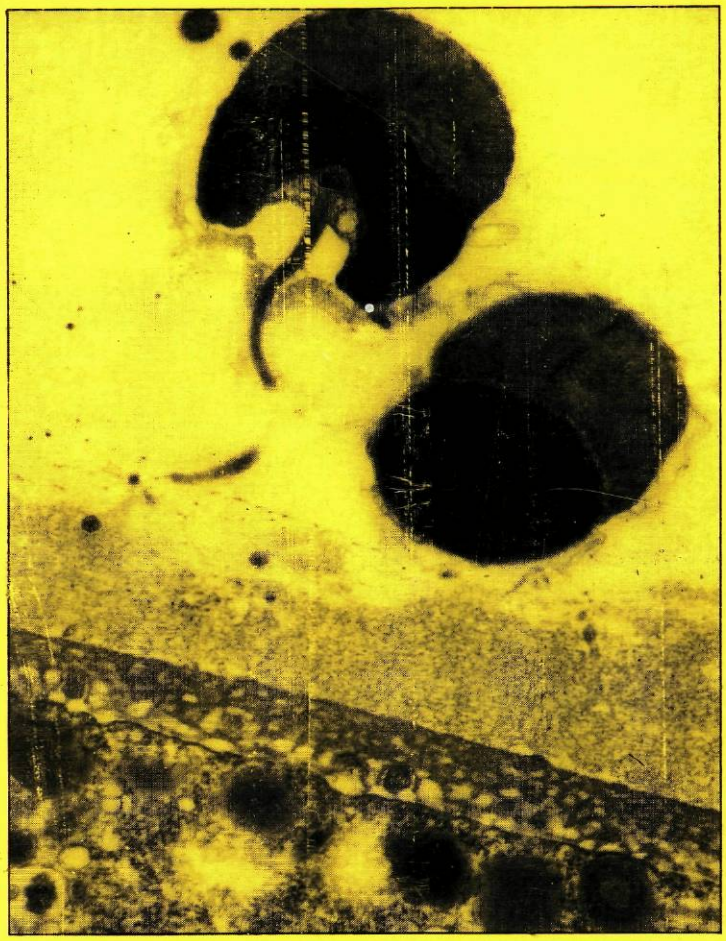


A FERTILIZAÇÃO em *Marthasterias glacialis* (Echinodermata, Asteroidea)

MÁRIO MANUEL DA SILVA LEITE SOUSA

Porto, 1991

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A FERTILIZAÇÃO em *Marthasterias glacialis* (Echinodermata, Asteroidea)

Dissertação de candidatura ao grau de Doutor em Biologia Celular apresentada ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

Laboratório de Biologia Celular
Instituto de Ciências Biomédicas de Abel Salazar
Universidade do Porto

PORTO 1991

Ao Professor Doutor

Carlos Azevedo

A meus pais

O trabalho experimental e a execução gráfica foram subsidiados parcialmente pelo INIC

PREFÁCIO

A obtenção do grau de Doutor corresponde, formalmente, ao reconhecimento da maturidade científica de um investigador. Porém, se é certo que a actividade científica desenvolvida para a obtenção desse grau assenta fundamentalmente num trabalho individual, esse mesmo percurso passa pelo envolvimento de toda uma equipa. O trabalho que desenvolvi necessitou, de facto, como base, de todo um apoio material e do conhecimento científico de múltiplas pessoas, sem os quais seria de todo impossível de elaborar.

O Professor Doutor Carlos Azevedo, Director do Laboratório de Biologia Celular do Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto, é o responsável pela minha formação académica e de investigador. A ele devo o convite para iniciar trabalho de investigação no seu Laboratório quando era aluno do 2º. ano da licenciatura de Medicina e, subsequentemente, o apoio firme e continuado até eu atingir a presente etapa. O Professor Carlos Azevedo ofereceu-me todas as possibilidades de trabalho e que assentaram sobretudo na disponibilização de um laboratório montado com os meios básicos para a execução do meu programa de investigação. No restante, tudo dependeu do meu esforço e dedicação. Perante este desafio intencionalmente provocado, revelei determinadas capacidades que criaram no espírito do meu orientador, uma segurança cada vez maior no nosso trabalho de equipa. Com trabalho realizado, tive assim oportunidade de ver facilitado tudo o que necessitei para desenvolver uma área de investigação que cedo me fora confiada. O rigor científico, os objectivos pragmáticos e a disciplina de trabalho

foram outros dos aspectos, de igual modo fundamentais, que o Professor Carlos Azevedo soube, aos poucos, incutir-me. É, pois, a ele que tudo devo e a ele compete-me, por isso, dedicar-lhe o meu maior apreço e amizade.

O meu trabalho dependeu também do apoio dos Técnicos do Laboratório de Biologia Celular, a quem sinceramente muito agradeço. O Sr. João Carvalheiro corresponde a uma pessoa chave em todo o meu trabalho, pois é fruto do seu esforço, dedicação e entusiasmo a passagem à realidade dos meus resultados, ou seja, a ele devo a excelente execução das fotografias publicadas. A Sra. Dª. Laura Corral foi quem me treinou em técnicas de microscopia electrónica e a ela também me compete agradecer por colaboração técnica na preparação de algumas experiências de citoquímica. O Sr. Vítor Oliveira ensinou-me as técnicas base de microscopia de luz e a ele também agradeço o apoio técnico durante a elaboração de trabalhos de histoquímica. Ainda a ele lhe devo o apoio com a manutenção dos aquários e dos animais. A Sra. Dª. Elsa Oliveira ajudou-me na incansável tarefa de pesquisa bibliográfica e a Sra. Dª. Ana Campos na limpeza de parte do material que nos trabalhos fui utilizando.

No início do meu trabalho em microscopia electrónica não foi no Laboratório de Biologia Celular do ICBAS que dispuz de um microscópio electrónico, mas sim no Instituto de Histologia e Embriologia Abel Salazar da Faculdade de Medicina da Universidade do Porto. Desejava pois, por este meio, agradecer nas pessoas dos Srs. Professores Doutores António Coimbra e Manuel Magalhães todos os apoios e facilidades que aí sempre dispus.

Com o tempo, verifiquei tornar-se absolutamente imprescindível fazer uso de outras técnicas de Biologia Celular que não as da microscopia. Para tal, encetei contactos com três outros laboratórios, e, do trabalho em comum, frutificaram novas publicações e avanços na pesquisa pretendida. Devo pois, a esses laboratórios uma boa fatia do meu trabalho. São eles o Laboratório de Bioquímica do ICBAS (Professor Doutor Pedro Moradas Ferreira), o Instituto de Antropologia da Faculdade de Ciências (Professor Doutor António Amorim) e o Laboratório de Enzimologia do Instituto de Genética Médica Jacinto de Magalhães (Dr. Rui Pinto). Em todos estes locais encontrei uma disponibilidade total, um grande interesse e entusiasmo pelos objectivos de trabalho e um apoio técnico e científico que permitiram ultrapassar os grandes obstáculos da experimentação e interpretação dos resultados. Neles tive acesso a um conjunto de novas técnicas da área da bioquímica, tendo aí aprendido e executado as experiências necessárias. Uma palavra de grande agradecimento por toda a atenção e disponibilidade durante a execução das técnicas bioquímicas cabe às Lic^{as}. Albertina Lemos, Amélia Amorim, aos Lic^{os}. Vítor Costa e Paulo Fernandes e à Técnica Ana Conceição do Laboratório de Bioquímica do ICBAS. Ainda às Técnicas Augusta Fontes e Helena do Laboratório de Enzimologia do Instituto de Genética Médica Jacinto de Magalhães agradeço o auxílio nas electroforeses em acetato de celulose.

À Sociedade Portuguesa de Microscopia Electrónica, sociedade científica *sui generis* que, pela sua dinâmica e elevada qualidade científica das suas reuniões, devo também um forte estímulo no desenvolvimento da qualidade e aferição crítica dos meus resultados e da minha formação.

A bibliografia representa, como se compreende, um dos aspectos mais sensíveis e primordiais da investigação. Nela colhi grande parte dos meus ensinamentos. Com ela aprendi como planear, como fazer, e como escrever. Assim, dispus sempre de fácil acesso nas mais variadas bibliotecas, nomeadamente do Instituto de Histologia e Embriologia da Faculdade de Medicina, do Centro de Citologia Experimental, na biblioteca Alberto Saavedra do ICBAS (e aqui expresso ao seu pessoal os meus sinceros agradecimentos pelas grandes facilidades obtidas), da Faculdade de Ciências e do Laboratório de Anatomia Patológica da Faculdade de Medicina, todos organismos da Universidade do Porto, do Hospital Geral de Santo António, do Instituto de Genética Médica Jacinto de Magalhães, bem como de várias outras bibliotecas de Coimbra e Lisboa.

Os meus agradecimentos estendem-se ainda a todos os anónimos revisores das nossas publicações científicas, pois aos nossos trabalhos emprestaram sempre um notável esforço no sentido de melhorarem a sua qualidade técnica e científica.

Gostava de incluir nestes agradecimentos uma palavra de apreço para com todos os meus professores e colegas médicos que tive ao longo da minha instrução até obter a graduação médica no ICBAS-HGSA. Deles sempre recebi, e continuo a receber, um forte estímulo e uma larga consideração, para não falar do apoio contínuo que deles ainda recebo na prática médica. Durante a minha formação recebi ainda um forte apoio da Fundação Calouste Gulbenkian, entidade a quem agradeço.

Ao Sr. Gualter Correia e colaboradores da Reprografia do ICBAS desejo expressar a minha gratidão no cuidado posto com a execução gráfica desta dissertação.

E, como não pode haver investigação sem auxílio financeiro, termino estes agradecimentos lembrando os apoios que recebemos, nomeadamente do INIC (Bolsa para Doutoramento no País, Processo Nº. 28105), do Centro de Morfologia Experimental da Universidade do Porto (Linha de Acção Nº. 6, Biologia da Reprodução), da JNICT (Projecto de Investigação) e da Fundação Eng. António de Almeida (Projecto de Investigação).

Pelo Decreto Lei nº 388/70, Art.º 8º, parágrafo 2, é permitido na elaboração da dissertação ao grau de Doutor, aproveitar total ou parcialmente os resultados dos trabalhos publicados. Foi com base nesta disposição legal que decidimos apresentar as secções dos Resultados, Materiais e Métodos, Discussão e Bibliografia como compilação dos artigos já publicados. Para nós, esta opção pareceu-nos a mais apropriada por vários motivos. Durante o tempo dedicado à investigação não fomos acumulando resultados para os apresentarmos de um modo integralmente original nesta dissertação, já que isso constituia um grave risco em termos de competição internacional no que diz respeito à publicação de novos resultados. Uma vez que fomos publicando os resultados da nossa investigação, pareceu-nos despropositado transcrevê-los e integrá-los num todo escrito em português. Assim, para nós, a apresentação dos resultados como compilação dessas publicações em revistas internacionais da especialidade, representa a melhor avalização das nossas capacidades técnicas e científicas.

Nesta dissertação englobo várias publicações, todas como primeiro autor, e nelas a minha contribuição pessoal foi a seguinte: em todos os casos coube-me o planeamento e execução das experiências, a preparação e observação do material, a análise dos resultados e a redacção dos artigos, sem prejuízo do acompanhamento crítico constante dos restantes coautores.

Como os artigos incluídos se reportam aos anos de 1983 a 1991, gostava de referir que os artigos de 1983 e 1985 foram elaborados enquanto aluno do curso de Medicina do ICBAS-HGSA, os artigos de 1986 e 1987 foram executados no período em que realizava o Internato Geral no HGSA e os artigos de 1988 a 1991 foram já elaborados durante a minha integração como Assistente Estagiário (1988) e depois como Assistente (1990) no Laboratório de Biologia Celular do ICBAS.

Neste prefácio incluo agora uma explicação no referente à justificação da investigação realizada, aos seus objectivos e à importância relativa dos resultados publicados.

O estudo da fertilização não é recente e engloba múltiplos aspectos que incluem não só os gâmetas, mas também a sua formação, a sua interacção no momento da fecundação, bem como o desenvolvimento embrionário subsequente à formação do ovo. O estudo da fertilização é fértil no seu impacto sobre a Vida, porque levou não apenas ao conhecimento dos factores morfogenéticos e fisiológicos envolvidos na formação e interacção dos gâmetas como ao controle *in vitro* das gerações. Simultaneamente, o estudo dos gâmetas, isolados ou

interagindo, mostrou que ambos constituem um rico modelo experimental para a compreensão de fenómenos biológicos mais gerais. Por exemplo, as descobertas dos mecanismos de activação e interacção celulares são sobretudo fruto dos estudos desenvolvidos em gâmetas. Não admira, portanto, que a gametogénese, a fertilização e a embriogénese sejam alvo de estudos intensivos com elevado impacto e aplicação prática. Neste respeito, cabe uma grande palavra aos equinodermes. Estes animais marinhos, de uso científico muito comum, constituem hoje, mais do que nunca, um elemento biológico chave no estudo dos diferentes mecanismos envolvidos na activação e interacção celulares. A sua utilização fica a dever-se ao facto de serem os seus gâmetas de fácil obtenção e isolamento, fornecendo enormes quantidades de células homogéneas, e devido ao facto da sua fertilização poder ser realizada *in vitro*, por simples mistura dos gâmetas em tubos contendo água do mar. A sua manipulação é, pois, relativamente simples e acessível. Para além deste aspecto, os embriões desenvolvem-se rápida e sincronizadamente, permitindo uma observação e manipulação dos complexos fenómenos temporais relacionados com a diferenciação e os ciclos de divisão celular. Este facto possui uma enorme importância, porquanto na maioria dos outros sistemas biológicos, esses fenómenos decorrem numa fracção temporal mais dilatada, tornando-os difíceis de interrelacionar.

Quando cheguei à investigação e me foi proposto o estudo do espermatozóide da estrela do mar da espécie *Marthasterias glacialis* (Echinodermata, Asteroidea), rapidamente me apercebi da volumosa bibliografia existente, bem como do rapidíssimo aparecimento de novas publicações. Que fazer perante tão apertada concorrência, caso

quizesse, de facto, introduzir novos dados científicos, tendo à mão apenas os recursos das microscopias óptica e electrónica e uma ausência total de uma equipa portuguesa altamente diferenciada nesta área ?

A resposta foi progressiva, e, hoje em dia, o tipo de trabalho que efectuámos adquiriu um estatuto internacional. Do estudo das publicações tornou-se cada vez mais notório que os investigadores americanos e japoneses, pioneiros e actualmente líderes na área em que me tentava introduzir, tinham, face aos grandes recursos técnicos e humanos de que dispunham, rapidamente optado pelo desenvolvimento do conhecimento nas áreas da fisiologia da reprodução, descurando um tanto nada a avaliação mais rigorosa do que a morfologia ainda podia trazer de relevante. Assim, aos poucos, o nosso trabalho foi divulgando que variados aspectos morfológicos e citoquímicos podiam trazer novos e interessantes dados, que, uma vez integrados com os estudos fisiológicos, lhes davam suporte e até suscitavam novas dúvidas. É, pois, essencialmente um trabalho de morfologia e citoquímica ultrastruturais o que apresento nesta dissertação, podendo observar-se que os nossos objectivos de estudo se detêm particularmente com a fase de interacção entre os dois gâmetas.

Como corolário da nossa investigação, passo a salientar quais as principais descobertas que fomos introduzindo na literatura da especialidade através dos artigos que foram por nós sucessivamente publicados:

(a) Ao estudar a ultrastrutura do espermatozóide e a sua formação, descrevemos com maior detalhe os aspectos e as relações morfológicas

dos diferentes organelos desta célula, sobretudo a nível do acrosoma. Descrevemos, nomeadamente, pela primeira vez, a existência de um novo organelo na linha germinal masculina dos equinodermes e demonstrámos a sua comparticipação na formação do acrosoma e de outras estruturas do citosqueleto do espermatozóide (artigos citados no índice com os nºs. 1-3).

(b) Utilizámos, pela primeira vez, de um modo sistematizado, a indução artificial da exocitose da vesícula acrosómica do espermatozóide pelo ionóforo A23187 e, colhendo o produto exocitado, nele podemos caracterizar várias enzimas. Assim, a nossa equipa foi a primeira a evidenciar com clareza a existência de vários sistemas hidrolíticos na vesícula acrosómica, já que, pelo contrário, a abordagem dos colegas estrangeiros faz uso da extracção total dos espermatozóides. Demonstrámos, deste modo, a existência de actividade fosfatase ácida, fosfatase alcalina, ATPásica Ca^{2+} e Mg^{2+} dependente, e de um sistema proteolítico complexo do tipo tripsina. A localização das enzimas foi corroborada com estudos citoquímicos. Em particular, a descoberta de um sistema pro-protease-protease veio relançar uma nova discussão a nível filogenético, já que se atribuía aos equinodermes a presença de uma protease tipo quimotripsina em oposição a um sistema complexo pro-protease-protease tipo tripsina nos mamíferos (artigos citados no índice com os nºs. 5-8 e 13).

(c) Os revestimentos externos do ovócito são outro tema dos nossos trabalhos. Esta matriz extracelular havia já sido isolada e caracterizada por outros investigadores, mas os resultados bioquímicos e fisiológicos careciam de suporte morfológico. Nós iniciámos o trabalho ao contrário, caracterizando, primeiro, esses materiais nos seus aspectos morfológico e citoquímico. Podemos, assim, constatar que é possível evidenciar determinados aspectos na ultrastrutura que suportam os achados bioquímicos mas que, por vezes, também sugerem a existência de outras macromoléculas que ficaram, muito provavelmente, por estudar. Por exemplo, os nossos resultados sugerem a existência de glicosaminoglicanos na composição da geleia do ovócito, facto que parece ter escapado à atenção de outros investigadores. Se este achado for comprovado no futuro próximo, virá trazer de novo à discussão os complexos problemas filogenéticos que aparentemente distanciam os estudos nos equinodermes dos executados nos mamíferos, já que, como se sabe, o "cumulus oophorus" apresenta uma matriz extracelular sobretudo rica em glicosaminoglicanos (artigos citados no índice com os nºs. 8, 10, 11, 13 e 14).

(d) A interacção espermatozóide-ovócito e a resposta deste à fusão com um espermatozóide são também temas já muito explorados por outros investigadores. Porém, coube-nos mostrar, pela primeira vez, a nível ultrastrutural, como é que o espermatozóide da estrela do mar reage durante a fertilização. Detectámos, também, onde se localizam as hidrolases e outras moléculas acrosómicas durante a penetração do espermatozóide através dos revestimentos externos do ovócito e estudámos o efeito da protease de origem acrosómica sobre a matriz extracelular do ovócito. Por fim, identificámos vários dos

constituintes moleculares contidos nas vesículas corticais do ovócito, bem como o seu destino após a exocitose das vesículas corticais, a qual ocorre em resposta à fusão com o espermatozóide (artigos citados no índice com os nºs. 4, 5 e 7-14).

Em termos de interacção espermatozóide-revestimentos externos do ovócito, podemos colocar, num extremo, o ovócito do ouriço do mar e o dos mamíferos e, noutro extremo, o da estrela do mar. Nos primeiros existe uma ampla matriz extracelular que o espermatozóide penetra sem reagir, guardando a sua ligação e reacção específica de espécie para a zona pelúcida, já muito perto do oolema. Na estrela do mar também os ovócitos dispõem de um ampla matriz extracelular, mas os espermatozóides ligam-se e reagem na sua periferia, bem distanciados do oolema. Esta separação permitiu-nos, por exemplo, separar as diferentes fases de interacção entre os gâmetas e sugerir, corroborando estudos electrofisiológicos, que a comunicação se pode efectuar entre as duas células antes da fusão das suas membranas (artigos citados no índice com os nºs. 4, 11 e 14).

Mas se poderá ser verdade que a obtenção do grau de Doutor corresponde em certa medida à maturação científica do candidato, já não é verdade que a dissertação ilustra um trabalho completo. Por isso, quando revejo os trabalhos aqui incluidos, sinto sempre a sensação de algo ainda incompleto e inacabado. Apesar de constituírem trabalhos aferidos pela comunidade científica, eles representam, para mim, apenas uma iniciação e, portanto, um compromisso para no futuro os desenvolver de um modo mais profundo. Por estas razões, a presente dissertação representa uma etapa no nosso esforço, estando incompleta e aberta à continuição.

Os nossos objectivos imediatos, a médio e a longo prazo, por esse motivo, serão agora enunciados:

(a) Isolar por cromatografia e depois caracterizar e imunolocalizar os diferentes componentes da vesícula acrosómica do espermatozóide, quer no gâmeta maduro quer durante a espermatogénese.

(b) Isolar as vesículas acrosómicas intactas para termos acesso aos constituintes da membrana da vesícula acrosómica. A importância deste isolamento é fundamental, na medida em que se sabe que é a membrana interna da vesícula acrosómica do espermatozóide e não a sua membrana citoplasmática que se funde com o oolema.

(c) Isolar por cromatografia, e depois caracterizar e imunolocalizar os diferentes componentes da matriz extracelular do ovócito, quer nos gâmetas maduros quer durante a ovogénese.

(d) Caracterizar enzimologicamente os constituintes das vesículas corticais dos ovócitos, seguindo-se a sua partição cromatográfica e imunolocalização quer no ovócito maduro quer durante a ovogénese.

(e) Partindo dos componentes isolados da vesícula acrosómica do espermatozóide, da matriz extracelular e das vesículas corticais dos ovócitos, conseguir sondas de ácidos nucleicos e, assim, seguir a sua expressão genética durante a espermatogénese e a ovogénese.

Por se tratar de um assunto complexo e os artigos incluidos apenas discutirem aspectos específicos da reprodução nos equinodermes, esta dissertação inclui também uma Introdução Geral, na qual é apresentada uma revisão completa sobre o estado actual dos conhecimentos nesta área da Biologia Celular.

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1. Sousa, M. and Azevedo, C. (1983) Fine structure of the spermatozoon of *Marthasterias glacialis* (Linnaeus) (Echinodermata; Asteroidea), with special reference to acrosomal morphology. *Internat. J. Invert. Reprod.*, **6**: 171-180. 84

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3. Sousa, M. and Azevedo, C. (1988) Silver staining of spermatogenesis in th e starfish *Marthasterias glacialis*. *Invert. Reprod. Develop.*, **15**: 105-108. 102

4. Sousa, M. and Azevedo, C. (1985) Acrosomal reaction and early events at fertilization in *Marthasterias glacialis* (Echinodermata: Asteroidea). *Gamete Res.*, 11: 157-167. 106

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12. Sousa, M. and Azevedo, C, (1990) Alkaline phosphatase and peroxidase activities in starfish cortical vesicles before and after cortical reaction. *J. Submicrosc. Cytol. Pathol.*, 22: 97-102. 170

13. Sousa, M. and Azevedo, C. (1989) Ultrastructural localization of calcium in the acrosome and jelly coat of starfish gametes. *Develop. Growth Differ.*, 31: 227-232. 176

14. Sousa, M., Pinto, R., Moradas Ferreira, P. and Azevedo, C. (1991) Histochemical studies of gametes of the starfish *Marthasterias glacialis* (Echinodermata, Asteroidea) before and after fertilization, with special reference to jelly coat. **(Submetido a publicação).** 182

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INTRODUÇÃO GERAL

A FERTILIZAÇÃO NOS EQUINODERMES, COM ESPECIAL REFERÊNCIA PARA A ESTRELA DO MAR *Marthasterias glacialis*

A fertilização pode ser definida como um fenómeno de múltiplas etapas que se inicia com a interacção e a subsequente fusão dos gâmetas. Este fenómeno culmina na associação dos grupos correspondentes dos cromosomas derivados de dois pronúcleos, um de origem maternal e outro de origem paternal, restaurando a condição diplóide necessária para o futuro desenvolvimento embrionário (Longo, 1973).

O nosso modelo de trabalho é a estrela do mar *Marthasterias glacialis* (Echinodermata, Asteroidea), um animal marinho com 5 braços braços e um reduzido corpo central, que atinge no animal adulto um diâmetro global de cerca de 15-20 cm. São animais muito sensíveis às condições ecológicas ambientais, pois encontram-se em zonas de água de maior salinidade, ± 38 ppm, e de *p*H mais elevado, *p*H = 8,0-8,3 (por exemplo, nas praias do Mindelo e Vila Chã, 30 Km a Norte da cidade do Porto, locais onde efectuámos todas as colheitas).

A partir da base de cada braço e de cada um dos lados do mesmo braço, formam-se as gónadas que, na época de plena maturação, ocupam todo o comprimento do braço (Fig. 1). A estrela do mar não possui dimorfismo sexual e tem um ciclo germinal anual. As gónadas começam a desenvolver-se por volta de Setembro/Outubro, apresentando-se

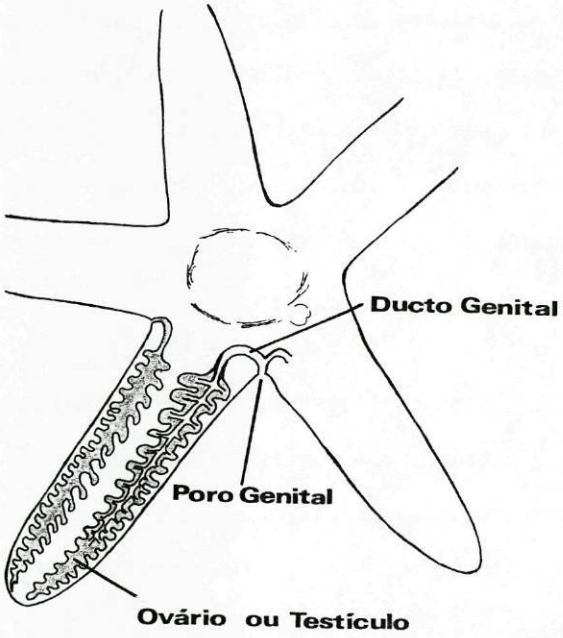


Fig. 1. Desenho esquemático representando os orgãos reprodutores na estrela do mar *Marthasterias glacialis*.

sexualmente indiferenciadas, em termos macroscópicos, durante vários meses. Em meados da Primavera, entre Abril e Maio, aparecem no lúmen das gónadas os gâmetas maduros, sendo nesta época que se verifica a reprodução destes animais. Durante o Verão verifica-se uma fase de degeneração do que resta da gónada madura. Nestes meses, assiste-se a uma fagocitose intensa no interior das gónadas, processo pelo qual os gâmetas que restaram são digeridos.

Na época da reprodução, as estrelas do mar fêmea e macho aproximam-se e, quase simultaneamente, ejaculam e ovulam para a água do mar. Este complexo comportamento depende de mecanismos ainda totalmente desconhecidos (Meijer e Guerrier, 1984; Smiley, 1990). Devido à proximidade física dos seus progenitores, os gâmetas interactuam de imediato. Em cativeiro observámos este fenómeno apenas uma vez, tendo notado que os animais faziam uso apenas de uma gónada por animal. Se na Natureza também assim for, isso quererá dizer que cada animal pode fecundar umas dez vezes. A gónada madura feminina tem um tom cor de rosa e a gónada masculina é esbranquiçada, cores que dependem exclusivamente da cor dos gâmetas. Assim, durante a ejaculação/ovulação surjem na água do mar duas "nuvens", uma esbranquiçada e outra cor de rosa, que rapidamente se misturam e a pouco e pouco se dissipam.

A estrela do mar é um animal de fecundação externa (decorre na água do mar), de modo que os seus gâmetas apresentam características que lhes permitem sobreviver nessas condições, durante algum tempo, antes de interagirem.

1. O ESPERMATOZÓIDE

O *phylum* Echinodermata (equinodermes) encontra-se actualmente subdividido em cinco classes, nomeadamente, Echinoidea (equinóides), Asteroide (asteróides), Ophiuroidea (ofiuróides), Holothuroidea (holoturóides) e Crinoidea (crinóides) (Fell, 1982). Destas cinco classes, praticamente apenas os equinóides e os asteróides têm sido objecto de intensas investigações na área da reprodução, pelo que nos reportaremos essencialmente aos dados obtidos, por nós e por outros investigadores, nestas duas classes.

De um modo geral a estrutura dos espermatozóides dos diferentes grupos taxonómicos depende do tipo de fertilização específica que possuem, interna ou externa, bem como das características dos revestimentos externos do ovócito a que se destinam (Afzelius, 1983; Koch e Lambert, 1990; Smiley, 1990).

Os espermatozóides podem ser divididos em dois grandes grupos, os primitivos e os modificados (Franzén, 1956, 1970). Os espermatozóides de tipo primitivo possuem um núcleo pequeno, arredondado ou cónico; um acrosoma simples no pólo apical, uma pequena peça intermediária no seu pólo posterior, compreendendo algumas mitocôndrias complanares rodeando dois centríolos; e um longo flagelo (Fig. 2). Os espermatozóides modificados são aqueles que se desviam deste arquétipo. Por exemplo, o espermatozóide humano é do tipo modificado porque a peça intermediária e o flagelo estão organizados de um modo mais complexo, nomeadamente devido à existência de uma baínha mitocondrial e de vários tipos de fibras acessórias em relação com o

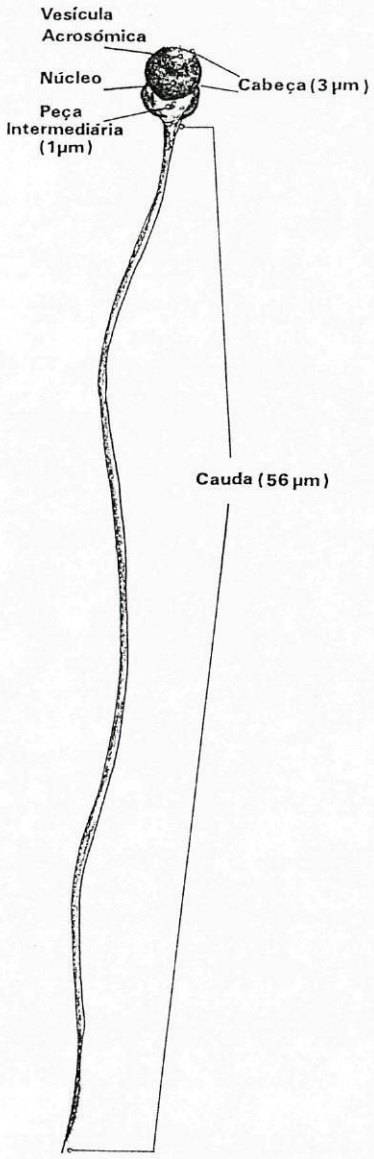


Fig. 2. Esquema do aspecto *in toto* do espermatozóide da estrela do mar *Marthasterias glacialis*.

axonema (Baccetti, 1984). De um modo geral, os espermatozóides primitivos fecundam externamente, enquanto que os modificados fecundam nos tractos genitais femininos. Porém, nem sempre é assim, pois existem equinodermes com espermatozóides modificados que fecundam externamente e equinodermes com espermatozóides de tipo primitivo que fecundam internamente (Atwood, 1975; Chia *et al.*, 1975; Holland, 1976, 1976b; Healy *et al.*, 1988; Eckelbarger *et al.*, 1989a, 1989b; Raff *et al.*, 1990). Nesta conformidade, Rouse e Jamieson (1987) reclassificaram os espermatozóides modificados em "ent-aquasperm" para os casos de fecundação externa, que exibem sobretudo um núcleo alongado como principal modificação e em "introsperm", para os casos de fecundação interna. Os espermatozóides de tipo primitivo e de fecundação externa recebem, por seu lado, a designação de "ect-aquasperm".

O espermatozóide da estrela do mar *M. glacialis* é do tipo primitivo (Fig. 3), sendo formado por um núcleo arredondado (d = ± 2 µm), contendo cromatina condensada. Este núcleo apresenta uma reentrância anterior (fossa acrosómica) onde se localiza o acrosoma.

O acrosoma é constituído por uma vesícula acrosómica e por um material denso que o rodeia, o material periacrosómico. A vesícula acrosómica tem o seu conteúdo diferenciado em três componentes principais: uma estreita zona periférica electronolucente, um componente periférico denso e um componente central claro. A membrana da vesícula acrosómica também se encontra diferenciada: apresentando a metade interna, ou basal, externamente revestida por uma fina camada de material denso, enquanto que a metade externa, ou anterior, está desprovida desse revestimento. A porção central da membrana externa da

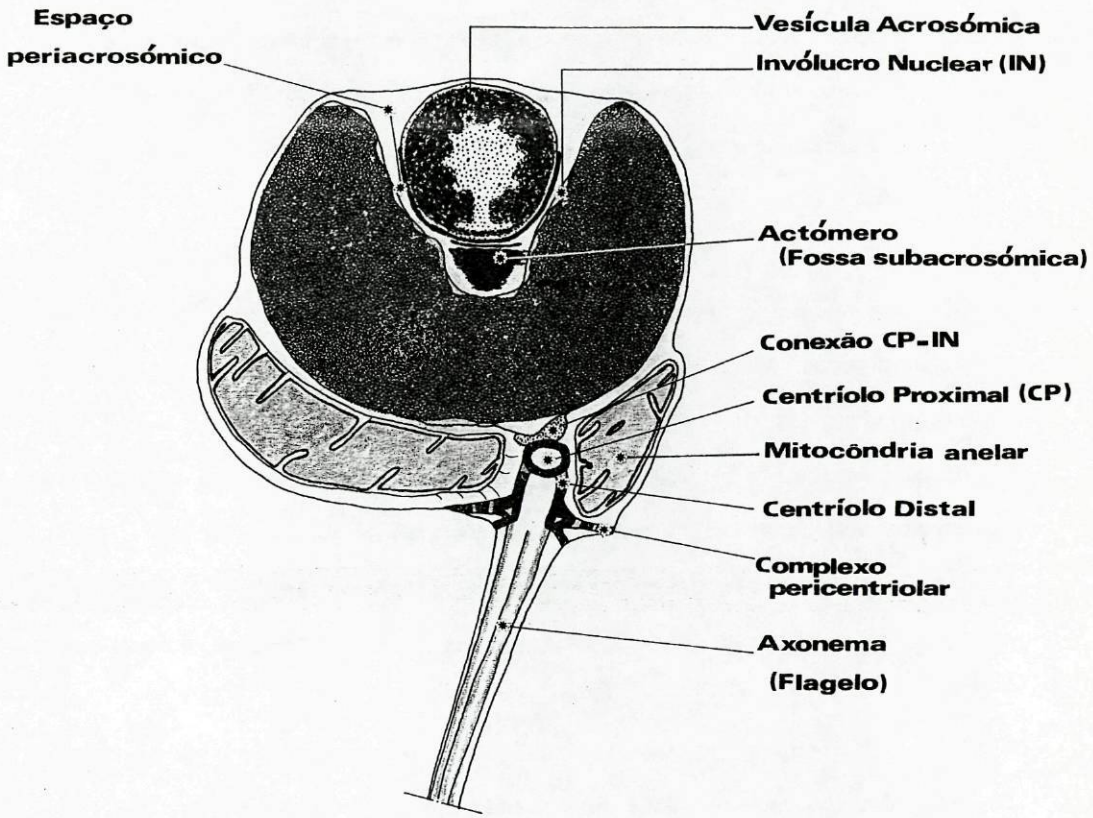


Fig. 3. Representação esquemática da ultrastrutura do espermatozóide da estrela do mar *Marthasterias glacialis*.

vesícula acrosómica encontra-se praticamente aderente à membrana citoplasmática, sendo nesta zona que as duas membranas se fundem, aquando da exocitose da vesícula acrosómica durante a fertilização. Na base da vesícula acrosómica, o espaço periacrosómico diferencia-se numa fossa subacrosómica, contendo um pequeno agregado de microfibrilas, o actómero, o qual, na realidade, corresponde a um centro polimerizador de microfilamentos (Fig. 3) (Tilney, 1978).

Na peça intermediária encontra-se uma volumosa mitocôndria anelar a rodear os dois centríolos. O centríolo proximal está aderente ao invólucro nuclear, enquanto que o centríolo distal adere à membrana citoplasmática por um complexo pericentriolar microfibrilar e estriado (Fig. 3).

O axonema (d = 0,2 - 0,3 µm) origina-se do centríolo distal e apresenta-se constituído por 9 pares periféricos de microtúbulos mais 2 simples centrais. O flagelo do espermatozóide está preenchido por este axonema rodeado da matriz flagelar (Fig. 3) (Sousa e Azevedo, 1983).

Nas diferentes classes dos equinodermes, a forma dos espermatozóides pouco difere, sendo praticamente todos do tipo primitivo (Fig. 4).

1.2. O ACROSOMA DO ESPERMATOZÓIDE

A. FORMAÇÃO DAS VESÍCULAS PROACROSÓMICAS

Em relação ao início da formação das vesículas proacrosómicas ao longo dos diferentes estadios da espermatogénese verifica-se uma

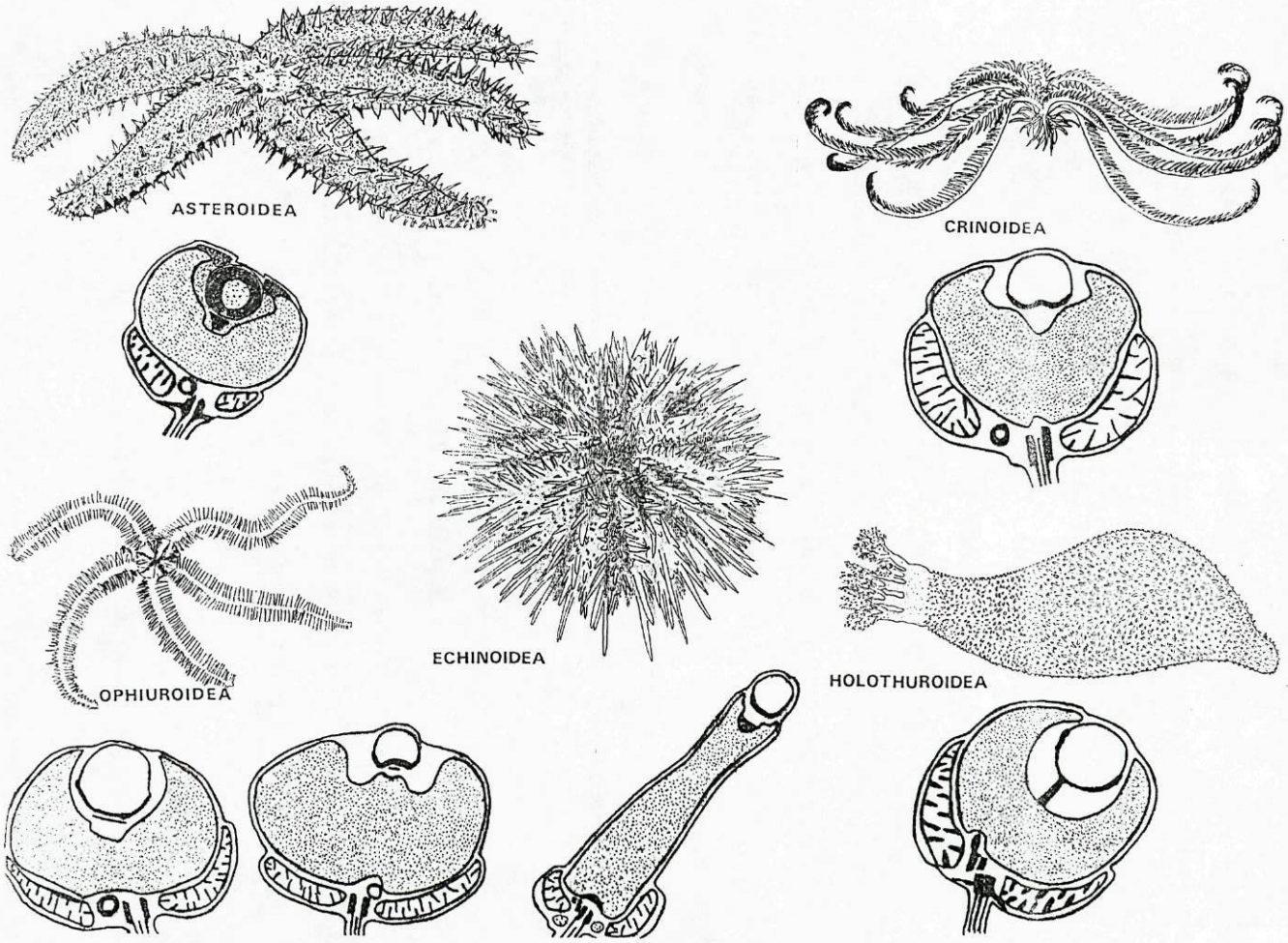


Fig. 4. *Phylum* Echinodermata, com as suas cinco classes e exemplos representativos dos espermatozóides respectivos.

grande variação entre as várias classes dos equinodermes. Nos asteróides as vesículas proacrosómicas foram primariamente reconhecidas nas espermatogónias (Sousa e Azevedo, 1988c), nos espermatócitos primários (Yamagata, 1988), ou nos espermatídeos (Dan e Sirakami, 1971; Janssen, 1984); nos equinóides elas foram encontradas apenas a partir do estadio de espermatídeo (Longo e Anderson, 1969; Sousa e Azevedo, 1988a); nos ofiuróides esses organelos, tanto podem aparecer, desde a fase de espermatogónia (Yamashita, 1983; Yamashita e Iwata, 1983), como apenas durante a espermiogénese (Buckland-Nicks *et al.*, 1984); nos holoturóides as vesículas proacrosómicas ou são inicialmente formadas durante a fase de espermatogónia (Atwood, 1974; Pladellorens e Subirana, 1975), ou apenas nos espermatídeos (Tilney, 1976a); finalmente, nos crinóides as vesículas proacrosómicas aparecem primeiramente nos espermatócitos primários (Bickell *et al.*, 1980), ou então nos espermatídeos (Afzelius, 1977). Independentemente de se saber quando é que se inicia, o desenvolvimento das vesículas proacrosómicas está sempre associado à presença de um dictiosoma bem desenvolvido. Yamashita e Iwata (1983) sugeriram que a presença de vesículas proacrosómicas em estadios mais precoces do que os espermatídeos, poderia expressar a incapacidade do tecido germinal em sintetizar os materiais necessários à produção rápida do acrosoma num período específico mais tardio da espermatogénese, tal como a espermiogénese. Sugeriu também, e alternativamente, que esse facto poderia expressar uma duração mais curta do estadio da espermiogénese, no qual existiria, consequentemente, uma incapacidade em produzir quantidades suficientes de material acrosómico. De acordo com estas sugestões, está o facto

de que no ouriço do mar a formação das vesículas proacrosómicas se inicia durante a espermiogénese (Sousa e Azevedo, 1988a, 1990b) e que o período de maturação é, nesta classe, prolongado. Contudo, o oposto ocorre na estrela do mar, em que se verifica, correspondentemente, um início precoce da formação das vesículas acrosómicas e um período curto de maturação dos gâmetas (Sousa e Azevedo, 1988c, 1990b).

B. DIFERENCIAÇÃO DA VESÍCULA ACROSÓMICA

A diferenciação antero-posterior da vesícula acrosómica completa-se nos espermatídeos jovens, imediatamente após a fusão entre si das vesículas proacrosómicas, para originar uma única vesícula acrosómica por espermatídeo. Durante a espermiogénese, a futura metade interna da membrana da vesícula acrosómica adquire características mais osmiofílicas, devido à sua interacção com o conteúdo da vesícula, bem como com os materiais periacrosómicos que entretanto surgem a rodear aquela. Esta metade mais densa da membrana da vesícula acrosómica irá ficar voltada para o núcleo no espermatozóide maduro, sendo sua característica a de permanecer resistente ao processo de fusão que ocorre durante a exocitose da vesícula no momento da fecundação. Para além disso, a membrana interna da vesícula acrosómica é aquela porção da membrana do espermatozóide que interagirá em primeiro lugar com o oolema. Este fenómeno deve-se ao facto desta porção da membrana da vesícula acrosómica sofrer uma evaginação devido à formação de uma fina expansão citoplasmática, preenchida de microfilamentos que, no seu conjunto, se denomina de perfuratório. A

metade externa da membrana da vesícula acrosómica, pelo contrário, mantém-se em contacto com a membrana citoplasmática e possui a capacidade de se fundir, quer com a membrana citoplasmática durante a fase de exocitose na reacção acrosómica, quer com as vesículas proacrosómicas de menor volume durante o processo de maturação (Sousa e Azevedo, 1985, 1988a, 1988c, 1990b).

