MOUSE GAMETE INTERACTIONS DURING FERTILIZATION IN VITRO

Chlortetracycline as a Fluorescent Probe for the Mouse Sperm Acrosome Reaction

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ABSTRACT

We have developed an assay for detecting the acrosome reaction in mouse sperm using chlortetracycline (CTC) as a fluorescent probe. Sperm known to be intact with nonreacted acrosomes show CTC fluorescence in the presence of Ca²⁺ over the anterior portion of the sperm head on the plasma membrane covering the acrosome. Sperm which have undergone the acrosome reaction do not show fluorescence on the sperm head. Mouse sperm bind to zonae pellucidae of cumulus-free eggs in vitro in a Ca²⁺-dependent reaction; these sperm are intact by the CTC assay. Intact sperm bind to mechanically isolated zonae under the same conditions: the egg is apparently unnecessary for this initial reaction. Sperm suspensions, in which >50% of the motile population had completed the acrosome reaction, were prepared by incubation in hyperosmolar medium followed by treatment with the divalent cation ionophore, A23187. Cumulus-free eggs challenged with such sperm suspensions preferentially bind intact sperm; acrosome-reacted sperm do not bind. We conclude that the plasma membrane of the mouse sperm is responsible for recognition of the egg's zona pellucida and that the obligatory sequence of reactions leading to fusion of mouse gametes is binding of the intact sperm to the zona pellucida, followed by the acrosome reaction at the zona surface, followed in turn by sperm penetration of the zona.

KEY WORDS acrosome reaction mammalian gamete recognition zona pellucidae mouse sperm chlortetracycline fluorescent probe

Although many studies have been aimed at elucidating the various reactions which sperm and egg must undergo during fertilization in mammals, agreement on the precise sequence of these reactions has not yet been reached (11, 27). The preliminary reactions for sperm can be grouped into two consecutive processes. The first process, capacitation (1, 14), normally occurs during the sperm's residence in the female reproductive tract. It is still poorly understood, primarily due to the
lack of easily identifiable morphological correlates. The process is reversible (15) and is thought to be an obligatory prerequisite for the second reaction, the acrosome reaction, in which dramatic and predictable structural alterations occur (3, 8, 10). During the acrosome reaction, a portion of the plasma membrane which previously surrounded the acrosome, a cap-like organelle overlying the anterior section of the sperm head, is discarded. The inner acrosomal membrane is then exposed to the external environment and becomes the apical limiting membrane of the sperm head. It is well established that sperm which have penetrated the zona pellucida of the egg and lie within the perivitelline space have undergone an acrosome reaction (2, 11, 16, 30, 40). However, the physiological starting point for the acrosome reaction has not yet been identified. Does the reaction occur before or after the sperm binds to the zona? If it occurs before binding, then the reaction by which the sperm recognizes the zona pellucida and binds to it—a species-specific reaction—occurs via the sperm's inner acrosomal membrane. If it occurs after binding, then the recognition reaction is effected by the plasma membrane.

Our previous investigations of the binding of mouse epididymal sperm to the zona pellucidae of mouse eggs in vitro demonstrated that the sperm's binding ability develops rapidly, requires extracellular Ca²⁺ and is reversed by EGTA (33). Ultrastructural studies indicated that only intact mouse sperm—those which had not undergone the acrosome reaction—were observed at the zona surface shortly after insemination, whereas limited numbers of fully acrosome-reacted sperm were bound to zonae recovered at much longer times after insemination (32). These observations led us to propose that a fertile mouse sperm binds to the zona in the intact configuration and, once bound, undergoes the acrosome reaction and penetrates the zona. In this scheme, it is the plasma membrane of the sperm which is responsible for specific recognition of the zona, not the inner acrosomal membrane. The scheme further predicts that mouse sperm, which have undergone the acrosome reaction and are limited anteriorly by the inner acrosomal membrane, are incapable of binding to zonae. This prediction can be tested by binding studies in which eggs are challenged with a suspension of motile mouse sperm containing a substantial fraction with reacted acrosomes. However, two experimental requirements must be met to obtain a definitive test. The first requirement is a reliable assay for the acrosome reaction in a suspension of motile mouse sperm. These gametes have a particularly small acrosome, and the acrosome reaction is difficult to observe and quantitate by standard optical methods. The second requirement is a method to induce consistently the acrosome reaction in a substantial fraction of the sperm in suspension. In this paper, we describe the development of methods to fulfill these requirements and their use in evaluating our prediction concerning the sequence of sperm membrane reactions immediately before fertilization.

