) anti-epididymal spermatozoa isoantibody fragments were substituted for the univalent anti-whole semen antibody fragments of Table 1.
Castro, 1972). The dispersed cumulus cells grew on the bottom of the culture dish forming a thin layer. The cumulus showed poor dispersion even after 24 hr of exposure to univalent antibody- (anti-whole semen or anti-epididymal) treated spermatozoa (Pl. 1, Figs 7 and 8). It was necessary to treat these cultures with commercial hyaluronidase (8 units/ml) to remove the bulk of the cumulus and permit examination of the ova for cleavage. Compared to the uninseminated controls, however, some cumulus dispersion did occur. The dispersed cells grew in round masses, not a thick layer, on the bottom of the culture dishes. The corona did not disperse. Phase-contrast examination of such ova after washing revealed that there were no spermatozoa in the corona, zona pellucida or perivitelline space.

The observed inhibition of fertilization in vitro by univalent antisperm isoantibodies is consistent with other observations that imply vulnerability of an essential step(s) in sperm–egg interaction to antibody inhibition. These observations include specific inhibition of sperm hyaluronidase, including cumulus-dispersing action, by univalent hetero-and isoantibodies to spermatozoa (Metz et al., 1972; Metz, 1973), poor dispersion of the cumulus following insemination of female rabbits isoimmunized with semen (Menge, 1971a), antibody inhibition of sperm attachment to the corona (Menge, 1971b; Metz, 1972) and antibody inhibition of fertilization following intratubal insemination (Metz, 1973).

Since the cumulus and corona were largely intact after 24 hr of exposure to antibody-treated spermatozoa and since no spermatozoa were found in the corona, zona pellucida or perivitelline space, it is concluded that antibody inhibited sperm–egg interaction at the cumulus and/or corona-penetration steps. Failure of the corona to disperse in the antibody-treated series is explained either by failure of univalent antibody-treated spermatozoa to penetrate the cumulus to the corona or by inhibition of the corona-dispersing enzyme of the spermatozoa (Zaneveld & Williams, 1970) by antibody. Further investigations will involve studies on insemination in vitro with univalent antibody-treated spermatozoa and ova from which the extracellular coats (cumulus, corona and zona) have been removed sequentially.

This research represents Contribution number 264 from the Institute of Molecular and Cellular Evolution, and was aided by grants from the Rockefeller Foundation and the Population Council. The authors are most grateful to Dr B. G. Brackett, University of Pennsylvania, for his advice and instruction concerning in-vitro fertilization techniques.

REFERENCES

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