Esta diferenciação da membrana da vesícula acrosómica deverá depender primariamente da sua composição molecular. Assim, moléculas diferentes nela contidas, poderão permanecer diferencialmente segregadas de acordo com as suas afinidades e/ou devido à sua interacção com outras moléculas, quer contidas na vesícula acrosómica, quer nos materiais periacrosómicos (Sousa e Azevedo, 1986, 1988a, 1988b, 1988c, 1988d, 1989a, 1989b, 1990b, 1991b; Yanagimachi, 1986; Yamagata, 1988; Garbers, 1989a Longo *et al.*, 1989).

Naqueles casos em que as vesículas proacrosómicas são formadas precocemente, duas situações se colocam: se a sua membrana também se diferencia precocemente, então as células necessitarão de algum factor que iniba a fusão entre elas até à fase de espermatídeo; nos casos em que a diferenciação não é precoce, mas apenas ocorre normalmente durante a espermiogénese, algum factor deverá então interactuar nesta fase com a vesícula acrosómica para induzir a sua diferenciação (Sousa e Azevedo, 1990b).

C. MIGRAÇÃO DA VESÍCULA ACROSÓMICA

Nos equinodermes, o transporte da vesícula acrosómica do pólo caudal para o pólo apical da célula é feito por variados meios: (1) a

vesícula acrosómica pode migrar pelo citoplasma aparentemente sem interagir com outros organelos, como ocorre em asteróides, ou interagindo com microtúbulos, como em holoturóides (Dan e Sirakami, 1971; Atwood, 1974; Pladellorens e Subirana, 1975; Tilney, 1976a; Sousa e Azevedo, 1988c); (2) durante a migração a futura metade interna da membrana da vesícula acrosómica pode ligar-se ao invólucro nuclear, situação que se verifica em equinóides, ofiuróides e crinóides (Bickell *et al.*, 1980; Yamashita, 1983; Yamashita e Iwata, 1983; Sousa e Azevedo, 1988a). Nestes casos, a deslocação da vesícula acrosómica pode ser explicada pela rotação do complexo núcleo--vesícula relativamente ao citoplasma. Este modelo, porém, não é adequado para o ouriço do mar, porque, neste caso, a região posterior do núcleo e da célula já se encontram determinados pela ligação dos dois centríolos ao núcleo e ao axonema exteriorizado (Sousa e Azevedo, 1988a). Para este caso, uma escalonada ruptura e reunião dos hipotéticos locais de ligação entre o invólucro nuclear e a vesícula acrosómica são pois necessários para que esta atinja a sua posição final (Sousa e Azevedo, 1988a); (3) a vesícula acrosómica pode também ligar-se à membrana citoplasmática pela sua futura porção anterior, tal como sucede em asteróides (Yamagata, 1988).

Independentemente do mecanismo de transporte, deverão com certeza existir moléculas com informação posicional nas membranas envolvidas para que, pela sua interligação, quer directa, quer por intermédio de outras moléculas exógenas, citoplasmáticas, a vesícula acrosómica se consiga deslocar do pólo posterior para o pólo anterior do futuro espermatozóide (Sousa e Azevedo, 1990b).

D. COMPOSIÇÃO DA VESÍCULA ACROSÓMICA

Até hoje, apenas o conteúdo da vesícula acrosómica conseguiu ser estudado, enquanto que a membrana deste organelo permanece por isolar e caracterizar. Nos equinóides a vesícula acrosómica contém vários produtos, nomeadamente: (1) enzimas hidrolíticas, que incluem as enzimas fosfatase ácida, fosfatase alcalina, fosfolipase, várias proteases, arilsulfatase, catalase e peroxidase (Vasseur, 1951; Brookbank, 1958; Isaka *et al.*, 1966; Anderson, 1968b; Conway e Metz, 1976; Hoshi *et al.*, 1979; Levine e Walsh, 1979, 1980; Green e Summers, 1980, 1982; Hoshi e Moriya, 1980; Yamada e Aketa, 1981; Boldt *et al.*, 1984; Schuel, 1984; Hoshi, 1985; Baccetti *et al.*, 1989; Matsumura e Aketa, 1989; Yasuhara *et al.*, 1991). As hidrolases acrosómicas parecem estar envolvidas, quer na exocitose da vesícula acrosómica, quer na penetração dos revestimentos externos do ovócito pelo perfuratório do espermatozóide. Os sistemas peroxidase/catalase exercem outras funções. Assim, a peroxidase utiliza o peróxido de hidrogénio que o ovo liberta após ser fecundado para criar produtos citotóxicos lesivos para o próprio espermatozóide (fenómeno de autodestruição), enquanto que a catalase destroi esse mesmo peróxido de hidrogénio numa tentativa de proteger o espermatozóide contra esse produto espermicida; (2) mucopolissacáridos ácidos (Summers e Hylander, 1974); e (3) a proteína bindina, que é responsável pela adesão do espermatozóide ao ovócito (Vacquier e Moy, 1977; Moy e Vacquier, 1979).

Nos asteróides, a vesícula acrosómica encontra-se compartimentada morfologica e funcionalmente, contendo a enzima Ca^{2+}-Mg^{2+}-ATPase,

cálcio e mucopolissacáridos ácidos no seu componente periférico denso e as enzimas fosfatase ácida e fosfatase alcalina no componente central bem como na região apical periférica, exactamente onde a membrana externa da vesícula acrosómica contacta com o plasmalema (Mabuchi e Mabuchi, 1973; Sousa e Azevedo, 1985, 1986, 1988d, 1989a; Sousa *et al.*, 1988, 1991b). Durante a fecundação, a vesícula acrosómica sofre exocitose e o seu conteúdo é então extravasado sobre os revestimentos externos do ovócito. Em termos morfológicos, o componente apical periférico, com as suas enzimas hidrolíticas, parece estar envolvido no processo de exocitose da vesícula acrosómica, enquanto que o componente central reveste a periferia do perfuratório para ajudar este a penetrar, por meio de lise enzimática, os revestimentos externos do ovócito. Por outro lado, o componente periférico denso da vesícula acrosómica permanece na periferia dos revestimentos externos do ovócito, assegurando a adesão do espermatozóide aos revestimentos externos do ovócito (Sousa e Azevedo, 1985, 1986, 1988d; Sousa *et al.*, 1991b). Nos asteróides também foi isolada da vesícula acrosómica, um sistema proteolítico com actividade tipo tripsina que parece ajudar o espermatozóide a penetrar os revestimentos externos do ovócito (Sousa *et al.*, 1991a). Contudo, a sua localização no interior da vesícula acrosómica ainda não foi determinada.

Nos ofiuróides a vesícula acrosómica contém polissacáridos ácidos (Hylander e Summers, 1975).

E. FORMAÇÃO E COMPOSIÇÃO DO MATERIAL PERIACROSÓMICO

Após a vesícula acrosómica atingir a sua localização definitiva, o núcleo invagina-se em torno da circunferência basolateral da vesícula acrosómica para formar a fossa acrosómica, que então se preenche de um material granulo-fibrilar, o material periacrosómico. Este material periacrosómico por vezes diferencia-se num material mais denso que se localiza nos ápices da fossa acrosómica e num material menos denso que se localiza em torno da face lateral da vesícula acrosómica.

Exceptuando os holoturóides, todos os outros equinodermes desenvolvem uma outra invaginação nuclear, de menores dimensões, sob a base da vesícula acrosómica, a fossa subacrosómica. Esta fossa contém um agregado de microfibrilas denominado actómero (Tilney, 1978).

Supõe-se que o núcleo se invagina em torno da vesícula acrosómica devido à interacção entre as duas membranas, a da vesícula acrosómica e a do invólucro nuclear e as moléculas do material periacrosómico e subacrosómico.

O material periacrosómico lateral foi estudado nos holoturóides e nos asteróides, tendo-se verificado que contém actina G associada com espectrina, fascina, profilina e precursores membranares (Tilney, 1976b; Maekawa e Sakai, 1982a, 1982b; Maekawa *et al.*, 1982). Durante a fecundação, a interacção da membrana do espermatozóide com os revestimentos externos do ovócito desencadeia a exocitose da vesícula acrosómica e, de seguida, a formação do perfuratório. O actómero, que na realidade é um centro polimerizador de microfilamentos, inicia a polimerização da actina G em actina F (Tilney, 1978). Enquanto que a espectrina e a profilina regulam a taxa de polimerização dos

microfilamentos, a fascina causa a agregação destes num estreito feixe (Tilney, 1976b; Tilney *et al.*, 1983). À medida que aumenta de comprimento, o feixe de actina F liga-se à membrana interna da vesícula acrosómica e, evaginando-a, forma um fino prolongamento celular (d = ± 0.1 µm) preenchido pelo feixe de microfilamentos, o perfuratório. Esta estrutura rígida penetra então os revestimentos externos do ovócito até contactar e depois se fundir com o oolema (Tilney *et al.*, 1973). Ao conjunto destas duas reacções, a exocitose da vesícula acrosómica e a formação do perfuratório, chama-se reacção acrosómica (Dan e Hagiwara, 1967).

Nos equinóides, a membrana que reveste o perfuratório deriva da simples evaginação da porção basal da membrana da vesícula acrosómica, já que o seu perfuratório apresenta um reduzido comprimento. Porém, nos não equinóides, nem a evaginação da membrana basal da vesícula acrosómica, nem o repuxamento para diante da membrana citoplasmática conseguem fornecer a quantidade necessária de membrana adicional requerida pela formação de um longo perfuratório, o qual pode atingir, nos asteróides, comprimentos de cerca de 20 µm (Sousa e Azevedo, 1985, 1986, 1988d; Sousa *et al.*, 1988) e, nos holoturóides, até 60 µm (Tilney e Inoué, 1982). Nestes casos, torna-se evidente que terá de existir uma fonte de precursores membranares, os quais se pensa existir no espaço periacrosómico lateral. São várias as evidências que suportam este ponto de vista: (1) nos ofiuróides e nos holoturóides verifica-se o aparecimento súbito de vesículas dentro do espaço periacrosómico durante a reacção acrosómica, as quais de seguida se fundem com a membrana que reveste o perfuratório, mais precisamente na sua base (Hylander e Summers, 1975; Tilney e Inoué,

1982); (2) existem lípidos no material periacrosómico lateral dos asteróides (Sardet e Tilney, 1977); (3) durante o processo de maturação do acrosoma nos asteróides, o material periacrosómico lateral dos asteróides incorpora cisternas de retículo endoplásmico liso (Sousa e Azevedo, 1988c).

Com a reacção acrosómica, o material periacrosómico lateral, contendo actina G, desvanece, uma vez que é integralmente consumido na produção do perfuratório. Pelo contrário, o material periacrosómico apical deverá conter uma outra composição pois que, durante a reacção acrosómica, esta estrutura se densifica e se mantém no ápice do espermatozóide. A permanência daquele material no ápice do espermatozóide é interpretada como conferindo resistência à célula nas suas intensas interacções com o outro gâmeta (Sousa e Azevedo, 1985).

F. ORIGEM DO MATERIAL PERIACROSÓMICO

No que diz respeito à origem do material periacrosómico, Tilney (1976a) sugeriu que as moléculas deste, à medida que eram sintetizadas, se associariam e ligariam a regiões especializadas da membrana da vesícula acrosómica e do invólucro nuclear. De facto, nos equinodermes não asteróides, o material periacrosómico parece acumular-se entre duas membranas, nomeadamente, entre a membrana interna da vesícula acrosómica e o invólucro nuclear (Atwood, 1974; Tilney, 1976a; Afzelius, 1977; Yamashita, 1983; Buckland-Nicks *et al.*, 1984; Sousa e Azevedo, 1988a). Contudo, no caso dos asteróides, o material periacrosómico parece acumular-se entre a membrana interna da vesícula acrosómica e cisternas do retículo

endoplasmático liso (Dan e Sirakami, 1971; Sousa e Azevedo, 1988c).

Alternativamente, o material periacrosómico poderia ser sintetizado pelo invólucro nuclear ou pelo retículo endoplásmico directamente envolvidos na ligação com a vesícula acrosómica. Porém, a presença de ribosomas nestas membranas apenas foi documentada numa espécie asteróide (Yamagata, 1988) e os estudos autoradiográficos, por outro lado, não demonstraram síntese proteica significativa na região acrosómica dos espermatídeos dos equinóides (Nicotra *et al.*, 1984).

Mais recentemente, surgiu um novo modelo para a origem do material periacrosómico (Sousa e Azevedo, 1988a, 1988c, 1989b, 1990b). Nesses trabalhos, os autores descreveram a presença de corpos densos citoplasmáticos que, sintetizados a partir da fase de espermatogónia, persistiam e aumentavam de número durante a espermatogénese, quer do ouriço do mar, quer da estrela do mar. Estes corpos densos interagem intimamente com a vesícula acrosómica durante a espermiogénese, parecendo que essas estruturas estão envolvidas na formação do material periacrosómico. Por outro lado, estes corpos densos citoplasmáticos interagem igualmente com outras estruturas citoplasmáticas, nomeadamente os microtúbulos dos centríolos e do axonema, o material pericentriolar, o material que liga o centríolo proximal ao invólucro nuclear e o material do anel contráctil que se forma durante a citocinese. Ou seja, os corpos densos citoplasmáticos parecem interagir com as várias estruturas citoplasmáticas que contêm elementos do citosqueleto. Esta relação morfológica foi ainda reforçada usando métodos citoquímicos que demonstraram que, quer esses organelos, quer os corpos densos citoplasmáticos, marcam

selectivamente pela prata (Sousa e Azevedo, 1988a, 1988c, 1989b, 1990b).

Os corpos densos citoplasmáticos haviam já sido descritos nas espermatogónias dos equinóides e dos ofiuróides, nos espermatócitos primários dos holoturóides e nos espermatídeos dos asteróides, mas a sua evolução ao longo da espermatogénese não fora investigada, sendo a sua descrição meramente passageira (Longo e Anderson, 1969; Dan e Sirakami, 1971; Atwood, 1974; Houk e Hinegardner, 1981; Yamashita, 1983; Yamashita e Iwata, 1983).

A coloração pela prata que Sousa e Azevedo (1988a, 1988b, 1988c, 1989b, 1990b) empregaram, é exactamente a utilizada para marcar o componente fibrilar e os organizadores nucleolares, técnica originalmente desenvolvida por Goodpasture e Bloom (1975) e Hernandez-Verdun *et al.*, (1982). Esta técnica marca especificamente uma proteína acídica e altamente fosforilada, a proteína C23 (ou nucleolina). A nucleolina liga-se à histona H1 e induz a descondensação da cromatina nucleolar nos centros fibrilares do nucléolo, permitindo a transcrição dos genes ribosomais (Erard *et al.*, 1988). Usando esta mesma técnica, tivemos então a oportunidade casual de verificar que a coloração pela prata, para além de marcar o nucléolo, também marcava determinadas regiões da periferia da heterocromatina, tendo sugerido que nesses locais também existiria cromatina descondensada e, portanto, a possibilidade de transcrição (Sousa e Azevedo, 1988a, 1989b, 1990b). Corroborando esta hipótese, verificámos igualmente que, à medida que a cromatina se condensava durante a espermiogénese, os depósitos de prata ficavam retidos nas regiões nucleares por condensar, desaparecendo, por fim,

quando o núcleo se condensava totalmente, exceptuando-se as regiões dos vacúolos nucleares que são regiões do núcleo maduro, nas quais persistem porções descondensadas de cromatina (Sousa e Azevedo, 1988a, 1989b, 1990b).

Voltando aos corpos densos citoplasmáticos, a argirofilia comum entre estes e o nucléolo não implica uma identidade molecular mas apenas uma similaridade reactiva e só estudos futuros nos permitirão saber qual, ou quais as moléculas responsáveis pela argirofilia citoplasmática. Os estudos por nós efectuados no ouriço do mar e na estrela do mar, sugerem que a argirofilia nos diferentes organelos citoplasmáticos se deve ao mesmo conjunto de proteínas argirofílicas estruturais. Assim, os corpos densos citoplasmáticos funcionariam como depósitos primordiais destes materiais argirofílicos necessários à formação de vários outros organelos argirofílicos. Alternativamente, os corpos densos poderiam representar um material tipo "nuage"[1], contendo as ribonucleoproteínas necessárias para formar os componentes dessas estruturas citoplasmáticas (Sousa e Azevedo, 1990b).

2. O OVÓCITO

O ovócito maduro de *M. glacialis* (Fig. 5) é redondo e tem *in vivo*, um diâmetro de cerca de 170 µm. Está rodeado por uma matriz extracelular que, *in vivo*, se apresenta com uma espessura de cerca de

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[1]Para uma definição morfo-funcional do termo "nuages" ver:

Azevedo, C. (1984). Development and ultrastructural autoradiographic studies of nucleolus-like bodies (nuages) in oocytes of a viviparous Teleost (*Xiphophorus helleri*). *Cell Tissue Res.*, 238: 121-128.

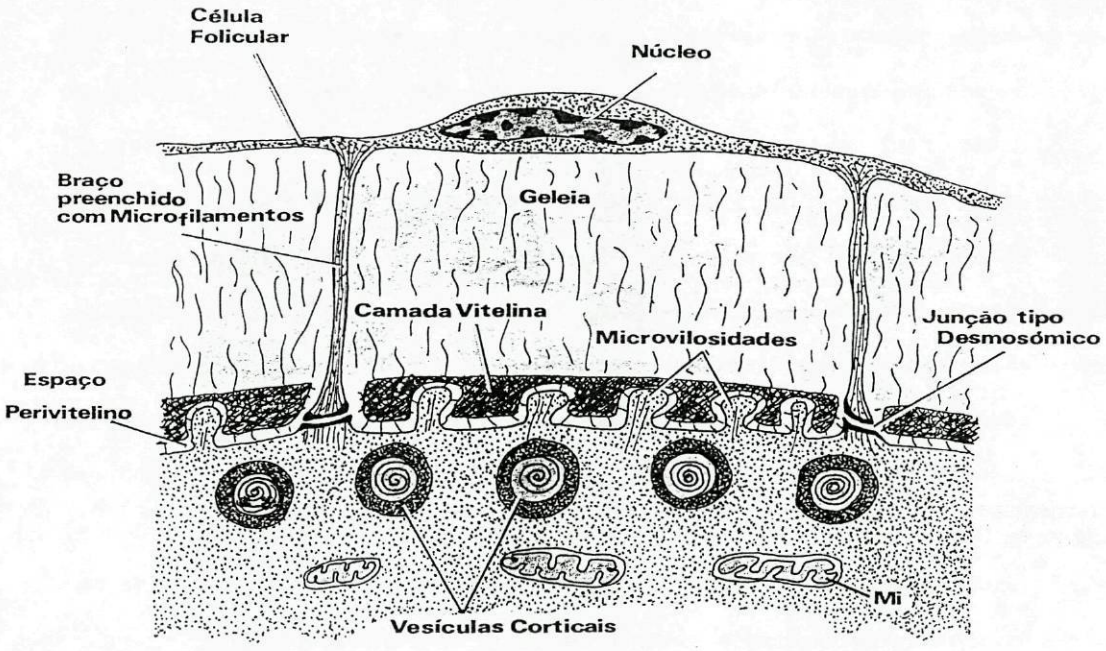


Fig. 5. Esquema da ultrastrutura do córtex e da matriz extracelular do ovócito da estrela do mar *Marthasterias glacialis*.

15-20 µm. Esta matriz extracelular encontra-se diferenciada em dois componentes principais: (1) a camada vitelina, com cerca de 0,3 µm de espessura, constitui a parte mais interna, é composta por um material fibrilar denso que se dispõe em torno das pequenas microvilosidades do ovócito, incluindo o seu ápice, e apresenta-se ligada ao oolema por finas expansões fibrilares. Esta estrutura é o equivalente à zona pelúcida dos mamíferos e de outros animais; e, (2) a geleia, que constitui o restante da matriz extracelular do ovócito e corresponde ao material extracelular contido no "cumulus oophorus" dos mamíferos. A geleia é formada por um fino material fibrilar laxo cuja ultrastrutura é de difícil preservação. A região cortical do citoplasma do ovócito possui vários organelos, nomeadamente as vesículas corticais (d = ± 1 µm), as vesículas vitelinas, retículo endoplasmático, vacúolos, vesículas em forma de bastonete com conteúdo fibrilar, mitocôndrias, ribosomas, glicogénio e microfilamentos (Holland, 1980; Longo *et al*, 1982; Luttmer e Longo, 1985; Sousa e Azevedo, 1987, 1988e; Sousa *et al.*, 1991b).

3. MATURAÇÃO, OVULAÇÃO E EJACULAÇÃO

No ovário, o ovócito da estrela do mar encontra-se parado em G2 da profase I e o seu metabolismo está dormente devido ao *p*H ligeiramente ácido do seu citoplasma (*p*H 6,8). Na altura da fecundação, o nervo radial liberta um neuro-péptido de acção hormonal, a substância estimuladora da gónada, que actua sobre as células foliculares que rodeiam o ovócito. As células foliculares libertam então uma outra hormona, a 1-metiladenina (1-MetAd). Esta substância

liga-se a receptores do oolema, desencadeando a maturação meiótica do ovócito e a retracção das células foliculares. Para além desta acção, a 1-MetAd difunde pelo ovário e, actuando sobre o sistema nervoso da parede gonádica, induz a ovulação por contracção desta mesma parede. Como consequência deste facto, os ovócitos são fecundados ainda durante a fase meiótica de profase I. Por seu lado, no testículo da estrela do mar as células de Sertoli também libertam a hormona 1-MetAd, desencadeando, por mecanismos semelhantes sobre a parede gonádica, a ejaculação. Ao contrário da estrela do mar, os ovócitos do ouriço do mar atingem a plena maturação ainda no interior do ovário, aí permanecendo em estado maduro durante alguns meses até sofrerem ovulação. É, pois, no lúmen do ovário, que estes ovócitos terminam as duas divisões meióticas, pelo que são fecundados num estadio mais avançado, ou seja, com o pronúcleo feminino já formado (Kanatani, 1969; Cloud e Schuetz, 1973; Ikegami, 1976; Kubota *et al.*, 1977; Meijer e Guerrier, 1984; Trimmer e Vacquier, 1986; Sardet e Chang, 1987; Pondaven *et al.*, 1989; Guerrier *et al.*, 1990; Peaucellier *et al.*, 1990; Smiley, 1990; Witchel e Steinhardt, 1990).

Como veremos, estas diferenças entre a estrela do mar e o ouriço do mar vão ditar uma sequência de acontecimentos diferentes durante a fecundação.

4. A FERTILIZAÇÃO

4.1. TRANSFORMAÇÕES DO ESPERMATOZÓIDE AO CONTACTAR COM A ÁGUA DO MAR

Os espermatozóides, após serem formados, ficam armazenados nos testículos, por vezes durante meses e o seu estado inactivo é mantido

por um *p*H interno (pH_i) de cerca de 7,2. É devido a uma elevada pressão parcial de CO_2 no sémen que o pH_i nos espermatozóides é mantido dentro deste nível. Sabe-se que um pH_i baixo inibe a motilidade flagelar e a respiração mitocondrial. Quando os espermatozóides são libertados na água do mar ocorre uma activação da bomba Na^+/H^+ existente na membrana do flagelo, pelo que se observa uma entrada de Na^+ e uma saída de H^+. A perda de H^+ eleva o pH_i dos espermatozóides para 7,6, de que resulta a activação da motilidade flagelar. Este permutador iónico da membrana do flagelo é insensível ao amilorido, mas as permutas são dependentes do potencial de membrana, a qual é modelada pelos iões Mg^{2+} e Zn^{2+}. A correlação entre o pH_i e a motilidade resulta da sensibilidade da dineína (ATPase), contida nos braços dos microtúbulos b do axonema, ao pH_i. A dineína hidroliza o ATP, fornecendo a energia necessária ao deslizamento microtubular, originando a motilidade do flagelo. A actividade desta enzima fica inibida se o pH_i for menor que 7,3, sendo, pelo contrário, estimulada se o pH_i ascender acima do limite dos 7,4. Correlacionada com a actividade da dineína está a respiração mitocondrial. A um pH_i de 7,2 ou inferior, a respiração encontra-se inibida devido aos elevados teores em ATP (que não é gasto no axonema) e à baixa concentração em ADP (que não é produzido por não haver hidrólise do ATP a nível do axonema). Quando a dineína é activada, os níveis de ATP diminuem e a concentração de ADP eleva-se, pelo que a respiração fica estimulada. O ATP é produzido na mitocôndria, sobretudo pela oxidação de ácidos gordos membranares. O transporte do ATP para o axonema faz-se por intermédio da fosforilcreatina e envolve a enzima creatina cinase. A motilidade depende não só da permuta

Na^+/H^+, mas também da actividade de uma bomba Na^+-K^+-ATPase contida na membrana do flagelo. Esta ATPase ajuda a manter o pH_i elevado, necessário à função da dineína, porque elimina do interior da célula o H^+ produzido durante a hidrólise do ATP no axonema (Ohtake, 1976; Shapiro e Eddy, 1980; Schatten, 1982; Christen *et al.*, 1983a; Johnson *et al.*, 1983; Trimmer e Vacquier, 1986; Vacquier, 1986; Mohri *et al.*, 1990).

4.2. A INTERACÇÃO DO ESPERMATOZÓIDE COM OS REVESTIMENTOS EXTERNOS DO OVÓCITO

A. A REACÇÃO ACROSÓMICA

Após a ovulação os ovócitos ficam livres na água do mar. Da geleia do ovócito libertam-se, então, pequenos péptidos que funcionam, quer como agentes quimiotácticos, quer como estimuladores da respiração e da motilidade dos espermatozóides. Deste modo, os espermatozóides são orientados e acelerados em direcção aos ovócitos (Epel, 1977; Epel e Vacquier, 1978; Metz, 1978; Shapiro e Eddy, 1980; Vacquier, 1981; Schatten, 1982; Shapiro *et al.*, 1982; Suzuki *et al.*, 1982; Miller, 1985; Ward *et al.*, 1985; Trimmer e Vacquier, 1986; Vacquier, 1986).

Na estrela do mar os espermatozóides colidem contra a geleia do ovócito e aderem à sua superfície externa (Figs. 6 e 7), de modo específico, através de receptores existentes na sua membrana. A geleia do ovócito da estrela do mar foi isolada e caracterizada bioquimicamente. Nela se detectaram, basicamente, três tipos de substâncias: uma glicoproteína sulfatada de elevado peso molecular (ARIS), um grupo de saponinas esteróides sulfatadas (Co-ARIS) e vários

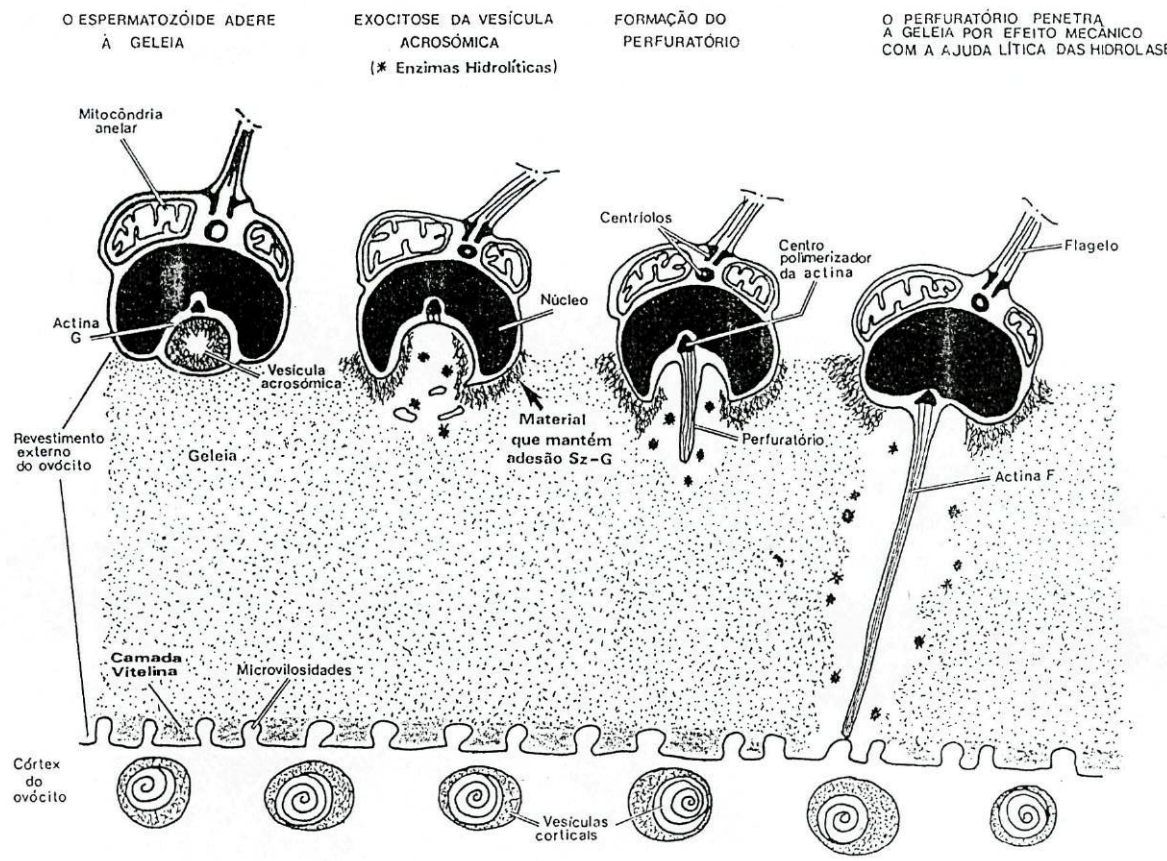


Fig. 6. Aspecto esquemático da interacção entre os dois gâmetas e da penetração do espermatozóide através da matriz extracelular do ovócito da estrela do mar *Marthasterias glacialis*.

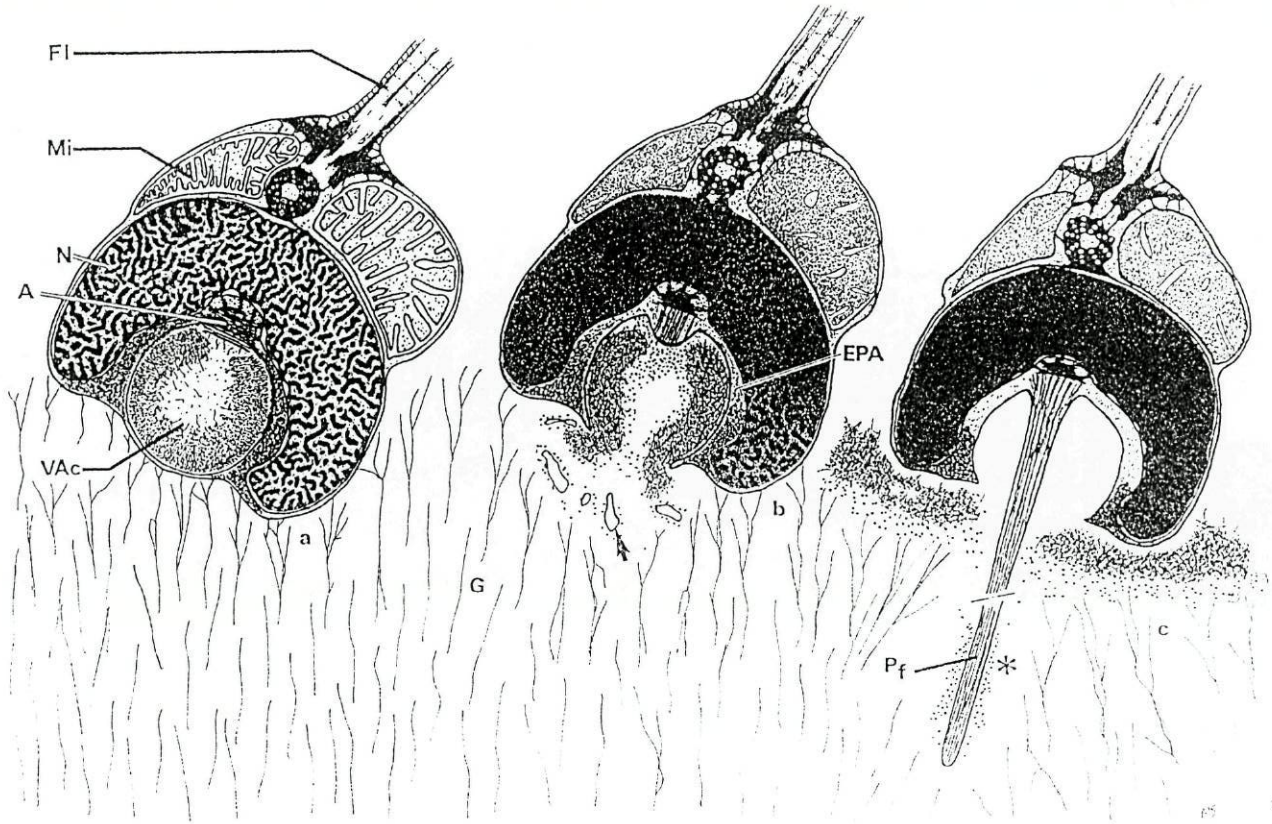


Fig. 7. Esquema da reacção acrosómica do espermatozóide (Sz) de *Marthasterias glacialis*. (a) O Sz adere à face externa da geleia (G) de que resulta (b) a fusão (seta) entre as membranas citoplasmática e acrosomal externa do Sz e a exocitose do conteúdo acrosómico, bem como a polimerização, ao nível do actómero (A), da actina G contida no espaço periacrosómico (EPA). (c) O feixe de microfilamentos evagina a membrana interna da vesícula acrosómica(VAc), formando o perfuratório (Pf). O componente denso periférico (**) da VAc adere o Sz à G, enquanto que o componente central (*), contendo hidrolases, reveste o Pf para ajudar à penetração. Note a progressiva condensação da mitocôndria (Mi) e do núcleo (N) do Sz (a-c). Fl, flagelo.

oligopéptidos (SAP). O espermatozóide possui receptores específicos na sua membrana citoplasmática para a ARIS e os SAP, mas não para os Co--ARIS, apesar destes últimos facilmente se integrarem na membrana citoplasmática do espermatozóide. A ligação da ARIS ao seu receptor membranar, tendo como adjuvante os Co-ARIS, estimula a abertura de canais para o Ca^{2+}, enquanto que a ligação dos SAP provoca a abertura de canais Na^{+}/H^{+} (Uno e Hoshi, 1978; Ikadai e Hoshi, 1981a, 1981b; Matsui *et al.*, 1986a, 1986b; Endo *et al.*, 1987; Nishiyama *et al.*, 1987a, 1987b; Hoshi *et al.*, 1990a, 1990b).

Assim, o espermatozóide, que, tal como o ovócito, é uma célula excitável, ao abrir os seus canais iónicos promove uma despolarização da sua membrana citoplasmática. A entrada do Ca^{2+} origina de imediato a exocitose da vesícula acrosómica, por fusão da sua membrana apical com a membrana citoplasmática adjacente (Summers e Hylander, 1974; Collins e Epel, 1977; Schackmann *et al.*, 1978; Tilney *et al.*, 1978; Schroeder e Christen, 1982). Supõe-se que o aumento dos teores em Ca^{2+} activa uma fosfolipase A2 membranar, provocando uma clivagem dos fosfolípidos membranares e um aumento nos teores em inositol 1,4,5--trisfosfato (IP3). Do resultado desta hidrólise resultam lisofosfoglicéridos que, em conjunto com os aumentos verificados nos teores em IP3 e Ca^{2+}, iniciam a fusão das membranas citoplasmática e acrosómica externa (Conway e Metz, 1976; Domino e Garbers, 1988, 1989; Domino *et al.*, 1989).

Deste modo, o conteúdo da vesícula acrosómica é derramado sobre a geleia do ovócito. O componente denso periférico da vesícula acrosómica da estrela do mar mantém a adesividade do espermatozóide à geleia, enquanto que o componente central, contendo essencialmente

enzimas líticas, vai ajudar o espermatozóide a penetrar os revestimentos externos do ovócito (Sousa e Azevedo, 1985, 1986, 1988d; Sousa *et al.*, 1988, 1991a).

Por outro lado, a alcalinização do meio interno do espermatozóide provocado pela saída do H^+, desencadeia a formação de um feixe de microfilamentos a partir do actómero e da actina G (Figs. 6 e 7) (Tilney, 1976a, 1976b, 1976c, 1978; Tilney *et al.*, 1973, 1978; Tilney e Kallenbach, 1979; Maekawa e Sakai, 1982a, 1982b; Maekawa *et al.*, 1982; Schroeder e Christen, 1982; Tilney e Inoué, 1982; Tilney *et al.*, 1983). Ao aumentar de extensão, o feixe de microfilamentos liga-se à membrana interna da vesícula acrosómica, empurra-a para diante e evagina-a, formando, deste modo, uma fina (± 0.1 µm) expansão celular denominada de perfuratório (Dan e Hagiwara, 1967; Sousa e Azevedo, 1985). O perfuratório penetra, então, rapidamente, os revestimentos externos do ovócito, quer por acção mecânica, quer com a ajuda das enzimas hidrolíticas acrosómicas que o revestem externamente (Figs. 6 e 7) (Sousa e Azevedo, 1985, 1986, 1988d; Sousa *et al.*, 1988, 1991a). Portanto, na estrela do mar, o espermatozóide reage mantendo-se na periferia da geleia, emitindo um longo prolongamento celular que perfura os revestimentos externos do ovócito até estabelecer contacto com o oolema (Fig. 8). Uma outra consequência da formação do perfuratório reside no facto de que a membrana que reveste a extremidade deste, originária da membrana interna da vesícula acrosómica, se destina a fundir-se com o oolema (Epel, 1977; Epel e Vacquier, 1978; Shapiro e Eddy, 1980; Vacquier, 1981; Shapiro *et al.*, 1982; Schuel, 1984; Trimmer e Vacquier, 1986).

No ouriço do mar, a reacção acrosómica apresenta uma outra sequência de acontecimentos. Quando o ovócito é lançado na água do mar a sua geleia expande-se. O espermatozóide, por seu lado, ao contactar com a geleia do ovócito não adere a ela, penetrando antes através dos seus interstícios, sofrendo a reacção acrosómica junto à camada vitelina (Afzelius e Murray, 1957; Bernstein, 1962; Dan *et al.*, 1964; Franklin, 1965; Tegner e Epel, 1973; Summers e Hylander, 1974; Summers *et al.*, 1975; Aketa e Ohta, 1977; Metz, 1978; Shapiro e Eddy, 1980; Usui *et al.*, 1980; Gache *et al.*, 1983). Desconhecem-se pormenores deste facto, mas trata-se de um processo aliás em tudo semelhante à fecundação nos mamíferos. Nestes, o espermatozóide penetra o "cumulus oophorus" sem reagir e, só após atingir a periferia da zona pelúcida, é que sofre a reacção acrosómica (Miller e Ax, 1990).

Deste modo, tudo indica que o espermatozóide do ouriço do mar também sofre a sua reacção acrosómica através da indução por macromoléculas existentes na geleia do ovócito. A geleia do ovócito do ouriço do mar também contém essencialmente três tipos diferentes de moléculas na sua composição: um **polímero de fucose sulfatada** (indutor da reacção acrosómica), uma **glicosialoproteína** (de função desconhecida), e vários **oligopéptidos** (de acção quimiotáctica para os espermatozóides, servindo também de cofactores para a reacção acrosómica, sendo igualmente capazes de activar a respiração e a motilidade flagelar) (Epel, 1977; Epel e Vacquier, 1978; Metz, 1978; SeGall e Lennarz, 1979; Garbers *et al.*, 1980, 1983; Shapiro e Eddy, 1980; Vacquier, 1981; Monroy e Rosati, 1982, 1983; Trimmer e Vacquier, 1986; Vacquier, 1986; Yamaguchi *et al.*, 1988, 1989; Mikami-Takei *et*

al., 1991).

O receptor da membrana citoplasmática do espermatozóide para o polímero de fucose sulfato da geleia do ovócito já foi isolado, tratando-se de uma glicoproteína com 210 kDa. A ligação destas duas moléculas desencadeia, tal como ocorre na estrela do mar, uma entrada de Ca^{2+} e Na^{+} e uma saída de K^{+} e H^{+}, sucedendo-se a despolarização da membrana citoplasmática, a exocitose da vesícula acrosómica e a formação de um pequeno perfuratório (Rossignol *et al.*, 1984; Christen *et al.*, 1983b; Trimmer e Vacquier, 1986; Trimmer *et al.*, 1986; Vacquier, 1986).

O aumento brusco dos níveis intracelulares de Ca^{2+} (Ca_i) causa outras alterações no espermatozóide. Este ião estimula também a adenilato ciclase existente na membrana citoplasmática da cabeça do espermatozóide, resultando um aumento da concentração intracelular em cAMP. O cAMP, por sua vez, estimula uma proteína cinase que vai fosforilar a histona H1 específica do espermatozóide (Sp-H1) (Garbers *et al.*, 1980, 1983; Kopf e Garbers, 1980; Porter e Vacquier, 1986; Trimmer e Vacquier, 1986; Vacquier, 1986).

A entrada de Ca^{2+} para o interior do espermatozóide origina também a acumulação deste ião no interior da mitocôndria, por activação de transportadores na sua membrana. O aumento da concentração deste ião na matriz mitocondrial desencadeia a activação de outros canais iónicos que, ao fazerem baixar o pH_i mitocondrial, estimulam a respiração mitocondrial e, portanto, a motilidade flagelar (Schackmann *et al.*, 1978; Lee *et al.*, 1983; Trimmer e Vacquier, 1986).

Por outro lado, os pequenos oligopéptidos da geleia do ovócito ligam-se a receptores existentes na membrana flagelar do

espermatozóide. Esta ligação provoca uma activação dos canais para o K^+, com saída deste ião. Esta alteração leva de imediato à activação da bomba Na^+/H^+, com saída de H^+ e entrada de Na^+. Em consequência, a membrana do flagelo despolariza e o pH_i eleva-se, activando, quer a respiração mitocondrial, quer a motilidade flagelar. Simultaneamente, verifica-se um aumento do Ca_i e dos teores em cAMP e cGMP por fosforilação da guanilato ciclase membranar que activa, em consequência, a adenilato ciclase. Deste modo, ficam activados, quer a respiração, quer a motilidade do espermatozóide, factores imprescindíveis ao avanço deste através dos interstícios da geleia (Kopf *et al.*, 1979; Hansbrough e Garbers, 1981; Suzuki *et al.*, 1981; Dangott e Garbers, 1984; Ward *et al.*, 1985; Lee e Garbers, 1986; Schackmann e Chock, 1986; Trimmer e Vacquier, 1986; Bentley *et al.*, 1987; Garbers, 1988, 1989a, 1989b; Suzuki, 1990).