MATERIALS AND METHODS

Media

For handling mouse gametes, two types of media were used. The first, a complete culture medium (CM), which supports mouse fertilization in vitro (25), was a modified Krebs-Ringer bicarbonate medium containing sodium pyruvate (1 mM), sodium lactate (25 mM), glucose (5.56 mM), and bovine serum albumin (20 mg BSA/ml); Fraction V, Sigma Chemical Co., St. Louis, Mo.) at pH 7.4. For some experiments, the NaCl content of CM was increased from 120 to 180 mM (HS-CM). The Ca²⁺ concentration for both CM and HS-CM was 1.7 mM. The other type of medium used was a simple buffer of Tris (20 mM) and NaCl (130 mM) at pH 7.4 (TN). TNC was a modified version of TN, which contained Ca²⁺ at a concentration of 1.7 mM; the NaCl concentration in TNC was decreased to 126 mM to maintain the same osmolality.

Guinea pig sperm were exposed to the minimal capacitating medium (MCM) of Barros (7), either in the presence of Ca²⁺ (MCM) or in its absence (CF-MCM). MCM is a completely defined medium containing NaCl (105 mM), NaHCO₃ (25 mM), sodium lactate (20 mM), sodium pyruvate (0.25 mM), and CaCl₂ (1.7 mM) at pH 7.4.

Media for both mouse and guinea pig gametes were sterilized by Millipore filtration (0.2-μm filter).

Gametes

Detailed descriptions of the handling of mouse gametes were reported previously (33). A brief summary is given here. Unfertilized tubal mouse eggs were incubated for 10 min in CM containing 0.1% hyaluronidase (Type I, Sigma) to disperse the cumulus. Any remaining cumulus cells were removed mechanically. Completely denuded eggs were washed twice and stored in CM under sterile silicone oil (Dow Corning Corp., Midland, Miss.) at 37°C until egg collection was completed. When required, zonae pellucidae were removed manually by forcing completely denuded eggs through narrow-bore micropipettes; these isolated zonae were washed five times and similarly maintained in CM. Insemination of mouse eggs in vitro was accomplished according to the method of Wolf and Inoue (39), using a final inseminating sperm concentration of 1 × 10⁴ cells/ml. Mouse sperm suspensions were prepared by mincing the excised cauda epididymides of a mature Swiss mouse in 0.4 ml TN. Particulate tissue was removed after allowing 10-15 min for sperm dispersion, and aliquots of the epididymal suspension were diluted 1:5 in the indicated medium (see text and figure legends).

Excised cauda epididymides of a mature guinea pig were held under silicone oil with a hemostat, which placed the epidid-
RESULTS

Fluorescence Assay for Mouse Sperm Acrosome Reaction

The antibiotic CTC is a fluorescent chelator of divalent cations, which markedly increases its fluorescence emission upon binding a divalent cation in a nonpolar environment (12, 13). It binds at low concentrations to mouse sperm and, in the presence of Ca$^{2+}$, emits fluorescence of variable intensity depending on its location. Fig. 1 shows the pattern of fluorescence displayed by sperm when CTC was applied to gametes recovered 15 min after insemination. Paired phase contrast and epifluorescent micrographs show that the mouse sperm bound at the zona surface (Fig. 1a) exhibited a compartmentalized pattern of CTC fluorescence (Fig. 1b). This distribution was observed consistently at this brief interval after insemination when sperm are known to be completely intact (32). Intense fluorescence was emitted from two areas, the midpiece and the area on the sperm head corresponding to the position of the acrosome, whereas the principal piece of the tail and the area corresponding to the post-acrosomal segment displayed very dim fluorescence. The intense CTC fluorescence emitted by the underlying egg cytoplasm was eliminated by rupturing the zona at several sites (Fig. 1a and b, arrows) and extruding the cytoplasm to create a darkened background for viewing the sperm.

The characteristic CTC fluorescence pattern shown by intact sperm enabled us to answer the important ancillary question: does the egg partic-
ipate in the binding of intact mouse sperm to the zona pellucida? The answer is that the egg is unnecessary, in contrast to findings obtained with hamster gametes (22, 24). Sperm bound to isolated zonae (Fig. 1c) showed the same fluorescence pattern on the anterior portion of the head (Fig. 1d) as sperm bound to zonae of intact eggs (Fig. 1b). The pattern was easier to see since there was no background fluorescence from the egg. The typical bright fluorescence of the midpiece can be seen at the sperm's neck in this micrograph (Fig. 1d), but the motion of the flagellum, which is unaffected by CTC, obscured photographic recording of fluorescence distally. The Ca\(^{2+}\) requirement for sperm binding to isolated zonae (Fig. 2; Table 1) is the same as those for binding to zonae of intact eggs. The use of the fluorescent probe in binding studies with isolated zonae defines this reaction in mouse gamete interaction as one which occurs between the surface of the intact membrane of the sperm head and the surface of the zona pellucida, with Ca\(^{2+}\) as the only exogenous factor required.

