Factors Affecting the Stability and Accuracy of the Bioassay for the Sperm Attractant Sirenin

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ABSTRACT

Optimal response of the sperm of Allomyces from the highly male strain M16 to the chemotactic agent, sirenin, was shown to occur when the sperm suspension contained 2 mM pipazine-N',N-bis[2-ethane sulfonic acid] buffer, 3 mM CaCl₂, and chelated trace elements. For the male strain M3, the CaCl₂ needed was 3.5 mM with the other two components the same as for M16. The inclusion in the sperm suspension of MgCl₂, KH₂PO₄, or NH₄Cl was without effect, except that under certain conditions phosphate was detrimental. The variability of 10 replicate assays was substantially reduced by using sperm in the bioassay at a concentration of 300,000 per ml rather than the former concentration of 100,000 per ml with a concomitant reduction in the concentration of sirenin above the membrane to which the sperm were attracted.

The establishment of the structure of sirenin followed by the synthesis of the racemate and, subsequently, of the separate enantiomorphs as well as certain analogues (see Reference 8 for citations to the pertinent literature) made it possible to determine the chemotactic activity of these substances as well as to initiate studies on the mechanism of action of sirenin. The assay, as described in the next section, was not accurate enough for such work and periodically became dysfunctional. This report covers work substantially resolving these defects and also constitutes the first detailed description of the bioassay with the substantive changes made since the original description (6) in 1958.

MATERIALS AND METHODS

The Bioassay Apparatus. The apparatus is fully described elsewhere (7). It is essentially a stainless steel tube with the bottom sealed with DuPont PD 150 uncoated cellophane, washed free of glycerol and other soluble impurities. The tube is supported by a tripod so that when resting in the 35 mm plastic Petri dish in which the assay is performed the bottom is 3 mm up from the floor of the dish. The mounted membranes were kept in aqueous solutions or distilled water at all times and replaced every 6 to 8 weeks when the response decreased because of deterioration of the membranes.

Preparation of Sperm Suspensions. Stock cultures of the highly male strain M3 were grown on Difco Emerson YpSs agar medium at 25 C with weekly transfers. From these, transfers were made to YpSs plates which, after drying for 3 to 4 days, had been layered with an 8-cm circle of uncoated DuPont PD 124 cellophane that had been autoclaved in 100 ml of glass-distilled water. After layering, the plates were dried for another 3 to 4 days, and then 5 transfers, plant side up, were made to each circle approximately 1.5 cm in from the edge of the circle and equidistant around the circumference. These were grown at 25 C, and the resulting “pads” of deep orange male plants were used when 11 to 13 days old.

Sperm suspensions were obtained by first washing five pads in 250 ml of distilled water with gentle shaking for 60 min with a change of water after 30 min. The pads were then transferred to 50 ml of DS solution (6) for sperm discharge, and then shaken for another 60 min. After removing the pads, the sperm concentration in the DS solution was determined with a hemocytometer using a sample of sperm which had been immobilized by adding 25 μl of 95% ethyl alcohol to 1.0 ml of sperm. The sperm suspension was then diluted with DS to 100,000 per ml.

Conduct of the Assay. The assay units, brought to room temperature after storage in the refrigerator, were sucked free of water inside and outside. The wells above the membranes were filled with test solutions until a positive meniscus formed, and they were then covered with a coverslip. The excess solution on the flange around the top of the well was removed by vacuum, and the unit was placed in a 35-mm plastic Petri dish into which 4.0 ml of the sperm suspension had been pipetted. Units were set up at 1-min intervals, with each variable replicated 10 times. Exactly 65 min after a given unit was set up, the number of sperm resting on the middle of the membrane was counted in 25 squares of an ocular micrometer using a compound microscope with a long working distance 5X objective and 20X wide-field eyepieces. The area counted represented 0.4624 mm² on the assay membrane. The strict timing was necessary because the number of sperm on the membrane increased with time for 75 to 90 min and then declined.

In experiments involving different solutes in the sperm suspension, 10 pads were washed for 1 hr as above, and then two pads were placed in 50 ml of different discharge media. After the 1-hr discharge period, the sperm concentration in each medium was determined and then diluted to 100,000 per ml with the discharge medium. In such experiments, as in the basic procedure described above, the sirenin was always dissolved in the same solution as that used for sperm discharge.