A reacção acrosómica do ouriço do mar também exibe particularidades distintas em relação à da estrela do mar (Epel, 1977; Epel e Vacquier, 1978; Shapiro e Eddy, 1980). Em primeiro lugar, a exocitose da vesícula acrosómica provoca a exteriorização do seu conteúdo sobre a camada vitelina. O conteúdo da vesícula acrosómica é sobretudo constituído pela proteína bindina (30,5 kDa). A bindina possui características de lectina e promove a ligação específica do espermatozóide a um proteoglicano da camada vitelina, o receptor do espermatozóide (Vacquier e Moy, 1977; Moy e Vacquier, 1979; Vacquier, 1980; Glabe *et al.*, 1982; Glabe, 1985a, 1985b; Trimmer e Vacquier, 1986; Vacquier, 1986; DeAngelis e Glabe, 1990). Curiosamente, a bindina liga-se aos glicosaminoglicanos, enquanto que é o eixo proteico do proteoglicano quem confere especificidade à ligação

(Vacquier, 1980, 1981; Rossignol e Lennarz, 1983; Rossignol *et al.*, 1984; Trimmer e Vacquier, 1986). Em segundo lugar, como o espermatozóide reage sobre a camada vitelina, a pouca distância do oolema, verifica-se que o seu perfuratório possui um reduzido comprimento, não necessitando para se formar, da adição de percursores membranares à membrana que o reveste. Para tal, basta-lhe, simplesmente, evaginar a membrana interna da vesícula acrosómica. Após esta ligação, o perfuratório, com a ajuda dos batimentos flagelares e das enzimas hidrolíticas acrosomais, penetra a camada vitelina até encontrar o oolema (Afzelius e Murray, 1957; Bernstein, 1962; Dan *et al.*, 1964; Franklin, 1965; Tegner e Epel, 1973; Summers e Hylander, 1974; Summers *et al.*, 1975).

B. A FUSÃO ENTRE AS MEMBRANAS DOS DOIS GÂMETAS

Quando as membranas dos dois gâmetas interagem, é então a vez do ovócito reagir (Fig. 8). A primeira reacção do ovócito consiste na despolarização da sua membrana (potencial de activação ou de fertilização), fenómeno que se traduz num bloqueio rápido mas transitório a novas e potenciais fusões do oolema com outros espermatozóides. É o chamado bloqueio rápido à polispermia (Steinhardt *et al.*, 1972; Epel e Carrol, 1975; Jaffe, 1976; Epel, 1977; Epel e Vacquier, 1978; Shapiro e Eddy, 1980; Dale e DeSantis, 1981; Dale *et al.*, 1981; Schuel, 1984; Trimmer e Vacquier, 1986; Obata e Kuroda, 1987).

Actualmente ainda se desconhece qual o mecanismo desta activação do

ovócito do ouriço do mar. Para alguns investigadores, a fusão entre as membranas do espermatozóide e do ovócito é que desencadeia o potencial de activação. Para outros investigadores, o ovócito é activado antes mesmo de ocorrer a fusão entre as membranas dos dois gâmetas (Longo, 1988, 1991; Longo *et al.*, 1986, 1990; Epel, 1990). Neste último caso, a activação do ovócito dependeria de um factor oriundo do próprio espermatozóide (Conway e Metz, 1976; Iwao e Jaffe, 1989; Dale e DeFelice, 1990; Kane, 1990; Whitaker e Crossley, 1990). Este factor poderia ser a própria bindina, para a qual muito recentemente se descobriram propriedades fusogénicas (Glabe *et al.*, 1982; Glabe, 1985a, 1985b; Monroy, 1985), ou a introdução/activação de canais para o cálcio no oolema (Shapiro e Eddy, 1980; Schatten, 1982; Epel, 1990).

4.3. A REACÇÃO DO OVO À FUSÃO COM O ESPERMATOZÓIDE

A. MECANISMOS MOLECULARES

Da fusão/ligação entre as membranas dos dois gâmetas resulta a activação de uma GTPase membranar (GTP binding protein ou G protein), que, por sua vez, activa a fosfolipase C membranar. Esta enzima vai clivar o fosfatidilinositol 4,5-bisfosfato (PIP2) em inositol 1,4,5-trisfosfato (IP3) e diacilglicerol (DAG). O IP3 induz a abertura de canais para o Ca^{2+}, existentes no oolema, e, difundindo para o citoplasma cortical, actua sobre o retículo endoplasmático, causando a libertação do Ca^{2+} que aí se encontra acumulado (Epel, 1977, 1990; Epel *et al.*, 1982; Eisen *et al.*, 1984; Eisen e Reynolds, 1985).

Estas alterações localizam-se inicialmente ao ponto de fusão entre os dois gâmetas, mas o aumento das concentrações intracelulares do ião Ca^{2+} desencadeia rapidamente uma propagação autocatalítica deste fenómeno por todo o ovo (Epel, 1977, 1990; Epel *et al.*, 1982; Jaffe, 1983; Trimmer e Vacquier, 1986; Hafner *et al.*, 1988). Este facto resulta da difusão do Ca^{2+} pelo citoplasma cortical do ovo, provocando a activação da fosfolipase C membranar, bem como a conversão do fosfatidil inositol (PI), molécula que constitui 25% dos fosfolípidos membranares, em PIP2, o substrato da fosfolipase C (Swann e Whitaker, 1986; Trimmer e Vacquier, 1986; Epel, 1990).

Por outro lado, o Ca^{2+}, o DAG e a porção α-GTP (Gs) da GTPase membranar activam os canais Na^+/H^+ do oolema por intermédio da activação de uma proteína cinase C situada no oolema (Epel, 1972, 1988, 1990; Epel *et al.*, 1978, 1982; Shen e Burgart, 1985; Shen e Sui, 1989).

Em conclusão, após a fusão entre os dois gâmetas assiste-se, em primeiro lugar, a um aumento localizado das concentrações livres em Ca^{2+} que, pelos mecanismos autocatalíticos anteriormente expostos, se propaga, como uma onda, a todo o ovo a partir do local de fusão celular. Em segundo lugar, devido à activação da bomba Na^+/H^+, observa-se um aumento progressivo no pH_i. Em terceiro lugar, a subunidade α-GTP liga-se à adenilato ciclase membranar, activando-a, do que resulta um aumento das concentrações em cAMP. Em consequência deste fenómeno, o cAMP difunde pelo citoplasma e activa várias proteínas cinases que, por fosforilação de enzimas chave e em conjunto com os aumentos do Ca_i e do pH_i, vão activar o metabolismo do

ovo, preparando-o para as divisões mitóticas subsequentes (Epel, 1977, 1990; Keller *et al.*, 1980; Vacquier, 1981; Schneider, 1985).

B. A INCORPORAÇÃO DO ESPERMATOZÓIDE

Os aumentos do Ca_i e do pH_i, apesar de estarem separados temporalmente, influiem de igual modo nas variadas transformações do citosqueleto do ovo que, de imediato, ocorrem após a fusão entre os dois gâmetas.

Imediatamente após a fusão entre as membranas dos dois gâmetas na zona de contacto, o ovo expande o seu citoplasma em direcção ao espermatozóide, para formar uma protusão citoplasmática, o cone de fertilização. A formação do cone de fertilização depende dos aumentos do Ca_i e do pH_i, e deve-se à polimerização rápida da actina G, armazenada no córtex do ovo, em microfilamentos (Tilney e Jaffe, 1980; Vacquier, 1981; Cline e Schatten, 1986; Longo, 1986b; Yonemura e Mabuchi, 1987; Hamaguchi e Mabuchi, 1988; Kyozuka e Osanai, 1988).

O mecanismo de entrada do espermatozóide no citoplasma do ovo, é ainda desconhecido, mas sabe-se que não depende dos batimentos flagelares, pois que, por videocinematografia, demonstrou-se que, nesta fase, o espermatozóide fica imóvel (Epel, 1977; Epel e Vacquier, 1978; Schatten, 1982). Pensa-se, pelo contrário, que, no cone de fertilização, a despolimerização rápida dos microfilamentos na sua face citoplasmática acoplada à polimerização dos mesmos na face voltada para o oolema ("treadmilling"), empurra o espermatozóide para dentro do ovo (Schatten, 1982).

O espermatozóide penetra totalmente no citoplasma do ovo, o que inclui os microfilamentos do perfuratório, o núcleo, os centríolos, a mitocôndria e o axonema. À medida que o espermatozóide penetra no córtex do ovo, o axonema recomeça os seus batimentos, o que também ajuda à finalização da sua incorporação. De seguida, o espermatozóide sofre uma rotação de 180°, pelo que o polo apical do núcleo fica voltado para o oolema, enquanto que os centríolos ficam em contacto com o interior do citoplasma (Epel, 1977; Schatten, 1982). A reacção cortical e a contracção cortical do ovo são dois outros mecanismos que também ocorrem na superfície do gâmeta feminino e que facilitam a incorporação do espermatozóide no córtex citoplasmático do ovo. Estas reacções são descritas de seguida.

C. A REACÇÃO CORTICAL

O aumento dos teores em Ca_i e em IP3, bem como a activação da "G--protein" e de proteases e da fosfolipase ligadas à membrana das vesículas corticais, desencadeado pela fusão entre o espermatozóide e o ovócito, induz a exocitose das vesículas corticais. Este fenómeno constitui a reacção cortical do ovo, que se inicia no local de fusão do espermatozóide com o oolema e se propaga rapidamente a todo o córtex celular (Fig. 8) (Epel e Carroll, 1975; Epel e Vacquier, 1978; Chandler e Heuser, 1979; Ferguson e Shen, 1984; Zimmerberg e Whitaker, 1985; Longo, 1986b, 1988; Turner *et al.*, 1986; Breckenridge e Almers, 1987; Jackson e Modern, 1990; Mohri e Hamaguchi, 1990).

A exteriorização do conteúdo das vesículas corticais exerce consequências dramáticas e irreversíveis sobre a superfície do ovo e a

evolução do embrião, constituindo um bloqueio definitivo à polispermia (Endo, 1961; Millonig, 1969; Holland, 1980; Longo *et al.*, 1982; Somers e Shapiro, 1989).

Das vesículas corticais libertam-se várias substâncias (Schuel, 1978, 1984; Shapiro e Eddy, 1980), nomeadamente: (1) uma enzima proteolítica, denominada delaminase, que corta as ligações da camada vitelina ao oolema, permitindo desse modo o seu afastamento da superfície do ovo (Carrol e Epel, 1975; Alliegro e Schuel, 1988); (2) materiais coloidais que, aumentando intensamente a osmolaridade do pequeno espaço situado entre a camada vitelina e o oolema, o espaço perivitelino, atraiem água para este local. A expansão do espaço perivitelino causa então um afastamento marcado, para longe do oolema, da camada vitelina, arrastando com ela os espermatozóides aí ligados (Schuel, 1978; Sousa e Azevedo, 1987, 1988e; Sousa *et al.*, 1991b); (3) uma enzima, denominada ovoperoxidase, que actua sobre a camada vitelina catalizando a formação de pontes entre tirosinas adjacentes, transformando-a, assim, numa estrutura rígida e impermeável a novas penetrações, a camada de fertilização (Foerder e Shapiro, 1977; Klebanoff *et al.*, 1979; Shapiro *et al.*, 1982; Heinecke e Shapiro, 1990; Sousa e Azevedo, 1990a). A camada de fertilização constitui, para além de uma barreira definitiva à penetração de outros espermatozóides, uma futura estrutura rígida que protegerá o embrião contra as agressões exógenas. A ovoperoxidase é uma enzima que utiliza como substrato o peróxido de hidrogénio, oriundo do citoplasma do ovo. O peróxido de hidrogénio, uma vez livre no espaço perivitelino, difunde também através da camada de fertilização, actuando como espermicida sobre os restantes espermatozóides aí

ligados (Boldt *et al.*, 1981; Coburn *et al.*, 1981). A ovoperoxidase é uma enzima dependente dos iões Ca^{2+} e Mg^{2+} e a sua ligação à camada vitelina faz-se na dependência da proteína proteoligandina ("proteoliaisin") (Weidman e Shapiro, 1987; Somers *et al.*, 1989); (4) uma outra enzima proteolítica, a hidrolase dos receptores dos espermatozóides, que actua sobre a camada de fertilização digerindo os receptores que aí existem para a ligação dos espermatozóides. Esta enzima promove a desinserção dos espermatozóides da camada de fertilização, induzindo a sua libertação da superfície do embrião (Carroll e Epel, 1975; Lois *et al.*, 1986); (5) a enzima β-1,3--glucanase de função desconhecida (Epel *et al.*, 1969; Moy *et al.*, 1983; Wessel *et al.*, 1987); (6) as enzimas fosfatase ácida e fosfatase alcalina (Sousa e Azevedo, 1989c, 1990a); (7) proteínas argirófilas (Sousa e Azevedo, 1987); e (8) a hialina, que, em conjunto com outras glicoproteínas, fica a revestir a superfície do ovo e depois a dos blastómeros, mantendo a adesão destes entre si (Fig. 8) (Wessel *et al.*, 1984; Cameron e Holland, 1985; Alliegro e McClay, 1988).

D. EXPANSÃO DAS MICROVILOSIDADES, ACTIVAÇÃO DA ENDOCITOSE E CONTRACÇÃO CORTICAL

Com a fecundação do ovócito pelo espermatozóide, o citosqueleto do ovo sofre várias outras transformações. Como vimos, o aumento do Ca_i e do pH_i no córtex do ovo causam a polimerização da actina G em microfilamentos. Esta rápida polimerização está implicada na formação do cone de fertilização, mas também provoca uma expansão das microvilosidades e a formação de um feixe rígido submembranar de

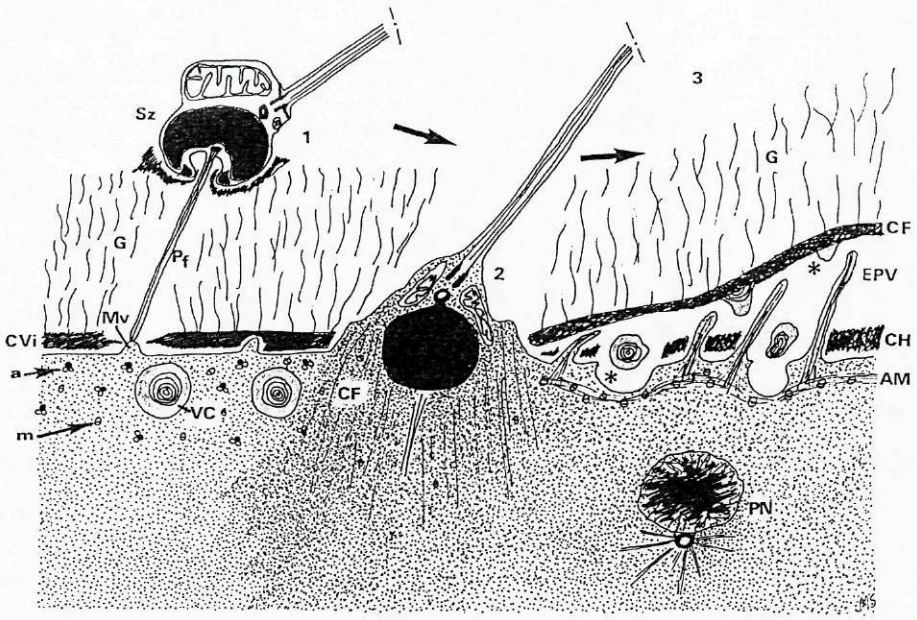


Fig. 8. Esquema da reacção do ovócito (Ov) à penetração do espermatozóide (Sz) em *Marthasterias glacialis*. (1) Quando a membrana que reveste o perfuratório (Pf) se funde com o oolema após ter penetrado a geleia (G) e a camada vitelina (CVi), o Ov projecta em sua direcção uma porção de citoplasma, o cone de fertilização (CF), para incorporar o Sz. (2) O CF é sustentado por feixes de microfilamentos (Mf) que se formam por polimerização da actina G (a). (3) De imediato ocorre a exocitose das vesículas corticais (VC). No espaço perivitelino (EPV) o conteúdo (*) das VC funde-se com a CVi, transformando-a na camada de fertilização (CF), e formam, junto ao oolema, a camada hialina (CH). Ao aumentar de volume, o EPV afasta a CF. As microvilosidades (Mv) sofrem um aumento de comprimento (dependente dos Mf) para obviar ao aumento de superfície membranar decorrente da reacção cortical. Sob o oolema, formam-se feixes de actomiosina (AM) (Mf + miosina, m) que originam uma forte contracção cortical. Entretanto, o núcleo do Sz inicia a sua transformação em pronúcleo masculino (PM).

microfilamentos (Fig. 8) (Harris, 1968; Burgess e Schoeder, 1977; Begg e Rebhun, 1979; Tilney e Jaffe, 1980; Vacquier, 1981; Begg *et al.*, 1982; Longo, 1988; Spiegel *et al.*, 1989, 1990). O alongamento das microvilosidades é de capital importância, pois que a fusão da membrana das vesículas corticais com o oolema aumenta, praticamente para o dobro, a superfície celular. Como após a fecundação o ovo pouco aumenta de volume, este súbito aumento de superfície da membrana é então contrariado pela extensão das microvilosidades (Vacquier, 1981; Schatten, 1982). Porém, esta extensão não é suficiente nem duradoura, pelo que também se assiste a um aumento dos processos de endocitose como meio de obviar ao aumento da superfície (Fisher e Rebhun, 1983; Carron e Longo, 1984; Sardet e Chang, 1987; Longo, 1988). Por sua vez, o feixe submembranar de microfilamentos, associado a moléculas de miosina, origina uma forte contracção da superfície do ovo na metade em que se verificou a penetração. Esta contracção, tal como a elevação da camada vitelina, promove definitivamente a incorporação do espermatozóide no citoplasma do ovo, para além de afastar ainda mais da sua superfície a camada de fertilização (Vacquier, 1981; Eisen *et al.*, 1984; Trimmer e Vacquier, 1986; Longo, 1988).

E. A EXPLOSÃO METABÓLICA DO OVO

A fase seguinte da resposta do ovo à penetração de um espermatozóide está dependente dos aumentos do Ca_i e do pH_i. A alcalinização do meio interno desencadeia uma explosiva activação metabólica, com desrepressão de todas as vias de síntese e abertura

de canais membranares, quer para iões, quer para aminoácidos (Schneider, 1985; Allemand *et al.*, 1986, 1987a, 1987b). A activação do ovo engloba ainda um aumento da síntese proteica, por desrepressão da tradução do ARNm armazenado no córtex (Colin *et al.*, 1987), um aumento da síntese do ADN, totalmente independente da síntese proteica, mas dependente da activação de vários sistemas enzimáticos (Whitaker e Steinhardt, 1981; Epel, 1990) e da formação das ciclinas (Epel, 1990) e uma activação de vários sistemas enzimáticos (Epel, 1977, 1988, 1990; Keller *et al.*, 1980; Shapiro e Eddy, 1980; Satoh e Garbers, 1985; Trimmer e Vacquier, 1986; Swezey e Epel, 1988).

Ao contrário do que sucede no ovo do ouriço do mar, estas transformações metabólicas não estão ainda bem delineadas na estrela do mar. A principal causa desta diferença reside no facto de, ao contrário do ouriço do mar, o ovócito da estrela do mar sofrer duas activações consecutivas. Numa primeira fase o ovócito da estrela do mar é activado pela 1-MetAd, substância que aumenta o pH_i, activa a síntese proteica e os sistemas enzimáticos e induz a maturação meiótica. Assim, quando o espermatozóide penetra o ovócito, já este se encontra parcialmente activado (Houk e Epel, 1974; Meijer e Guerrier, 1984; Chiba *et al.*, 1990). A fusão dos dois gâmetas desencadeia, portanto, o potencial de activação, um aumento do Ca_i, a formação do cone de fertilização, a reacção cortical, a expansão das microvilosidades e a contracção cortical. Aparentemente, porém, não se observa um aumento do pH_i. Pensa-se que, para ocorrerem aquelas transformações dependentes dos microfilamentos, deverão, pelo menos, ocorrer aumentos localizados do pH_i. Supõe-se também

que, no caso da estrela do mar, mais importante do que o aumento global do pH_i, será factor determinante a sua variação em locais específicos do citoplasma do ovo (Schuetz, 1975; Peaucellier e Doree, 1981; Johnson e Epel, 1982; Eisen e Reynolds, 1984; Meijer e Guerrier, 1984; Sardet e Chang, 1987).

Consequente à explosão metabólica do ovo, libertam-se produtos tóxicos resultantes do aumento de consumo de oxigénio (Perry e Epel, 1985), nomeadamente os radicais de oxigénio, os quais são convertidos em peróxido de hidrogénio (Heineck e Shapiro, 1989; Heineck *et al.*, 1990). Este, por sua vez, é libertado para o espaço perivitelino, funcionando como substrato para a ovoperoxidase. A partir do espaço perivitelino o peróxido de hidrogénio difunde através da camada de fertilização, actuando directamente sobre os espermatozóides como espermicida (Boldt *et al.*, 1981; Coburn *et al.*, 1981). O peróxido de hidrogénio tem a sua origem nas reacções que levam ao aumento do Ca_i. Assim, o Ca^{2+} livre, em conjunto com a proteína calmodulina, activa a cinase do NAD que vai converter o NAD citoplasmático em NADH e NADPH (Epel *et al.*, 1978, 1981; Eisen *et al.*, 1984; Heineck e Shapiro, 1989; Epel, 1990; Heineck *et al.*, 1990). Estes cofactores activam a NADPH oxidase que então forma o peróxido de hidrogénio. A formação daqueles cofactores reduzidos é, portanto, outro dos estímulos que permitem a activação metabólica do ovo (Epel, 1977).

F. FORMAÇÃO, MIGRAÇÃO E FUSÃO DOS PRONÚCLEOS

Os centríolos do espermatozóide mantêm-se ligados ao núcleo pelo

centríolo proximal e transformam-se em ásteres após a entrada completa do espermatozóide no citoplasma do ovo (Fig. 8) (Longo, 1973, 1988, 1991; Summers *et al.*, 1975; Schatten, 1981; Vacquier, 1981). Os microtúbulos nascidos destes ásteres expandem-se então progressivamente pelo citoplasma do ovo. Devido a esta expansão, acabam por contactar com o oolema, pelo que o núcleo do espermatozóide vai sendo arrastado para o centro do ovo, local onde se equilibram as forças radialmente opostas criadas pela expansão dos microtúbulos contra o oolema. Na sua expansão, os microtúbulos acabam também por se ligar ao invólucro nuclear do pronúcleo feminino, arrastando-o igualmente para o centro do ovo (Fig. 9). Este movimento do pronúcleo feminino depende de um processo complexo. Após a ligação ao invólucro nuclear do pronúcleo feminino, essa extremidade dos microtúbulos pára de sofrer polimerização, enquanto que a extremidade junto aos centríolos do pronúcleo masculino mantém uma fase de rápida despolimerização. Este facto, em conjunto com a acção de uma dineína (ATPase), como que puxa o pronúcleo feminino para as proximidades do áster, ou seja, para o centro do ovo e para a periferia do pronúcleo masculino (Mar, 1980; Bestor e Schatten, 1981; Schatten, 1981, 1982; Schatten *et al.*, 1985, 1986, 1988).

Em conclusão e em relação ao citosqueleto, pode-se pois, distinguir duas fases distintas. Primeiramente assiste-se a um fenómeno dependente da acção dos microfilamentos corticais (formação do cone de fertilização, penetração do espermatozóide, expansão das microvilosidades e onda contráctil) para, de seguida, se observar uma fase dependente dos microtúbulos (migração dos pronúcleos).

Facto curioso, mas bastante generalizado, é o de serem os

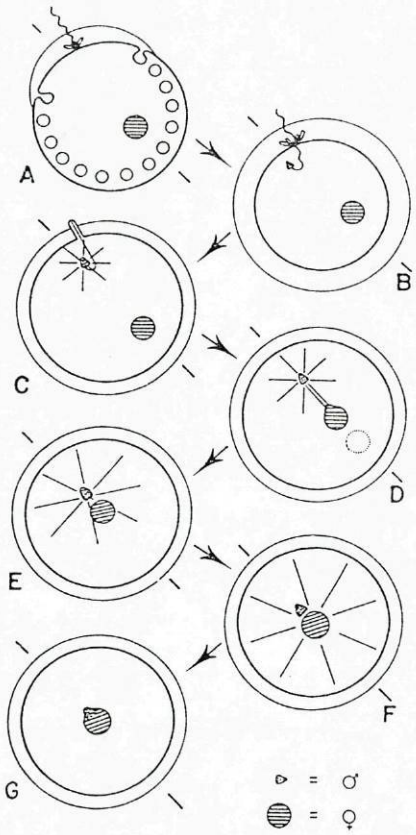


Fig. 9. Esquema da incorporação do espermatozóide, migração e fusão dos pronúcleos no ouriço do mar.

centríolos do espermatozóide e não os do ovócito, os responsáveis pelos ásteres, pois que os ásteres do ovócito sofrem uma despolimerização e, consequentemente, desaparecem. Por exemplo, na estrela do mar, quando o espermatozóide penetra no ovócito este ainda possui os seus próprios centríolos e deles fará uso para finalizar as duas divisões meióticas. Porém, após se formar o pronúcleo feminino, os ásteres maternos despolimerizam, deixando aos centríolos paternos a responsabilidade das migrações dos dois pronúcleos e a formação dos futuros fusos mitóticos. A causa destes fenómenos é ainda desconhecida (Hirai *et al.*, 1981; Schuetz e Longo, 1981; Schroeder e Otto, 1984; Sardet e Chang, 1987; Sluder *et al.*, 1989; Kato *et al.*, 1990; Shirai *et al.*, 1990).

O núcleo do espermatozóide inicia a sua transformação em pronúcleo masculino ainda enquanto se encontra no córtex do ovo. No ouriço do mar, o núcleo do espermatozóide perde, por fragmentação, o seu invólucro nuclear. A perda do invólucro nuclear deve-se à fosforilação das laminas. Esta perda, porém, não é total, pois as membranas localizadas nas fossas centriolar e acrosómica mantêm-se íntegras. De imediato, a cromatina começa a descondensar, de um modo progressivo, da periferia para o centro, fenómeno que depende da interacção da cromatina com factores citoplasmáticos oriundos do citoplasma do ovo. Esta dispersão da cromatina, de facto, coincide com a fosforilação da histona H2 específica do espermatozóide (Sp-H2) (lembremo-nos que a Sp-H1 já havia sido fosforilada durante a reacção acrosómica) e a substituição das histonas Sp-H1 e Sp-H2 por histonas básicas dadas pelo citoplasma do ovo, as H1, H2A e H2B. Simultaneamente, o núcleo do espermatozóide aumenta de volume. Após se completar a dispersão da

cromatina, verifica-se a reformação do invólucro nuclear, para finalmente se constituir o pronúcleo masculino. A reformação do invólucro nuclear faz-se pela adesão de vesículas do retículo endoplasmático, oriundo do citoplasma do ovo, à cromatina do espermatozóide e depende da desfosforilação da laminas. Pensa-se que a reformação dos poros nucleares, por seu lado, se deve à manutenção dos seus componentes na periferia da cromatina do espermatozóide (Longo, 1973, 1983, 1988, 1991; Summers *et al.*, 1975; Kunkle *et al.*, 1978; Kunkle, 1982; Porter e Vacquier, 1986; Luttmer e Longo, 1987).

Uma vez chegados ao centro do ovo, as membranas nucleares dos dois pronúcleos fundem-se entre si, restaurando a diploidia, pelo que de imediato se segue a primeira divisão mitótica (cerca de 2 horas após a fecundação) (Fig. 9) (Longo e Anderson, 1968; Longo, 1973).

Este mecanismo de evolução dos pronúcleos verifica-se para o ouriço do mar, mas não para a estrela do mar. Esta diferença deve-se ao facto do ovócito da estrela do mar ser fecundado quando ainda não existe um pronúcleo feminino. Por isso, na estrela do mar, enquanto o ovócito reage à incorporação do espermatozóide no citoplasma e se forma o pronúcleo masculino, o núcleo do ovócito sofre, primeiro, as duas divisões meióticas, libertando dois glóbulos polares, e só então se transforma em pronúcleo feminino. Esta dessincronização verificada na estrela do mar, é responsável por uma evolução diferente, em relação ao ouriço do mar, no que diz respeito à formação do pronúcleo masculino e ao tipo de fusão subsequente entre os dois pronúcleos. Verifica-se, por exemplo, que o invólucro nuclear do núcleo do espermatozóide não sofre qualquer rutura, e que a cromatina se dispersa de um modo global e não da periferia para o centro. Por outro lado, apesar da migração central, os invólucros nucleares dos

dois pronúcleos não se fundem entre si. Observa-se que ambos perdem as suas membranas para de imediato os cromosomas se juntarem na futura placa metafásica da primeira divisão embrionária (Longo, 1991).

Os destinos dos outros componentes do espermatozóide no interior do ovo são vários. A mitocôndria e o axonema, por exemplo, vão progressivamente degenerando, apesar dos seus constituintes só desaparecerem por completo na fase de 2-4 blastómeros (Anderson, 1968a; Longo e Anderson, 1968; Longo, 1973, 1991). Um outro componente do espermatozóide que se integra totalmente no ovo, é a sua membrana citoplasmática. Estudos de imunofluorescência demonstram a persistência de componentes da membrana do espermatozóide no local de fusão dos dois gâmetas até fases tardias da embriogénese (Shapiro e Eddy, 1980), enquanto que estudos por imunolocalização demonstraram que efectivamente tal não sucede (Longo, 1988). Parece, pois, que os componentes da membrana do gâmeta masculino rapidamente difundem e se dispersam no oolema, num fenómeno em tudo semelhante ao verificado com os componentes das membranas das vesículas corticais (Longo, 1981, 1986a, 1986b, 1988, 1989a, 1989b; Gunderson *et al.*, 1982, 1986; Nishioka *et al.*, 1989).

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Fine structure of the spermatozoon of *Marthasterias glacialis* (Linnaeus) (Echinodermata; Asteroidea), with special reference to acrosomal morphology

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The sperm of *Marthasterias glacialis* (Linnaeus) was studied by light and electron microscopy. It is a long uniflagellated cell of the "primitive" type. The head has a spherical shape and contains a nucleus with a spheroid acrosome lying in a cup-shaped anterior fossa. The acrosome is formed by an acrosomal vesicle surrounded by the periacrosomal material. The basal specializations of the acrosomal vesicle show a clear differentiation of its constituents resembling the structure of membrane. The midpiece contains a very large annular mitochondrion which encircles two perpendicular centrioles. The distal centriole is in close association with a pericentriolar radial complex. The tail, containing a common microtubular axoneme, is projected to a variable position.

ultrastructure, spermatozoon, acrosome, Asteroidea

Introduction

Ultrastructural studies of spermatozoa in different marine animal groups are considered of great importance as they may contribute to the knowledge of the phylogenetic relationships between other similar groups [1–4] and may serve as a basis for taxonomic classification in species ecologically alike [5–7].

On the other hand, it seems of considerable interest to study the ultrastructure of the spermatozoa from the echinoderm groups, particularly the asteroids, of which there have been few studies [8–13] in contrast to the vast literature on spermatozoa in other taxonomic groups [14].

In the present work we have studied the ultrastructure of the *M. glacialis* spermatozoon with particular reference to acrosomal morphology.

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Materials and methods

Several specimens of *M. glacialis* (Linnaeus) were collected monthly from the intertidal zone of the northern coast of the Portuguese Atlantic. After dissection, testes were fixed in Bouin's fluid for light microscopy studies. For electron microscopy, small testis pieces and isolated spermatozoa were fixed in 2.5% glutaraldehyde, rinsed in buffer and post fixed in 2% OsO4, each for 2 h at 4°C; 0.2 M sodium cacodylate, pH 7.2, was used throughout. After dehydration in alcohol, they were embedded in Epon. For light microscopy, semithin sections were stained in 1% toluidine blue. Ultrathin sections were double stained with uranyl acetate and lead citrate and examined in a JEOL 100B electron microscope, operated at 80 kV.

For whole mounts, mature isolated spermatozoa were sedimented by centrifugation when they were in the dehydration steps. The pellet was shaken and the spermatozoa floating in ethanol were removed from below on Formvar-coated grids [15].

Results

Light microscopy

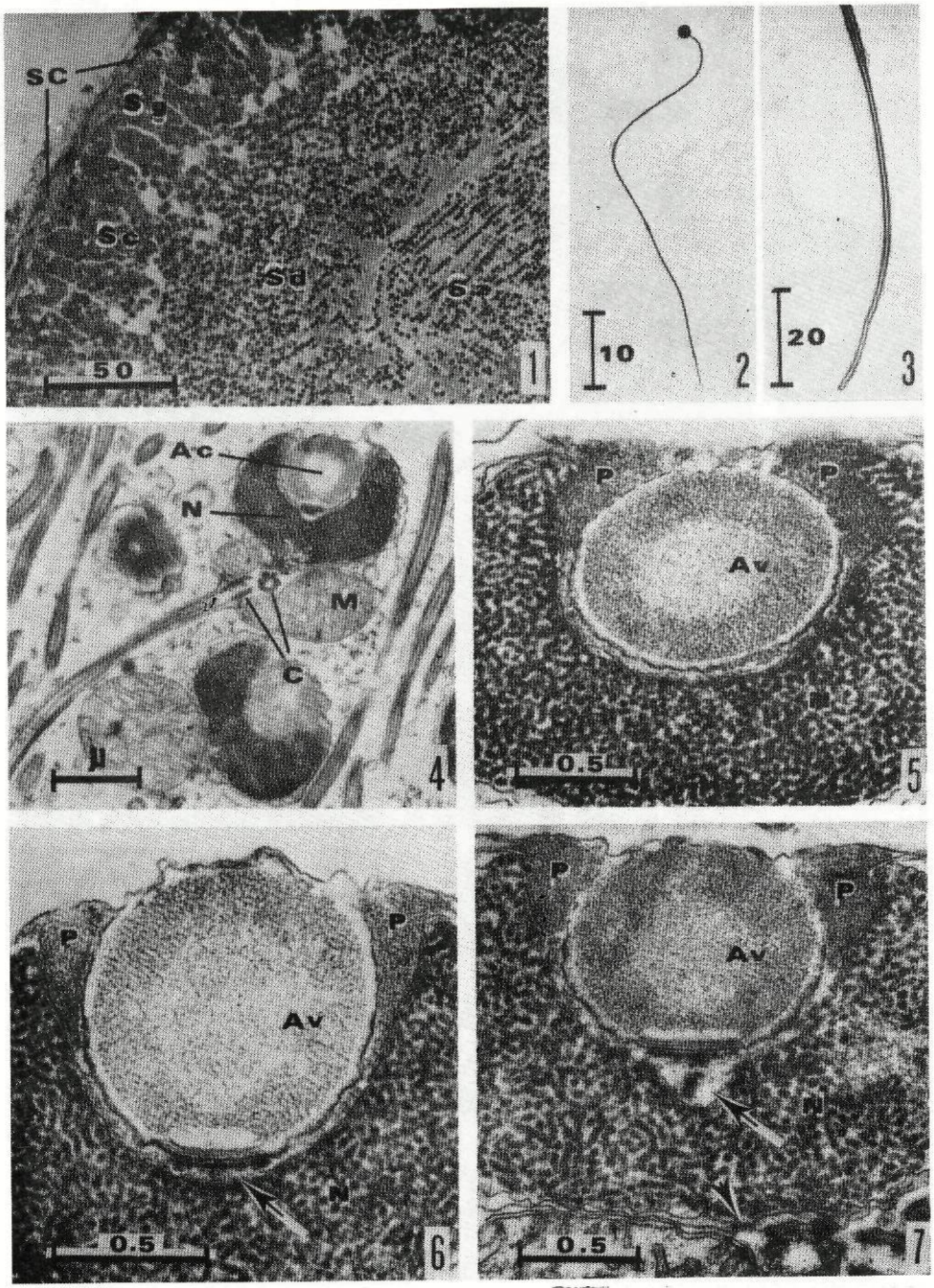
Germ cells are disposed in columns and maturation proceeds centripetally. Mature sperm and spermatids fill the lumen of the unspawned testes (Fig. 1).

Electron microscopy

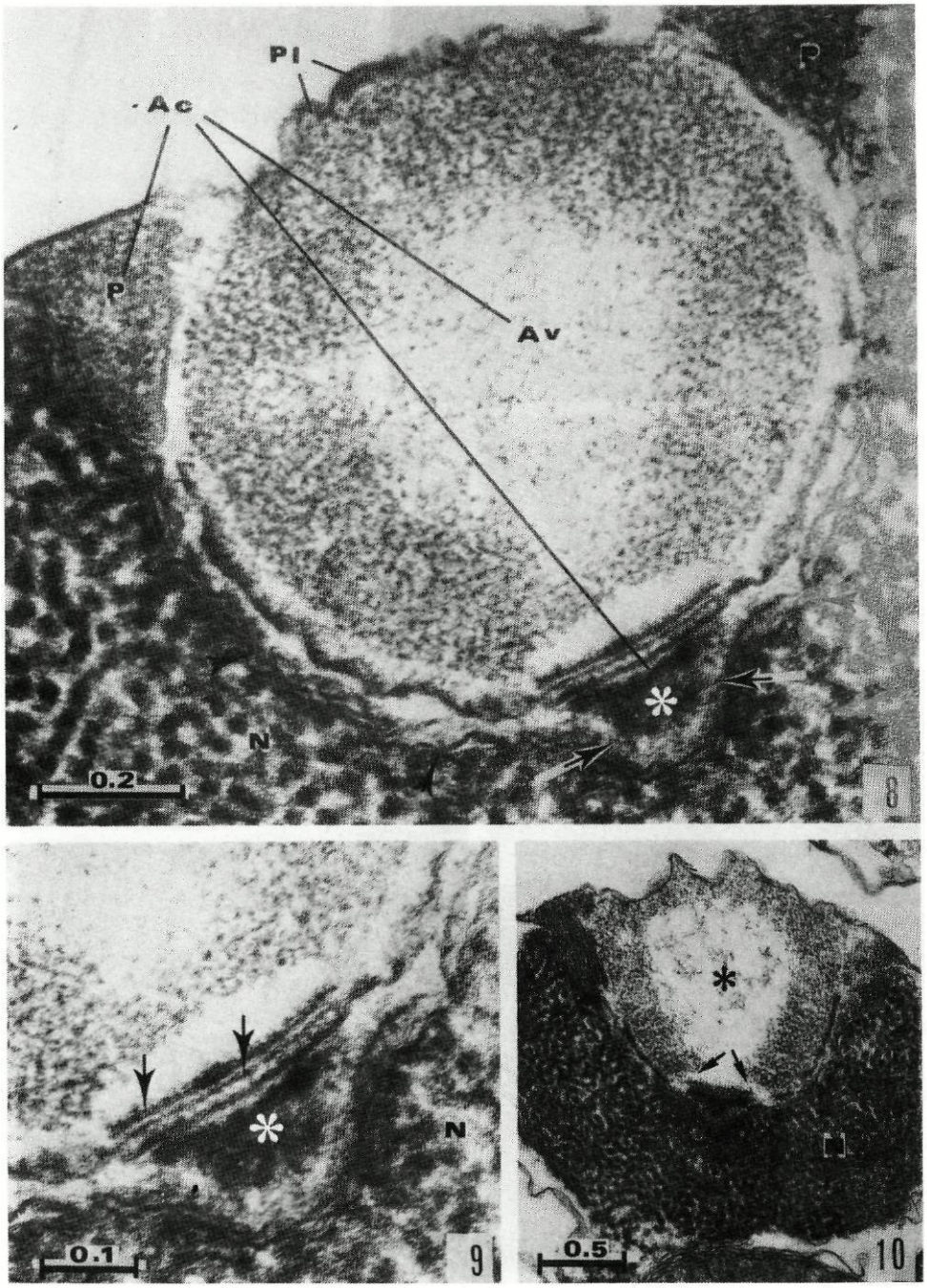
The mature spermatozoon of *M. glacialis* has a total length of ~ 61.7 μm when observed in whole mounts (Fig. 2). It is a uniflagellated cell, composed of a head, midpiece and tail (Figs. 2–4).

Head

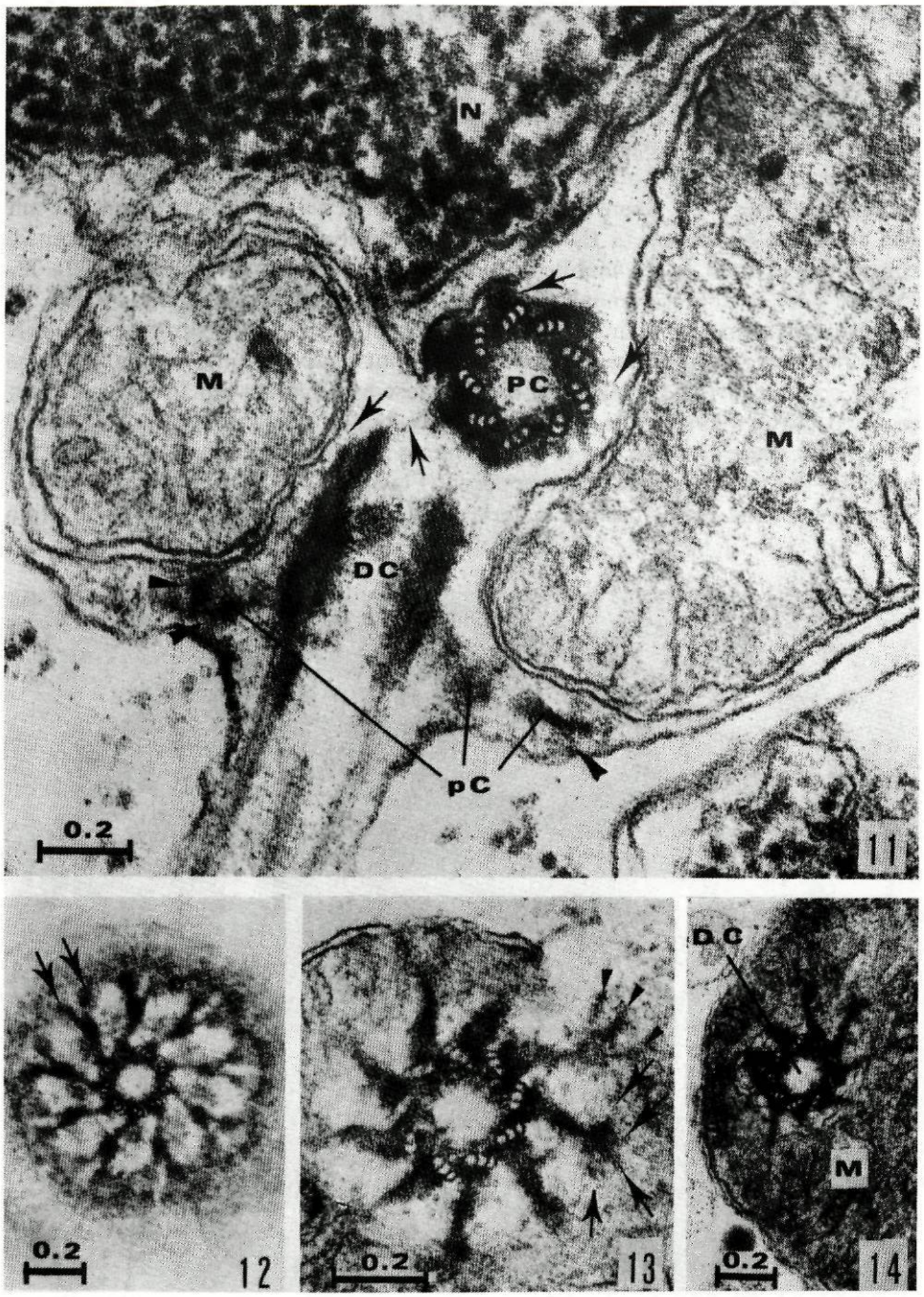
This is a round structure approximately 2.2 μm in diameter containing, in an anterior nuclear fossa, the acrosomal complex (Fig. 4). The nucleus presents a nucleoplasm formed by coarse, branching or anastomosing strands of chromatin, ~ 300 Å in diameter, which display no recognizable order in their packing (Figs. 7 and 8). The nuclear envelope seems devoid of pores. In the external concave face, where it presents a larger perinuclear space, a clear approximation of the two membranes is seen at the place where the outer nuclear membrane connects the proximal centriole (Fig. 7). The acrosome is composed of a spheroid acrosomal vesicle ~ 1 μm in diameter, surrounded by periacrosomal material. The acrosomal vesicle (Figs. 5–10) is composed of three concentric portions, the external one being the narrowest and lightest and the middle one the most electron dense. The lighter regions seem to be made by fibrous expansions of the middle and denser portion (Fig. 8). Light expansions of the central portion are also seen into the middle zone. In the flattened basal region of the acrosomal vesicle, there is a specialization of the



Figs. 1–7. 1. Semithin section of the testis, showing external somatic cells (SC), spermatogonia (Sg), spermatocytes (Sc), spermatids (Sd) and spermatozoa (Sz). 2 and 3. Spermatozoon and end piece, respectively, observed as whole mounts in the electron microscope. 4. In the head the acrosomal complex (Ac) is composed of an acrosomal vesicle, periacrosomal material and subacrosomal material, and the nucleus (N). The midpiece is composed of one mitochondrion (M) and two centrioles (C). 5–7. Serial sections of the acrosomal complex, composed of an acrosomal vesicle (Av) and surrounding periacrosomal material (P) which apically covers the acrosomal vesicle only at its lateral sides. In Figs. 6 and 7 a complex subacrosomal fossa is evident (arrow). The nucleus (N) surrounds these structures. In Fig. 7 the arrowhead points to an approximation of the two nuclear membranes. Scale bar = μm.