Previous ultrastructural studies of several mammalian species (2, 11, 16, 30, 40), including the mouse (42), have demonstrated that sperm which have undergone the perivitelline space have undergone an acrosome reaction. In contrast, only intact mouse sperm which have not undergone the acrosome reaction were noted within the perivitelline space (22). The pattern of CTC fluorescence of sperm located within the perivitelline space was therefore examined for comparison with that observed in sperm bound to the zona surface (Fig. 1b and d). For these experiments, mouse eggs were inseminated in the presence of the divalent cation ionophore, A23187 (3 μM), a treatment which permits as many as 50 sperm to penetrate the zona and enter the perivitelline space.

Under the usual conditions for fertilization in vitro, few extra sperm enter the perivitelline space; the use of A23187 greatly increases the probability of finding perivitelline sperm for examination. The mechanism of the ionophore effect is currently unknown, but its presence does not appear to alter markedly the kinetics of zona penetration by the sperm or the gamete's ultrastructure (32). Gametes were recovered 2 h after insemination and thoroughly washed to remove any sperm bound to the zona. Exposure to CTC produced a distribution of fluorescence notably different from that seen in sperm bound to zonae (Fig. 1): the bright fluorescence formerly localized in the anterior portion of the sperm head was no longer visible (Fig. 3b). The midpiece retained its intense fluorescence, thereby serving as a convenient internal control. Since the background fluorescence of the egg cytoplasm was unavoidable in this situation, the zona was again ruptured to permit the escape of the egg cytoplasm. The inset in Fig. 3a demonstrates that this sperm was located well within the perivitelline space: the sperm's tail is being carried through the tear in the zona by the outward flow of the egg cytoplasm.

CTC can therefore be used as a fluorescent probe for the acrosome reaction in motile mouse sperm. Intact sperm show bright fluorescence on the anterior portion of the sperm head corresponding to the position of the acrosome. Acrosome-reacted sperm do not show this fluorescence; the sperm head remains dark. The difference is sufficiently marked that the assay is reliable and readily quantitated with regard to percent sperm which have undergone the acrosome reaction.

Experiments were undertaken to extend this assay to guinea pig sperm, which have prominent acrosomes visible by phase contrast optics. The acrosome reaction in these sperm is readily induced in vitro (9, 36, 37). After exposure to CTC, the fluorescence pattern observed in guinea pig sperm was analogous to that in the mouse, with the difference that ~50% of the acrosome-reacted sperm appeared to acquire bright fluorescence in the post-acrosomal region. In all samples examined, however, the inner acrosomal membrane of the reacted sperm was nonfluorescent.

The occurrence of the acrosome reaction in suspended mouse sperm can be monitored by use of CTC in the absence of eggs. If mouse sperm were incubated for increasing lengths of time in either CM or TNC and then exposed to CTC, a predictable sequence of fluorescence patterns, reflecting the successive stages of the acrosome reaction, was observed. The sperm seen in Fig. 4a and b was recovered after a 10-min incubation and is completely intact, as indicated by the bright and uniform fluorescence limited to the acrosomal area of the sperm head. The first alteration in this CTC fluorescence pattern was the superimposition of discrete, intensely fluorescent spots on the already fluorescent acrosomal area (Fig. 4c and d). These spots appeared to coalesce gradually into larger

1 Dr. Don P. Wolf, University of Pennsylvania School of Medicine, personal communication.
units with the simultaneous loss of the bright acrosomal background fluorescence (Fig. 4e and f). In the terminal phase of the reaction (Fig. 4g-j), all of the CTC fluorescence was lost from the sperm head.

It was not possible to predict in advance the actual number of mouse sperm in a given suspension that would display this sequence of fluorescence changes. The variability appeared to reflect the individuality of sperm suspensions prepared from different males. This held true to a limited extent for the time course over which these changes occurred, although some generalizations about this parameter can be made. Fluorescence patterns similar to the sperm in Fig. 4b comprised the overwhelming majority of sperm observed during the first hour in suspension. During the next 2 h, variable numbers of sperm exhibited fluorescence patterns similar to those seen in Fig. 4d and f. Fluorescence patterns corresponding to fully reacted sperm (Fig. 4h and j) were rarely seen before 3 h of incubation.