Organisms. The Ceylon strain of Allomyces arbuscula (N = 16) was used as the female parent and the Burma 3 strain of Allomyces macrogyrus (N = 28) as the male parent in crosses from which new highly male strains were selected (3). Most of the work was done with the new male strain M16

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and the balance with the original male strain M3 (6). This change became necessary when M16 sharply decreased in its maleness.

Reagents. DS solution (6), originally introduced to provide a very dilute ionic environment for the various swarmers of *Allomyces*, was 0.5 mm with respect to KH₂PO₄, K₂HPO₄, and (NH₄)₂HPO₄, and 0.1 mm with respect to MgCl₂ and CaCl₂ with a final pH of 7.0. In part of the work to be described, DS concentrations as multiples of that just described were used and are referred to as DS, 5 DS, etc. Subsequently, the effects of the major ions in DS were investigated. In these experiments, separate stock solutions of KH₂PO₄, NH₄Cl, MgCl₂, and CaCl₂ were used with the pH of the final solution adjusted with KOH, Cl⁻ and K⁺ concentrations were not investigated and varied depending on the concentrations of the salts used. The trace element solution used is described elsewhere (5). When the stock solution was used at the rate of 20 ml/liter of final nutrient solution, it is referred to as TE with higher concentrations indicated as multiples, i.e., 2 TE, 3 TE, etc.

Sirenin was available dissolved in methylene chloride. The solutions, initially natural l-sirenin, then synthetic dl-sirenin, and finally synthetic l-sirenin, were all prepared in the laboratory of Professor Henry Rapoport in the Department of Chemistry of the University of California at Berkeley. To prepare solutions for assay purposes, an appropriate number of microliters was placed in a 125-ml Erlenmeyer flask and evaporated to dryness while covered with a flowing stream of argon. When dry, the same medium as used for the sperm discharge was added, and the flask was then gently shaken for 15 to 30 min to dissolve the sirenin fully.

RESULTS

Variability. After a prolonged and apparently irreversible dysfunction of the assay, response was restored when the DS sperm discharge medium was replaced with 5 DS plus 2 TE with these concentrations established as optimum. Initial experiments to identify the deficient or inhibitory concentrations of the components of 5 DS (except K⁺ and Cl⁻) failed because results were not reproducible. The difficulty was found to be a very high SD. The average mean of five identical assays using 100 nm natural l-sirenin with each mean based on the standard 10 replicate assays was 127 ± 28 for M3 sperm.

In experiments using solution DM16, the optimum discharge medium for M16 sperm (see later), it was found that when the assay was run with 100,000 sperm per ml with natural l-sirenin at 100 nm and 5 squares counted, the average mean was 136 ± 26; with 500,000 sperm per ml, the sirenin at 10 nm and two squares counted, the average mean was 105 ± 10. With a standard deviation of 10, using the t test for small samples, a 90% level of significance requires a difference between means exceeding only 8, whereas a SD of 26 requires a difference exceeding 21.

After the composition of DM16 was established, a series of experiments were done using natural and synthetic l-sirenin as well as synthetic dl-sirenin at various concentrations varying the number of squares counted to give means ranging from 90 to 164. The results are reported in Table I. The variability under those conditions was a function of the density of the sperm on the membrane as evidenced by the sharp increase in SD as 10 or more squares had to be counted to obtain counts in the range of approximately 100 to 150.

The Composition of Sperm Discharge Media. Systematic variation of the concentrations of the major ions in 5 DS and of the level of TE resulted in the discharge medium DM16

### Table I. Relationship of Standard Deviations to Density of Sperm on the Membranes in Response to Sirenin

<table>
<thead>
<tr>
<th>Area Counted</th>
<th>No. of Means</th>
<th>Avg. of Means</th>
<th>Avg. SD</th>
<th>SD of Avg. SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>squares</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>115</td>
<td>9</td>
<td>2.7</td>
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<tr>
<td>3</td>
<td>6</td>
<td>120</td>
<td>11</td>
<td>2.9</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>105</td>
<td>9</td>
<td>2.1</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>137</td>
<td>16</td>
<td>3.8</td>
</tr>
<tr>
<td>25</td>
<td>4</td>
<td>132</td>
<td>23</td>
<td>5.9</td>
</tr>
</tbody>
</table>

### Table II. The Effect of the Media on Which M3 Male Plants Were Grown on the Response of the Sperm to 5 nm Synthetic l-Sirenin in Various Discharge Media