Figs. 8–10. 8. Detail of the acrosomal complex (Ac) composed of acrosomal vesicle (Av), periacrosomal material (P) and subacrosomal fossa (*) and surrounded by the nucleus (N). The dense material which fills the subacrosomal fossa has clear connections with the nuclear envelope (arrows). The two nuclear membranes seem to contact each other in several regions (arrowheads). The apical zone of the acrosomal vesicle is in close contact with the plasmalemma (Pl). 9. Detail of the subacrosomal fossa. The two dense layers at the basal portion of the acrosomal vesicle have a membrane-like morphology (arrows). The material situated in the subacrosomal fossa is fibrillar (*). 10. The central vesicular component (*) shows its communication with the basal vesicular region (arrows) of the subacrosomal complex. Scale bar = μm.



Figs. 11–14. 11. Longitudinal section through the middle piece, showing the nucleus (N), mitochondrion (M) and centrioles (PC, proximal and DC, distal). Clear connections are seen between all the organelles of the middle piece (arrows). The pericentriolar complex (pC) in close association with the distal centriole, shows some connections with the mitochondrion and the plasmalemma (arrowheads). 12. A transverse section through the pericentriolar complex. It exhibits the classical morphology: there are two secondary arms (arrows) that seem to terminate at the plasma membrane. 13. In an oblique section through the pericentriolar complex it seems that there are more than two (arrowheads) and even more than three (arrows) secondary arms. 14. The mitochondrion (M) is circular, having at its center the centrioles. Scale bar = μm.

contents of the vesicle, which is formed by the narrowing of the middle component of the vesicle and also by the presence of two dense lamellae which have a membrane-like morphology. At its extremities the inner lamella seems in continuity with the middle region of the vesicle and the outer one with the basal vesicle membrane (Figs. 6–9). There is a less dense material between the two lamellae and also from the outer one to the basal vesicle membrane. From the inner lamella longer fibers depart to the middle and central zones of the vesicle (Figs. 6–9). The concave part of the acrosomal vesicle membrane is thicker than the convex one because the former contains additional material at its inner surface. The periacrosomal material is homogeneous and similar but denser than the middle acrosomal vesicle component, and appears to have a close association with the vesicle membrane. It covers the acrosomal vesicle apically, but not at its central portion where the cellular space between the two membranes is not large enough to contain any material: the two membranes are opposed (Figs. 7 and 8). The acrosomal nuclear fossa receives the periacrosomal material in three distinct parts, an upper portion,

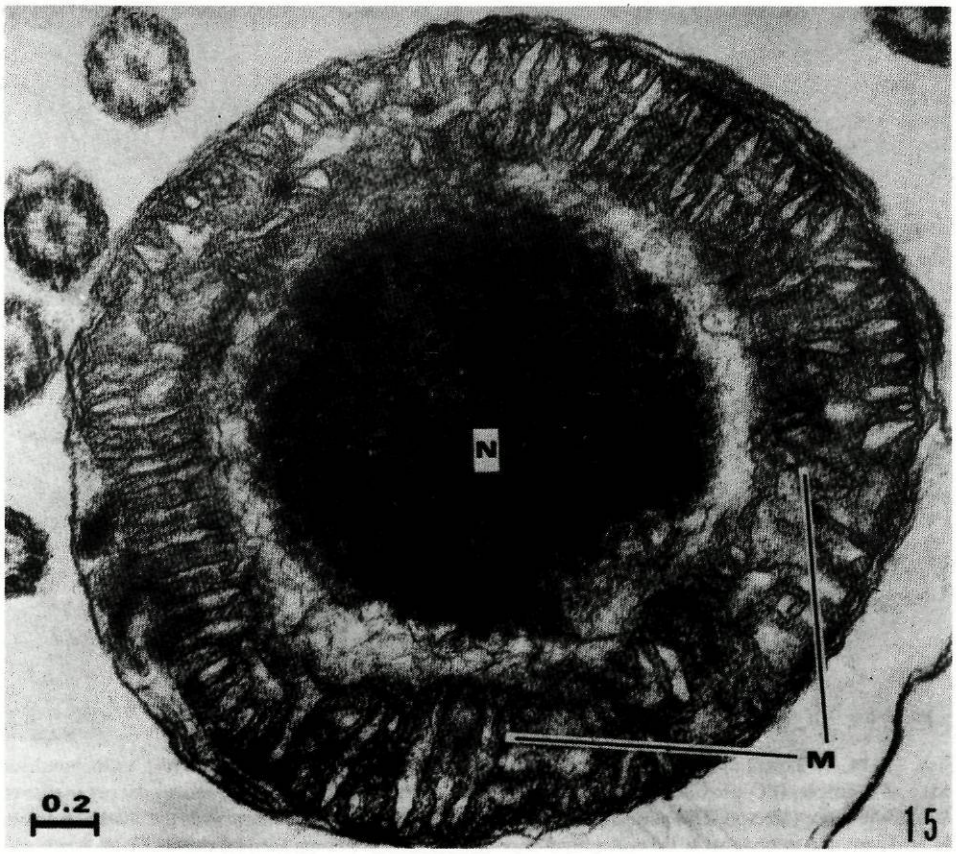


Fig. 15. The circular mitochondrion (M) and the basal part of the nucleus (N). Scale bar = μm.

the largest, lying in an apical nuclear depression, a lateral portion which is the narrowest, and a basal portion which rests in a subacrosomal nuclear fossa and contains a specialization of the periacrosomal material (Figs. 6–8). The subacrosomal material seems to be formed by parallel and dense lamellae or fibers forming a 90° angle with the long axis of the sperm. The fibers that connect the mass with the vesicle membrane and the nuclear envelope extend from this aggregate (Fig. 8). The nuclear envelope in its apical fossa seems to present pores or zones of apparent approximation of the two membranes (Fig. 8).

Midpiece

In the basal zone of the nucleus there is a large annular mitochondrion ~ 2.1 μm in diameter (Figs. 11, 14 and 15). In the center of this organelle there are two centrioles which are perpendicular (Fig. 11), the proximal one making a 90° angle with the long axis of the sperm (Fig. 11). Since the tail is projected to a variable position it seems that the mitochondrion has the capacity of moving its mass and so, the centrioles can be positioned more or less excentrically through the mitochondrion (compare Fig. 13 with Fig. 14). The interconnections between the elements of the midpiece are very clear. Consequently, we can see a close association between the outer nuclear membrane and the proximal centriole, and a system of interrelations between the mitochondrion and the outer nuclear membrane, between the two centrioles, and between the centrioles and the mitochondrion (Fig. 11). A pericentriolar complex is associated with the distal centriole. Along its length it gives rise to some material, that from the superior side ending at the mitochondrion membranes, whereas that from the inferior side terminates in the plasmalemma (Fig. 11). In a very oblique cut, each primary arm of the complex seems to divide itself into various secondary arms in contrast with the bifurcation seen in transversal sections (Figs. 12 and 13).

Tail

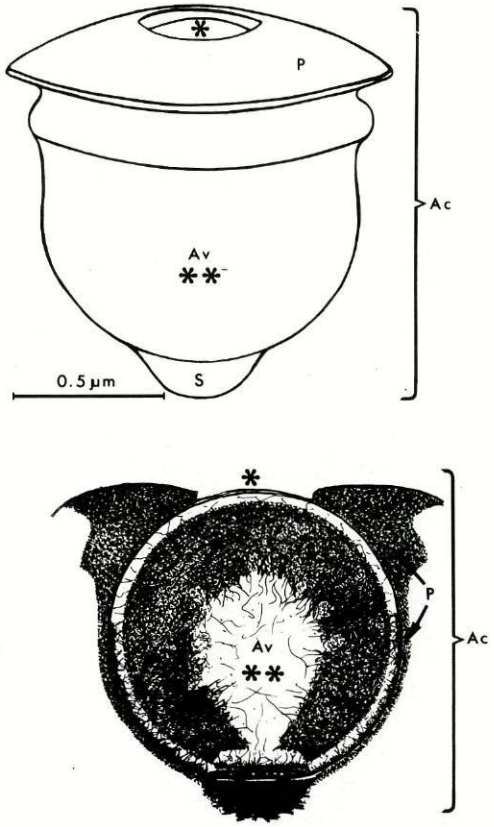
The tail is in continuity with the distal centriole and is projected in variable directions in relation to the longer axis of the sperm. It is about 59.9 μm long with a common 9 + 2 microtubular pattern with the central microtubular pair originating in the tail immediately below the termination of the tail's expansion of the pericentriolar complex (Figs. 2–4 and 11). We followed the tapering of the tail and observed that it is caused by the disappearance of the central pair and reduction in the number of the peripheral ones (Fig. 3).

Discussion

The sperm of *M. glacialis* is of the "primitive" type and belongs to the spheroidal spermatozoa of the *Echinodermata phylum.* Spheric head sperms of the Echinoderms do not present any relevant ultrastructural differences except in the acrosomal morphology. In this discussion we are going to deal only with this type of spermatozoa.

The acrosomal vesicle does not change in shape among asteroids, ophiuroids, holothuroids and crinoids, but it is extremely variable in its contents [9–11, 13, 16–21]. In our material the central zone of the vesicle communicates with the peripheral portion of the basal pole, which seems similar in *Asterias amurensis* [9], *Comanthus japonica* [16] and *Asterias forbesi* [10]. In *Luidia clathrata*, the central zone contacts apically with the most peripheral portion of the three concentric zones of the acrosomal vesicle [13]. Perhaps the variations in organization of the interior of the acrosomal vesicle are a way of receiving and communicating stimuli and interacting more rapidly and efficiently with the basal portions of the acrosome during the acrosomal reaction.

The periacrosomal material always seems identical in structure, varying in shape. In asteroids and crinoids the periacrosomal material is more or less arranged into apical, lateral and basal agglomerations, but in the other spheroidal sperms of the



Figs. 16 and 17. A three-dimensional model and a semischematic illustration of the acrosome complex (Ac), showing: acrosomal vesicle (Av) seen in the central (* *) and apical zones (*); periacrosomal material (P); subacrosomal fossa (S).

phylum, this material is much less subdivided appearing unique and/or larger [10,11,16].

In our study, the ultrastructural organization of the basal specializations in the acrosomal vesicle has been elucidated. This structure seems typical of sea stars and may exist, with some differences, in other classes [11,16,18]. In the subacrosomal fossa, the dense material looks like lamellae or fibers that are parallel to each other and orthogonal to the sperm axis. This mode of organization is common to starfishes whose ultrastructure has been studied, with the exception of *Pisaster ochraceus* where the material is made up of small dense granules arranged in rows [12].

In this study we show the relationships between the components of the midpiece, which perhaps function as supporting elements [22] and as carriers and transformers of energy [23, 24]. The apparent connection of the outer nuclear membrane to the proximal centriole is interesting. We still do not know if it has a role in pronuclear movements, attachment of the centrioles or some other function. In Fig. 13, we propose a modification to the model of the pericentriolar complex [23] in that the primary arms do not bifurcate, but instead divide into several secondary branches. We also show the connections of this complex with the mitochondrion and the plasmalemma.

In Figs. 16 and 17, we present a model of the acrosomal complex that perhaps represents and unifies information provided by other members of Asteroidea [9–11,13], although there are minor varations and exceptions that must have some importance in adaptation and phylogeny of this class.

Acknowledgements

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MÁRIO SOUSA and CARLOS AZEVEDO

FINE STRUCTURAL STUDY OF THE ACROSOME FORMATION IN THE STARFISH *MARTHASTERIAS GLACIALIS* (ECHINODERMATA, ASTEROIDEA)

Keywords: Asteroidea, spermatogenesis, acrosome formation

ABSTRACT. The fine structure of the spermatogenic cells in the starfish *Marthasterias glacialis* was studied regarding acrosome formation. The main finding in the spermatogenesis of *M. glacialis* is that the formation of the pro-acrosomal vesicles seems to be initiated in late spermatogonia. Small dense bodies resulting from the division of large granulofibrillar masses were also observed in the cytoplasm of late spermatogonia. During spermiogenesis the inner acrosomal vesicle membrane becomes coated first with dense materials originated from the cytoplasmic dense bodies and then with cisternae of endoplasmic reticulum. Both coating materials are incorporated in the periacrosomal space of the mature acrosome. Besides being involved in the genesis of the periacrosomal material, cytoplasmic dense bodies were also seen in close relationship with intercellular bridges and midpiece structures of spermatids. These findings are discussed in comparison with other echinoderm spermatogenesis.

Introduction

The structure and development of spermatozoa have been studied in several species of echinoderms (Longo and Anderson, 1969; Dan and Sirakami, 1971; Atwood, 1974; Bickell *et al.*, 1980; Yamashita, 1983; Buckland-Nicks *et al.*, 1984).

Starfish spermatozoon presents an acrosome which has different structural and functional components (Mabuchi and Mabuchi, 1973; Sousa and Azevedo, 1983, 1985, 1986), being a suitable model for studying the interaction between those different components and the oocyte at fertilization. Basically to any attempt in the evaluation of the processes initiated by the interaction of the oocyte and the sperm is a clear understanding of the ontogeny of each gamete. However, there has been no detailed fine structural investigation of the male germinal cells less mature than spermatids for any asteroid. Although the beginning of the acrosome formation in the asteroids is presumed to occur in the spermatids, the exact beginning is still uncertain, because only spermiogenesis has been observed (Dan and Sirakami, 1971; Janssen, 1984).

Therefore, the purpose of the present study is to describe the fine structure of the spermatogenesis of an asteroid with special reference to the formation of the different acrosome components.

Materials and Methods

Several specimens of *Marthasterias glacialis* were collected at the Portuguese intertidal North Atlantic coast. Small pieces of testis were fixed with 2·5% glutaraldehyde, washed, post-fixed with 2% OsO_4 and in some cases *en bloc* stained with 0·5% uranyl acetate (for 2 hr at 4°C, each). Several buffers were tried: 0·2 M sodium cacodylate, pH 7·4, with or without 7% sucrose; filtered sea water, pH 8; 0·2 M sodium cacodylate-filtered sea water (1:3, 1:1 or 3:1) with or without 2·5 mM $CaCl_2$; fixation with sodium cacodylate-filtered sea water, followed by washing and post-fixation with 1·25% sodium bicarbonate. The material was dehydrated in ethanol and embedded in Epon. Ultrathin sections were double stained with 3% uranyl

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acetate and lead citrate and observed in a JEOL 100CXII eletron microscope operated at 60 kV.

Results

The fine structure of the germinal cells of *M. glacialis* was found difficult to preserve and is very sensitive to the buffer system used. The mixture of 0·2 M sodium cacodylate with filtered sea water (1:3) produced the best results when compared with several others that were tried.

The general description of the spermatogenesis of *M. glacialis* is identical to the other echinoderm species, so only those aspects related with acrosomogenesis will be focused. The cytoplasm of spermatogonia contains two special components, the dense vesicles and the dense bodies (Fig. 1). Large dense vesicles are presumably the result of the fusion of smaller vesicles which are originated in the Golgi complex. At first, the small vesicles have an electron-lucent content but then acquire a dense core (Fig. 2). In the cytoplasm of early spermatogonia a few large granulofibrillar masses were seen in close contact with clusters of mitochondria (Fig. 3). Later, however, those masses divide into smaller dense bodies (Fig. 4). The cytoplasm of spermatocytes contains the same organella found in the spermatogonia but there is an accumulation of dense vesicles and small dense bodies (Figs 5, 6).

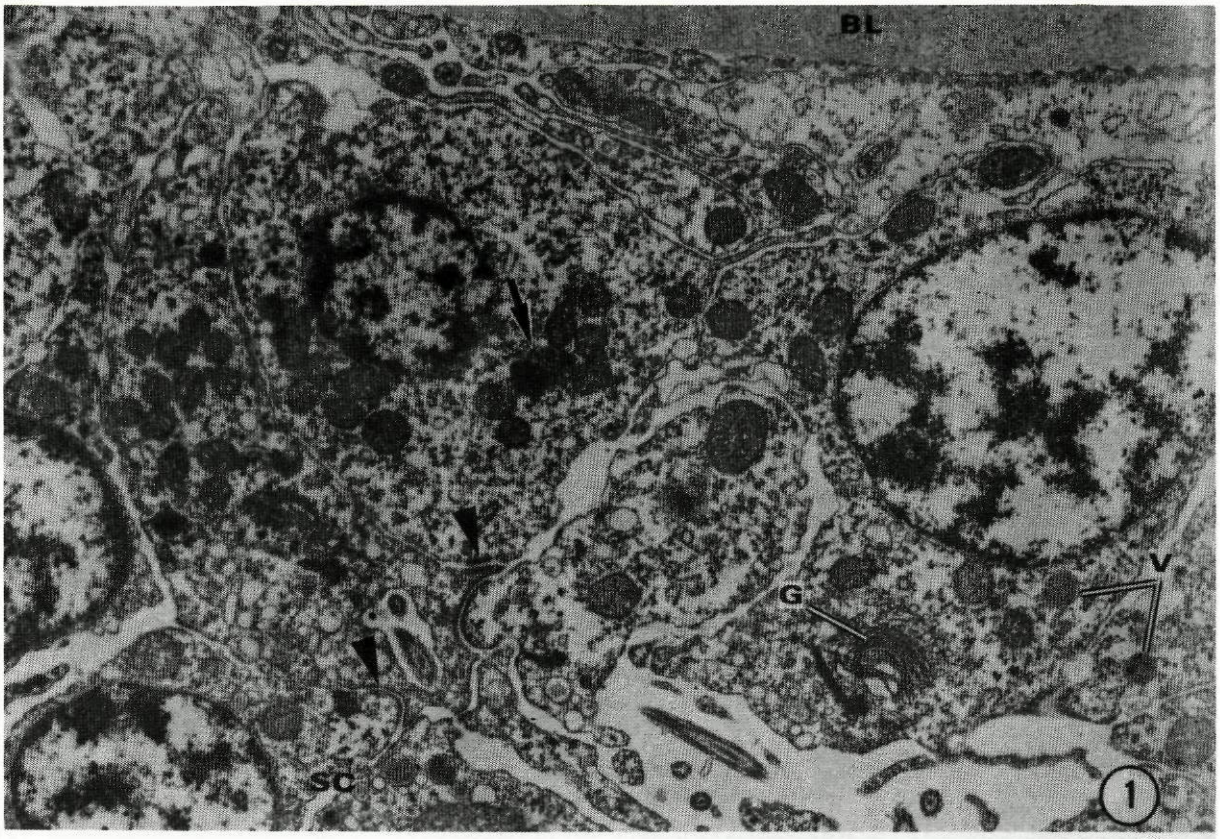
During spermiogenesis the dense vesicles disappear and one large acrosomal vesicle is formed. The contents of the acrosomal vesicle are fibrillar in appearance and segregate into an outer dense layer which surrounds a light core. At this phase, the outer side of the future inner acrosomal vesicle membrane becomes coated with a dense material which seems to be originated from the cytoplasmic-dense bodies (Fig. 7). At the acrosomal vesicle base it also formed the fibrillar material which corresponds to the developing actomere (Fig. 7). The inner acrosomal vesicle membrane with its dense coat is then surrounded by endoplasmic reticulum cisternae (Fig. 8). When the acrosomal complex arrives at the anterior region of the spermatid, the acrosomal vesicle sinks into the nucleus with its two coats (Fig. 9). At this phase, cytoplasmic-dense bodies are still observed in close relationship with the acrosomal vesicle base (Fig. 9). The cisternae of endoplasmic reticulum become lodged between the acrosomal vesicle base and the nuclear envelope as the nucleus invaginates further to receive the acrosomal complex (Fig. 10). When the apicolateral portions of the periacrosomal material appear, the acrosome condenses and the cisternae no longer become distinguishable (Fig. 11). Cytoplasmic-dense bodies were also seen in close relationship with the centrioles, the pericentriolar complex and the intercellular bridges of spermatids (Figs 11–13). Cytoplasmic dense bodies progressively decrease in number during spermiogenesis and finally disappear in late spermatids.

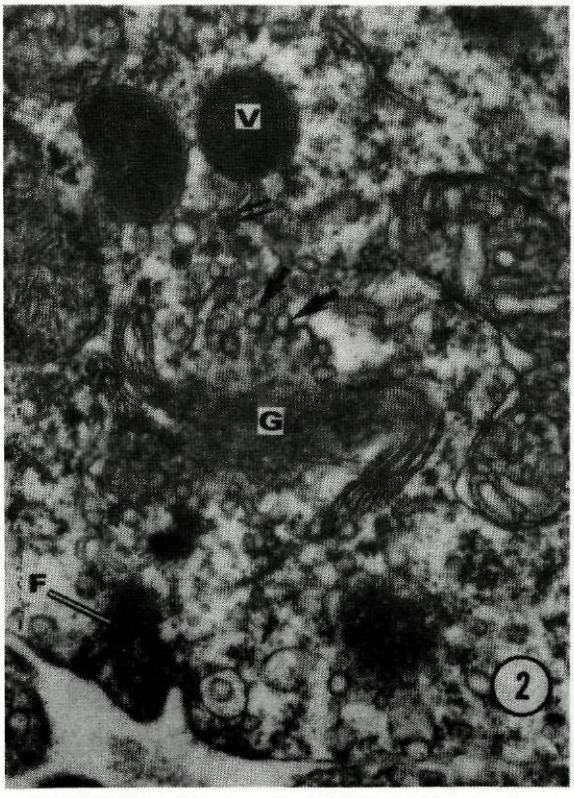
Fig. 1. Cross-section through the testicular wall, showing the basal lamina (BL), an early spermatogonium containing a large granulofibrillar mass (arrow) surrounded by mitochondria, and a late spermatogonium with a well-developed Golgi complex (G) and dense vesicles (V) in the cytoplasm. Primary spermatocytes (SC) and spermatogonia are connected by desmosome-like junctions (arrowheads). ×12,000.

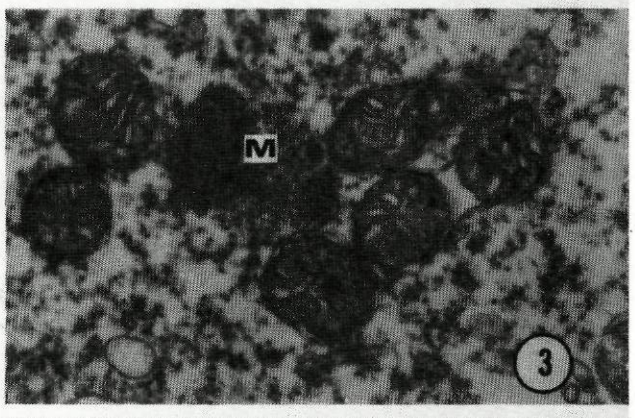
Fig. 2. Late spermatogonium. Small vesicles with light contents (arrows) are originated in the Golgi complex (G), then acquire a dense core (double arrow), and finally give origin to large dense vesicles (V). F, flagellum. ×31,000.

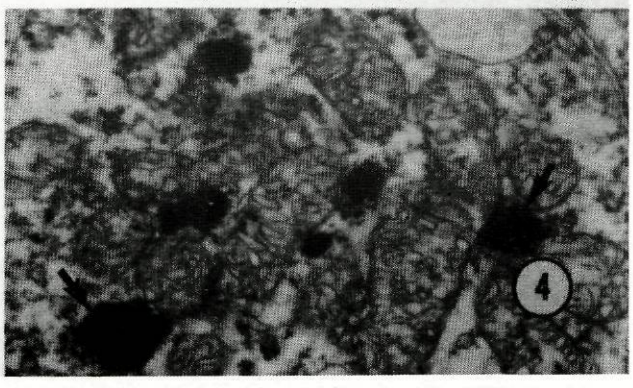
Fig. 3. Early spermatogonium, showing a large granulofibrillar mass (M) in close contact with a cluster of mitochondria. ×35,000.

Fig. 4. Late spermatogonium, showing small dense bodies (arrows) associated with a cluster of mitochondria. ×18,000.


BL
V
G
SC
1


V
G
F
2


M
3


4

Discussion

We studied all the spermatogenic steps of the starfish *M. glacialis* and two main original findings were evidenced: (a) starfish acrosomogenesis begins in late spermatogonia and not during spermiogenesis; (b) cytoplasmic-dense bodies seem to be storage structures, delivering materials to the periacrosomal material and probably to other cell organelles as well.

In starfishes, acrosome formation is believed to be initiated during the spermiogenesis (Dan and Sirakami, 1971; Janssen, 1984) but less mature stages have not been observed. In *M. glacialis*, cytoplasmic-dense vesicles originated from the Golgi complex were observed since late spermatogonia. Similar vesicles were also found in spermatocytes and spermatogonia of other echinoderm species and were interpreted as pro-acrosomal vesicles (Atwood, 1974; Pladellorens and Subirana, 1975; Bickell *et al.*, 1980; Yamashita and Iwata, 1983). In *M. glacialis*, the contents of the dense vesicles are rather different from those of the acrosomal vesicle, and as no fusion between dense vesicles were observed there is no conclusive evidence that they correspond to pro-acrosomal vesicles. However, as no other putative pro-acrosomal vesicles were found there is a strong probability that dense vesicles are in fact the precursors of the acrosomal vesicle. Immunocytochemical studies are now in progress to verify this hypothesis.

The acrosomal vesicle of starfishes was shown to be precociously surrounded by cisternae of endoplasmic reticulum and it was suggested that these were involved in the synthesis of the dense material which accumulates on the acrosomal vesicle base (Dan and Sirakami, 1971). In the spermatids of *M.*

Fig. 5. Primary spermatocyte, showing a synaptinemal complex (SC) in the nucleus, and several dense vesicles (V) and small dense bodies (arrow) in the cytoplasm. ×21,000.

Fig. 6. Primary spermatocyte, showing a cytoplasmic cluster of small dense bodies (arrows). ×41,000.

Fig. 7. Mid-spermatid. The outer side of the inner acrosomal vesicle membrane is coated by dense material (double arrows) which is similar to the associated cytoplasmic dense body (arrow). Some fibrillar material is also observed at the acrosomal vesicle (AV) base (arrowheads). ×108,000. Inset: Cytoplasmic dense body (arrow) associated with the acrosomal vesicle base. ×30,000.

Fig. 8. Mid-spermatid. An endoplasmic reticulum cisternae (arrowhead) surrounds the coated basal half (arrows) of the acrosomal vesicle (AV). ×43,000.

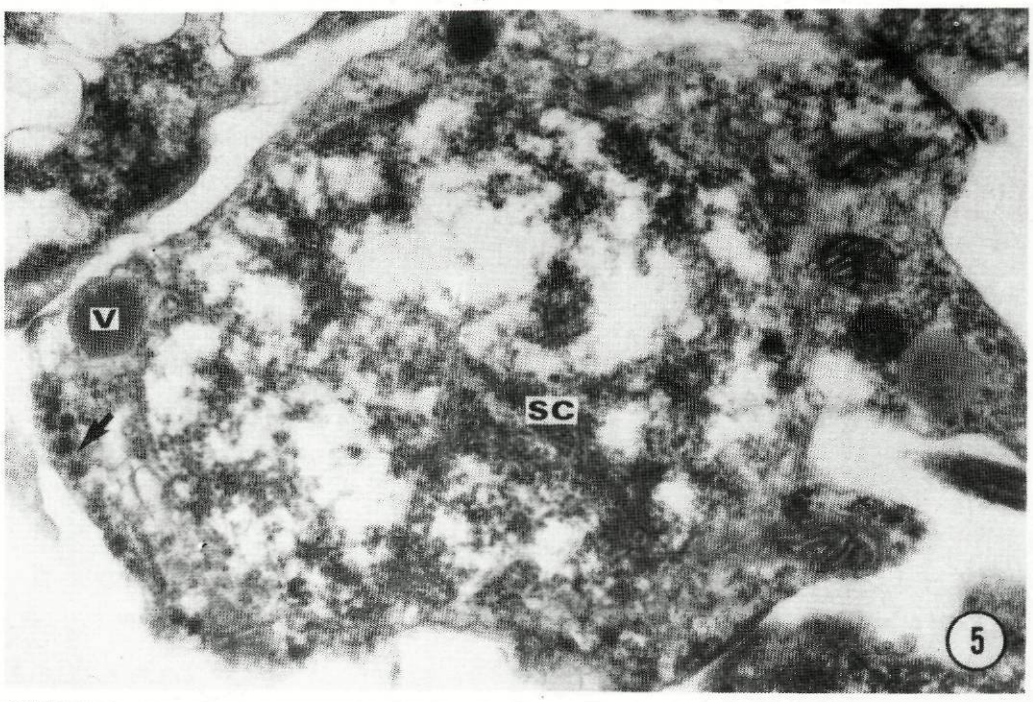
Fig. 9. Mid-spermatid. The acrosomal vesicle (AV) is beginning to sink into the anterior nuclear region (N). The basal half of the acrosomal vesicle is surrounded by some dense material (double arrows) and endoplasmic reticulum (arrowheads). A cytoplasmic-dense body (arrow) is observed in close relationship with the acrosomal vesicle base. ×46,000.

Fig. 10. Mid-spermatid. the acrosomal vesicle (AV) is completely surrounded by the nucleus (N). The endoplasmic reticulum cisternae (arrowheads) is observed between the coated acrosomal vesicle base (arrow) and the nuclear envelope (NE). ×46,000.

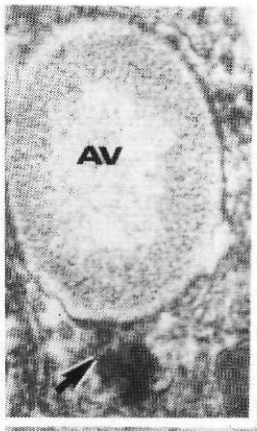
Fig. 11. Late spermatid, showing in the condensed acrosome the apicolateral portions of the periacrosomal material (arrowheads). At the nuclear base, a cytoplasmic-dense body (arrow) is interacting with both centrioles (C). F, flagellum; M, mitochondrion. ×24,000.

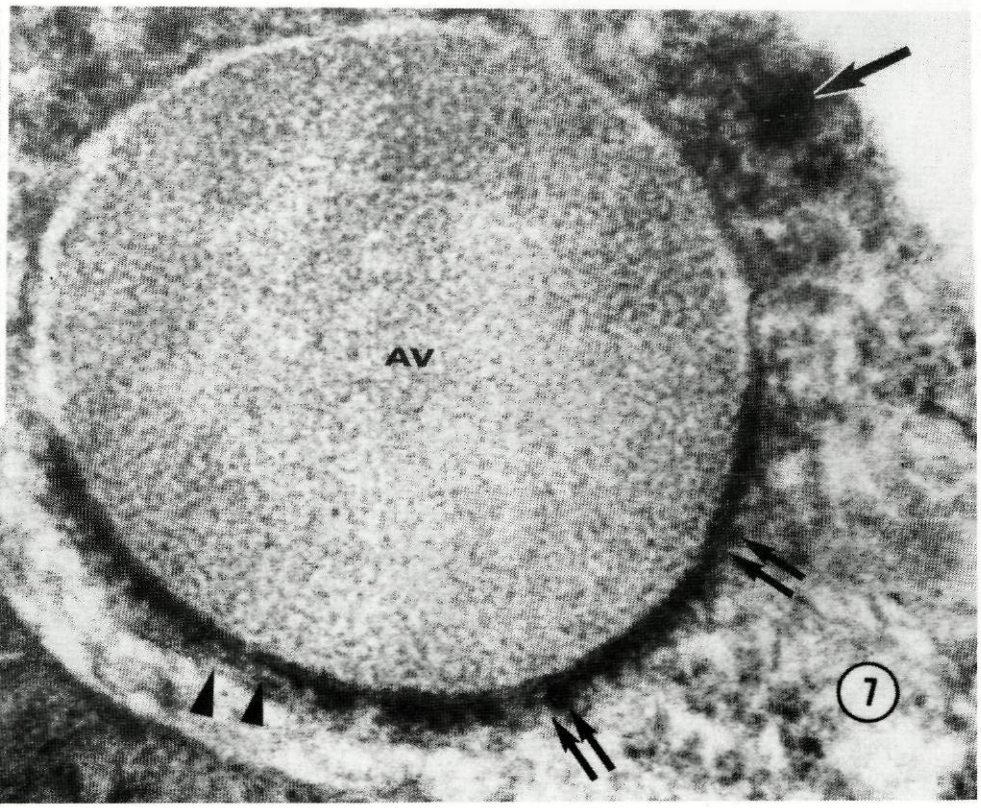
Fig. 12. Late spermatid, showing a cytoplasmic-dense body (arrow) associated with the distal centriole complex (DC). PC, proximal centriole; G, Golgi complex; F, flagellum. ×58,000.

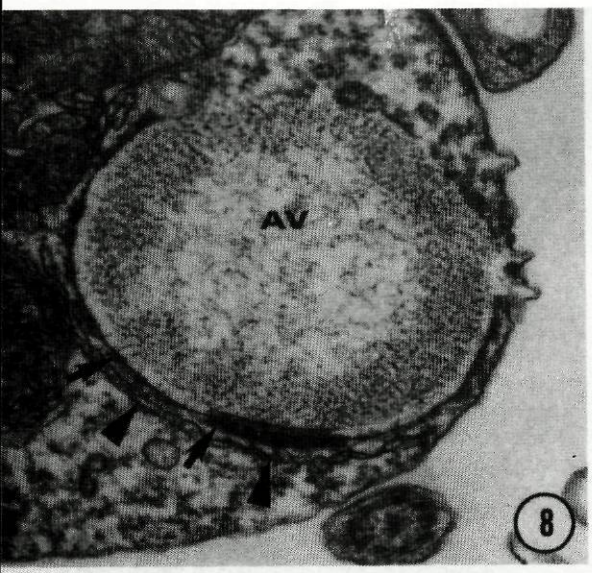
Fig. 13. Late spermatid, showing a cytoplasmic-dense body (arrowhead) in close relationship with the cytoplasmic-dense coat of an intercellular bridge (arrow). ×56,000.

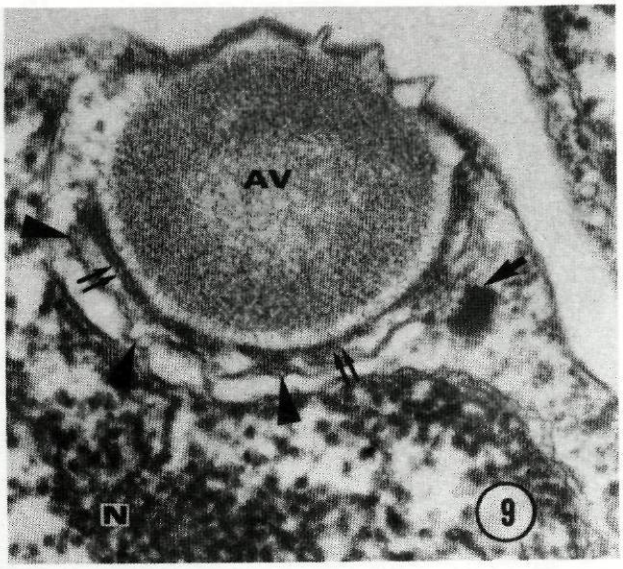

V
SC
5

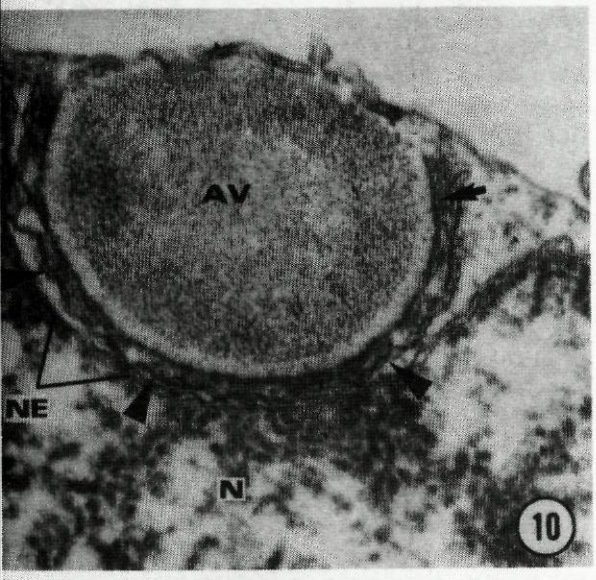

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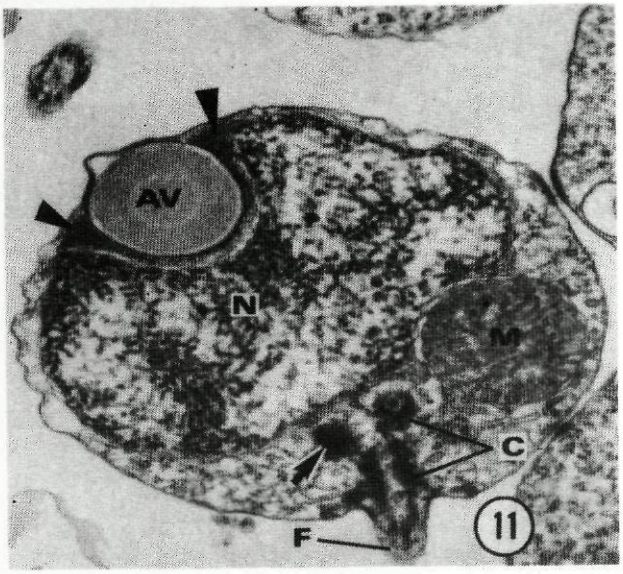

AV

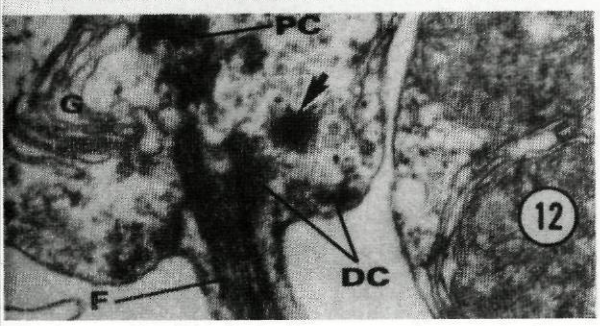

AV
M
7

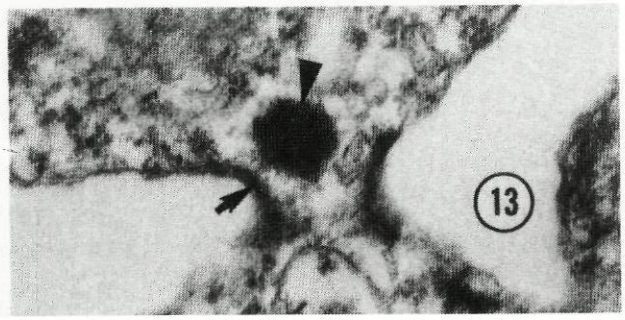

AV
8


AV
N
9


AV
NE
N
10


AV
N
M
C
F
11


PC
G
DC
F
12


13

glacialis the acrosomal vesicle is also surrounded by endoplasmic reticulum cisternae but it is our suggestion that these represent the membrane precursors of the acrosomal process membrane which were shown to be accumulated in the periacrosomal material (Hylander and Summers, 1975; Sardet and Tilney, 1977). Furthermore, we show that the subacrosomal material appears in advance of the cisternae and is formed from the cytoplasmic-dense bodies.

Cytoplasmic-dense bodies were described in spermatogonia of echinoids and ophiuroids (Longo and Anderson, 1969; Houk and Hinegardner, 1981; Yamashita and Iwata, 1983), in the spermatocytes of holothuroids (Atwood, 1974), and in the spermatids of asteroids (Dan and Sirakami, 1971). Similar structures were also seen near centrioles and intercellular bridges in the annelids (Bertout, 1976; Rice, 1981; Eckelbarger, 1984). Although several authors suggest that cytoplasmic-dense bodies are nuages, their origin and fate are unknown (Houk and Hinegardner, 1981; Rice, 1981; Eckelbarger, 1984). In *M. glacialis*, cytoplasmic-dense bodies were first observed in spermatogonia and seem to result from the division of 'nuage-like' materials. After that stage, their involvement in the formation of the periacrosomal material, their association with other cell organelles, and their progressive disappearance during spermiogenesis, suggest that they may be storage-structures containing materials that will be needed for the formation of those cytoplasmic organelles, but cytochemical studies are necessary to support further this hypothesis.

Acknowledgements

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Silver staining of spermatogenesis in the starfish *Marthasterias glacialis*

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Summary

Spermatogenesis of the starfish *Marthasterias glacialis* was studied by electron microscopy with the application of the silver nitrate method. In the nucleus, silver stained the nucleolus and specific regions at the periphery of chromatin. In the cytoplasm, silver deposits appear as argyrophilic bodies, on midpiece and tail structures, and on the dense materials which line the intercellular bridges and the inner acrosomal vesicle membrane.

Keywords: Asteroidea, spermatogenesis, silver staining

Introduction

Silver staining has been demonstrated in somatic mammalian cells to be due to an acidic, highly phosphorylated protein, C23, which seems to be involved with decondensed regions of chromatin (Hernandez-Verdun and Derenzini, 1983; Spector et al. 1984). The silver technique has also been shown to stain several nuclear and cytoplasmic cytoskeletal structures of germ cells (Czaker, 1985; Bertout, 1986; Azevedo et al., 1986; Amikura et al., 1987; Sousa and Azevedo, 1987; 1988 a,b; Azevedo and Oliveira, 1988).

In this ultrastructural study we show that silver nitrate stains cytoplasmic dense bodies as well as other components of the acrosome and midpiece. A possible relationship between the argyrophilic bodies and the other stained cytoplasmic structures is discussed. Results also evidence the strong similarity between the asteroid and echinoid spermatogenesis (Sousa and Azevedo, 1988b).