The question of a Ca\(^{2+}\) requirement for the mouse acrosome reaction could not be answered with this assay. Exposure of sperm to CTC in the absence of added Ca\(^{2+}\) resulted in considerably reduced fluorescence emission (Fig. 4k and l). In this situation, the midpiece displayed weak fluorescence, while the sperm head was virtually invisible. The lack of fluorescence did not change as a function of length of incubation; mouse sperm exposed to CTC at different times in the absence of added Ca\(^{2+}\) exhibited an extremely faint fluorescent image. Addition of EGTA changes the fluorescence pattern observed with CTC plus Ca\(^{2+}\) to that observed with CTC in the absence of Ca\(^{2+}\). The same dull image was obtained after incubation of sperm in TN plus 2 mM Mg\(^{2+}\). Although CTC will chelate Mg\(^{2+}\) as readily as Ca\(^{2+}\) and emit an intense fluorescent signal in a nonpolar environment (13), no perceptible fluorescence was displayed by mouse sperm in the presence of this divalent cation after exposure to CTC.

Several years ago, tetracycline was proposed as a fluorescent probe for the occurrence of capacitation in rabbit sperm (18, 19). The use of this antibiotic for that purpose was later questioned (38) on the basis that some of the observed tetracycline fluorescence changes did not correspond to the changes predicted to occur within the appropriate interval at known sites of capacitation. The question of whether tetracycline and CTC give similar results in the assay described here was therefore examined. Mouse sperm were exposed to tetracycline under conditions identical to those used for CTC. No fluorescent image resulted after the exposure of mouse sperm to 10 \(\mu\)M tetracycline, the concentration of CTC routinely used. Application of tetracycline at 170 \(\mu\)M provided only a faint fluorescent image of the midpiece alone; the sperm head generally did not exhibit any fluorescence with this compound. An occasional sperm displayed dull, uniform fluorescence over the entire sperm head; however, no regional fluorescence patterns were detected with tetracycline. Finally, these patterns did not change as a function of the length of sperm incubation. Tetracycline does not give the same results as CTC and is unsuitable as a fluorescent probe for the acrosome reaction.

**Conditions for the Occurrence of the Acrosome Reaction in Mouse Sperm In Vitro**

Since unpredictable and relatively small num-

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**Figure 1** Paired phase contrast and epifluorescent micrographs of sperm bound to zonae and exposed to CTC. (A and B) Mouse sperm bound at the surface of the zona pellucida. The gametes were recovered 15 min after insemination by the stop-fix technique in the absence of glutaraldehyde and exposed to 10 \(\mu\)M CTC. The intense fluorescence of the egg cytoplasm often prohibited epifluorescent visualization of the sperm head. Creation of a small rupture (arrows) in the zona by the application of pressure to the top of the coverslip allowed the cytoplasm to escape, and provided a dark background for improved visualization. Note the bright, uniform fluorescence on the sperm head localized to the area occupied by the acrosome, in contrast to the dimly fluorescent adjacent area of the sperm head corresponding to the post-acrosomal segment. (C and D) Mouse sperm bound at the surface of a manually isolated zona pellucida. Sperm and isolated zonae were recovered 15 min after sperm addition, washed with a wide-bore micropipette and exposed to 10 \(\mu\)M CTC. The edge of the zona, which is difficult to resolve visually, is indicated by the arrows. The pattern of CTC fluorescence is identical to that seen in sperm bound to intact zonae recovered at this same interval after sperm addition and is readily visualized here due to the lack of background fluorescence contributed by the egg cytoplasm. Z, zona pellucida; C, egg cytoplasm; A, acrosomal region of the sperm head; P, post-acrosomal region of the sperm head. Bar, 10 \(\mu\)m.
FIGURE 2 (A and B) Phase contrast micrographs of manually isolated zonae pellucidae incubated with mouse sperm in the absence (A) or presence (B) of 1.7 mM Ca++. Zonae were washed thoroughly in CM after isolation to eliminate contamination by the eggs. After incubation with sperm for 15 min, zonae were recovered, washed gently with a wide-bore micropipette, and examined for sperm binding. Sperm were preincubated for 60 min in (A) TN or (B) TNC. ×600.

Numbers of mouse sperm appeared to undergo the acrosome reaction when incubated in either CM or TNC alone, as assayed by the distribution of CTC fluorescence, a method was required for the consistent induction of the acrosome reaction in a large proportion of suspended sperm for use in zona binding determinations. In other mammalian systems, the addition of the ionophore A23187 to a sperm suspension results in the occurrence of the acrosome reaction in high numbers (21, 36, 37). The ionophore alone was not effective in the induction of the acrosome reaction in large numbers of mouse sperm incubated in either CM or TNC. Use of an incubation medium with increased salt concentration in combination with A23187, however, was consistently effective. Sperm were incubated in CM in which the NaCl concentration had been raised from 120 to 180 mM (HS-CM) for a minimum of 60 min, followed by the addition of A23187 (3 μM). Exposure of these sperm to CTC after a further incubation interval of 30 min consistently resulted in the occurrence of the acrosome reaction in at least 50% of the motile sperm population, as assayed with CTC. In some of the sperm suspensions examined, as many as 80% of the motile population had undergone the reaction. Neither HS-CM nor A23187 alone were capable of consistently producing this proportion of reacted mouse sperm.