<table>
<thead>
<tr>
<th>Discharge Media</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Difco Yps</td>
</tr>
<tr>
<td>DM3</td>
<td>100</td>
</tr>
<tr>
<td>DM3-TE</td>
<td>74</td>
</tr>
<tr>
<td>DM3-Ca²⁺</td>
<td>51</td>
</tr>
<tr>
<td>DM3-Ca²⁺ and TE</td>
<td>32</td>
</tr>
</tbody>
</table>

Each mean was the average of 10 replicate assays in which the number of sperm on the areas of membrane indicated were counted. Each SD was for the 10 replicates. Each square of the micrometer eyepiece was equal to 0.0185 mm² of membrane.

with no component being deficient or inhibitory. This medium consisted of 3 mm CaCl₂, 2 mm PIPES (4), and 2 TE at a pH of 6.8, the pKₐ of PIPES. The pH, when varied in 0.5 pH increments from 5 to 7, showed no significant effect on response from pH 5.5 to 7.0, but the response dropped off at pH 5.0. Omissions of NH₄⁺, PO₄³⁻, and Mg²⁺, all components of 5 DS, were without effect and hence unnecessary. PIPES buffer was substituted for phosphate to avoid precipitates in the presence of the high CaCl₂ and because under certain conditions phosphate was inhibitory. When the principal media used were compared, the results were: DM16, 100%; 5 DS + 2 TE, 62%; and DS, 47%.

To test whether Ca²⁺ and TE had to be present both during sperm discharge and in the sperm suspension during the assay, sperm were discharged in DM16 and DM16 lacking all but PIPES buffer. To part of the latter suspension, after discharge, the missing components were added for an hour, and the assay was then run with all three sperm suspensions. The readings were: DM16, 127; DM16 lacking Ca²⁺ and TE, 61; and sperm discharged in DM16 lacking Ca²⁺ and TE and then exposed to these for 1 hr, 126. Adequate Ca²⁺ and TE have to be present during the actual response to sirenin.

The final experiment was to see whether Ca²⁺ and TE in the growth medium of the pads would affect the need for these in the sperm suspension. Unfortunately at this time, M16 underwent a change in which the percentage of male gam-
etangia dropped from 95 to 75%. Male strain M3 (6), which was 92% male, was therefore used. In DM16 it gave the same response to 5 nM l-sirenin as had M16. Each of the components of DM16 was tested for both deficiency and toxicity. The concentrations of PIPES and TE were found to be the same as for M16, but the CaCl₂ had to be increased to 3.5 mM. The slightly changed medium was designated DM3. It was also demonstrated that Mg²⁺, NH₄⁺, and PO₄³⁻ were without effect when added to DM3.

The effect of the pad growth medium on the subsequent response to sirenin was tested by growing pads on YpSs medium modified to contain the concentrations of Ca²⁺ and TE used in DM3 as well as on standard Difco YpSs. The results in Table II are the averages of six tests of responses of sperm to modified YpSs and five Difco YpSs controls. Again, Ca²⁺ and TE must be present in the sperm suspension during the actual response to sirenin.

DISCUSSION

This work has resulted in a medium for the discharge of Allomyces sperm which evokes the best chemotactic response so far obtained. Further, the use of high sperm concentrations in the bioassay with quite low sirenin concentrations using a reasonable number of replicates reduced the variability to the point where the bioassay can be used quantitatively for determining the concentration of sirenin as well as the effects of various treatments on this chemotactic system. This made possible the study of the enantiomorphs of sirenin and certain analogues as well as the uptake of sirenin by sperm (1). The revised assay has worked consistently for 2 years. The variability of the assay when using a sperm concentration of 500,000 per ml and low concentrations of sirenin apears to be a function of the concentration of sperm attracted to the membrane through which the sirenin diffused. It is suggested that the sirenin is not at a uniform concentration on the lower side of the membrane. Thus, with relatively few sperm coming to rest on the membrane, the actual number in a section of the middle of the membrane varied highly from assay unit to assay unit. With high concentrations of sperm on the membrane these differences appear to be erased, possibly through the overlapping of sperm attracted to specific spots on the membrane.

The facts that Ca²⁺ and trace elements are needed for maximum response of the sperm to sirenin are suggestive of future avenues of investigation. When the trace element solution is dissected, one or more of the elements may be found to be the active components and become indicative of enzymes involved in the interaction of the sperm and sirenin. Calcium is involved in many cell functions (2) and more specifically in various aspects of sperm behavior and fertilization (9). No evidence is available at present for its role in the response of sperm to sirenin.

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LITERATURE CITED