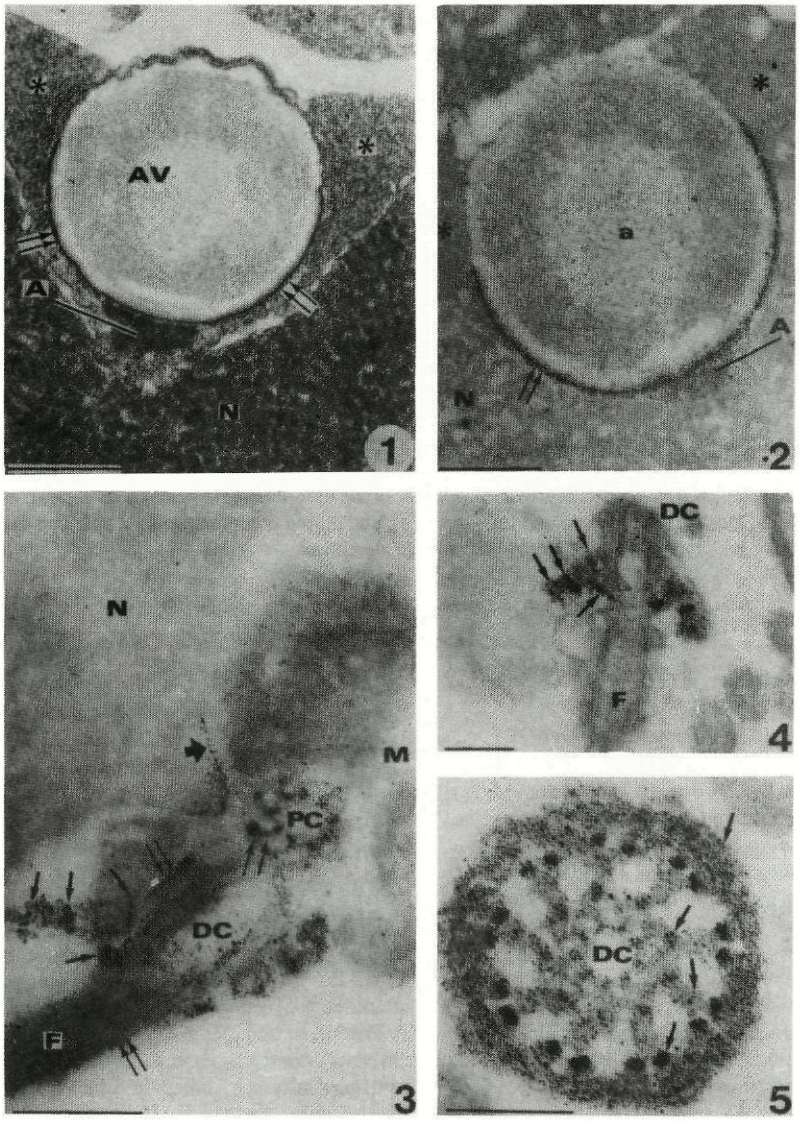
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Materials and Methods

Specimens of *Marthasterias glacialis* (Echinodermata, Asteroidea) were collected in the intertidal zone of the Portuguese North Atlantic coast. Small fragments of testis were fixed in 2.5% glutaraldehyde in filtered sea water / 0.2 M Na-cacodylate (3:1) for 2 h at 4°C, rinsed in the same buffer, postfixed with buffered 2% OsO_4 under the same conditions, dehydrated in graded ethanol series and embedded in Epon. Ultrathin sections were double contrasted with uranyl acetate and lead citrate.

For silver staining, pieces of testis were fixed and rinsed as above, postfixed in Carnoy's solution for 12 min, gradually rehydrated in ethanol and rinsed in distilled water for 30 min. They were then exposed to 70% $AgNO_3$ under a photoflood (2300 K bulb) for 20 min in such a way as to reach 55°C. Finally, the specimens were rinsed in distilled water, incubated in an ammoniacal silver-formalin solution (Goodpasture and Bloom, 1975) for 6 min, rinsed in distilled water, dehydrated in ethanol and embedded in Epon. Ultrathin sections were observed without contrast.

Grids were examined in a JEOL 100 CXII TEM operated at 60 KV.



Figs. 1–5. Mature sperm. Bar = 0.25 μm.
Fig. 1. The outer surface of the inner acrosomal vesicle membrane is coated by a thin dense layer (double arrows). A, actomere; (*), periacrosomal material; AV, acrosomal vesicle; N, nucleus.
Fig. 2. Silver stains the outer surface of the inner acrosomal vesicle membrane (double arrows). The light central component of the acrosomal vesicle (a) and the actomere (A) appear slightly stained. Periacrosomal material (*); N, nucleus.
Figs. 3–5. Silver stains the nuclear basal fossa (large arrow), the outer surface of centriolar and axonemal microtubules (double arrows), and specific regions of the pericentriolar complex (small arrows). N, nucleus; M, mitochondrion; PC, proximal centriole; DC, distal centriole; F, flagellum.

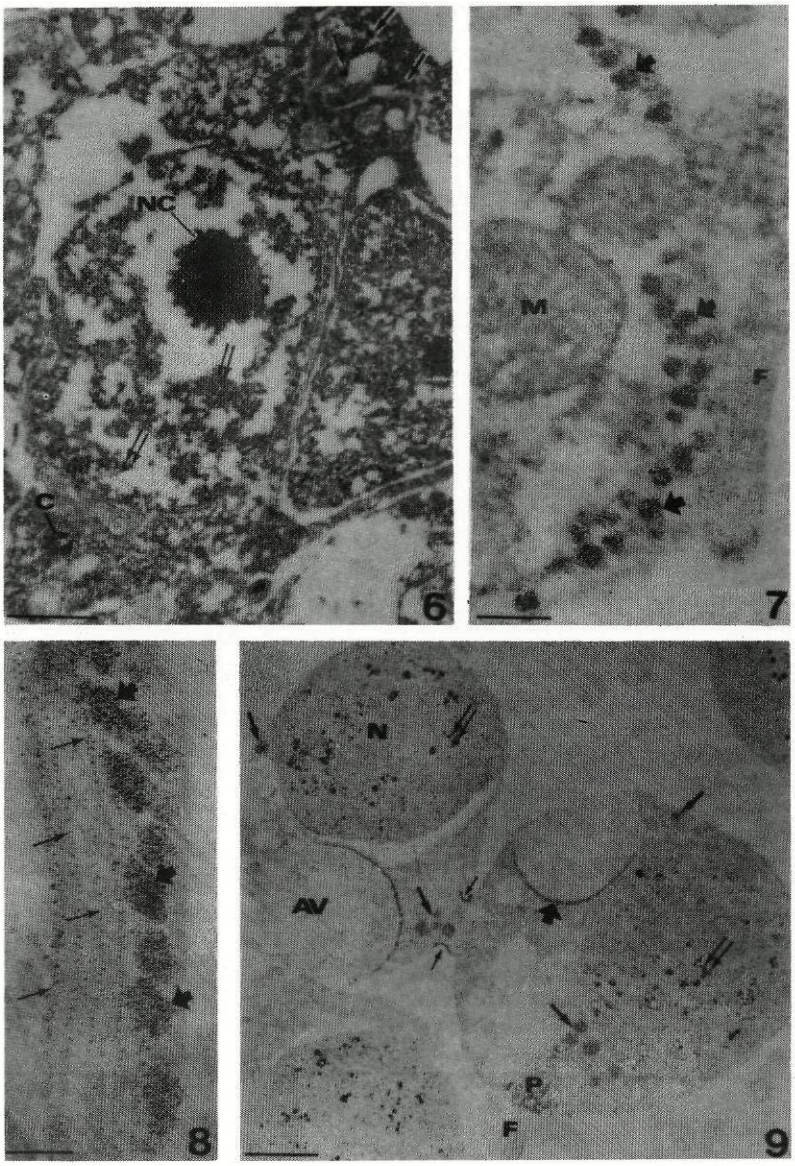


Fig. 6. Spermatogonium. Silver stains the nucleolus (Nc), specific sites at the heterochromatin periphery (double arrows), cytoplasmic dense bodies (arrows) and centrioles (C). Bar = 1 μm.
Fig. 7. Spermatocyte. Silver stains the cytoplasmic dense bodies (arrows). M, mitochondrion; F, flagellum. Bar = 0.25 μm.
Fig. 8. Spermatocyte. Silver stained cytoplasmic dense bodies (large arrows) interact with axonemal microtubules (arrows). Bar = 0.1 μm.
Fig. 9. Midspermatids. Silver granules at chromatin periphery accumulate in those nuclear spaces not yet condensed (double arrows). In the cytoplasm, silver stains the outer surface of the inner acrosomal vesicle membrane (large arrow), cytoplasmic dense bodies (arrows) and intercellular bridges (small arrows). AV, acrosomal vesicle; N, nucleus; F, flagellum; P, pericentriolar complex. Bar = 0.5 μm.

Results

In mature sperm, silver stains the dense material which lines the inner acrosomal vesicle membrane (Figs. 1,2), the nuclear basal fossa, the periphery of centriolar and axonemal microtubules, and the pericentriolar complex (Figs. 3–5).

In spermatogonia, silver deposits were found on the nucleolus, as argyrophilic patches scattered along the periphery of heterochromatin, on microtubular structures, and as cytoplasmic argyrophilic bodies (Fig.6). In the spermatocyte cytoplasm, argyrophilic bodies increase in number and were observed in close association with microtubules (Figs. 7,8).

During spermiogenesis, as chromatin condenses, argyrophilic granules at the chromatin periphery accumulate in those nuclear areas not yet condensed (Fig. 9). After being formed in the basal pole of the cell, and as it migrates towards the nuclear apex, the acrosomal vesicle becomes coated by some silver stained material at its basal half (Fig. 9). The dense cytoplasmic coat of intercellular bridges are also silver stained (Fig. 9). Argyrophilic bodies were seen in close association with the acrosomal vesicle, midpiece structures and intercellular bridges (Fig. 9).

Discussion

Results obtained in the present work with the silver staining technique support our previous structural observations concerning the relationship between argyrophilic bodies and other cytoplasmic structures during spermatogenesis in *M. glacialis* (Sousa and Azevedo, 1988c).

In *M. glacialis*, Ag-staining of cytoplasmic structures may actually be due to a common set of argyrophilic proteins. The morphological association between argyrophilic bodies and other cytoplasmic structures in the acrosome and midpiece suggests that argyrophilic bodies may be primordial storage depots containing argyrophilic proteins that will be needed for the formation of other argyrophilic organelles. Very similar results were also recently demonstrated for sea urchin spermatogenesis (Sousa and Azevedo, 1988b).

Silver staining had already been used to study the mature sperm of another starfish, *Asterina pectinifera* (Amikura et al., 1987). It is interesting to note, however, that while silver did not stain the subacrosomal material in *A. pectinifera*, it stained the subacrosomal material in two other starfishes, *Asterias rubens* and *M. glacialis* (Sousa and Azevedo, 1988a; and this report).

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Acrosomal Reaction and Early Events at Fertilization in Marthasterias glacialis (Echinodermata: Asteroidea)

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When the spermatozoon of M glacialis contacts the mature oocyte jelly it adheres to it. Following this, there is a slight tumefaction of the acrosome, which is followed by the disruption of the apical acrosomal vesicle and cytoplasmic membranes. Acrosomal vesicle contents are liberated and spread along the outer surface of the oocyte jelly. Meanwhile, the acrosomal process begins to extend, penetrates all the jelly extension, then the vitelline layer, and finally contacts the cytoplasmic egg membrane. Nevertheless, the sperm cell continues lying at the outer border of the jelly. From the beginning of the acrosome reaction the dense and finely fibrillar subacrosomal material is connected, by some expansions, to the basal acrosomal vesicle membrane. Both nuclear and mitochondrial diameters have diminished.

Key words: acrosomal reaction, fertilization, Asteroidea

INTRODUCTION

There have been extensive works on Echinoderm artificial acrosomal reaction [Dan et al, 1964; Dan and Hagiwara, 1967; Hylander and Summers, 1975; Summers et al, 1975] but only in the Echinoidea and Holothuroidea classes was fertilization shown by electron microscopy [Summers and Hylander, 1974]. In the class Asteroidea, the work of Dan and Hagiwara [1967] is the latest information on acrosomal reaction. In the meantime, electron microscopy techniques have improved and we shed a new light on the early events occurring during acrosomal reaction in M glacialis, a member of the Asteroidea class, as well as on fertilization in these Echinoderms, which have a thicker outer jelly layer covering the mature oocyte. We also describe the relationship between the subacrosomal dense material and the basal acrosomal vesicle membrane before the reaction, which we consider worth discussing. The subject is also well developed by Tilney et al [1983].

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MATERIALS AND METHODS

Several specimens of M glacialis were collected at the Portuguese intertidal North Atlantic coast. The oocytes were still surrounded by the follicular cells, and so they were left for 20 minutes in continuously stirred filtered sea water (FSW), pH 8, until their spontaneous maturation, which is characterized by the rupture of follicular cells and germinal vesicle breakdown [Cloud and Schuetz, 1973; Hirai, 1976; Hirai et al 1981]. The spermatozoa were slightly centrifuged and stored at 4°C until being used (not longer than half an hour). Several samples of this sperm pellet were then diluted in FSW until an ideal density was obtained. All the mature oocytes were monofertilized and all of them developed synchronously. The insemination was effected by adding 2 ml of spermatozoa to 2 ml of mature oocyte solutions. Between 2 and 6 seconds after gamete mixing, 4 ml of 6% glutataldehyde in FSW was then added to each tube. The oocyte sediment with their spermatozoa at the bottom of the tubes, but with manipulation a great number of sperms were liberated in the supernatant. We isolated these supernatant spermatozoa by concentrating them as a sticky mass through filtration in filtering paper after glutaraldehyde fixation. Electron microscopy procedures are described in Sousa and Azevedo [1983], except that FSW was used as buffer. For studying mature spermatozoa, small pieces of testis were prepared as described in the above-cited paper. Ultrathin sections were examined in an electron microscope JEOL 100B operated at 80 Kv.

RESULTS

Acrosomal Reaction in Isolated Spermatozoa

The mature spermatozoon of M glacialis possess the typical ultrastructure of the rounded head sperms of the Echinodermata phylum (Fig. 1). The mitochondrial and nuclear contents show clearly visible crests in the former and a granular or chordal pattern in the latter. It is also important to note the dense material that fills the subacrosomal fossa (Fig. 2) [Sousa and Azevedo, 1983]. In the first phase of the acrosomal reaction there is a tumefaction or disarrangement of the acrosome and the establishment of some connections between the material of the subacrosomal fossa and the basal part of the acrosomal vesicle membrane (Fig. 3). Then, it is observed the disruption of the apical portion of the acrosomal vesicle and cytoplasmic membranes that proceeds with consequent vesiculation and ultimate loss towards the exterior of the cell (Fig. 4). After this the continuity of the cytoplasmic membrane with that of the acrosomal vesicle is evident on both sides of the rim of dehiscence as it is the densification of the apical portion of the periacrosomal material (Fig. 5). In the following steps there is the extrusion of the acrosomal vesicle contents and the progressive extension of the acrosomal process (Fig. 5–7). The acrosomal process is completely filled with microfilaments that are implanted in the subacrosomal fibrillar material (Figs. 5–7). The acrosomal vesicle contents do not seem to cover the acrosomal process but rather spread over the apical portion of the cell (Fig. 6). The flagellum of the reacted spermatozoon has not changed its position in relation to the principal cell axis (compare Fig. 1 with Fig. 7). The nuclear and mitochondrial transverse diameters varied, before the acrosomal reaction, between 2.1 and 2.2 μm but after the reaction they diminish to 1.6 μm. Therefore, there is a reduction in the diameters of these two organelles.

Acrosomal Reaction at Fertilization

The mature oocyte of M glacialis is spherical and coated with two external layers. The proximal or vitelline layer is denser and narrower (Fig. 14). The distal layer or jelly is 8–10 μm in thickness, has a very loose structure, and by routine electron microscopic preparations its detail is lost (Fig. 15). The oocyte cytoplasmic surface is very rich in small microvilli that penetrate through the vitelline layer nearly up to its external surface (Fig. 8). The oocyte cortex also contains numerous cortical granules (Fig. 8, 9).

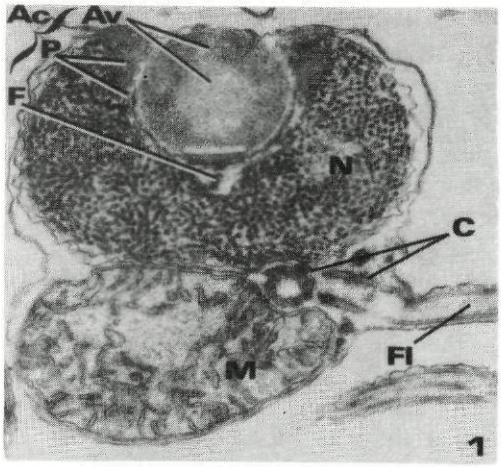
The spermatozoa swim vigorously until they collide with the oocyte jelly in which they stick (Fig. 8). Immediately thereafter they begin the apical membrane rupture and vesiculation (Figs. 9–11). Then the acrosomal vesicle contents are progressively expelled and spread along the outer surface of the oocyte jelly at the point of sperm attachment (Figs. 12–15). At the same time the extension of the acrosomal process begins, progressively traversing all the jelly thickness and then the vitelline layer; the sperm, however, continues at the outer border of the jelly (Figs. 12–15). The contact between the acrosomal process and the vitelline layer suggests that penetration uses some kind of lysis, because at the connection site the vitelline layer is thinner (Fig. 16). Also, until this moment there is not a complete eversion of the remmant acrosomal membrane.

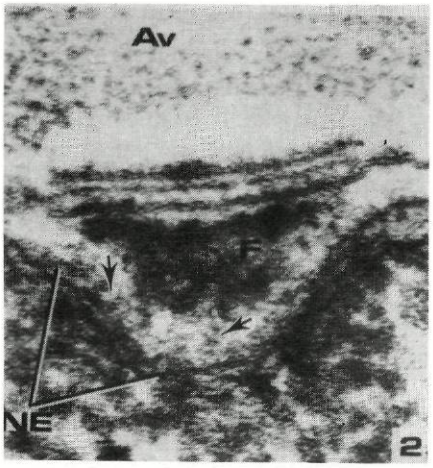
DISCUSSION

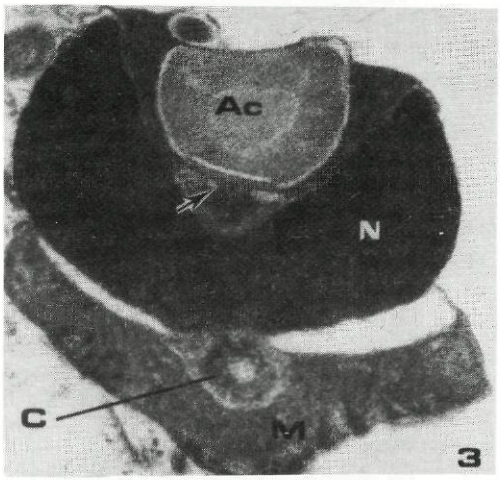
This is the first investigation on fertilization at ultrastructural level in the class Asteroidea. We have shown that the spermatozoon of M glacialis swimming vigorously collides with the oocyte jelly to which it adheres. After that, it initiates an acrosomal swelling [as it was predicted for Thyone by Inoué and Tilney, 1982] which is followed by the progressive membrane fusion of the apical acrosomal vesicle and cytoplasmic membranes, a phenomenon which takes the form of vesiculation. In echinoids apical membrane dehiscence begins at a unilateral site and the fused complex is moved aside, resulting in exposure of the acrosomal vesicle contents. Later, the remmant of the apical membrane is lost [Dan et al, 1964; Summers and Hylander, 1974]. In Thyone and Saccoglossus membrane fusion is not shown but some vesicles were seen at the sperm apical region [Colwin and Colwin, 1963; Colwin et al, 1975]. In ophiuroids, Hydroides, and in bivalve molluscs the apex of the vesicle membrane fuses centrally with the overlying plasmalemma. The portions of these membranes remaining lateral to the point of fusion form a membranous collar around the opening. The edge of this collar may undergo vesiculation, since vesicles are observed in proximity to it [Colwin et al, 1957; Hylander and Summers, 1975, 1977]. In Asterias it was shown a membrane vesiculation in all the acrosome apical region [Dan and Hagiwara, 1967]. In this work we show that in Marthasterias there is an initial unilateral fusion followed by the total apical membrane vesiculation.

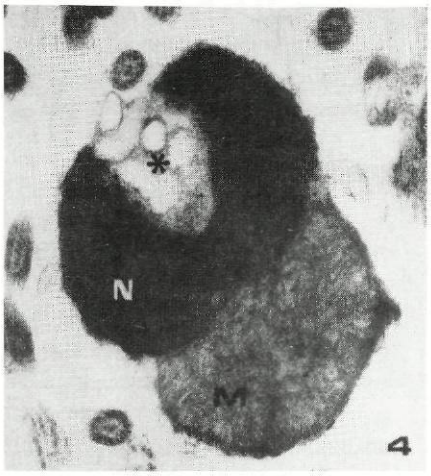
In our material, acrosomal vesicle contents are slowly exteriorized as was also shown in other asteroids [Dan and Hagiwara, 1967], in echinoids [Dan et al, 1964], in enteropneusta [Colwin and Colwin, 1963], and in holothuroids [Inoué and Tilney, 1982].

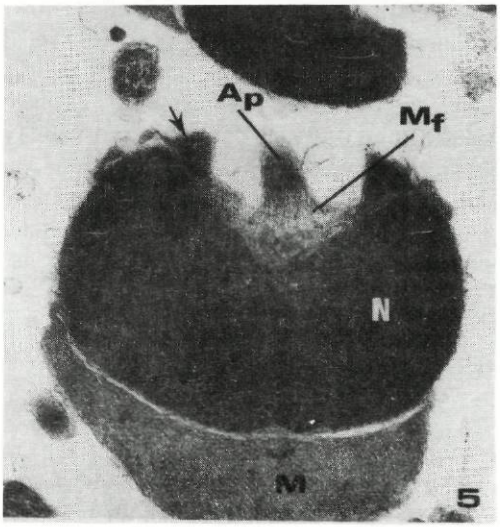
Concerning the destiny of the acrosomal vesicle contents, it is noted in echinoids they stay attached to the sperm head and as the acrosomal process extend they cover it continuously till gamete fusion [Summers and Hylander, 1974]. In holothuroids,

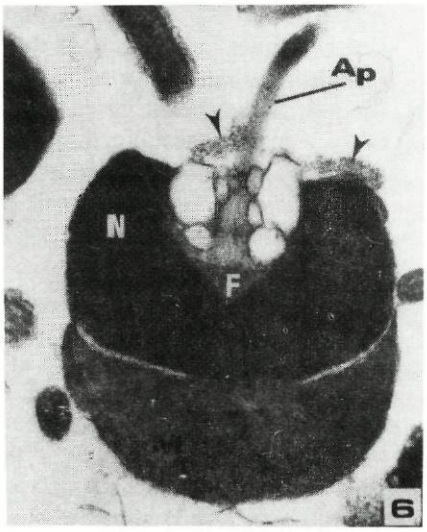

Ac
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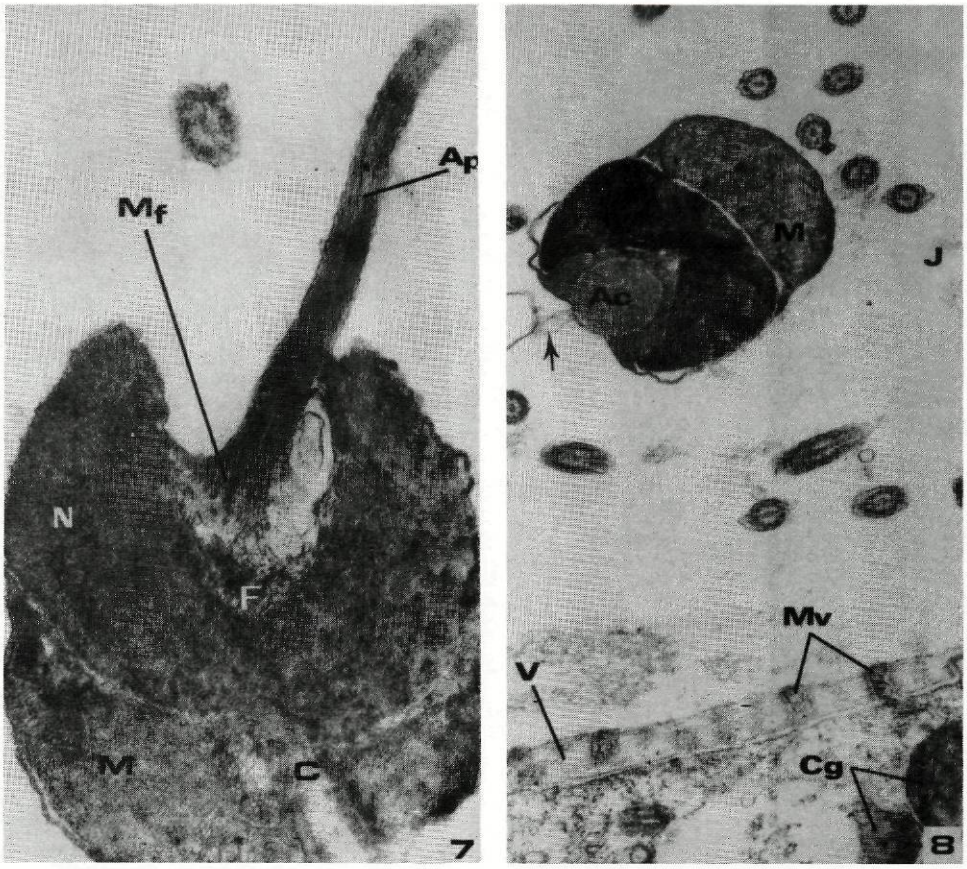

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Figs. 1, 2. Mature sperms observed in testis.

Fig. 1. The head of the spermatozoon is composed of the acrosome (Ac) and the nucleus (N). The acrosome contains an acrosomal vesicle (Av), a periacrosomal material (P) and a subacrosomal material (F). The midpiece is filled with an annular mitochondrion (M) and two centrioles (C). The flagellum (Fl) arises from the distal centriole. ×20,000.

Fig. 2. Detail of the subacrosomal material (F). It is dense, finely fibrillar, and tightly parallel to the basal acrosomal vesicle membrane (Av). It is also connected to the nuclear envelope (NE) by some expansions (arrows). ×151,200.

Figs. 3–7. Acrosomal reaction in isolated sperms.

Fig. 3. Initial phase of acrosomal reaction. There is swelling of the acrosome (Ac) that deforms the normal morphology (compare with the regular form in Fig. 1), and the establishment of connections between the subacrosomal material and the basal vesicle membrane (arrow). ×34,800.

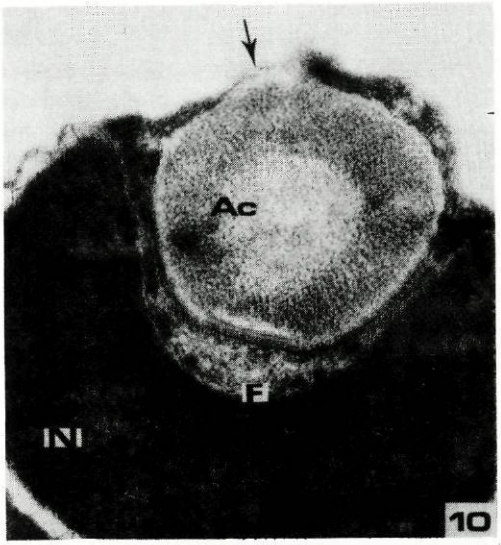
Fig. 4. Progressive vesiculation (*) as a consequence of the fusion of the apical cytoplasmic and acrosomal vesicle membranes. The cell is shown in a very tangential section, which leads to no visualization of the acrosomal vesicle contents. ×24,000.

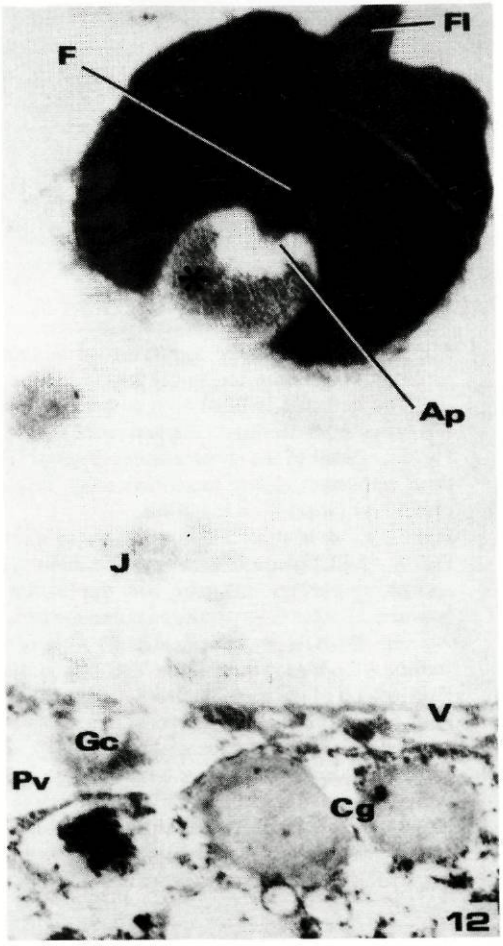
Figs. 5–7. The acrosomal process (Ap) grows progressively by polymerization of microfilaments (Mf) that arise from the subacrosomal material (F). In Figure 6, the exteriorized acrosomal contents surround the apical region of the sperm (arrowheads). ×35,300, ×25,200, and ×40,000, respectively.

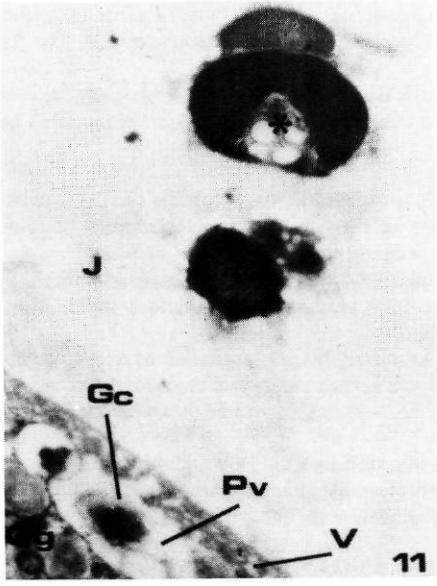
Figs. 8–16. Initial fertilization events.

Fig. 8. A spermatozoon has attained the outer surface of the oocyte jelly (J) and seems to be initiating the acrosome reaction (arrow) although there is no clear membranar disruption. Vitelline layer (V), microvilli (Mv), and cortical granules (Cg) are also shown. ×19,300.


M
Fl
Ac
J
V
Cg
9


Ac
Fl
N
10


Fl
F
Ap
J
V
Gc
Pv
Cg
12


J
Gc
Pv
V
11

ophiuroids, and asteroids, acrosomal contents spread over the sperm head and over the outer surface of the oocyte jelly [Colwin et al, 1975; Hylander and Summers, 1975, and this paper]. No evidence is shown about the acrosomal process covering by this acrosomal vesicle material except when the ruthenium red technique was used [Hylander and Summers, 1975]. In holothuroids, after being discharged over the jelly surface, the acrosomal vesicle contents rapidly disappear [Colwin et al, 1975]. In enteropneusta, acrosomal contents gradually disappear as they are dispersed over the oocyte jelly [Colwin and Colwin, 1963]. In bivalve molluscs the apical component of the acrosomal vesicle of Chama also disappears after acrosomal reaction but the basal ring of the vesicle is not dispersed, remaining closely adherent to the anterior sperm surface through the entire reaction. This basal ring also plays a role in gamete binding and fusion as it forms a morphological association with the fibrous tufts on the tips of the microvilli of the oocyte [Hylander and Summers, 1977]. In this paper we show that acrosomal contents do not disappear immediately after the reaction, as they can still be seen 6 seconds after gamete mixing when the acrosomal process is already connected to the oocyte membrane.

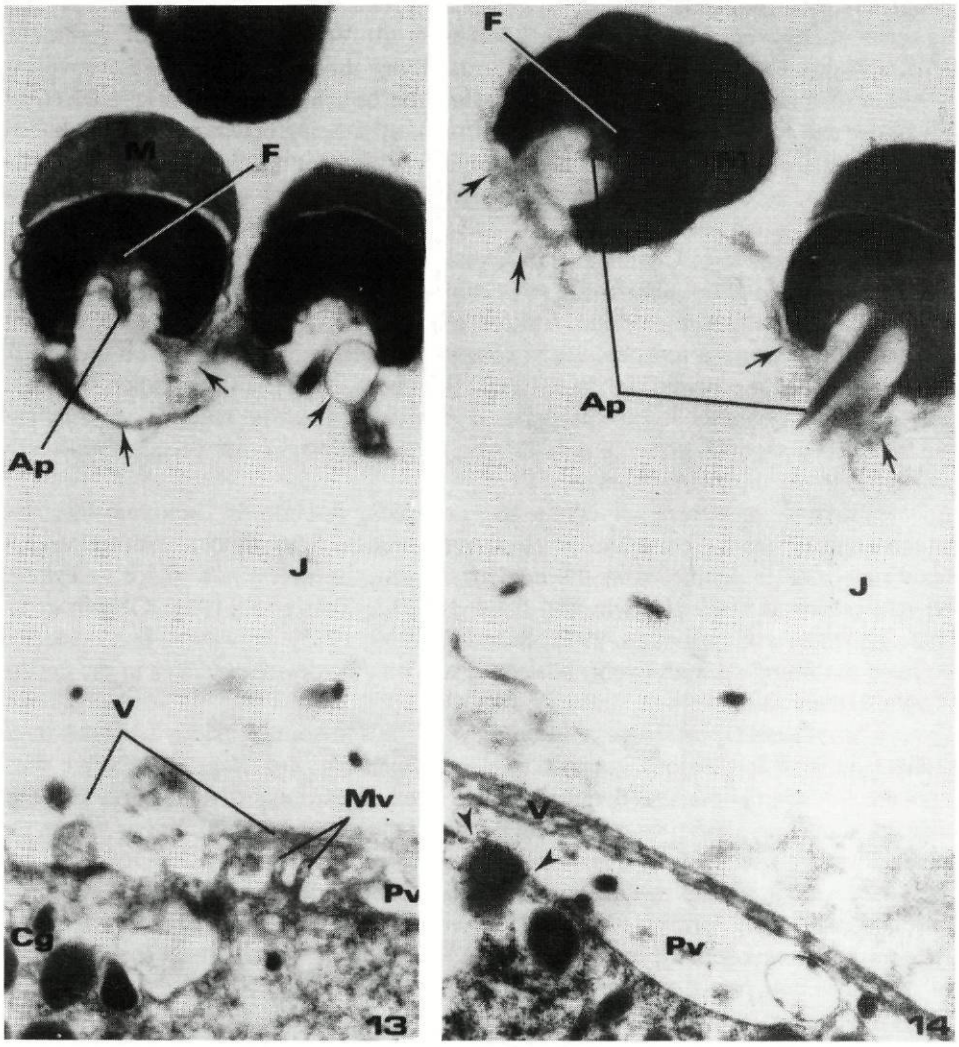
In several specimens of echinoids, asteroids, ophiuroids, holothuroids, and enteropneusta there is a complete eversion of the remaining or basal acrosomal vesicle membrane, and in echinoderms the nucleus acquires as well a new shape as it loses the subacrosomal fossa [Colwin and Colwin, 1963; Dan et al, 1964; Colwin et al, 1975; Hylander and Summers, 1975; Summers et al, 1975]. However, in M glacialis we have not found such an event, but perhaps it may be developed later in the course of sperm penetration.

In holothuroids, asteroids, and ophiuroids the acrosomal vesicle is lodged in an anterior nuclear depression. In echinoids, enteropneusta, annelids, and bivalve molluscs the acrosomal vesicle is over the nuclear apex. In echinoderms the periacrosomal material shows no special organization except in the subacrosomal region, where the actomere can be seen [as in Tilney's nomenclature: Tilney, 1978]. In enteropneusta no subacrosomal specialization could be found, but in some annelids and molluscs there are previously formed acrosomal processes, with or without preformed microfilaments. The acrosomal process extension seems to happen in those sperms which do not have any preformed process or any specialized oocyte extensions. In this aspect Hydroides seems nearer the mollusca species with nonextending acrosomal process, while the enteropneusta show more similarities with the echinoderms [Colwin et al, 1957; Summers et al, 1975; Hylander and Summers, 1975, 1977; Lewis et al, 1980, and this paper].

In some annelids, bivalve molluscs, and echinoids, the acrosomal vesicle contents are undoubtedly implicated in gamete binding [Colwin et al, 1957; Summers and Hylander, 1974; Hylander and Summers, 1977]. In nonechinoids, in abalone, and in enteropneusta sperms, the acrosomal vesicle contents are not clearly involved in gamete adhesion as they quickly disappear [Colwin and Colwin, 1963; Colwin et

Figs. 9–11. The apical cytoplasmic and acrosomal vesicle membranes begin to degenerate (arrow in Figs. 9 and 10) till complete vesiculation (*in Fig. 11). The perivitelline space (Pv) possesses some cortical granule contents (Gc). ×17,300, ×48,800, and ×12,200, respectively.

Fig. 12. When acrosomal vesicle contents (*) are almost at the outside, the acrosomal process (Ap) formation begins (as it can also be seen on the left side cell in Fig. 14, where the apex membrane of the acrosomal process is clearly seen). ×30,500.



Figs. 13–15. Despite the progressive oocyte jelly penetration by the acrosomal process (Ap), the sperm and its acrosomal vesicle contents (arrows) remain at the outer surface of the jelly (J). The perivitelline space (Pv) contains cortical granules contents (Gc) which are derived from the exocytosis (arrowheads) of the cortical granules (Cg). In this place the vitelline layer (V) is elevated from the oocyte cytoplasmic surface. ×18,800, ×17,400, and ×12,000, respectively.

al, 1975; Sakai et al, 1982]. However, we have shown that in Marthasterias the acrosomal contents remain at the region where the sperm adheres to the oocyte jelly.

In Hydroides and Saccoglossus some pits were observed in the oocyte external coats in relation to sperm penetration, and they were suggested as being of a lytic origin [Colwin et al, 1957]. In bivalve molluscs, Hylander and Summers [1977] have not observed any morphological alteration of the vitelline envelope in proximity to reacted sperms that could suggest lytic activity. However, it is suspected that the apical component of the acrosomal vesicle is lytic in nature. In the prosobranch Haliotis (Archaeogastropoda), the oocyte envelopes are dissolved after total release of the acrosomal substance [Sakai et al, 1982]. In echinoids a lytic component in the

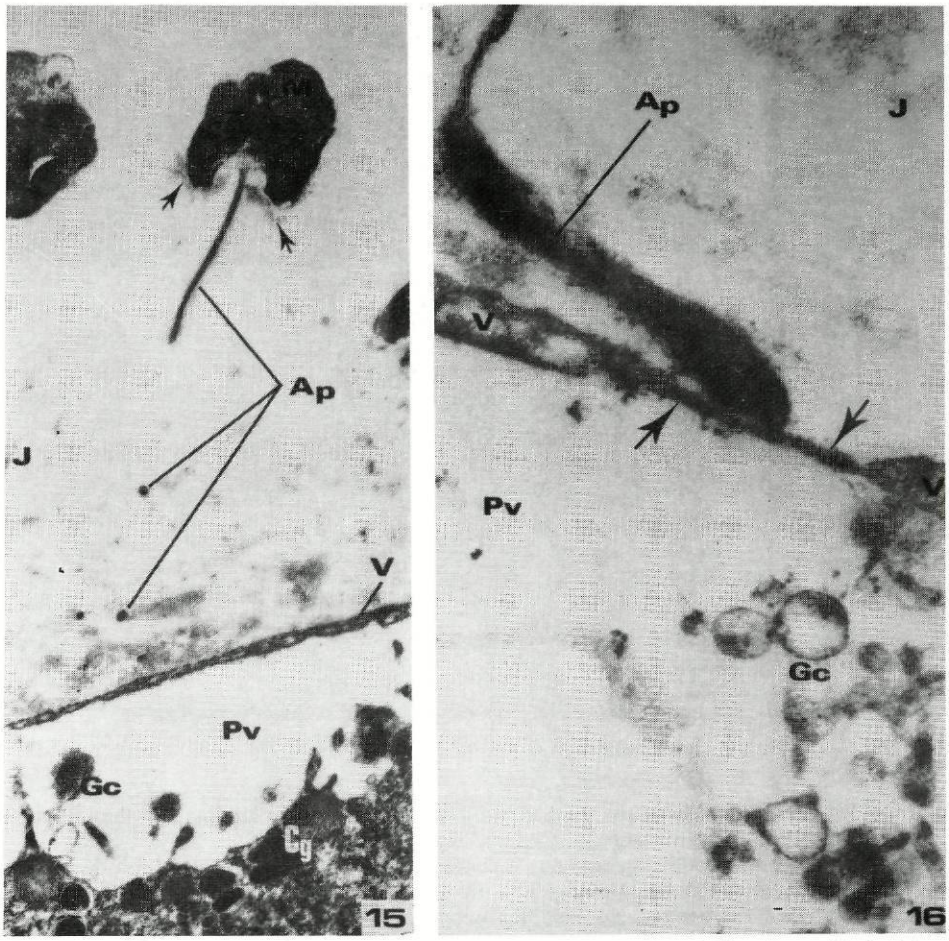


Fig. 16. At the contact region with the acrosomal process (Ap) the vitelline layer (V) is thinner (arrows). ×44,300.

acrosomal granule was demonstrated [Anderson, 1968]. In our material, we have shown that the vitelline layer is thinner at the contact region with the acrosomal process, probably also as a result of lytic action. In Marthasterias we have found that both nucleus and mitochondrion show reduced dimensions after acrosomal reaction. We have also shown that the actomere connects to the basal portion of the acrosomal vesicle membrane by means of several expansions. As this event is observed for the first time and as we do not have any micrograph to show if this attachment will persist, we can not now draw conclusions concerning its importance in the discussion of microfilaments polymerization.

In Figure 17 a summary is shown of the acrosome reaction at fertilization in M glacialis.

ACKNOWLEDGMENTS

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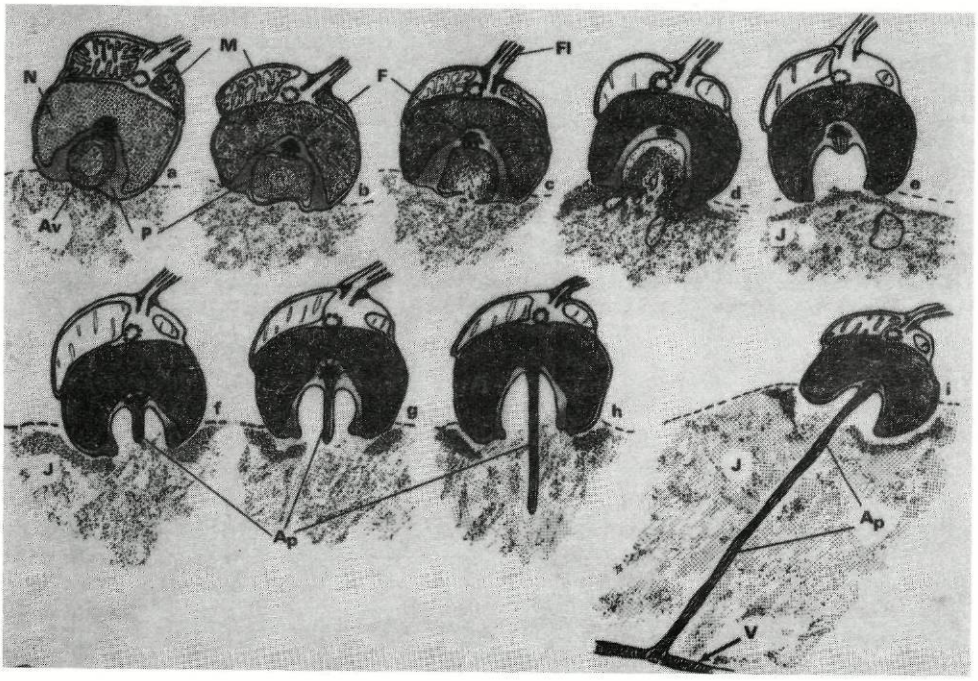


Fig. 17. A schematic illustration summarizing the early events in M glacialis fertilization.