Will Acrosome-Reacted Mouse Sperm Bind to the Zona Pellucida?

The development of the CTC fluorescence probe assay for the acrosome reaction and of the method for induction of this reaction in suspension with high salt medium plus A23187 permitted us to test the prediction that reacted mouse sperm will not bind to the zona of the egg. The rationale underlying the test was the following: If the particular membrane surface over much of the sperm head is irrelevant for recognition of and binding to the zona surface, then the proportions of intact and acrosome-reacted sperm bound to the zona should reflect the proportions of each in the population. Alternatively, if a specific membrane surface is required for this process, then those sperm

| Medium* | Mean number of sperm/zona  |
|---------|---------------------------|
| CM      | 8.0 ± 0.70 (62)           |
| TNC     | 9.5 ± 0.76 (66)           |
| TN      | 0.07 ± 0.03 (75)          |

* The same medium was used for both sperm preincubation (60 min) as well as for sperm and zona incubation.

† Binding was assayed 15 min after sperm addition. Values are given ±SEM. Figures in parentheses give the actual number of zonae counted.

Data compiled from three replicate experiments.
having that membrane surface will be selectively bound, independent of their proportion in the population. Evidence that the latter case holds true for hamster gametes was obtained using scanning electron microscopy (22), although in those experiments the challenging sperm population consisted almost solely of acrosome-intact sperm.

Eggs were inseminated with sperm from one of three possible preincubation regimes: incubation for 90 min in TNC or CM, or in HS-CM for 60 min plus 3 μM A23187 for 30 min to induce the acrosome reactions. Eggs were recovered by the stop-fix technique in the absence of glutaraldehyde, at 30 min post-insemination, and exposed to

**Figure 3** (A and B) Paired phase contrast and epifluorescent micrographs of a mouse sperm located within the perivitelline space. Gametes were inseminated in the presence of 3 μM A23187 and recovered 2 h after insemination. Exposure to 20 μM CTC resulted in emission of intense fluorescence from the sperm midpiece, while the entire sperm head is only barely visible with epifluorescence. The inset shows more clearly the position of the sperm within the perivitelline space; arrows point to the rupture in the zona, created to eliminate the background CTC fluorescence of the egg cytoplasm. Z, zona pellucida; M, sperm midpiece; H, sperm head. Bar, 10 μm.
FIGURE 4  Paired phase contrast and epifluorescent micrographs of motile mouse sperm in suspension exposed to CTC. Panels A through J show the successive stages of the acrosome reaction from the fully intact to the fully reacted state. Throughout the reaction, the CTC fluorescence of the midpiece remains quite bright. (A and B) Completely intact sperm displaying the CTC fluorescence distribution characteristic for that state. Note the bright and uniform fluorescence emitted from the acrosomal area of the sperm head, in contrast to the poorly fluorescent post-acrosomal segment. (C and D) An early stage of the...
TABLE II

Status of the Acrosome of Mouse Sperm Bound to Zonae

| Insemination medium | Sperm preincubation | CTC fluorescence category |
|---------------------|---------------------|---------------------------|
|                     |                     | 1 | 2 | 3 | 4 |
| A. CM               | HS-CM/A23187        | 93 | 6 | 1 | 0 |
| B. CM               | CM                  | 91 | 7 | 2 | 1 |
| C. TNC              | TNC                 | 92 | 6 | 1 | 1 |

Eggs recovered by the stop-fix technique in the absence of glutaraldehyde at 50 min postinsemination and exposed to 10 μM CTC. The values represent the percentage of sperm in the CTC fluorescence category, as defined below, for the stated combination of insemination medium and sperm preincubation conditions in the experimental sets A, B, and C. The total number of gametes examined in each experimental set were as follows: A, 305 sperm, 81 eggs; B, 281 sperm, 76 eggs; C, 227 sperm, 68 eggs.

* Sperm were preincubated for 90 min in (A) HS-CM for 60 min, plus 3 μm A23187; (B) CM; or (C) TNC.

$CTC fluorescence category: (1) Completely intact sperm, with bright uniform fluorescence over the acrosomal area abruptly adjacent to very dim fluorescence over the post-acrosomal segment (see Fig. 4b). (2) Sperm in the initial phase of the acrosome reaction, with intensely fluorescent spots of fluorescence superimposed upon the already fluorescent acrosomal area, giving rise to a mottled appearance of the acrosome (see Fig. 4d). (3) Sperm in a very late stage of the acrosome reaction—with few bright spots of fluorescence lying on a weakly fluorescent acrosomal area (see Fig. 4f). (4) Completely reacted sperm, with very weakly fluorescent or nonfluorescent sperm head (see Fig. 4h and 4j).