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Original article

Cytochemical study on the spermatozoon and at early fertilization in *Marthasterias glacialis* (Echinodermata, Asteroidea)

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Using cytochemical methods we have detected, at the ultrastructural level, an ATPase activity in the peripheral component of the acrosomal vesicle in the mature spermatozoon of *Marthasterias glacialis*, but no acid phosphatase activity could be demonstrated in the acrosome. Early in fertilization, we showed that the acrosomal process was externally coated by acid phosphatase, as a positive reaction for its activity could be followed on the acrosomal process path through the external layers of the oocyte. These results suggest that the starfish spermatozoon has a lytic enzyme whose function cannot be detected till the beginning of the acrosomal reaction, perhaps because it is stored in an inhibited state.

Asteroidea — cytochemistry — acrosome — fertilization

INTRODUCTION

The acrosomal vesicle is extraordinarily variable in shape and in the morphological appearance of its contents, but it exhibits a common way of reacting and functioning [3, 13, 19, 20, 23, 24, 26, 27, 29].

The acrosomal vesicle has been shown to contain various lytic enzymes [2, 5, 12, 14, 15, 17, 21, 25], ATPases [16] and a binding protein named bindin [28].

It has been suggested that the morphological compartmentalization of the acrosomal vesicle contents is related to different functional activities, but this has never been demonstrated [6, 8, 13, 19, 29].

In starfishes, there is a morphological differentiation of acrosomal vesicle contents [7, 11, 22, 24]. Also, and contrarily to what was demonstrated in sea urchins [2], it has been shown that the acrosome of sea stars does not possess any acid phosphatase activity [16]. In this work we present morphological evidence which suggests that different enzymatic activities are located in distinct acrosomal vesicle components.

MATERIALS AND METHODS

Electron microscopy procedures are described elsewhere [22]. Handling of gametes for fertilization experiments follows the methods described previously [23].

Acid phosphatase activity was determined by the method of Paavola [18]. Material was pre-fixed with 2.5 glutaraldehyde in 0.067 M Na cacodylate, pH 7.2, for 2 hr at 4°C, washed in 0.1 M Na cacodylate, pH 7.2, 5% sucrose, for 2 hr at 4°C, and pre-incubated in 40 mM Tris-maleate, pH 5, 5% sucrose, for 30 min at 4°C. Incubation medium consisted in 11.5 mM Na β-glycerophosphate and 2.4 mM lead nitrate in 40 mM Tris-maleate, pH 5, 5% sucrose. This was filtered and then allowed to reach 37°C before used. Incubation time was 40 min with or without continuous agitation. The material was then washed in 0.05 M acetic acid-acetate, pH 5, 7% sucrose, four times, 5 min each, at room temperature, and post-fixed with 1% OsO^4 in 0.14 M Palade buffer, pH 7.4, for 1 hr at 4°C. In some experiments, final washings were made with 40 mM Tris-maleate, pH 7.4, 5% sucrose, two times for 5 min each, followed by two other washes of 5 min each in 0.1 M Na cacodylate, pH 7.4, 5% sucrose. In this latter case, post-fixation was developed with 1% OsO^4 in 0.1 M Na cacodylate, pH 7.4. To study mature spermatozoa, very small pieces of the testis or isolated cells were used.

Calcium and magnesium-dependent ATPase activities were detected as in Mabuchi and Mabuchi [16], always using isolated cells. Spermatozoa were pre-fixed with 0.3% glutaraldehyde in filtered sea water for 30 min in an ice bath, washed twice in chilled 50 mM Tris-maleate, pH 7.8, dialysed against the same buffer solution for 1 hr at 4°C, and incubated at 25°C for 20 min in an incubation medium containing 4 mM ATP, 80 mM Tris-maleate, pH 7.8, 10 mM $CaCl_2$ or $MgSO_4$, and 0.2 mM $Pb(NO_3)_2$. Then, cells were washed in distilled water and post-fixed with 1% OsO_4 in 0.1 M Na cacodylate, pH 7.4.

For controls, samples were incubated without substrate. Ultrathin sections with material for the determination of acid phosphatase activity at early fertilization were observed with and

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without final contrast with uranyl acetate (3% aqueous solution) for 10 min and with lead citrate (Reynolds) for 5 min.

Ultrathin sections were cut with glass or diamond knives on an LKB 8800 ultrotome III and observed in a Jeol 100 B electron microscope operated at 80 KV.

The substrates used were sodium β-glycerophosphate from Merck and ATP disodium salt from Sigma.

RESULTS

The mature spermatozoon of *Marthasterias glacialis* is of the primitive type. In the head, a cup-shaped nucleus contains, in its apical indentation, the acrosome. The acrosome is constituted by periacrosomal material which surrounds an acrosomal vesicle. The midpiece is formed by an annular mitochondrion which encircles two centrioles. The pericentriolar complex and the axoneme project from the distal centriole. The relevant findings are the morphological compartmentalization of the acrosomal vesicle contents in peripheral and central regions, and also the fact that the central one is in direct contact with the basal specializations of the acrosomal vesicle [22].

Spermatozoa treated for cytochemical demonstration of acid phosphatase activity show a strong positive reaction in the nuclear envelope (~ 26% of 27 cells counted) and a diffuse weak reaction in the mitochondrion (~ 70% of 26 cells counted) (Fig. 1), but not in the acrosome. Occasionally, a positive reaction could be observed in the central component and at the apical zone of the acrosomal vesicle (Fig. 2). Controls show no lead phosphate deposits in any structure of the spermatozoon (Fig. 3).

A positive reaction for ATPase activity was found in the peripheral component of the acrosomal vesicle (in almost all cells), in the mitochondrion (~ 24% of 73 cells counted), in the centrioles and pericentriolar complex (~ 5% of 30 cells counted), and in the axoneme of spermatozoa (~ 56% of 32 cells counted) (Figs. 4—6). Calcium-dependent ATPase activity appears exclusively at the peripheral component of the acrosomal vesicle and with a greater density of lead phosphate deposits than Mg^{2+}-dependent ATPase activity. Magnesium-dependent ATPase was detected in the peripheral component of the acrosomal vesicle and the other structures listed above. No lead phosphate precipitates were found in the central region of the acrosomal vesicle or in the subacrosomal fossa. In controls, no lead phosphate deposits were seen in the spermatozoon structures (Figs. 7 and 8).

At gamete binding, the apical zone of the spermatozoon contacts the outer surface of the oocyte jelly (Fig. 9). Immediately thereafter, the spermatozoon begins the acrosomal reaction with the fusion of the apical cytoplasmic membrane and the apical portion of the acrosomal vesicle membrane. This elicits the exocytosis of the acrosomal vesicle contents. While the acrosomal process extends through the external oocyte layers, the spermatozoon remains at the outer border of the oocyte jelly, maintaining its adherence to the oocyte by the peripheral component of the acrosomal vesicle (Fig. 9). After acrosomal reaction, the central component of the acrosomal vesicle is no longer visible. In some zones, the oocyte jelly has lost its structure, especially at the spermatozoon contact zone and along the penetration path made by the acrosomal process (Fig. 9).

At gamete binding, observing material without a final contrast, a positive reaction for acid phosphatase activity could be seen in the exteriorized contents of the acrosomal vesicle and along the acrosomal process path through the external

FIGURE 1. — Acid phosphatase activity in an unreacted sperm prefixed in a small piece of testis. Deposits of lead phosphate are seen in the nuclear envelope (NE) and in the mitochondrion (Mi), but not in the acrosome (Ac). N, nucleus; Cm, cell membrane. Uncontrasted section; × 17,400.

FIGURE 2. — Acid phosphatase activity in an unreacted sperm prefixed as isolated cell. Deposits of lead phosphate are seen in the central (c) and in the apical *(arrows)* regions of the acrosomal vesicle. N, nucleus. Uncontrasted section; × 44,700.

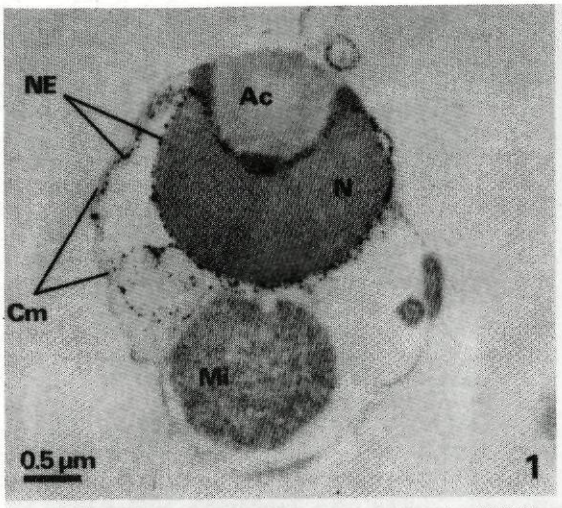
FIGURE 3. — Control for acid phosphatase activity. There are no lead phosphate deposits. Ac, acrosome; N, nucleus; Mi, mitochondrion; Cm, cell membrane. Uncontrasted section; × 28,300.

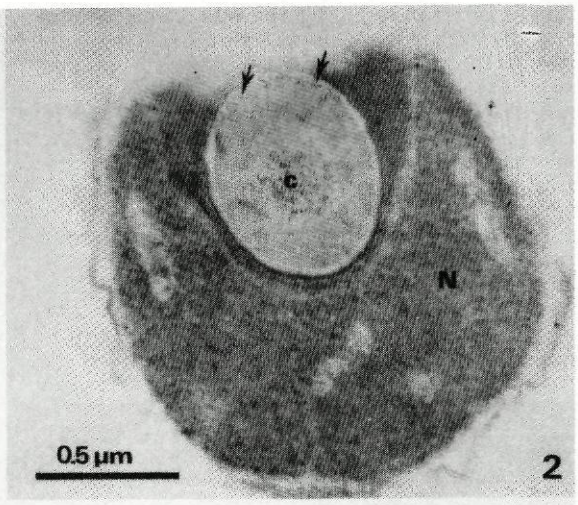
FIGURE 4. — Mg^{2+}-dependent ATPase activity. Lead phosphate deposits are found in the peripheral region of the acrosomal vesicle (p) and in the mitochondrion (Mi), but not in the central region of the acrosomal vesicle (c) and in the subacrosomal fossa (F). N, nucleus. Uncontrasted section; × 21,500.

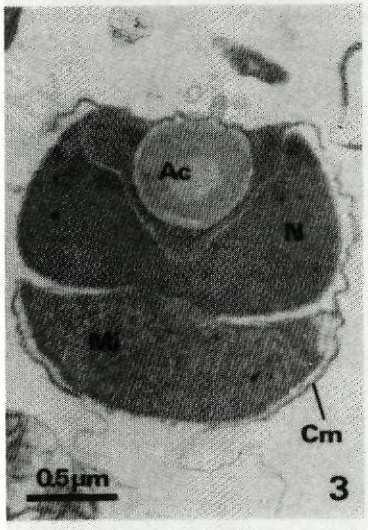
FIGURE 5. — Mg^{2+}-dependent ATPase activity. Deposits of lead phosphate can be seen in the distal centriole (Cd), along the pericentriolar complex *(arrows)* and delimiting the microtubules of the flagellum (Mt). Uncontrasted section; × 36,000.

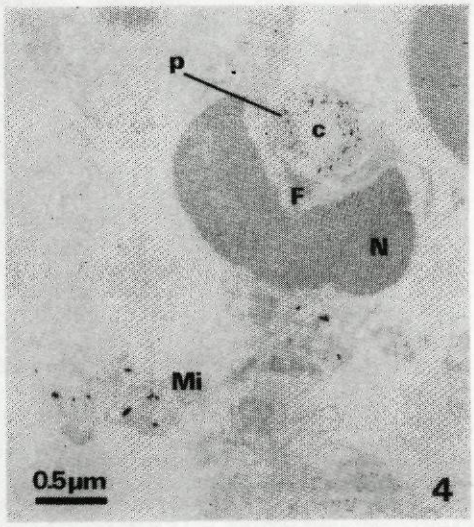
FIGURE 6. — Ca^{2+}-dependent ATPase activity. Lead phosphate deposits are exclusively found in the peripheral region of the acrosomal vesicle (p). N, nucleus; c, central region of the acrosomal vesicle; Mi, mitochondrion; F, subacrosomal fossa. Uncontrasted section; × 31,500.

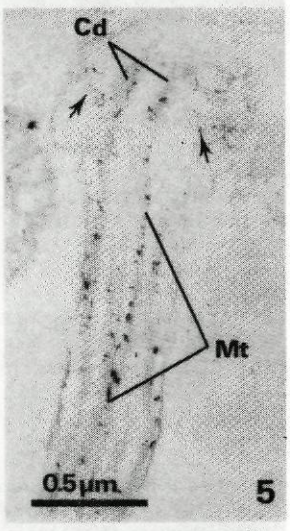
FIGURES 7 and 8. — Controls for ATPase activity. There are no lead phosphate deposits. Ac, acrosome; F, subacrosomal fossa; N, nucleus; Mi, mitochondrion; Cd, distal centriole; pericentriolar complex *(arrows)*; Mt, microtubules of the flagellum. Uncontrasted sections; × 17,000 and × 27,000.

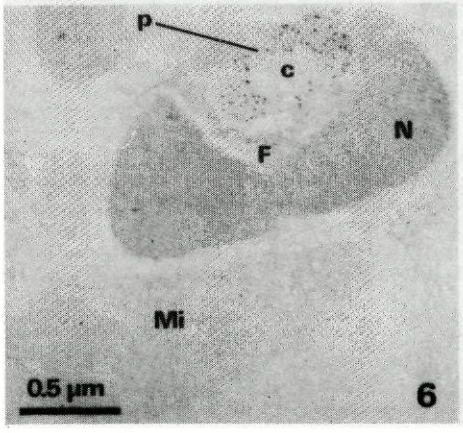

NE
Ac
N
Cm
Mi
0.5 µm
1

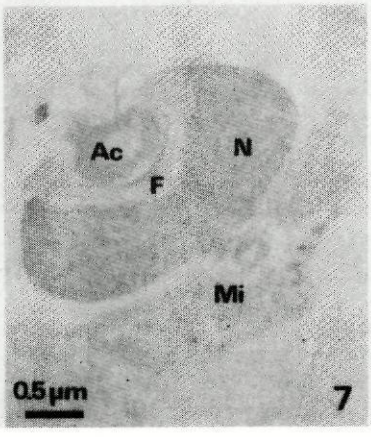

c
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2

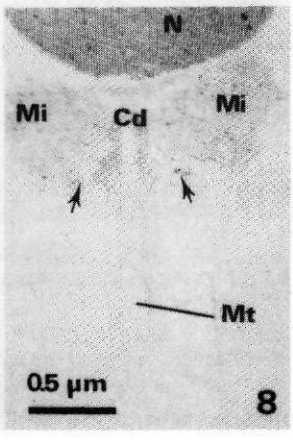

Ac
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Mi
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0.5 µm
3


p
c
F
N
Mi
0.5µm
4


Cd
Mt
0.5µm
5


p
c
N
F
Mi
0.5 µm
6


Ac
N
F
Mi
0.5µm
7


N
Mi
Cd
Mi
Mt
0.5 µm
8

coatings of the oocyte (Fig. 10). Also, some cavities in the oocyte jelly could be found in the neighbouring regions of the spermatozoon attachment and acrosomal process penetration (Fig. 10). When observing material with a final contrast, in order to visualize the oocyte jelly structure, lead phosphate deposits could be followed clearly along with the acrosomal process. As the structure of the oocyte jelly was now visible, it could be seen that its structure was lost around the penetrating acrosomal process, forming clear areas in those regions (Figs. 11 and 12). Lead phosphate precipitates delimited these clear areas. Lead phosphate deposits were also observed in the membranous vesicles formed by the vesiculation which occurred as a consequence of the acrosomal reaction (Fig. 14), and they were also present at the external face of the peripheral component of the acrosomal vesicle (Fig. 13). Controls for acid phosphatase activity showed no lead phosphate precipitates (Fig. 15).

DISCUSSION

In several invertebrate animal groups, the content of the acrosomal vesicle presents two distinct morphological components. One has been proposed to have a lytic function, while the other would play a role in gamete adhesion and contain bindin [28]. In these species, the lytic component once exteriorized is no longer morphologically visible, while the adhesive component remains detectable at the junction between the gametes [13, 19, 20].

Acid phosphatase is a lytic enzyme and it has been found in the acrosomes of several species [2, 4]. Several authors have also demonstrated the existence of lytic enzymes in acrosomes which are activated only at fertilization, and have suggested they participate in the dissolution of the oocyte coats or in the exocytosis of the acrosomal vesicle [9, 10, 15].

In this report we have demonstrated an acid phosphatase activity in the nuclear envelope and in the mitochondrion of the unreacted spermatozoon, but not in the acrosomal vesicle. However, in fertilization experiments, an acid phosphatase activity appears in the exteriorized contents of the acrosome and along the acrosomal process. This enzymatic activity is related to the lysis of the oocyte external coats, but we do not know if the enzyme is acting directly or if it is activating other enzymes. As we have not detected an acid phosphatase activity in the acrosome and in the oocyte jelly before fertilization, we think that this enzyme is stored in an inhibited state, being activated only at fertilization. At this moment we cannot be sure about the localization of this enzyme as it could exist in the acrosomal vesicle or in the oocyte jelly. However, we support the idea that the enzyme is located in the acrosomal vesicle because of several evidences. First, the occasional detection of the enzymatic activity in the acrosomal vesicle of the unreacted spermatozoa is considered to be specific and not an artifact as it has not been found in the control experiments. The simple explanation for this detection is an occasional state of incomplete enzymatic inhibition or that the treatment of the cells has inhibited an abnormal acrosomal reaction. Second, the enzymatic activity was found in the spermatozoon membranes which are liberated by the exocytosis of the acrosomal vesicle during the acrosomal reaction.

FIGURE 9. — Early steps in fertilization. The sperm is at the external surface of the oocyte jelly (J) and is maintained adherent to it by the peripheral component of the acrosomal vesicle (p). The acrosomal process (Ap) has penetrated all the jelly thickness. At the neighbouring regions of the sperm and of the acrosomal process the oocyte jelly structure is lost. V, vitelline layer; O, ooplasm. Contrasted section; × 13,700.

FIGURE 10. — Acid phosphatase activity at early fertilization. Lead phosphate deposits are seen in the exteriorized contents of the acrosomal vesicle and at the surface of the acrosomal process *(arrows)*. Near lead phosphate deposits there are cavities in the jelly structure *(arrowheads)*. Mi, mitochondrion; N, nucleus; Ap, acrosomal process; J, oocyte jelly; V, vitelline layer; O, ooplasm. Uncontrasted section; × 24,600.

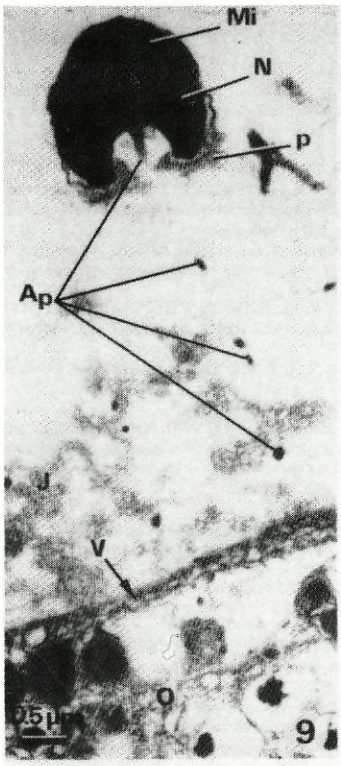
FIGURE 11. — Acid phosphatase activity at early fertilization. The acrosomal process (Ap) has already penetrated the external egg coats. Through its path clear areas delimited by lead phosphate deposits *(arrows)* can be seen. Mi, mitochondrion; N, nucleus; J, oocyte jelly; V, vitelline layer; O, ooplasm. Contrasted section; × 10,800.

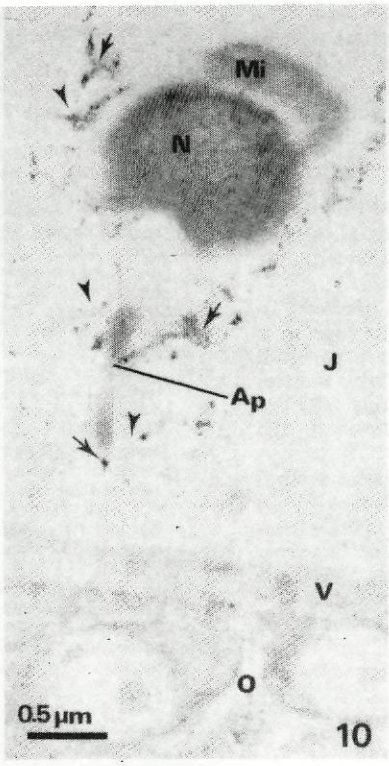
FIGURE 12. — Amplification of the distal region of the acrosomal process (Ap) shown in Fig. 10. Lead phosphate deposits are seen delimiting the clear areas and the acrosomal process surface *(arrows)*. J, oocyte jelly; V, vitelline layer; O, ooplasm. Contrasted section; × 27,000.

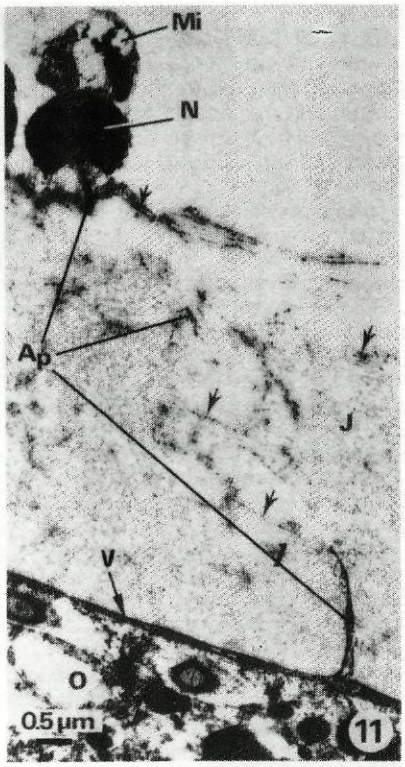
FIGURE 13. — Acid phosphatase activity at early fertilization. The contents of the acrosomal vesicle are being exteriorized. Lead phosphate deposits are seen at the external surface *(arrows)* of the dense component of the acrosomal vesicle (p). N, nucleus; Ap, acrosomal process; J, oocyte jelly. Contrasted section; × 14,400.

FIGURE 14. — Acid phosphatase activity at early fertilization. Lead phosphate deposits are seen in the membranous vesicles *(arrows)* liberated as a consequence of the vesiculation which occurs in the exocytosis of the acrosomal vesicle. N, nucleus; J, oocyte jelly. Contrasted section; × 50,600.

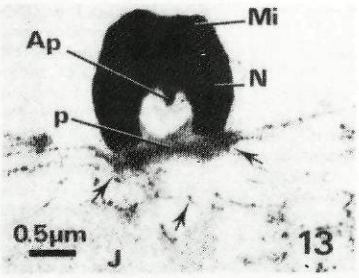
FIGURE 15. — Control for acid phosphatase activity at early fertilization. No lead phosphate deposits are seen. Fl, flagellum; Mi, mitochondrion; N, nucleus, Ap, acrosomal process; J, oocyte jelly; V, vitelline layer; O, ooplasm. Uncontrasted section; × 11,700.


Mi
N
p
Ap
J
V
O
0.5µm
9

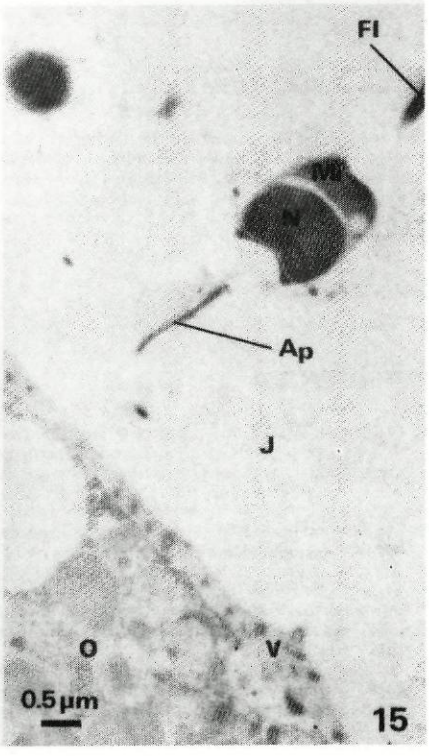

Mi
N
J
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V
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0.5 µm
10


Mi
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11


J
Ap
V
O
0.5µm
12


Mi
Ap
N
p
0.5µm
J
13


N
J
0.5 µm
14


Fl
Mi
N
Ap
J
O
V
0.5 µm
15

Our results show that if the enzyme is in the acrosomal vesicle then it is stored in the central component and in the apical portion of the vesicle. The presence of this acid phosphatase in the central component of the acrosomal vesicle would be of strategic value for fertilization because it is this portion of the vesicle which lies in direct contact with the extending acrosomal process. Also, the localization of this enzyme in the apical portion of the acrosomal vesicle would have a special interest because it is this region which will fuse with the cytoplasmic membrane.

The aim of our report was directed at demonstrating morphologically the existence of a lytic action in the penetration of the acrosomal process through the external layers of the oocyte. Experiments are in progress to determine whether this enzyme is exogenous or endogenous to the spermatozoon.

An ATPase activity has been detected in several spermatozoon structures [1, 2, 16], but only in *Asterias* and *Mytilus* has the enzyme been demonstrated in the acrosome [16]. In *Marthasterias* we have found an ATPase activity in the same structures except that we have not detected any reaction in the subacrosomal fossa and that Ca^{2+}-dependent ATPase is confined to the peripheral component of the acrosomal vesicle, while Mg^{2+}-dependent ATPase is also localized in other spermatozoon structures.

ACKNOWLEDGEMENTS

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Letter to the Editor

Starfish acrosomal acid phosphatase: a cytochemical and biochemical study

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The acrosome reaction of spermatozoa from the starfish *Marthasterias glacialis* was induced with the ionophore A23187. Reacted cells were then processed for acid phosphatase ultrastructural cytochemistry, but significant enzyme activity was not detected. However, when the supernates from suspensions of ionophore-treated sperm were assayed for acid phosphatase, a net enzyme activity was observed. Supernatant proteins were run in starch gel electrophoresis and fluorescent zymograms revealed a single band of acid phosphatase. SDS-PAGE of proteins eluted from the active spots of starch gels showed one major band of about 63 kDa. The results obtained support the hypothesis that the acid phosphatase whose activity has been detected only at the time of binding of sperm and egg originates from the sperm acrosome.

asteroidea — acrosome — acid phosphatase

INTRODUCTION

The acrosomal vesicle of the starfish spermatozoon possesses distinct morphological components to which various enzymatic activities have been related [14, 21–23]. In order to study the nature of the interaction between starfish sperm and oocyte molecules, a definition of the components in the sperm acrosomal vesicle is of first importance. However, most studies on acrosomal vesicle contents of Echinodermata have been carried out in echinoid sperm [1, 3, 4, 8–10, 12, 25–27].

In starfish, at fertilization, acid phosphatase activity has been observed surrounding the sperm acrosomal process during penetration through the external layers of the oocyte [23]. Acid phosphatase may have some jelly-lytic activity, because the oocyte jelly structure is lost around the penetrating acrosomal process. Acid phosphatase activity, however, was not found in unreacted gametes, which led to the suggestion that the enzyme was stored in an inhibited state inside the acrosomal vesicle. In this study we present morphological and biochemical evidence suggesting that the acid phosphatase detected during starfish fertilization is endogenous to the sperm acrosomal vesicle.

MATERIALS AND METHODS

Specimens of *Marthasterias glacialis* (Echinodermata, Asteroidea) were collected at the Portuguese intertidal North Atlantic coast. Semen was obtained by squeezing gonads through gauze and stored at 4°C. Spermatozoon concentration was determined using a haemocytometer. Sperm was diluted to 0.93–18.5 10^8 cells/ml with artificial seawater (ASW, 17). Ionophore A23187 (Boehringer-Mannheim) was dissolved 1 mg/ml in ethanol, then diluted in the sperm suspensions to concentrations of 19–57 μM. Sperm were left to react with the ionophore for 5 min in semi-darkness [19, 24]. Electron microscopy and acid phosphatase cytochemistry procedures are described elsewhere [21–23]. After fixation sperm suspensions were centrifuged at 2,500 rpm for 5 min. For controls the material was incubated without substrate or in complete medium with 10 mM NaF. Unstained sections were examined in a JEOL 100CXII transmission electron microscope operated at 60 kV.

To obtain acrosomal vesicle proteins, suspensions of reacted spermatozoa were centrifuged at 5,000 rpm for 10 min at 4°C in a Sorvall RC-SB centrifuge with an SS34 rotor. The supernates were kept in an ice bath for immediate use or otherwise stored at −80°C. Protein was determined by the method of Lowry *et al.* [13]. Acid phosphatase activity was assayed according to Barrett and Heath [2]. Enzyme units are expressed in micromoles of 4-nitrophenol released per milligram of protein per 30 min. Supernates of ASW with or without ionophore or cells were used as controls.

Starch gel electrophoresis (Connaugh, 15%) was performed with cooling (8°C) at 6 V/cm over 15 h for the following systems: (*a*) 0.25 M Tris and 0.067 M citric acid pH 7.6, as bridge buffer; gel buffer diluted 1: 8. (*b*) 0.4 M citric acid and 1.13 M NaOH pH 6, as bridge buffer; gel buffer: 0.02 M histidine hydrochloride and 0.01 M NaOH pH6. (*c*) 0.2 M maleic anhydride and 0.136 M NaOH pH 5.6, as bridge buffer; gel buffer diluted 1: 10. The zymogram of acid phosphatase was obtained by incubating a strip of the gel with a staining solution containing 4-methylumbelliferyl dihydrogen phosphate (BDH) (1 mg/ml) in 1 M acetate buffer pH 4.8 at 37°C. Fluorescence was inspected after incubation under UV light at 366 nm. To extract the protein from starch gels we used the stained strips as reference, and the desired regions were cut out of the unstained remaining gel and macerated in 10 mM Tris-HCl pH

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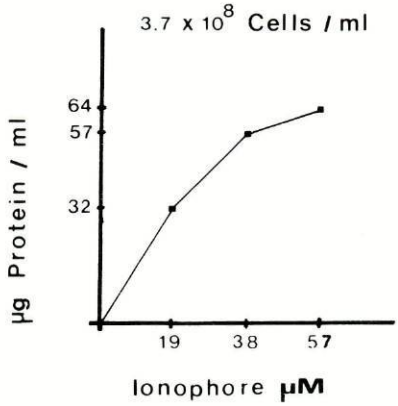


FIGURE 4. – Effect of different A23187 concentrations on the amount of proteins released from a fixed concentration of spermatozoa. Each point represents the mean of 3 experiments, each in duplicate.

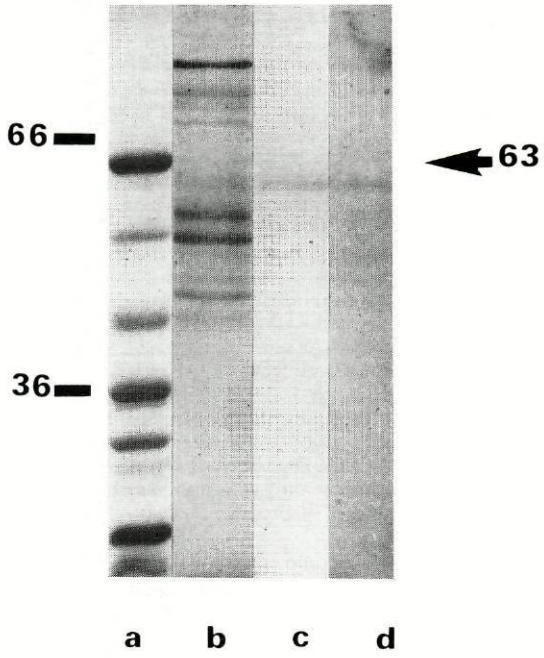


FIGURE 6. – SDS-PAGE of molecular weight standards (**a**), total acrosomal proteins (about 25 µg protein was loaded in lane **b**), and of proteins eluted from starch gels after electrophoresis at pH 6 (**c**) and pH 7.6 (**d**). Molecular weights are indicated in kilodaltons (*bars*). Coomassie blue R staining.

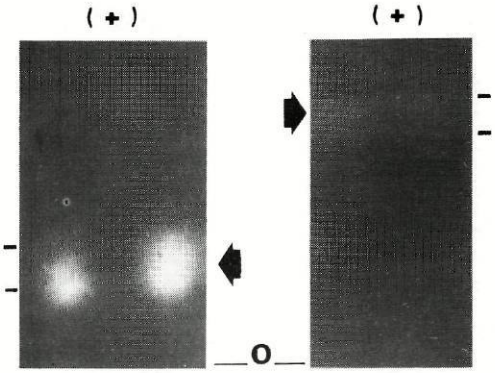


FIGURE 5. – Fluorescent zymograms of acid phosphatase (*arrows*) using starch gel electrophoresis at pH 5.6 (*left*) and pH 7.6 (*right*). About 50 µg of acrosomal proteins was applied to each lane. **O**, origin; (+), anode; bars indicate the strip width used for enzyme elution.

band of acid phosphatase with increasing mobility towards the anode with increasing pH (Fig. 5). The electrophoretic SDS-PAGE pattern of proteins eluted from the active spots of starch gels shows one major band at about 63 kDa (Fig. 6). The electrophoretic pattern of total exocytic proteins reveals the presence of several proteins whose molecular weights are predominantly above 45 kDa (Fig. 6). None of the acrosomal proteins revealed positive staining with the PAS method.

DISCUSSION

In a previous report, acid phosphatase activity was cytochemically localized along the acrosomal process of starfish sperm during penetration of the oocyte external coat [23]. As no enzyme activity was detected in the unreacted gametes, it was suggested that before fertilization, the enzyme was stored in an inhibited state inside the acrosomal vesicle [23]. In the present study, acid phosphatase activity was found in supernates of solutions containing artificially reacted starfish spermatozoa. This result demonstrates that the acid phosphatase found at the site of starfish sperm-oocyte binding originates from the sperm acrosome.

Although several enzymes are known to be associated with the sperm acrosome of echinoderms [1, 3, 4, 8–10, 12, 14, 25, 27], it is the first time that an acid phosphatase has been demonstrated in echinoderm acrosomes by biochemical assay. In mammalian species, acid phosphatase is also released into the surrounding medium after capacitation [15, 16, 18, 20]. By comparison with our results, however, the sperm of some mammalian species have higher acid phosphatase activities [5, 15, 20]. Curiously, acrosomal acid phosphatases of both *M. glacialis* and mammals were detected after the acrosomal reaction [15; this report], but cytochemical methods failed

to locate the enzyme in mature acrosomes [18, 23]. As in the case of mammals, our experiments with artificially reacted sperm seem to rule out the necessity of some oocyte jelly factor for activating the acrosomal acid phosphatase. The specific mechanism of enzyme inhibition and activation, however, remains unknown.

Acid phosphatases isolated from mammalian spermatozoa have pH optima between 4.5 and 5.5, and molecular weights between 45 and 123 kDa, but none was shown to be stored in the acrosomal vesicle (5–7, 16, 18). Preliminary results obtained with a crude method are reported in this paper, which allowed demonstration of a single acid phosphatase with an apparent molecular weight of 63 kDa in the acrosomal vesicle of the starfish sperm. In fact, although other contaminating proteins are evidenced in SDS-PAGE, one of the minor bands (>63 kDa) is present only in lane (*d*). Therefore, it cannot be responsible for acid phosphatase activity. On the other hand, the second possible contaminant (<63 kDa) shows different intensities in lanes (*c*) and (*d*), thus legitimizing the conclusion that the only common band (also in intensity, 63 kDa) is indeed responsible for the acid phosphatase activity detected.

The purification and characterization of starfish acrosomal acid phosphatase are under further investigation.

ACKNOWLEDGEMENTS

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PRESENCE OF ATPase AND ALKALINE PHOSPHATASE ACTIVITIES IN THE STARFISH SPERM ACROSOME

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Abstract

ATPase activity was cytochemically detected in the peripheral acrosomal component of ionophore-reacted sperm, while alkaline phosphatase activity was demonstrated in the upper and central components of the acrosome and, at fertilization, at the site of sperm-oocyte binding. Supernatants of ionophore treated sperm suspensions were assayed for ATPase, alkaline and acid phosphatase activities. Results suggest that alkaline phosphatase may be involved both in the acrosomal reaction and oocyte jelly lysis but the function of the acrosomal ATPase remains unknown.

Introduction

The cytochemical detection of ATPase activity inside the acrosomal vesicle has only been achieved in the Mytilus and starfish sperm (Mabuchi and Mabuchi,1973; Sousa and Azevedo, 1986). This is an enigmatic localization since in other species sperm head ATPases have been found in the cytoplasmic and outer acrosomal membranes where they are supposed to be involved with ion pumping at fertilization (Gordon and Barrnett, 1967; Anderson, 1968; Gonzales and Meizel, 1973; Usui and Yanagimachi, 1986). Our enzyme assay experiments confirm the presence of the starfish acrosomal ATPase and give a partial characterization of the enzyme. On the other hand, acrosomal alkaline phosphatase has only been cytochemically detected in the sea urchin (Anderson, 1968; Morton, 1976). Our present experiments show that the starfish acrosomal vesicle also contains an alkaline phosphatase whose localization, activation and physiological role in the unreacted sperm appear identical with the starfish acrosomal acid phosphatase (Sousa and Azevedo, 1986). Slight differences, however, were noticed to exist between the two enzymes during sperm penetration through the oocyte investments.

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Materials and Methods

Collection and handling of Marthasterias glacialis gametes are described in Sousa and Azevedo (1985). Ionophore A23187 (Boehringer Mannheim) was prepared (Summers et al., 1976) and dissolved in artificial sea water (ASW, the MBL's formulae in Meijer et al., 1984) at a final concentration of 19 μM. To obtain acrosomal vesicle proteins, suspensions of ionophore treated spermatozoa were centrifuged at 5,000 rpm for 10 min at 4°C in a Sorvall RC-SB centrifuge with a SS34 rotor (Sousa et al., 1988). Supernatants were then assayed for ATPase and AlPase activities using 2-amino-2-methyl-1,3-propandiol as buffer (Lowry, 1957). and for AcPase activity using the acetate buffer system (Barrett and Heath, 1977). Supernatants of ASW with or without ionophore or cells were used as controls. Protein was determined using bovine serum albumin as standard by the method of Lowry et al. (1951). For the cytochemical detection of Ca^{2+}-ATPase activity, ionophore treated sperm were fixed with 0.3 % glutaraldehyde in ASW for 30 min at 4°C (Mabuchi and Mabuchi, 1973) and then concentrated through filtering paper. Incubation conditions are defined in Mughal et al. (1987). AlPase activity was determined according to Hugon and Borgers (1966). In both cases postfixation mediums included 1.5 % potassium hexacyanoferrate (II) trihydrate GR (Merck), and for controls the complete mediums without substrates were used. Unstained sections were examined in a JEOL 100CXII TEM operated at 60 KV.

Results and Discussion

Cytochemistry : In mature sperm no AlPase activity was found in the acrosomal vesicle of M.glacialis. At early fertilization some of the still unreacted acrosomes, however,exhibited lead deposits in the upper and central light components of the acrosomal vesicle (Fig.1). This indicates that acrosomal AlPase is activated only at fertilization. A similar localization and mode of activation has been found for starfish acrosomal AcPase (Sousa and Azevedo, 1986). The upper enzyme localization suggests that AlPase may be involved in the process of acrosomal vesicle exocytosis, while the enzyme in the central component will coat the acrosomal process. After acrosomal reaction AlPase activity was found at the sperm--oocyte binding interface surrounding lysed jelly areas into which the spermatozoon head sinks (Fig.2). Some lead deposits could also been found along the acrosomal process (Fig.2). A similar localization of AcPase activity has been found at starfish fertilization (Sousa and Azevedo, 1986) but in the

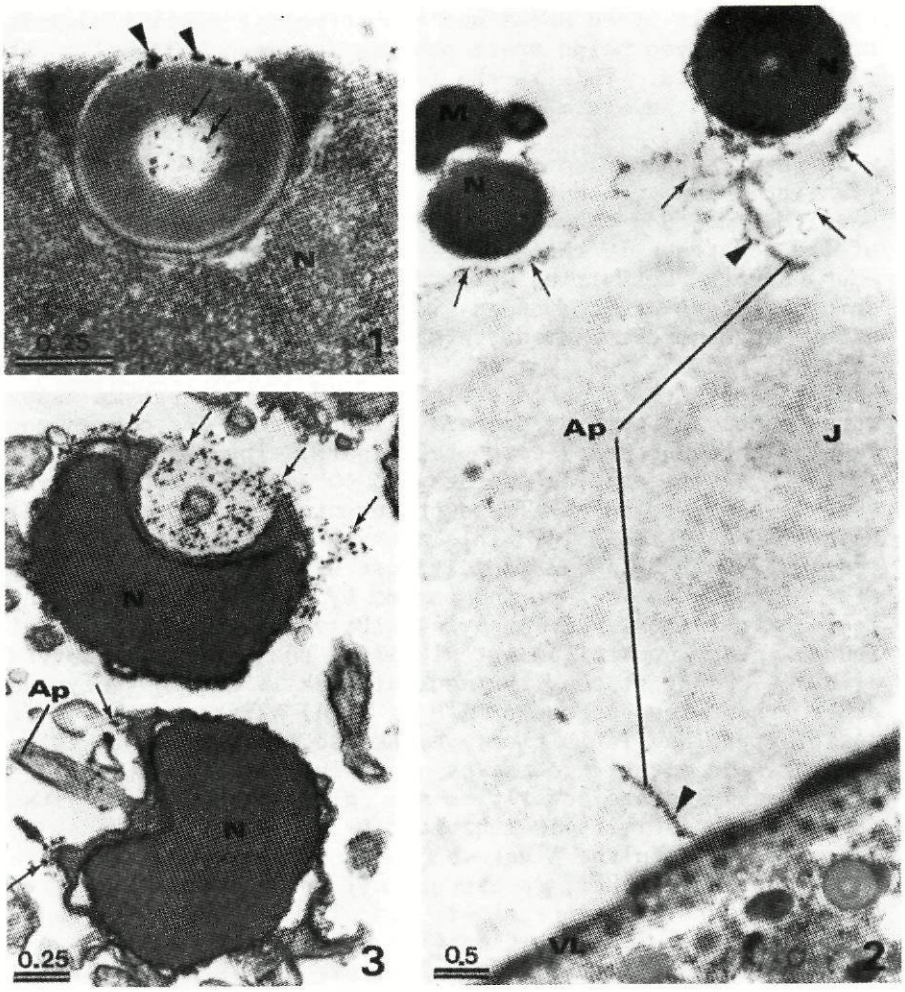


Fig.1. AlPase activity is demonstrated in the upper (arrowheads) and central (arrows) light components of the acrosomal vesicle. N, nucleus. Bar = μm.

Fig.2. AlPase activity is demonstrated at sperm-oocyte binding sites surrounding lysed jelly areas (arrows), and around (arrowheads) the acrosomal process (Ap). N, nucleus; M, mitochondrion; J, jelly layer; VL, vitelline layer. Bar = μm.

Fig.3. Ionophore treated sperm. Ca++- ATPase activity is demonstrated in the peripheral dense component of the acrosomal vesicle (arrows). N, nucleus; Ap, acrosomal process. Bar = μm.

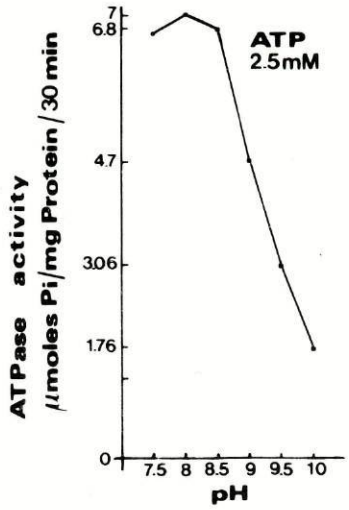
case of AlPase lead deposits predominantly accumulate at the outer jelly region. The association of AlPase activity both with the jelly lysed areas and the acrosomal process suggests that the enzyme helps sperm penetration through the oocyte external coats. This is the first report on precise AlPase localization in sperm acrosomes and its distribution during fertilization.

As starfish acrosomal ATPase is inactivated by routine fixation, low concentrations of glutaraldehyde have to be used (Mabuchi and Mabuchi, 1973) precluding the preservation of the oocytes. In the unreacted sperm of M.glacialis, Ca^{2+}--Mg^{2+}-ATPase activities were found in the peripheral dense acrosomal vesicle component (Sousa and Azevedo, 1986), and after ionophore treatment this exocytosed component remains labeled (Fig.3).

Enzyme assays : In our experimental conditions enzyme assays were done in complex protein mixtures and in consequence results should be viewed as preliminary. The pHs optima for ATPase, AlPase and AcPase are 8, 10 and 4.4, respectively (Figs.4-6). At pH 8 ATPase preferentially hydrolyses ATP (ATP≫**G**TP>**A**DP)(Table I). This activity is completely inhibited by 2.5 mM EDTA and rapidly lost with freezing (Table I). All attempts to isolate the enzyme by starch gel electrophoresis have failed. When assaying AlPase activity 2 peaks were found, near neutral and at alkaline pHs (Fig.5). Several evidences suggest that the neutral peak is due to enzyme(s) other than the AcPase or ATPase : a) AlPase activity is resistant to NaF inhibition; b) AcPase activity is inhibited by NaF and clearly decreases near neutral pH (Fig.6); c) AlPase and AcPase activities are glutaraldehyde resistant while ATPase activity is inhibited by the fixative; and, d) at pH 8, and in the ATPase incubation conditions, several other substrates (PPi, p-nitrophenyl phosphate, β-glycerophosphate) are much more slowly hydrolysed and activities are resistant to EDTA inhibition (Table I). These results also give evidence that the acrosomal ATPase splits ATP to ADP+Pi.

Because assays were done using enzyme mixtures in ASW, definite characterization of the ATPase and AlPase was not achieved. Nevertheless, experiments enabled us to further substantiate the presence of a non-membranous ATPase activity and to find an AlPase activity in the acrosomal vesicle of the starfish sperm. The purification and specific characterization of the enzymes are under further investigation.

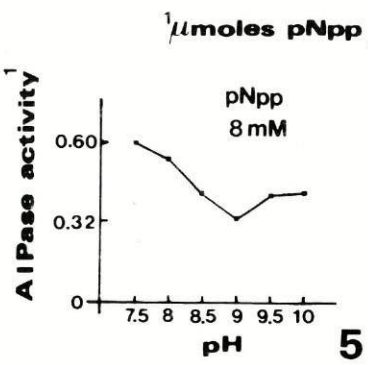
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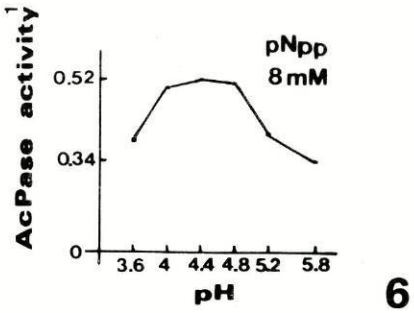
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incubation condition (pH 8, 37°C, 30 min)	enzyme activity (µmoles Pi/mg Protein)
2.5 mM ATP	7
2.5 mM GTP	2.7
2.5 mM ADP	2
2.5 mM PPi	0.65
5.0 mM βGPNa	0.53
5.0 mM pNpp	0.74
2.5 mM ATP + 2.5 mM EDTA	0
2.5 mM ATP after 72h at -80°C	1.26

Table I



5



6

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Presence of a Trypsin-Like Protease in Starfish Sperm Acrosome

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short title: Starfish acrosomal protease

one Table and two Figures

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ABSTRACT

Marthasterias glacialis sperm cells were treated with ionophore A23187, centrifuged and the supernatants assayed for esterase activity. With N-benzoyl-L-arginine ethyl ester-HCl (BAEE) as substrate, a net activity was determined which was not detectable when N-acetyl-L-tyrosine ethyl ester (ATEE) was used. The BAEE trypsin-like activity was inhibited by soybean trypsin inhibitor (SBTI), N-α-p-tosyl-L-lysine chloromethyl ketone-HCl (TLCK) and phenyl methyl sulfonyl fluoride (PMSF), but not by L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK). The presence of proteolytic activity in acrosomal exudates was further demonstrated by gelatin-sodium dodecyl sulfate-polyacrylamide gel electrophoretic zymography (gelatin-SDS-PAGE). The presence of several bands of low proteolytic activity and of one band of high proteolytic activity, which also has the lower molecular weight, together with the fact that all are inhibited by benzamidine, suggests the existence of a trypsin-like proteinase system. The effect of the acrosomal exudate on the oocyte jelly coat was investigated by SDS-PAGE analysis. All jelly proteins appeared to be digested by the acrosomal enzymes. Furthermore, if SBTI is added shortly after insemination, the sperm fail to fertilize the oocytes. These results indicate that the starfish sperm acrosomal vesicle contains a trypsin-like protease which may be involved in sperm penetration through the oocyte jelly coat.

A proacrosin-acrosin system has been detected in the mammalian sperm acrosome and was suggested to be involved in the acrosomal reaction, gamete binding and penetration of the oocyte zona pellucida by the spermatozoon (Green, '78; Parrish and Polakoski, '79; Huneau et al., '84; Jones et al., '88).

Several lytic enzymes have been described in the sea urchin sperm (Vasseur, '51; Brookbank, '58; Isaka et al., '66; Hoshi and Moriya, '80; Hoshi, '85), but there are contradictory results about the existence of a chymotrypsin-like (Hoshi et al., '79; Yamada and Aketa, '81; Green and Summers, '82; Matsumura and Aketa, '89) or a trypsin-like enzyme (Levine and Walsh, '79; Green and Summers, '80). Ascidians occupy a phylogenetic position between vertebrates and echinoderms. The activities of both chymotrypsin-like and trypsin-like enzymes are found in all ascidians tested. For sperm penetration, however, only chymotrypsin-like activity seems responsible in the Enterogona while both activities are required in the Pleurogona (Hoshi et al., '81; Sawada et al., '83,'84; Hoshi, '85; Pinto et al., '90). Recently, however, acrosin has been shown by immunocytochemistry to be present in several species belonging to various animal phyla, including the sea urchin spermatozoon (Baccetti et al., '89).

In order to elucidate the mode of action of the lysin system of starfish sperm, characterization of a putative acrosomal protease has been attempted in our laboratories. In previous studies, we identified in the starfish sperm acrosome acid and alkaline phosphatases which appeared to be involved in acrosomal vesicle exocytosis and penetration of the oocyte jelly layer by the acrosomal process (Sousa and Azevedo, '86, '88; Sousa et al., '88). In the

present paper, we describe evidence that the acrosomal vesicle of the starfish spermatozoon also contains a trypsin-like protease system which possibly participates in oocyte jelly coat penetration at fertilization.

MATERIALS AND METHODS

Specimens of *Marthasterias glacialis* (Echinodermata, Asteroidea) were collected at the Portuguese intertidal North Atlantic coast. The ionophore A23187 (Boehringer-Mannhein) (60 µM final concentration) was added to sperm suspensions (2-3 x 10^9 cells/ml) made in Millipore (0.2 µm) filtered sea water (FSW) (Summers et al., '76; Schroeder and Christen, '82). After 5 min in darkness, suspensions were centrifuged at 10,000 rpm (Sorvall refrigerated ultracentrifuge, with a SS-34 rotor) for 15 min at 4°C (Sousa et al., '88). Supernatants of untreated sperm suspensions were used as controls. BAEE and ATEE esterase activities were assayed as described by Yamada and Aketa ('81), except that for BAEE activity the assays were performed in 50 mM Tris-HCl, pH 8, containing 3 mM CaCl , 0.5 mM BAEE and 0.3 ml of the acrosomal supernatant in a final volume of 3 ml. PMSF, TLCK, SBTI and TPCK were diluted in the assay solution and preincubated for 5 min with the substrate before addition of the acrosomal supernatant (Polakoski and McRorie, '73; Beynon, '88). Enzyme units are expressed as the amount giving an absorbance change of 1 OD unit during the first minute, and specific activity as enzyme units per mg of protein (Polakoski et al., '73). Protein concentrations were determined by the method of Lowry et al. ('51).

Gelatin-SDS-PAGE was done at 4° C according to Siegel et al. ('86a,b). Briefly, the 12.5% acrylamide gels (0.75 mm) were prepared and cast as described by Laemmli ('70) except that a final concentration of 0.1% gelatin was copolymerized in the resolving gel. The stacking gel contained no gelatin. Acrosomal supernatants were concentrated in an Amicon B15 at 4°C and mixed with non-reducing sample buffer (125 mM Tris-HCl, 1% SDS, 10% glycerol and 0.001% bromphenol). Electrophoresis was performed at a constant current of 18 mA at 4°C. The gels were then shaked at room temperature for 1 h in 2.5% Triton X-100 in water to remove the SDS. After this, the gels were washed with 200 ml of distilled water 3-4 times to remove the Triton X-100, then incubated in 50 mM Tris-HCl pH8 for 2-3 h at 37°C. The gels were fixed and stained for at least 1 h in a 0.1% solution of amido black in methanol:acetic acid:water (30:10:60) and destained in methanol:acetic acid:water (30:10:60).

For SDS-PAGE analysis under reducing conditions (Laemmli, '70), samples were prepared as follows. Acrosomal supernatants were concentrated in an Amicon B15 at 4°C or in a speed vacuum concentrator (Savant). In the latter case, samples were then dialysed against 0.9% NaCl at 4°C and immediately used, or frozen in liquid nitrogen vapour and stored at -70°C until use. The oocyte jelly coat was isolated by the acid method of SeGall and Lennarz ('79). After incubation at room temperature up to 21 h, samples containing oocyte jelly coat, acrosomal exudate, as also mixtures of oocyte jelly coat with acrosomal exudate or commercial trypsin (180 µg) (Sigma type III) (Yamada and Aketa, '81), were precipitated with 10% trichloroacetic acid (TCA), dissolved in reducing sample buffer (2%

SDS, 5% β-mercaptoethanol) and boiled. Gels were electrophoresed as above and then stained with coomassie blue R (Fluka AG), silver (Merril et al., '81) or alcian blue (Krueger and Schwartz, '87). Molecular weight standards were from Pharmacia and all chemicals were of analytical grade.

RESULTS

When supernatants of ionophore-treated sperm suspensions were assayed for esterase activity, hydrolysis was observed with BAEE as substrate but not with ATEE, indicating the presence of a trypsin-like protease in acrosomal exudates. The observation that the enzyme activity could be inhibited with PMSF and SBTI suggested that the enzyme was a serine protease. Inhibition of the enzyme by TLCK provided evidence that an active site histidine was also involved in enzymatic catalysis. The failure of TPCK to inhibit the enzyme further supports the belief that this was not a chymotrypsin-like enzyme (Table I). The specific activity of the BAEE trypsin-like activity was 0.73 U / mg protein.

On gelatin-SDS-PAGE zymographs, one band of low proteolytic activity and one band of high proteolytic activity, with a migration distance close to that of commercial trypsin, were observed (Fig. 1 A, B). The higher molecular weight (Mr) band of digestion corresponded to an acrosomal glycoprotein of about 144 kDa, whereas the lower Mr band, which also exhibited the highest proteolytic activity, had around 21 kDa (Fig. 1 B-E). In some cases we were able to identify five other bands of low proteolytic activity with apparent Mr of 89, 74, 61, 51

Table I

INHIBITOR	% INHIBITION
PMSF (mM)	
1	49
2	89
TLCK (mM)	
0.1	71
0.3	87
0.6	97
SBTI (mg/ml)	
0.15	47
0.30	64
0.45	86
0.80	100
TPCK (mM)	
0.1	0

Table I. Percentages represent the mean of three experiments each with two replicates.

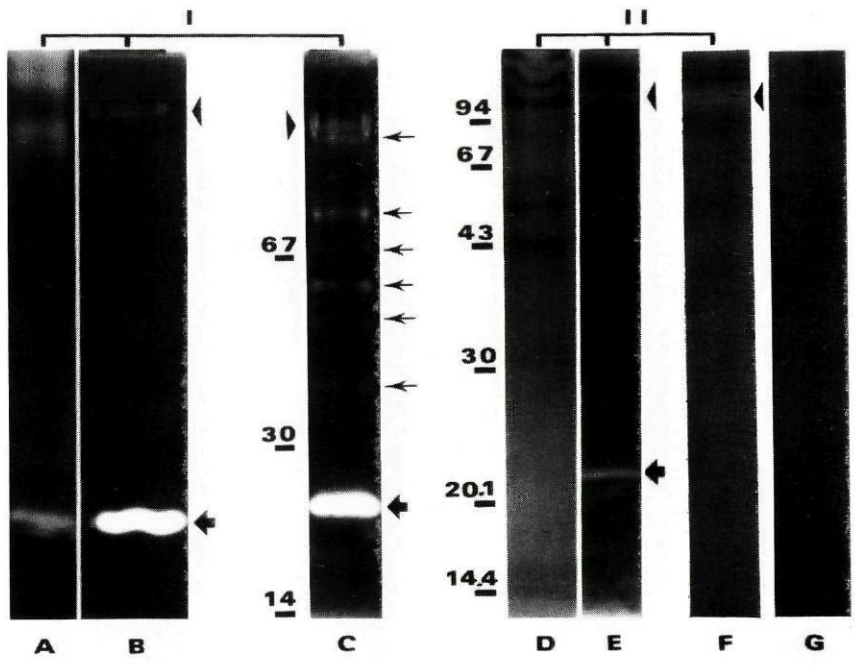


Fig. 1. Gelatin-SDS-PAGE zymographs in 10% (lanes A and B) and 12.5% (lanes C-F) gels. Panel I compares proteinase digestion profiles between trypsin (1.5 µg in lane A) and acrosomal exudates (90 µg protein was loaded in lane B, 46 µg in lane C). Large arrows point to trypsin and to the most active acrosomal proteinase, small arrows to intermediate bands of proteinase digestion, and arrowheads to the putative zymogen. Panel II shows the correspondence between acrosomal exudate proteins (150 µg) under reducing conditions (lane D) and their proteolytic activities under non-reducing conditions (10 µg protein) before (lane E) or after (lane F) freeze-thawing. No bands of digestion are observed if gels are washed and incubated in the presence of benzamidine and p-aminobenzamidine (lane G). The three components with molecular masses above 94 kDa in lane D are further analysed in figure 2 (lanes C and F). Molecular weights are indicated in kilodaltons (bars).

and 37 kDa. In these cases, the 144 kDa component exhibited a doublet pattern of activity and the Mr of the two forms differed by about 10 kDa (Fig. 1 C). Aging solutions or freeze-thawing caused the loss of all bands of activity except the one of higher Mr (Fig. 1 F). When benzamidine (50 mM) and p-aminobenzamidine (1mM), inhibitors for enzymes with trypsin-like specificity, were incubated with the gel wash and incubation buffers (Siegel et al., '86a), all digestion bands were completely inhibited (Fig. 1 G).

To study the effect of the acrosomal exudate on the oocyte jelly coat, this material was chemically isolated and then analysed by SDS-PAGE before and after incubation either with trypsin or with acrosomal exudate. SDS-PAGE analysis of acrosomal exudates revealed the presence of five high molecular weight proteins with apparent Mr of 144, 180, 221 and 254 kDa and one greater than 330 kDa (Fig. 2 C). Alcian blue stained the 43, 144 and 180 kDa proteins and only faintly those of 221 and 254 kDa (Fig. 2 F). In contrast, the oocyte jelly coat separated into five main proteins with Mr of 44, 109, 123, 149 and 202 kDa when stained with coomassie blue, but with silver staining several others appeared (Fig. 2 A, B). Except for the 44 kDa component, alcian blue stained all these proteins as also three other components with Mr greater than 330 kDa (Fig. 2 E). These very high molecular weight components seem to be glycoproteins as they appeared susceptible to digestion by commercial trypsin although they stained only faintly with silver after long term development (Fig. 2 A, arrows). After incubation with acrosomal exudates, all the oocyte jelly coat proteins appeared digested and only the acrosomal protein bands remained visible in gels (Fig. 2 D, G). Preliminary results

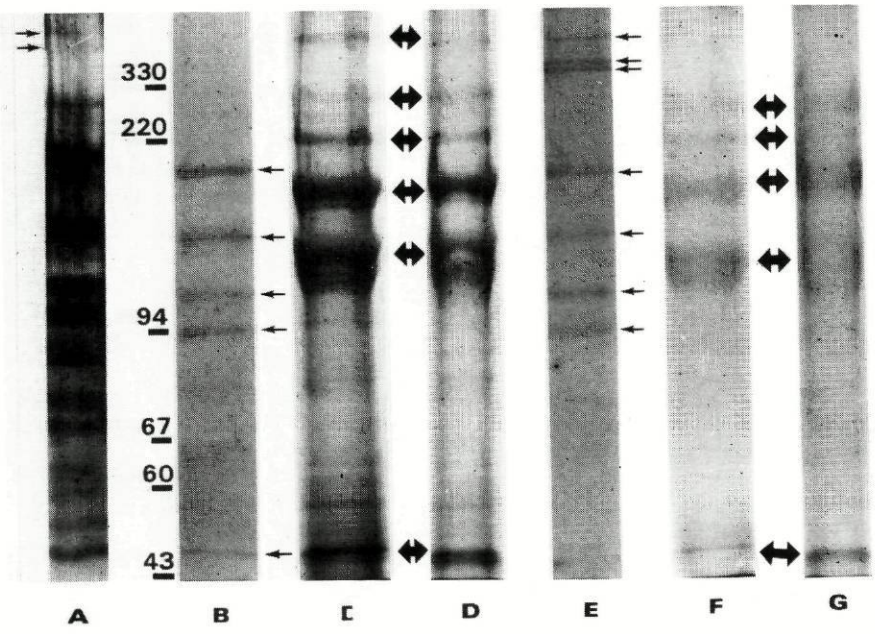


Fig. 2. SDS-PAGE analysis (7 % gels) of the oocyte jelly coat (0.5-1 mg)(lanes A, B and E), acrosomal exudates (250 µg)(lanes C and F), and of the proteins remaining after incubating the oocyte jelly coat with acrosomal exudates (1 mg)(lanes D and G). Gels were stained with silver (lane A), coomassie blue (lanes B-D), or alcian blue (lanes E-G). Arrows indicate major components of jelly coat (arrows) and acrosomal exudate (double arrows). Molecular weights are indicated in kilodaltons (bars).

also show that the addition of SBTI (1mg/ml) 2 seconds after insemination (after acrosomal reaction but before gamete fusion) inhibits fertilization.

DISCUSSION

The starfish sperm acrosome reaction, can be artificially elicited by A23187 (Summers et al., '76; Schroeder and Christen, '82), releasing to the surrounding medium the acrosomal vesicle contents (Sousa and Azevedo, '88; Sousa et al., '88).

In the present work, an esterase activity has been detected in the starfish acrosomal exudate. This enzyme had a trypsin-like serine protease activity on the basis that it hydrolyses BAEE but not ATEE, and BAEE hydrolysing activity was inhibited by PMSF, TLCK and SBTI but not by TPCK. When samples of the acrosomal exudate were analysed on gelatin-SDS-PAGE, several bands of proteolytic activity were identified, all being inhibited by benzamidine. These results suggest the presence of a trypsin-like protease system in the starfish sperm acrosome that may be similar to the proacrosin-acrosin system of the mammalian sperm (Siegel et al., '86b). Although the zymogen has not been extracted and its conversion into the active protease specifically assayed, the data obtained suggest that the higher Mr band of activity (144 kDa) is the proenzyme. Evidence to support this belief is as follows. Firstly, mammalian proacrosin is a glycoprotein and no other acrosomal proteinase has been found either with a higher Mr or with a doublet pattern of activity (Parrish and Polakoski, '79). The same appears to be true of the putative *M. glacialis* zymogen.

Secondly, the bands of intermediary Mr having protease activity were not always observed, which suggest that they represent intermediate enzymatic forms. Finally, all bands of activity, except the one of higher Mr, disappeared following long term incubation or freeze-thawing, conditions which are known to destroy acrosin activity (Polakoski et al., '73). The lower Mr band of activity (21 kDa) exhibited the highest proteolytic activity and was considered to correspond to the active protease form. Because only one band of intense activity was found on zymographs, it is conceivable that the starfish sperm acrosomal vesicle does not contain other trypsin-like serine proteases like those demonstrated to be present in mammals and ascidians (Sawada et al., '84; Siegel et al., '86b). The activity bands of intermediary Mr found in some gels suggest, however, that the putative starfish zymogen is converted into the active protease through limited sequential proteolytic degradations as happens in mammals (Parrish and Polakoski, '79).

The chemical composition of the starfish oocyte jelly coat is only known for two species and has been demonstrated by gel filtration to consist of a very large sulfated, fucose-rich glycoprotein, a high mannose neutral glycoprotein, and several asterosaponins and oligopeptides which activate the sperm and induce the acrosomal reaction (Uno and Hoshi, '78; Ikadai and Hoshi, '81; Nishiyama et al., '87; Hoshi et al., '90). In the present work, SDS-PAGE analysis of *M. glacialis* oocyte jelly coat revealed the presence of several proteins and glycoproteins, three of which had Mr greater than 330 kDa.

After insemination, the starfish spermatozoon attaches to the outer

border of the jelly coat and there remains until the acrosomal process makes contact with the oolemma, which happens in about 6 seconds (Sousa and Azevedo, '85). In the present work, as jelly coat proteins, but not acrosomal proteins, appeared susceptible to digestion by acrosomal exudates, and because sperm failled to fertilize oocytes if SBTI was added 2 seconds after insemination, the results suggest that, in the starfish *M. glacialis*, the acrosomal trypsin-like protease may participate in penetration of the oocyte investments at fertilization.

ACKNOWLEDGMENTS

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Silver staining of the cortical reaction in oocytes of *Marthasterias glacialis* (Echinodermata, Asteroidea)

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SOUSA, M., and AZEVEDO, C. 1987. Silver staining of the cortical reaction in oocytes of *Marthasterias glacialis* (Echinodermata, Asteroidea). Can. J. Zool. **65**: 2607–2611.

Morphologically, cortical granules of the starfish *Marthasterias glacialis* present a lamellar structure within a dense matrix. However, with silver nitrate staining, the matrix is subdivided into argyrophilic and argyrophobic materials. Just before exocytosis the cortical granules swell. After being released in the perivitelline space, the unstained matrix disperses while the other components are incorporated into the vitelline layer. When cells are incubated with pronase before exposure to silver nitrate, no silver deposits are found.

SOUSA, M., et AZEVEDO, C. 1987. Silver staining of the cortical reaction in oocytes of *Marthasterias glacialis* (Echinodermata, Asteroidea). Can. J. Zool. **65** : 2607–2611.

Les granules corticaux de l'astérie *Marthasterias glacialis* présentent une structure lamellaire incluse dans une matrice dense. Cependant à la coloration au nitrate d'argent, la matrice se divise en substances argyrophile et argyrophobe. Peu avant l'exocytose, les granules enflent. Une fois les granules libérés dans l'espace périvitellin, la partie argyrophobe de la matrice se disperse, alors que les autres structures sont incorporées dans la couche vitelline. Si les cellules sont incubées en présence de pronase avant la coloration au nitrate d'argent, il ne se fait pas de dépôts d'argent.

[Traduit par la revue]

Introduction

Silver nitrate has been used to demonstrate the nucleolus organizer regions of chromosomes (Goodpasture and Bloom 1975; Hernandez-Verdun and Derenzini 1983) whose staining reaction is due to acidic proteins (Schwarzacher et al. 1978; Spector et al. 1984). Recently, a modified silver nitrate technique has also been used to demonstrate cytoplasmic argyrophilic organelles in germ cells (Azevedo et al. 1985; Czaker 1985).

Several studies have been undertaken to cytochemically characterize the cortical granules of echinoderm eggs (Bal 1970; Hylander and Summers 1982; Yamashita 1984; Cameron and Holland 1985). However, there have been few fine structure studies (Holland 1980; Longo et al. 1982) and no histochemical characterization of cortical granules in the class Asteroidea.

In this study we used the silver nitrate reaction to better characterize the structure of cortical granules in an attempt to search for distinct constituents and their specific fate during the cortical reaction of a starfish.

Materials and methods

Marthasterias glacialis specimens were collected from the intertidal zone of the Portuguese North Atlantic coast. Female gonads were washed and cut in filtered sea water (FSW). The liberated oocytes were collected and left in FSW until their spontaneous maturation. Some of these oocytes were then fixed with 2.5% glutaraldehyde in FSW for 2 h at 4°C. Spermatozoa were collected dry by cutting and teasing gonads through gauze. After being diluted they were added to a suspension of mature oocytes. All studied eggs were from samples that developed synchronously. At 2, 5, and 15 s after insemination, eggs were fixed as above. For fine structure studies, cells were then washed in FSW for 2 h at 4°C, postfixed with 1% OsO_4 in FSW for 2 h at 4°C, dehydrated in ethanol and then in propylene oxide, and embedded in Epon (Sousa and Azevedo 1983, 1985). Ultrathin sections were cut in an LKB Ultratome III and double stained with 3% aqueous uranyl acetate for 20 min and lead citrate for 15 min.

For histochemical studies the technique of Goodpasture and Bloom (1975) was modified as follows. After fixation and washing, oocytes and eggs were postfixed with Carnoy's fluid for 12 min at room temperature, rapidly rehydrated in ethanol, and rinsed in distilled water for 30 min. Then cells were immersed in 70% $AgNO_3$ under a photoflood (2800-K bulb) for 20 min at 55°C, rinsed in distilled water, and incubated in an ammoniacal silver – formalin solution for 6 min. After being rinsed in distilled water, cells were dehydrated and embedded as above. Some ovary fragments and isolated oocytes were fixed and washed as described, immersed in 0.5% pronase E (70 000 PUK/g, Merck) aqueous solution at 40°C for 45 min, washed in distilled water, postfixed in Carnoy's fluid and processed for the silver nitrate technique. Ultrathin sections were observed with and without double contrast. As contrast does not alter the specificity of the histochemical staining, micrographs from briefly contrasted sections (10 and 5 min) were preferred.

Grids were observed in a JEOL 100B electron microscope operated at 80 kV.

Results

Morphologically, cortical granules have very dense contents but in favourable sections a lamellar structure can be observed within a dense matrix (Fig. 1). Shortly after insemination cortical granules swell, their contents being moved away from the membrane (Fig. 2). After granule exocytosis in the perivitelline space, the lamellar component is transformed into a reticulated mass, while within the matrix some light spaces appear (Figs. 2–4). The lamellae are seen to fuse with the vitelline layer, but the destiny of the matrix materials could not be followed (Figs. 5, 6).

When stained with silver nitrate the matrix appears subdivided into argyrophilic and argyrophobic materials (Fig. 7). The matrix surrounding the lamellae penetrates through the interior of the granule and gives rise to the matrix cores (inset of Fig. 7). The lamellae are not labelled by silver nitrate (Fig. 7). With fertilization and after exocytosis, the unstained matrix is no longer observable (Fig. 8), while the argyrophilic matrix and the lamellae fuse with the vitelline layer (Figs. 9–13).

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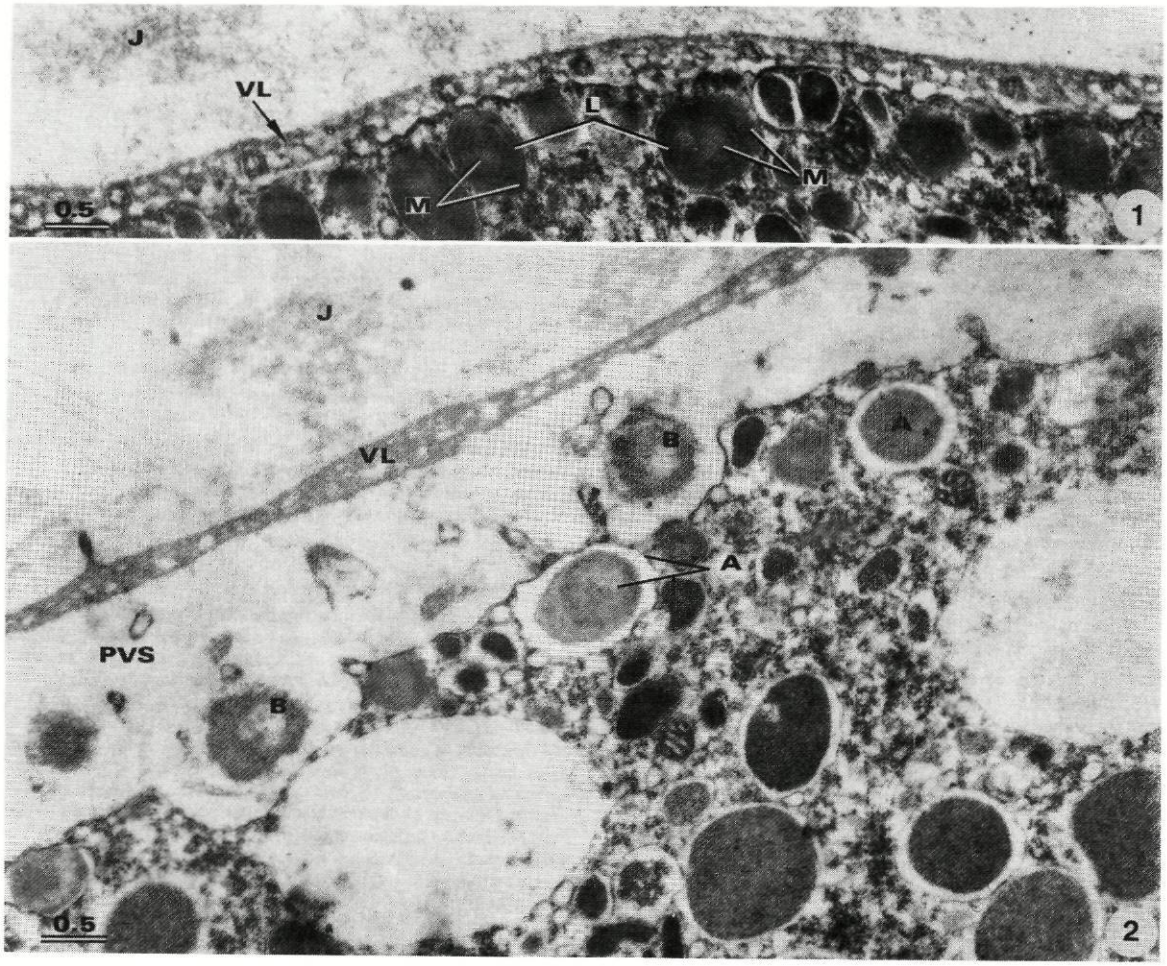


FIG. 1. Oocyte cortex. Cortical granules have a dense matrix (M) in which a lamellar structure (L) can be observed. VL, vitelline layer; J, oocyte jelly. FIG. 2. General view of the cortical reaction. First, cortical granules swell (A) and then they are released (B) into the perivitelline space (PVS). Scale bars are in micrometres.

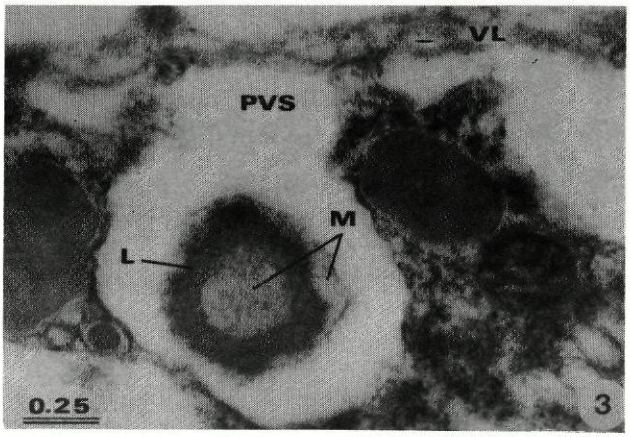
When oocytes are incubated with pronase before silver nitrate staining no silver deposits can be found (Figs. 14, 15).

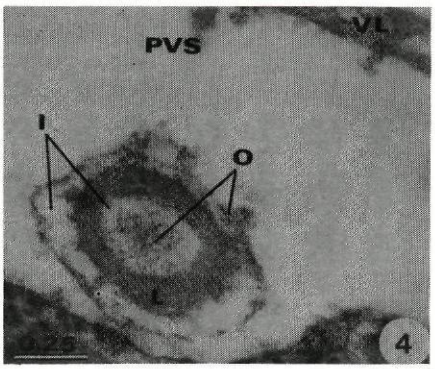
Discussion

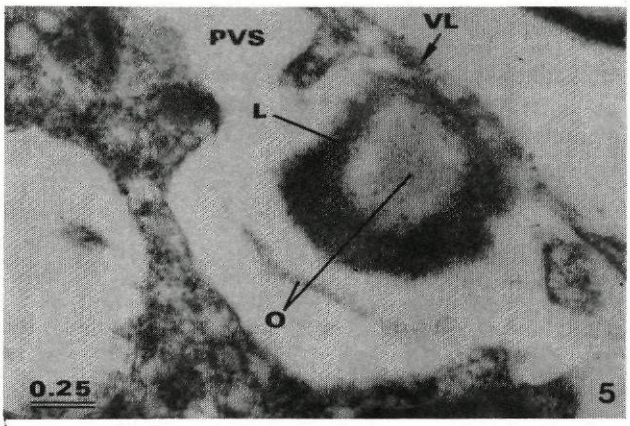
Fine structural changes in the eggs of echinoderms undergoing the cortical reaction have been studied, but it has not been possible to analyse with certainty the specific fate of all cortical granule components (Anderson 1968; Holland 1979, 1980; Longo et al. 1982; Yamashita 1984). Indeed, the exact correspondence of the components of cortical granules with those of the vitelline layer, perivitelline space, or hyaline layer is not always clear. Cytochemical studies can play a major role in the elucidation of the destinies of granule components, but they have only been carried out at the ultrastructural level in echinoids and ophiuroids (Bal 1970; Hylander and Summers 1982; Yamashita 1984; Cameron and Holland 1985).

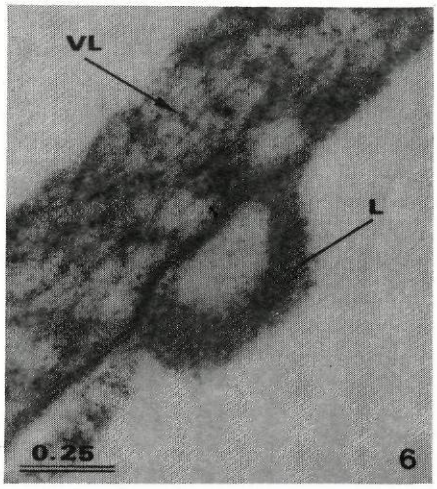
Silver nitrate staining of the cortical granules in the starfish *M. glacialis* revealed materials that were indistinguishable on

FIGS. 3 and 4. Cortical granules in the perivitelline space (PVS). Initially, the matrix (M) is intact (Fig. 3). After that, some light spaces appear inside the matrix (I), while the remainder of the matrix is preserved (O) (Fig. 4). The lamellar component (L) is transformed into a reticulated mass. VL, vitelline layer. FIGS. 5 and 6. Fusion of cortical granules with the vitelline layer (VL). FIG. 7. Oocyte cortex. The matrix surrounding the lamellar component is subdivided into argyrophilic (large arrows) and argyrophobic (I) materials after silver impregnation, and it penetrates through the interior of the granule (* in inset) to give origin to the granule matrix cores (arrowheads). Lamellae (L) are linked by fine fibrillar bridges (small arrows). All scale bars are in micrometres.

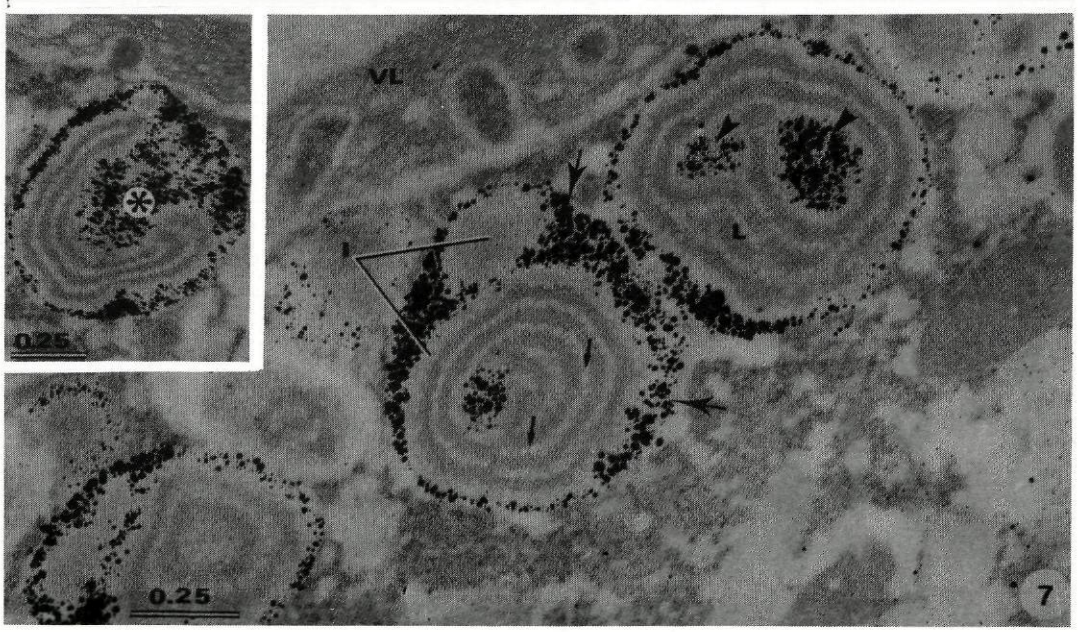

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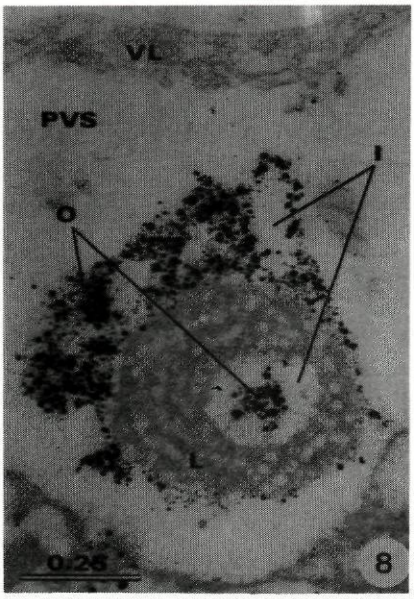

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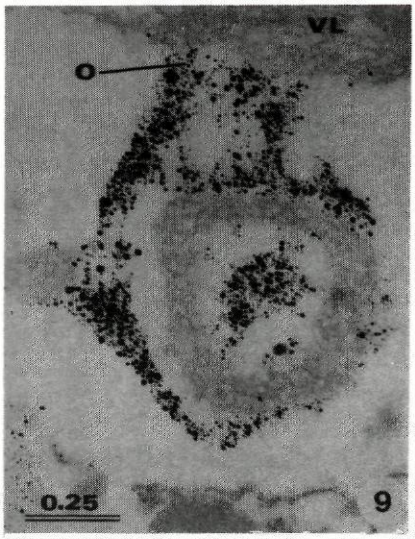

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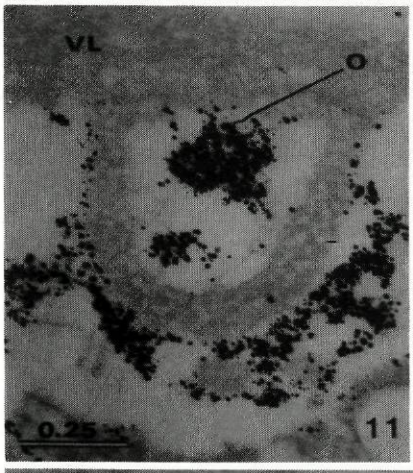
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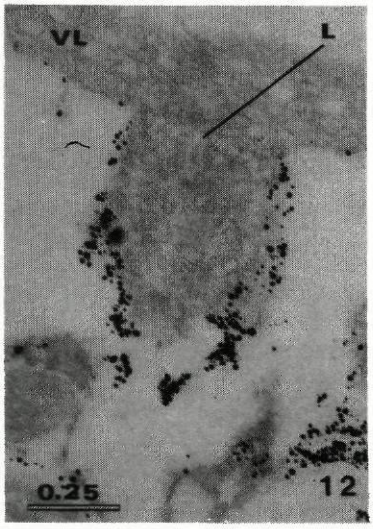

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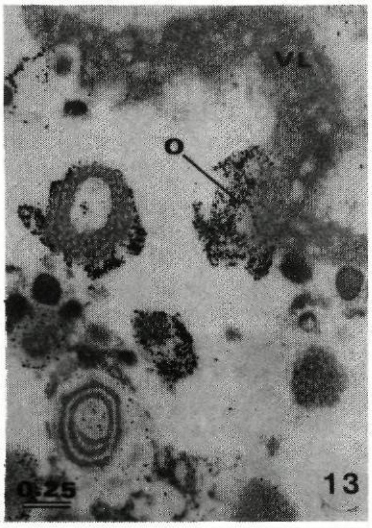

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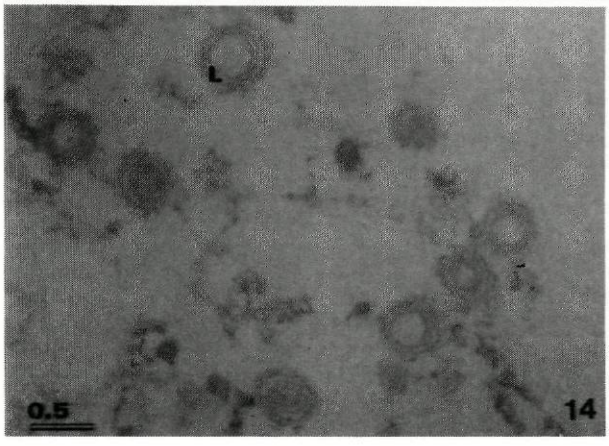

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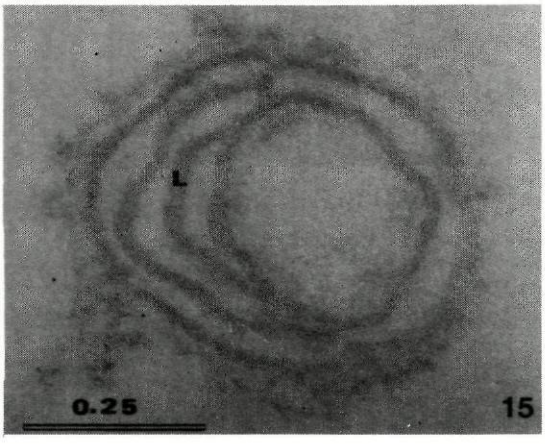

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VL
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VL
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0.5
14


L
0.25
15

fine structure observation, and allowed the changes and specific fate of each granule component during the cortical reaction to be followed.

Two special events occur during the cortical reaction of *M. glacialis*. First, just before exocytosis the cortical granules swell, and this may correspond to the irreversible osmotic swelling of sea urchin cortical granules caused by calcium, which drives the fusion of the granules' membrane with the oolema (Zimmerberg and Whitaker 1985). Second, in spite of their incorporation into the vitelline layer, the granules' argyrophilic materials do not transfer their staining pattern to it, perhaps because the reactive sites of argyrophilic proteins are changed.