DISCUSSION

The utility of CTC as a fluorescent probe for assaying the acrosome reaction results from the localized fluorescence of the probe bound to the intact mouse sperm head in the presence of Ca²⁺. The fluorescence disappears when the sperm undergoes the acrosome reaction. The intense fluorescence signal of CTC is elicited upon chelation of a divalent cation, such as Ca²⁺ or Mg²⁺, in a nonpolar environment (12, 13); The probe actually reports the location of divalent cations in membranes. In mouse sperm, the physiological divalent ion is Ca²⁺. Fluorescence in the absence of added divalent cation was very weak (Fig. 4k and l); either endogenous Ca²⁺ or trace levels of Ca²⁺ in TN may be responsible for the dim fluorescence emitted from the sperm's midpiece. The addition of Mg²⁺ to sperm in TN did not increase the CTC fluorescence over that seen in TN, while EGTA in amounts sufficient to chelate the Ca²⁺ in TNC entirely prevented the CTC fluorescence. Any proposal for the mechanism of fluorescence loss must account for the observation that intense fluorescence is emitted only when the CTC-Ca²⁺ complex is membrane-bound (12). Loss of CTC fluorescence could result from the release of membrane-bound Ca²⁺ from the sperm head, due to loss of specific Ca²⁺ binding groups on the membrane. An alternative proposal, that better takes into consideration the regionalization of the CTC fluorescence, is that the CTC-Ca²⁺ complex binds.
specifically to the plasma membrane overlying the acrosomal, but not post-acrosomal, region. Given the close apposition of the plasma and outer acrosomal membranes, the CTC-Ca\(^{2+}\) complex may also be bound to the latter (31). Loss of fluorescence would then result quite simply from the loss of these membranes which occurs as the main feature of the acrosome reaction. Such a restricted localization of the chelate-cation complex is feasible due to the highly compartmentalized organization of spermatozoa (20).

A similar suggestion regarding the localization of Ca\(^{2+}\) in the sperm head has been made by Singh et al. (34) to account for the consistent disappearance during the acrosome reaction of a portion of \(^{40}\)Ca\(^{2+}\) associated with bovine sperm. Additional support for this localization of the CTC-Ca\(^{2+}\) complex overlying the acrosome is found in micrographs of reacting guinea pig sperm exposed to pyroantimonate (20). This agent also chelates cations, but then forms an insoluble precipitate rather than a fluorescent compound. In reacting sperm, all of the cation-pyroantimonate precipitate is located within membranous vesicles surrounding the sperm head. Although this reagent does not have a strict cation specificity, the experiments nonetheless demonstrate the absence of cation binding sites on the newly exposed inner acrosomal membrane.

Use of CTC as a fluorescent probe also showed a striking localization of the Ca\(^{2+}\)-CTC complex in the midpiece region of mouse and guinea pig sperm. This observation agrees closely with the Ca\(^{2+}\) distribution in bull sperm exposed to 0.17 mM Ca\(^{2+}\), as determined by Babcock et al. (6), using electron microanalysis. They found that the midpiece was 3.5–8 times increased over background, while the content of the other regions was near background. The question of whether the Ca\(^{2+}\) was actually located in the mitochondria under these conditions was answered in the affirmative, based on both electron microanalysis (6) and previous biochemical studies (4, 5). Yet the sperm plasma membrane in rabbit (35) and human (29) sperm seems quite impermeable to Ca\(^{2+}\). Further investigation is required to clarify this problem; CTC should prove to be a useful fluorescent probe in this investigation.

The effectiveness of the HS-CM medium in the induction of the acrosome reaction in mouse sperm in vitro was not altogether unexpected, since media of high ionic strength (28) have been utilized previously to enhance mouse sperm membrane destabilization. Nor was the need for A23187 surprising, since this ionophore has been employed for the induction of the acrosome reaction in other mammalian systems (22, 36, 37), and the dependency of the acrosome reaction upon extracellular Ca\(^{2+}\) in both invertebrate (see reference 17) and mammalian (37, 41) sperm has been well established. However, it was not anticipated that sequential exposure of mouse sperm to these conditions would be required, although a fairly similar system has very recently been reported by Lui and Meizel (26) for the synchronous induction of the acrosome reaction in hamster sperm. If influx extracellular Ca\(^{2+}\) is the event common to all sperm for the initiation of the acrosome reaction, then it is not unreasonable to suspect that this same goal may be attained by slightly different routes demanding very particular experimental conditions for gametes from different species.

The selective binding of intact sperm to zona, particularly when the eggs were confronted with a large population of motile, acrosome-reacted sperm, allowed the identification of the plasma membrane as the sperm surface responsible for recognition of and binding to the zona pellucida. This finding has several interesting implications. It defines the obligatory sequence for the membrane reactions of fertile mouse sperm immediately before sperm-egg fusion: binding of the sperm to the zona surface is followed by the acrosome reaction, and then zona penetration. The acrosome reaction, for the successful spermatozoon, occurs at the zone surface. The occurrence of the reaction at this location neither implies nor negates the activity of a specific zonal or vitelline initiating factor. The interaction at the zona could be restricted to the binding reaction. The acrosome reaction would then be viewed as the culmination of endogenous processes initiated before binding to the zona. The zona pellucida is the site of a major barrier to interspecific fertilization (23, 40), suggesting the existence of a specific receptor for the zona in sperm. We have demonstrated here that this putative receptor resides in the mouse sperm plasma membrane.

The authors acknowledge with thanks the assistance of Mr. Richard Seifert (Optical Apparatus Co.) and Mr. Steve Mahdessian (Farrand Optical Co.) during the complex installation and initial operation of the optical system. We are grateful to Drs. Keith Burridge, Linda Heffner, and Don Wolf for their helpful suggestions and critical comments, and to Ms. Patricia Park for excellent secretarial aid.
This work was supported by U. S. Public Health Service grant HD-06274 and National Science Foundation grant PCM-77-15965.

Received for publication 16 March 1979, and in revised form 27 August 1979.

REFERENCES

1. Austin, C. R. 1951. Observations on the penetration of the sperm into the mammalian egg. Am. J. Anat. 86:501-516.
2. Austin, C. R. 1975. Membrane fusion events in fertilization. J. Reprod. Fertil. 44:155-166.
3. Austin, C. R. 1977. Spermatozoa and the role of membranes in the fertilization process. In Mammalian Cell Membranes, Vol. III. G. A. Jamieson and D. M. Robinson, editors. Butterworths, London. 206-230.
4. Babcock, D. F., N. L. First, and H. A. Lardy. 1975. Transport mechanisms for succinate and phosphate localized in the plasma membrane of bovine spermatozoa. J. Biol. Chem. 250:6404-6410.
5. Babcock, D. F., N. L. First, and H. A. Lardy. 1976. Action of ionophore A23187 at the cellular level. Separation of effects at the plasma and mitochondrial membranes. J. Biol. Chem. 251:3881-3886.
6. Babcock, D. F., D. M. Stameriohn, and T. Hutchinson. 1978. Calcium redistribution in individual cells correlated with ionophore action on motility. J. Exp. Zool. 204:391-400.
7. Barros, C., J. M. Bedford, L. E. Franklin, and C. R. Austin. 1967. Membrane vesiculation as a feature of the mammalian acrosome reaction. J. Cell Biol. 34:547-557.
8. Barros, C., J. J. Herrera, and B. T. Storey. 1975. Capacitation in vitro of guinea pig spermatozoa in a saline solution. J. Reprod. Fertil. 34:191-200.
9. Beddow, J. M. 1968. Ultrastructural changes in the sperm head during fertilization in the rabbit. Am. J. Anat. 122:29-38.
10. Beddow, J. M., and G. W. Cooper. 1978. Membrane fusion events in the fertilization of vertebrate eggs. Cell Surface Reviews. 6:5-12.
11. Caswell, A. H., and J. D. Hutchinson. 1971. Visualization of membrane-bound cations by a fluorescent technique. Biochem. Biophys. Res. Commun. 42:41-49.
12. Caswell, A. H., and J. D. Hutchinson. 1971. Selectivity of cation chelation to tetracyclines: evidence for special conformation of calcium chelate. Biochem. Biophys. Res. Commun. 43:625-630.
13. Chang, C. M. 1951. Fertilizing capacity of spermatozoa deposited in the Fallopian tubes. Nature (Land.). 168:697.
14. Chang, C. M. 1957. A detrimental effect of seminal plasma on the fertilizing capacity of sperm. Nature (Land.). 179:256-259.
15. Chang, C. M., and R. H. F. Junter. 1975. Capacitation of mammalian sperm: biological and experimental aspects. Handb. Physiol. 5:339-351.
16. Epple, D., and V. C. Vaucouleurs. 1978. Membrane fusion events during in vitro fertilization. Cell Surface Reviews. 61:5-63.
17. Ericson, R. J. 1967. A fluorometric method for measurement of sperm capacitation. Proc. Soc. Exp. Biol. Med. 125:1115-1118.
18. Ericson, R. J., and V. F. Barker. 1967. Binding of tetraacycline to mammalian spermatozoa. Nature (Land.). 214:610-616.
19. Ericson, R. J., and V. F. Barker. 1967. Binding of tetraacycline to mammalian spermatozoa. In Immunology of Gametes. M. Eddin and M. H. Johnson, editors. Cambridge University Press, Cambridge. 5-30.
20. Green, D. P. L. 1978. The induction of the acrosome reaction in guinea-pig sperm by the divalent metal cation ionophore A23187. J. Cell Sci. 22:157-161.
21. Gwatkin, R. B. L., H. W. Carter, and H. Patterson. 1976. Association of mammalian sperm with the cumulus cells and the zona pellucida studied by scanning electron microscopy. In Scanning Electron Microscopy. Vol (VI). Proceedings of the Workshop on SEM in Reproductive Biology, H. T. Research Institute, Chicago, Ill. 374-384.
22. Hanada, A., and M. C. Chang. 1972. Penetration of zona-free eggs by spermatozoa of different species. Biol. Reprod. 6:300-309.
23. Hanada, A., and M. C. Chang. 1972. Penetration of zona-free eggs by spermatozoa of different species. Biol. Reprod. 6:300-309.
24. Hartmann, J. H., R. L. C. Gwatkin, and C. F. Hutchinson. 1972. Early contact interactions between mammalian gametes in vivo: evidence that the vitellus influences adherence between sperm and zona pellucida. Proc. Natl. Acad. Sci. U.S. 68:2797-2799.
25. Inoue, M., and D. P. Wolf. 1975. Sperm binding characteristics of the murine zona pellucida. Biol. Reprod. 13:434-436.
26. Lui, C. W., and S. Meier. 1979. Further evidence in support of a role for hamster sperm hydrolytic enzymes in the acrosome reaction. J. Exp. Zool. 207:173-186.
27. Mezies, S. 1978. The mammalian sperm acrosome reaction, a biochemical approach. In Development in Mammals, Vol. 3. M. Johnson, editor. Elsevier/North Holland Biomedical Press, Amsterdam. 61-64.
28. Oldshue, G., and B. G. Bracken. 1973. Capacitation of mouse spermatozoa in media with elevated ionic strength and reversible decapacitation with epididymal extract. Fertil. Steril. 26:49-95.
29. Peterson, R. M., and M. Frand. 1976. Relationship between motility and the transport and binding of divalent cations to the plasma membrane of human spermatozoa. Fertil. Steril. 27:1301-1307.
30. Pezo, L. 1969. Gamete structure and sperm entry in mammals. In Fertilization. C. B. Metz and A. Monroy, editors. Academic Press, Inc., New York. 2323-2343.
31. Rodman, G. M. 1975. Calcium binding to the acrosomal membrane of human spermatozoa. Exp. Cell Res. 96:123-130.
32. Salin, P. M., J. J. Cowin, and D. P. Wolf. 1978. Calcium-dependent binding of mouse epididymal spermatozoa to zona pellucidae in vitro: sequential relationship to the acrosome reaction. J. Exp. Zool. 204:229-238.
33. Salin, P. M., B. T. Storey, and D. P. Wolf. 1978. Calcium-dependent binding of mouse epididymal spermatozoa to the zona pellucida. Dev. Biol. 60:515-525.
34. Singh, J. P., D. F. Babcock, and H. A. Lardy. 1978. Increased calcium-ion flux is a component of capacitation of spermatozoa. Biochem. J. 172:549-556.
35. Storey, B. T. 1975. Energy metabolism of spermatozoa. IV. Effect of calcium on respiration of mature epididymal sperm of the rabbit. Biol. Reprod. 13:1-9.
36. Summers, R. G., P. I. Talbot, E. M. Kooi, B. L. Hyland, and L. E. Franklin. 1976. Ionophore A23187 induces acrosome reactions in sea urchin and guinea pig spermatozoa. J. Exp. Zool. 196:381-385.
37. Talbot, P. R. G. Summers, B. L. Hyland, E. M. Kooi, and L. E. Franklin. 1976. The role of calcium in the acrosome reaction: an analysis using ionophore A23187. J. Exp. Zool. 190:383-393.
38. Vaidya, R. A., J. M. Binford, R. H. Glass, and J. M. Morris. 1969. Evaluation of the removal of tetracycline fluorescence from spermatozoa as a test for capacitation in the rabbit. J. Reprod. Fertil. 19:483-489.
39. Wolf, D. P., and M. Inoue. 1976. Sperm concentration dependency in the fertilization and the zona sperm binding properties of some eggs inactivated in vitro. J. Exp. Zool. 196:27-38.
40. Yanagimachi, R. 1977. Specificity of sperm-egg interactions. In Immunology of Gametes. M. Eddin and M. H. Johnson, editors. Cambridge University Press, Cambridge. 255-265.
41. Yanagimachi, R., and N. Itoh. 1974. Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. Exp. Cell Res. 89:161-174.
42. Zamboni, L. 1971. Fine Morphology of Mammalian Fertilization. Harper and Row, New York. 154-155.