The silver nitrate technique has been used to demonstrate argyrophilic proteins in cytoplasmic organelles, but the chemical nature of the reaction has not been elucidated. In this work we show that argyrophilia in cortical granules is inhibited by pronase pretreatment, a result which suggests protein involvement in the staining reaction.

Acknowledgments

This work was supported by the University of Oporto under contract No. 108/85 and by the Eng. A. Almeida Foundation.

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FIGS. 8–13. Silver staining of the cortical reaction. In the perivitelline space (PVS) the lamellar component (L) is transformed into a reticulated mass, the argyrophobic matrix (I) appears to be empty space, and the argyrophilic matrix (O) remains present (Fig. 8). Remaining granule components progressively fuse with the vitelline layer (VL) (Figs. 9–13). FIGS. 14 and 15. Oocytes incubated with pronase before silver nitrate staining. No silver nitrate deposits can be observed. L, lamellar component. All scale bars are in micrometres.

J. Submicrosc. Cytol. Pathol., **20** (3), 629-633, 1988

Ultrastructural and histochemical observations of the cortical reaction in *Marthasterias glacialis* (Echinodermata, Asteroidea)

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SUMMARY - When fixed in the presence of alcian blue the jelly coat of *M. glacialis* oocytes is seen to be made up of two regions: a large inner unstained layer and a thin outer densely stained layer from which some fibres extend through the inner jelly region. The contents of cortical granules present 3 distinct materials which, after the cortical reaction, fill the fertilization space and fuse with the fertilization envelope. Once in the extracellular space one of these cortical granule components stained by alcian blue. The elevated fertilization envelope was demonstrated to be formed by several different layers. When the fertilization envelope is further moved away from the egg surface the material filling the fertilization space forms a distinct hyaline layer.

Key Words *Asteroidea - cortical reaction - ultrastructure - histochemistry*

INTRODUCTION

The outer coats of echinoderm eggs have been described for several species (Schuel, 1978; Shapiro and Eddy, 1980). In starfishes, however, the fine structure of the fertilization envelope has not yet been resolved in detail and the existence of a hyaline layer remains controversial (Holland, 1980; Longo *et al.*, 1982; Crawford and Abed, 1986).

Extracellular molecules containing proteins and carbohydrates similar to those surrounding eggs are better preserved when cationic dyes are included in the primary fixative. The present study describes the fine structure of the egg outer coats of the starfish *Marthasterias glacialis* preserved in fixative containing alcian blue, before and after fertilization. The new findings are discussed in comparison with other echinoderm species.

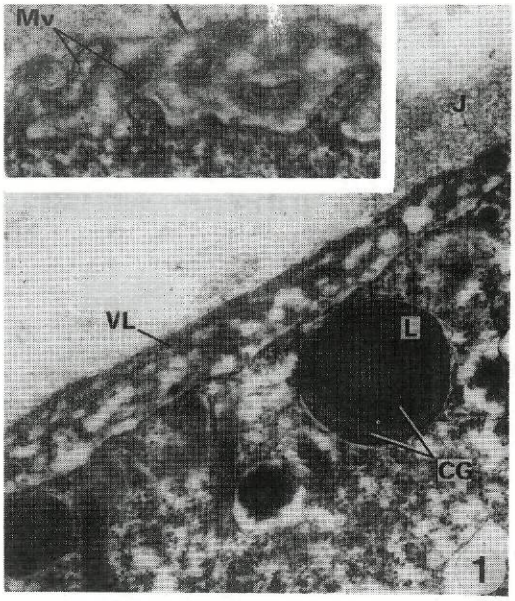
MATERIALS AND METHODS

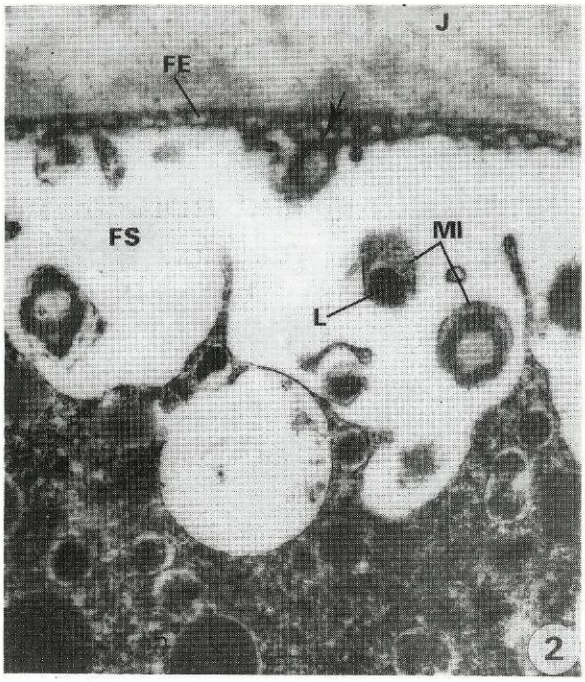
Specimens of *Marthasterias glacialis* were collected at the Portuguese intertidal North Atlantic coast. Handling of gametes and electron microscopy procedures are described elsewhere (Sousa and Azevedo, 1985, 1986). Alcian blue staining was done according to Crawford and Abed (1986): fixation for 3 h with 78 parts 1.3% alcian blue in filtered sea water (mixed for 2 h and filtered), 4 parts 25% glutaraldehyde, and 18 parts distilled water (pH 6.9, filtered); rinsing for 2 h with filtered 1.25% sodium bicarbonate, pH 7.2, and poststained in 2% OsO_4 for 1 h in the same buffer; staining for 1 h with filtered 2% aqueous uranyl acetate. Grids were examined in a JEOL 100CXII electron microscope operated at 60 KV.

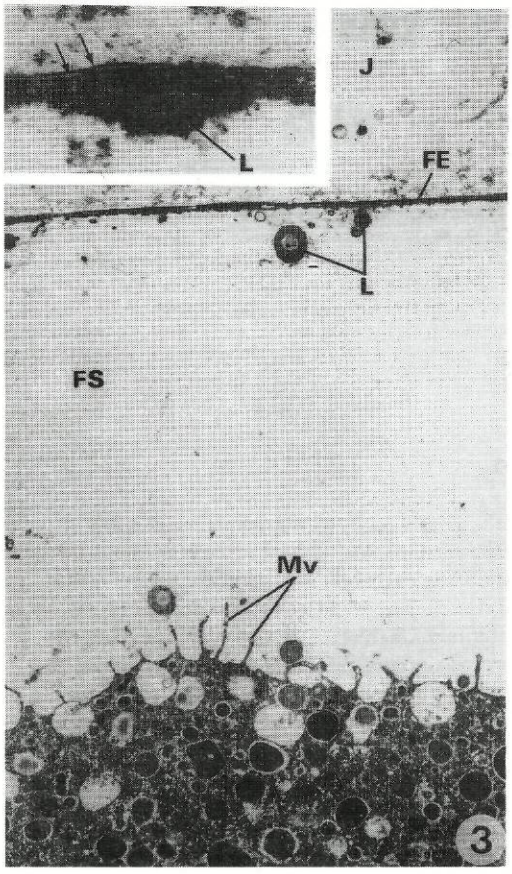
RESULTS

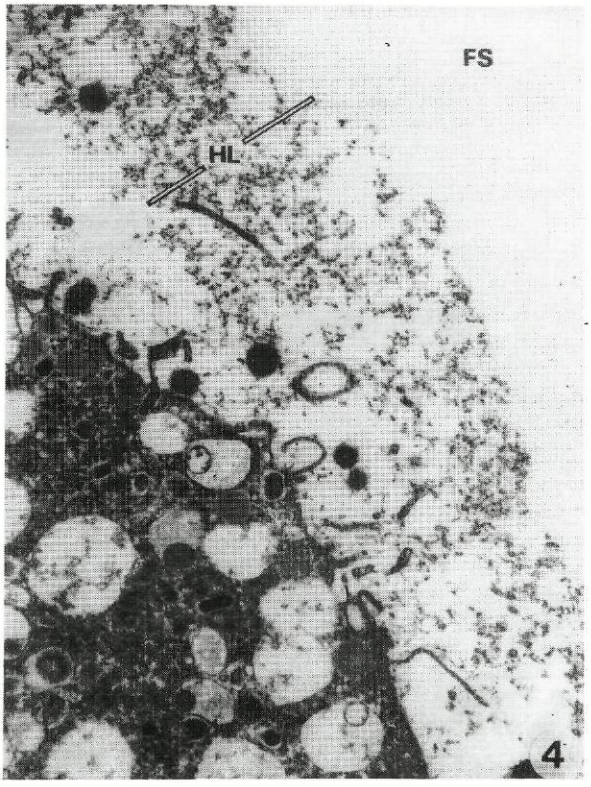
In *M. glacialis* oocytes the jelly coat is made of a fine fibrillar material, the vitelline layer is a fibrous meshwork surrounding microvilli, and cortical granules contain a lamellar body (Fig. 1). After cortical granule exocytosis into the fertilization space, the lamellar body appears as a dense mass surrounded by some matrix material, and fuses with the fertilization envelope (Fig. 2). When the fertilization envelope is further moved away from the egg surface it becomes denser and externally coated by a thin trilaminar structure (Fig. 3). Except in a small number of eggs (Fig. 4), a hyaline layer was not detected in the fertilization space (Fig. 3).

In oocytes, alcian blue stains the outer region of the jelly coat and some fibres which extend from it (Fig. 5). With


Mv
J
VL
L
CG
1


J
FE
FS
MI
L
2


L
J
FE
L
FS
Mv
3


FS
HL
4

fertilization, the unstained jelly material is solubilized (Fig. 6). Immediately after the beginning of the cortical reaction, the elevated fertilization coat differentiates into a narrow dense inner layer and a large outer fibrillar layer (Figs. 6 and 7). The outer portion of the latter is further differentiated into a dense zone and a saw-like dense line which are connected by some fibrillar bridges (Fig. 7). When the fertilization envelope is further pulled apart from the egg surface a hyaline layer is formed (Fig. 8). Later, the hyaline layer becomes thicker and positioned slightly above the egg surface (Fig. 9).

DISCUSSION

Alcian blue binds to anionic sites of molecules, and has been used to stain acid glycosaminoglycans of extracellular materials (Reale *et al.*, 1986). Alcian blue was shown to stain the jelly coat of starfishes and crinoids, and the vitelline layer of echinoids (Kidd, 1978; Holland, 1977; Crawford and Abed, 1986). Ruthenium red was similarly used and stains the jelly coat of echinoids and the vitelline layer of echinoids and ophiuroids (Kidd, 1978; Yamashita, 1984). In *M. glacialis*, however, only the outer jelly region appeared stained by alcian blue. At starfish fertilization it is to this particular jelly region that the sperm adheres and in which the acrosomal reaction begins. Induction of the acrosomal reaction by jelly acid polysaccharides has also been biochemically demonstrated in starfishes (Matsui *et al.*, 1986).

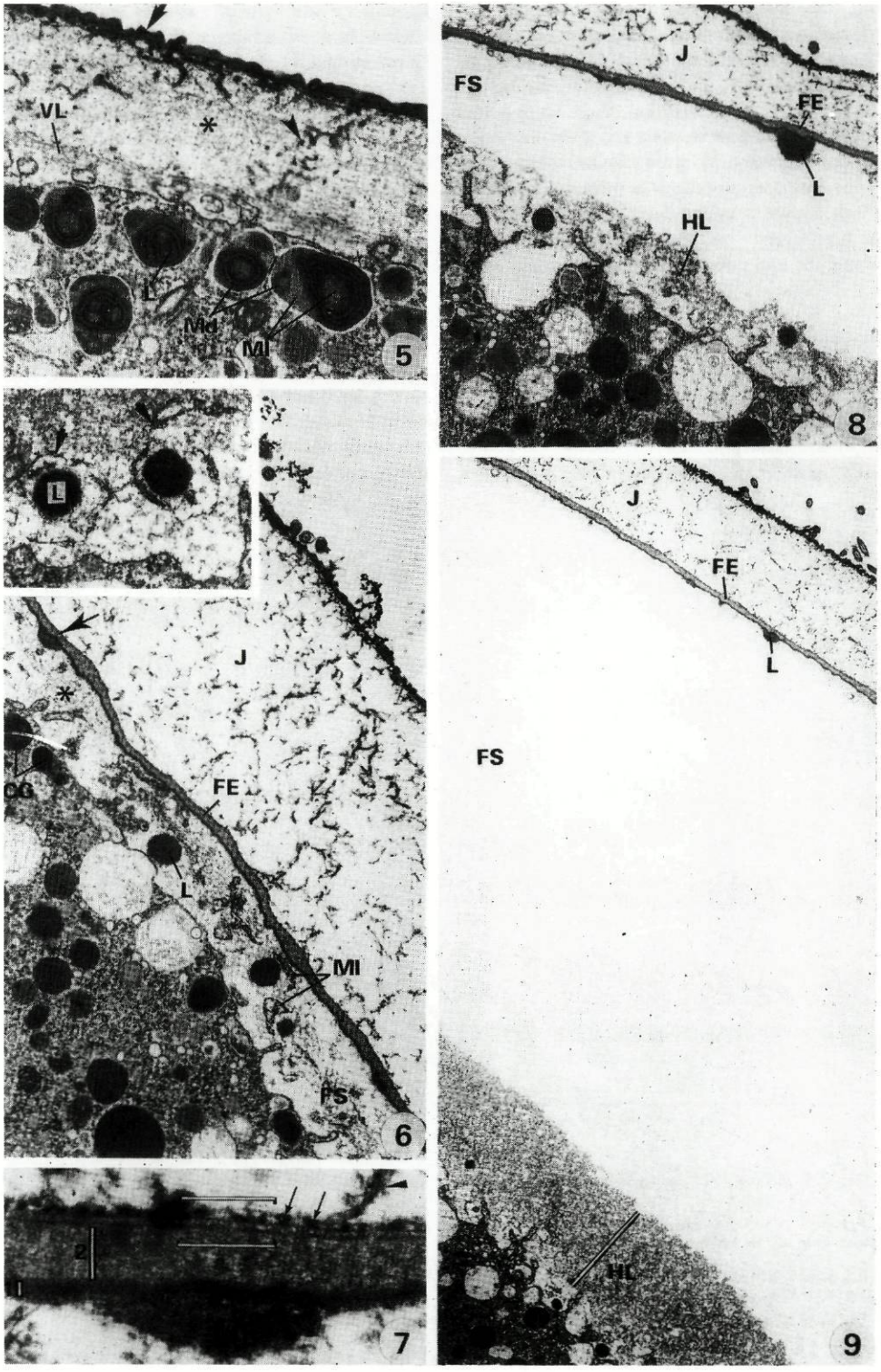
The differentiation of the fertilization coat into several layers is supposed to play a role in its hardning process (Chandler and Heuser, 1980). The fine structure of the starfish fertilization coat has been found difficult to preserve (Holland, 1980; Longo *et al.*, 1982), but in the present study all layers were fully preserved and rendered clearly visible by the use of alcian blue.

FIGURE 1 Oocyte. J: jelly coat; VL: vitelline layer (arrow in inset); Mv: microvilli; CG: cortical granule with lamellar body (L). × 31,000, inset: × 29,000.

FIGURE 2 Egg fixed 15 sec after insemination. In the fertilization space (FS) the lamellar body (L) and some zones of the matrix (Ml) from cortical granules seem to fuse (arrow) with the fertilization envelope (FE). × 20,000.

FIGURE 3 Egg fixed 10 min after insemination. The fertilization envelope (FE) is densified and externally coated by a trilaminar structure (arrow). × 5,000, inset: × 53,000.

FIGURE 4 Egg fixed 10 min after insemination. Over the egg surface there is a hyaline layer (HL). × 9,000.


VL
*
L
Md
MI
5
L
J
*
CG
FE
L
MI
FS
6
2
1
7
FS
J
FE
L
HL
8
J
FE
L
FS
HL
9

Eggs of echinoids and ophiuroids have a hyaline layer while those of crinoids and holothuroids do not (Afzelius, 1956; Holland, 1977, 1979, 1981). Asteroids were supposed not to have a hyaline layer, but recently it was demonstrated in *Pisaster* eggs at hatching (Crawford and Abed, 1986). Our observations made on alcian blue stained *M. glacialis* eggs show, however, that a hyaline layer is formed soon after the cortical reaction. Although the hyaline layer of echinoids and of *Pisaster* were stained by alcian blue (Cameron and Holland, 1985; Crawford and Abed, 1986) that of *M. glacialis* was not. In conclusion, we think that the addition of alcian blue to primary fixatives can be a valuable technique in demonstrating those extracellular materials of eggs which are not preserved in routine electron microscopy.

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Figures 5 to 9 Material fixed in the presence of alcian blue.

Figure 5 The outer region of the oocyte jelly coat is stained (arrow) and from it some fibres project (arrowhead) through the unstained inner portion of the jelly coat (asterisk). The matrix of cortical granules is subdivided into dense (Md) and light (Ml) portions; L: lamellar body. × 16,000.

Figure 6 With fertilization, the unstained jelly material is solubilized (J). After cortical granule (CG) exocytosis into the fertilization space (FS) the light matrix regions (Ml) become stained (arrows in inset), the dense matrix regions fill the fertilization space (asterisk), and the lamellar bodies (L) fuse with the fertilization envelope (FE). × 8,000, inset: × 16,000.

Figure 7 Layers of the fertilization envelope: 1) cortical granule material; 2) densified vitelline layer; outer trilaminar structure (area between brackets). The stained jelly fibres (arrowhead) end on some stained globular structures which lie over the fertilization envelope (small arrows). × 56,000.

Figure 8 Egg fixed 30 sec after insemination. HL: hyaline layer. × 7,000.

Figure 9 Egg fixed 10 min after insemination. HL: definitive hyaline layer. × 4,000.

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Starfish sperm-oocyte jelly binding triggers functional changes in cortical granules

A study using acid phosphatase and ruthenium red ultrastructural histochemistry

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Accepted May 19, 1988

Summary. Starfish oocytes were examined before fertilization, immediately after insemination, and during the cortical reaction by means of acid phosphatase and ruthenium red ultrastructural histochemistry. Oocyte cortical granules are composed of a lamellar body and a surrounding matrix which is subdivided into dense and light portions. In unfertilized oocytes cortical granules are not stained by ruthenium red but show a weak acid phosphatase activity in the light portion of the granule matrix. Immediately after the adhesion of the spermatozoon to the oocyte jelly coat, the light matrix portion of cortical granules appears stained by ruthenium red and shows a strong acid phosphatase activity. During the cortical reaction, cortical granules are released into the perivitelline space and the lamellar body, surrounded by the stained matrix, fuses with the fertilization envelope. Our data suggest that membrane permeability changes and enzyme activation occur in the egg when the spermatozoon binds to the oocyte jelly coat.

Introduction

Recent studies have shown that sea urchin sperm-egg binding causes the opening of ion channels in the oolemma (Dale and De Santis 1981; Longo et al. 1986). If these membrane changes which occur before fusion of the sperm and egg plasma membranes are inducers of egg activation remains unknown.

Previous attempts to define the time of attachment of sperm to the egg and of fusion of sperm and egg plasma membranes in sea urchins were hampered by the fact that these events occur in a very short time period. While in the echinoids gamete binding occurs near the oolemma (Gache et al. 1983; Rossignol et al. 1984; Acevedo-Duncan and Carrol 1986), in the asteroids sperm-oocyte initial binding occurs several micrometers away from the oolemma (Sousa and Azevedo 1985). The larger width of the outer coats of the starfish egg delays the contact between gamete membranes allowing to consider as zero-time for ultrastructural observations the moment of mixing of sperm and egg suspensions. This fact enabled us to morphologically distinguish the moment of sperm attachment from the time the cortical reaction is initiated following gamete membrane fusion.

The investigation reported in this paper was undertaken to morphologically determine if sperm-egg jelly binding is responsible for the activation of the egg. We have fixed starfish eggs before fertilization, at the moment the spermatozoon adheres to the egg jelly coat, and during the cortical reaction, and studied the histochemical properties of the cortical granules at those different times. Results suggest that membrane permeability changes and enzyme activation occur in the starfish egg when spermatozoa bind to the jelly coat.

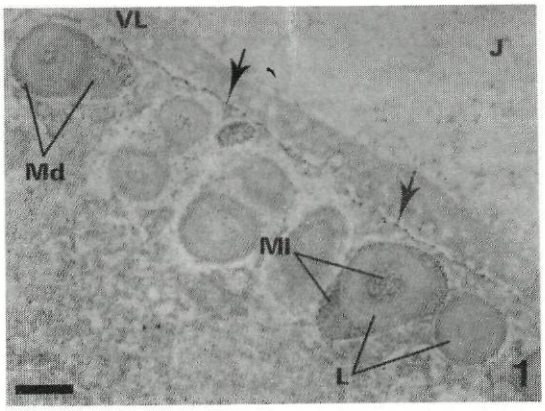
Materials and methods

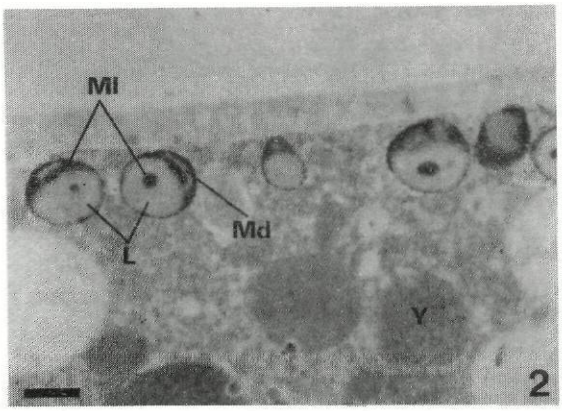
Obtaining and handling of gametes. Several specimens of *Marthasterias glacialis* (Echinodermata, Asteroidea) were collected at the Portuguese North Atlantic coast. Male gonads were cut, squeezed through gauze, and the obtained semen was stored at 4° C as dry sperm. Female gonads were cut in filtered sea water (FSW), and the liberated oocytes were then left in continuously stirred FSW until their spontaneous maturation (Sousa and Azevedo 1985). Sperm suspensions were prepared by the addition of 10 ml FSW to 0.1 ml dry sperm. Insemination was carried out by adding 2 ml of sperm suspensions to 2 ml of oocytes collected from the bottom of goblets where they had sedimented.

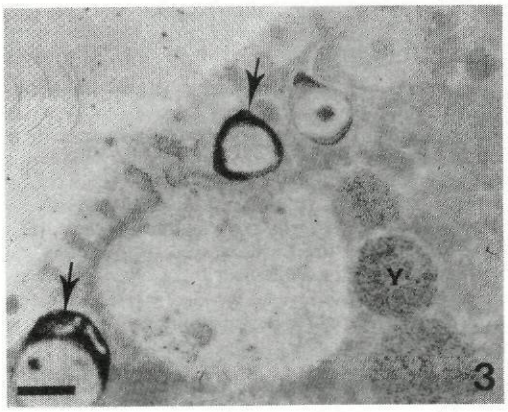
Ruthenium red staining. Suspensions of oocytes and fertilized eggs were fixed for 2 h at 4° C by the addition of an equal volume of 5% glutaraldehyde in 0.2 *M* sodium cacodylate, pH 7.2. Fertilized eggs were fixed at several seconds after insemination. After washing for 2 *h* at 4° C in 0.2 *M* sodium cacodylate, pH. 7.2, the cells were postfixed for 1 h 30 at 4° C with 2% OsO_4 in 0.1 *M* sodium cacodylate, pH 7.2, with 0.1% ruthenium red (Summers and Hylander 1974). Cells were then washed several times with 0.1 *M* sodium cacodylate, dehydrated in an ascending ethanol series to propylene oxide, and embedded in Epon.

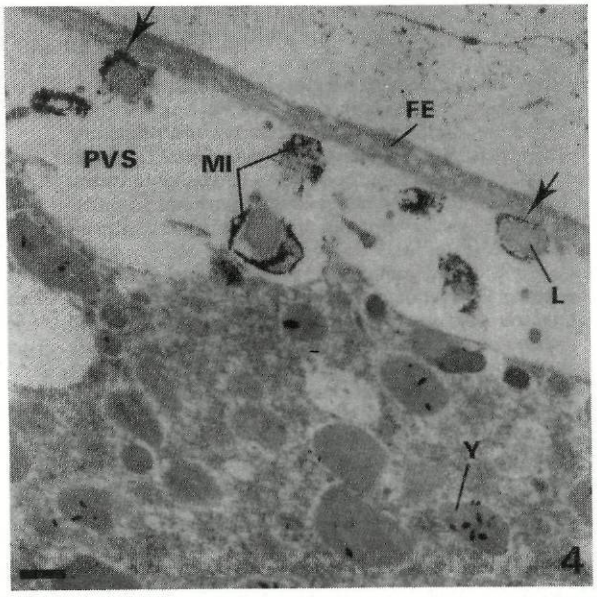
Acid phosphatase histochemistry. Acid phosphatase activity was determined according to Sousa and Azevedo (1986). Briefly, suspensions of oocytes and fertilized eggs were fixed for 30 min in an ice bath by the addition of an equal volume of 5% glutaraldehyde in FSW. Cells were then washed in 0.2 *M* sodium cacodylate, pH 7.4 with 5% sucrose, for 2 × 15 min, at 4° C, followed by preincubation in 40 m*M* *Tris*-maleate, pH 5, with 5% sucrose, for 2 × 15 min at 4° C. The incubation medium consisted of 11.5 m*M* sodium-B-glycerophosphate and 2.4 m*M* lead nitrate in 40 m*M* *Tris*-maleate, pH 5, with 5% sucrose. This was filtered and then

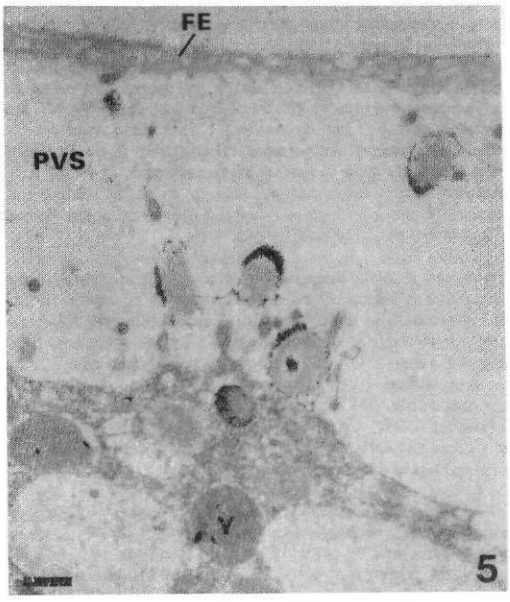
* To whom offprint requests should be sent

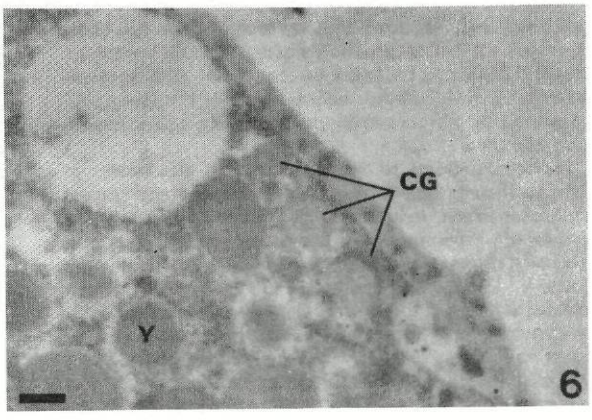








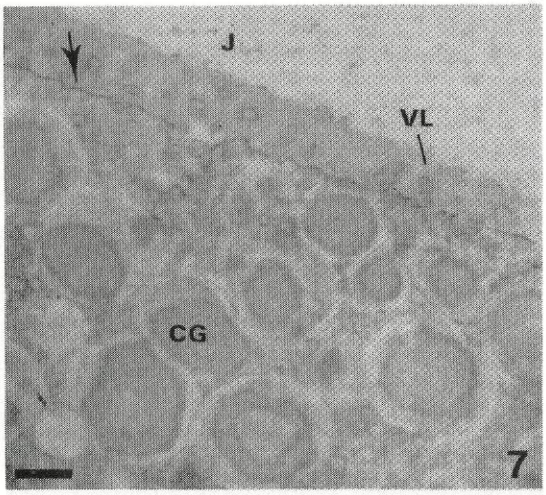


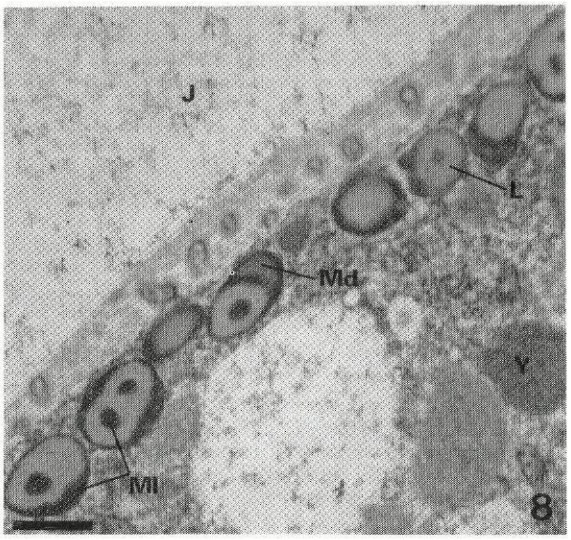


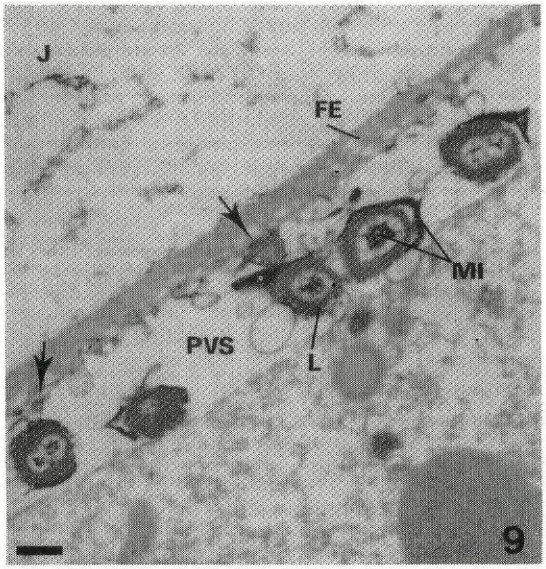
Figs. 1–5. Material processed for the ultrastructural detection of acid phosphatase activity. *Bars* = 0.5 μm

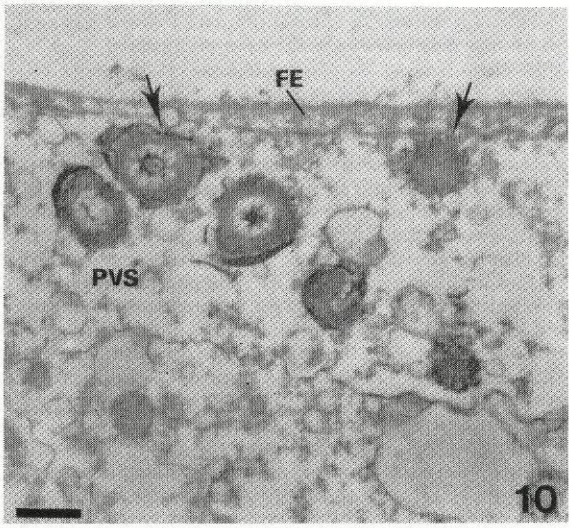
Fig. 1. Unfertilized oocyte. Lead phosphate deposits are observed on the oolemma (*arrows*) and in the light matrix component of cortical granules (*MI*). Lamellar body (*L*) and dense matrix portion (*Md*) of cortical granules; *VL*, vitelline layer; *J*, jelly coat. × 18000

Fig. 2. Egg fixed immediately after insemination. Lead phosphate deposits are seen in the light matrix portion of cortical granules (*MI*) and in yolk granules (*Y*). × 17000









Figs. 7–10. Material processed for the ultrastructural demonstration of extracellular acidic mucopolysaccharides using ruthenium red. *Bars* = 0.5 μm

Fig. 7. Unfertilized oocyte. The oolemma (*arrow*) and fibrils of the jelly coat (*J*) appear stained. *VL*, vitelline layer; *CG*, cortical granules. × 18000

Fig. 8. Egg fixed immediately after insemination. The light matrix portion (*Ml*) of cortical granules appears stained. Lamellar body (*L*) and dense matrix portion (*Md*) of cortical granules. × 25000

Figs. 9 and 10. Eggs fixed at 10 s (**Fig. 9**) and 20 s (**Fig. 10**) after insemination. In the perivitelline space (*PVS*) the lamellar body (*L*) and the stained matrix portion (*Ml*) of cortical granules fuse (*arrows*) with the fertilization envelope (*FE*). No staining of this latter structure is noticed. **Fig. 9** × 14000 and **Fig. 10** × 20000 respectively

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Fig. 3. Egg fixed 6 s after insemination. Lead phosphate deposits are seen in cortical granules (*arrows*) and yolk granules (*Y*). × 18000

Fig. 4. Egg fixed 15 s after insemination. In the perivitelline space (*PVS*) the lamellar body (*L*) and the light matrix portion (*Ml*) of cortical granules fuse (*arrows*) with the fertilization envelope (*FE*). Lead phosphate deposits are only seen in *Ml*. × 14000

Fig. 5. Egg fixed 30 s after insemination. No lead phosphate deposits are observed in the elevated fertilization envelope (*FE*). × 15000

Fig. 6. Control of acid phosphatase activity. No lead phosphate deposits are observed in cortical granules (*CG*) and yolk granules (*Y*) in egg fixed 6 s after insemination

allowed to reach 37° C before use. Incubation time was 40 min. The cells were then washed in 40 m*M* *Tris*-maleate, pH 7.4, with 5% sucrose, for 2 × 15 min at 4° C, and after that in 0.2 *M* sodium cacodylate, pH 7.4, with 5% sucrose, for 2 × 15 min at 4° C. Post-fixation was developed with OsO_4 in 0.2 *M* sodium cacodylate, pH 7.4, for 2 h at 4° C, and dehydration and embedding was as described above. For controls, the material was incubated without substrate or with 10 m*M* NaF.

Results

The cortical granules of *M. glacialis* oocytes are composed of a lamellar structure and a surrounding matrix which is subdivided into light and dense portions. Unfertilized oocytes processed for the ultrastructural detection of acid phosphatase activity show, in about 50% of cortical granules, some lead phosphate deposits in the light portion of the cortical granule matrix (Fig. 1), and patchy lead phosphate deposits on the oocyte plasma membrane (Fig. 1). In eggs fixed immediately after insemination, spermatozoa have stuck to the egg jelly coat and are beginning the acrosomal reaction. At this very early fertilization step, the acid phosphatase activity in cortical granules is increased and the plasma membrane deposits have disappeared (Fig. 2). After sperm binding, the acrosomal process takes some seconds to penetrate the egg coats and fuse with the oolemma, triggering the cortical reaction. When this exocytic step begins, the intensity of the enzymatic activity in the nearer cortical granules reaches its maximum (Fig. 3). After cortical granule release into the perivitelline space, the lamellar body and the matrix region containing lead phosphate deposits fuse with the fertilization envelope (Fig. 4). Acid phosphatase activity, however, has not been found in the elevated fertilization coat (Fig. 5). Acid phosphatase activity was also found in yolk granules. Before the cortical reaction, yolk granules appear full of fine lead phosphate deposits of low density (Fig. 2). After the exocytic event, however, yolk granules just contain a few number of high density, coarse lead phosphate deposits (Fig. 4). In controls no lead phosphate deposits were found (Fig. 6).

In unfertilized oocytes processed for the ultrastructural detection of extracellular acid mucopolysaccharides, ruthenium red did not stain cortical granules or any other intracellular structure, and of the extracellular coats only the jelly fibrils appeared stained (Fig. 7). In eggs fixed immediately after insemination, however, the light portion of the cortical granule matrix appears stained by ruthenium red (Fig. 8). After exocytosis into the perivitelline space, the lamellar body and the stained matrix of cortical granules fuse with the fertilization envelope, but no staining of this latter structure was noticed (Figs. 9 and 10).

Discussion

In echinoderm eggs yolk granules are known to contain the enzyme acid phosphatase (Ferrand 1984). Acid phosphatase activity, however, has never been reported in cortical granules. Unfertilized oocytes of *M. glacialis* show a weak acid phosphatase activity in about 50% of the cortical granules. Immediately after insemination, however, a strong acid phosphatase activity is then demonstrated in almost all of the cortical granules, suggesting that sperm-jelly binding induces the activation of the enzyme. Sperm-jelly binding seems also to cause the disappearance of the patchy acid phosphatase activity from the oolemma, while the increase in yolk granules acid phosphatase activity was only noticed after the cortical reaction.

Ruthenium red has been used to stain the acid mucopolysaccharide extracellular coats of cells (Luft 1971; Anderson 1968), and it stains the jelly fibrils in the unfertilized oocytes of *M. glacialis*. Immediately after insemination, however, the egg plasma membrane becomes permeable to ruthenium red as cortical granules appear stained with this dye, suggesting that sperm-jelly binding induces permeability changes in the oolemma.

These histochemical findings fit with previous electrophysiological studies (Dale and De Santis 1981; Longo et al. 1986), and represent the first morphological observations which demonstrate that when some sperm factor interacts with the extracellular oocyte coats triggers the activation of the oocyte. This activation is of a functional type because the cortical reactions begins only after gamete membrane fusion.

Histochemical investigations on echinoderm cortical granules have only been carried out at the ultrastructural level in echinoids and ophiuroids, and showed them to contain acidic mucopolysaccharides (Bal 1970; Yamashita 1984; Cameron and Holland 1985), ovoperoxidase (Klebanoff et al. 1979), and hyalin (Hylander and Summers 1982). In the present study we demonstrate that in the starfish *M. glacialis*, oocyte cortical granules contain acidic mucopolysaccharides and acid phosphatase.

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Alkaline phosphatase and peroxidase activities in starfish cortical vesicles before and after cortical reaction

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SUMMARY - We have investigated at the ultrastructural level the presence of alkaline phosphatase and peroxidase activities in *Marthasterias glacialis* cortical vesicles both before and after the cortical reaction. Cortical vesicles are composed of a central hollow body and a surrounding matrix. In unfertilized oocytes, cortical vesicles do not show alkaline phosphatase or peroxidase activities. With cortical reaction, however, cortical vesicles matrixes stain for alkaline phosphatase whereas peroxidase activity becomes detectable in the central bodies. As cortical vesicles materials fuse with the elevated vitelline layer, alkaline phosphatase activity then appears as fine scattered lead deposits while peroxidase activity is confined to the inner layer of the fertilization coat.

Key Words *Asteroidea - cortical reaction - alkaline phosphatase - peroxidase*

INTRODUCTION

Echinoderm cortical vesicles are exocytosed when the oocyte is fertilized by a spermatozoon. The products then liberated remain in the fertilization space to protect the embryo during early development or fuse with the fertilization coat to prevent polyspermy (Schuel, 1978, 1984; Shapiro and Eddy, 1980).

Biochemical and cytochemical methods have been used to elucidate the chemical composition and the destinies of echinoid cortical vesicle components, and studies have demonstrated them to contain sulfated acid mucopolysaccharides (Bal, 1970; Schuel *et al.*, 1972; Cameron and Holland, 1985), hyalin (Hylander and Summers, 1982), proteases (Schuel *et al.*, 1973; Lois *et al.*, 1986; Alliegro and Schuel, 1988), catalase (Katsura and Tominaga, 1974), peroxidase (Foerder and Shapiro, 1977; Klebanoff *et al.*, 1979; Somers *et al.*, 1989), and glucanase (Epel *et al.*, 1969).

As starfishes are also important animals in basic reproduction studies we have undertaken some investigations in order to morphologically characterize the chemical nature and the fate of starfish cortical vesicle contents. Recently, we have demonstrated in starfish cortical vesicles the presence of acid mucopolysaccharides, argyrophilic proteins, and acid phosphatase activity (Sousa and Azevedo, 1987, 1988, 1989). In the present work we show that cortical vesicles of starfish oocytes also contain the enzymes alkaline phosphatase and peroxidase.

MATERIALS AND METHODS

Several specimens of *Marthasterias glacialis* (Echinodermata, Asteroidea) were collected at the Portuguese intertidal North Atlantic coast. Male gonads were cut, squeezed through gauze, and the obtained semen was stored at 4 °C as dry sperm. Female gonads were cut in filtered sea water (FSW), and the liberated oocytes were then left in continuously stirred FSW until their spontaneous maturation (Sousa and Azevedo, 1985). Sperm suspensions were prepared by the addition of 10 ml FSW to 0.1 ml dry sperm. Insemination was carried out by adding 2 ml of sperm suspensions to 2 ml of oocytes collected from the bottom of goblets where they had sedimented.

For AlPase histochemistry, oocytes and fertilized eggs were prefixed with 2.5% glutaraldehyde in FSW - 0.1 M Pipes buffer (3:1), pH 7.2, 1% sucrose, for 1 h at 4 °C, washed in Pipes buffer, 5% sucrose, for 2 × 1 h at 4 °C, and preincubated in 0.2 M Tris-maleate buffer, pH 9, 5% sucrose, for 1 h at 4 °C. The incubation medium consisted of 10 ml 0.2 M Tris-maleate buffer, pH 9, 10 ml 1.25% Na-β-glycerophosphate, 23.5 ml distilled water, 6.5 ml 1% lead nitrate and 3.25 g sucrose; the

pH of the solution was adjusted to 9 with 1 N NaOH and then filtered through 2 sheets of Whatman paper 50 (Hugon and Borgers, 1966); incubation was carried out for 45 min at 37 °C. Cells were then washed with Tris-maleate buffer for 2 × 15 min at 4 °C, followed by 2 × 30 min washes with Pipes buffer. Postfixation was done with 1% OsO_4 in Pipes buffer with 1.5% potassium hexacyanoferrate (II) trihydrate GR, for 2 h at 4 °C. For controls the material was incubated without substrate. Some oocytes and eggs were also incubated with thiamine pyrophosphate or Na-β-glycerophosphate at pH 7.2 (Essner, 1973), and ATP at pH 8.5 (Mughal *et al.*, 1987).

For peroxidase histochemistry, oocytes and fertilized eggs were incubated for 10 min at room temperature in 5.6 mM 3,3' - diaminobenzidine, 0.45 M NaCl, 0.1 M Tris-HCl buffer, pH 8, and 6 mM hydrogen peroxide (Klebanoff *et al.*, 1979). Cells were then washed in FSW for 15 min at 4 °C and prefixed with 2.5% glutaraldehyde in FSW for 2 h at 4 °C. After being washed with FSW and 0.1 M Pipes buffer, pH 7.2, 5% sucrose, cells were postfixed with 1% OsO_4 in Pipes buffer. For controls, cells were incubated without hydrogen peroxidase or incubated only after having been prefixed; in this latter case, cells were incubated for 30 min at 4 °C followed by other 30 min at room temperature (Graham and Karnovsky, 1966; Essner, 1974).

After dehydration in ethanol and embedding in Epon, the material was examined without counterstaining in a JEOL 100CXII TEM at 60KV.

RESULTS

Unfertilized oocytes show no alkaline phosphatase activity in cortical vesicles (Fig. 1). Inseminated oocytes that underwent the cortical reaction, however, exhibit lead deposits in matrix portions of discharged cortical vesicles. As the exocytosed materials fuse with the fertilization coat alkaline phosphatase activity then appears in it as scattered lead deposits (Figs. 2 and 3). No lead deposits were found in cells incubated without substrate (Fig. 4). Also, no lead deposits were found in cortical vesicles of cells incubated with TPP at pH 7.2, Na-β-glycerophosphate at pH 7.2 (positivity was found in yolk vesicles), or ATP at pH 8.5 (positivity for Ca-ATPase was only found in mitochondria).

In unfertilized oocytes processed for the ultrastructural detection of peroxidase activity, no staining was noticed in cortical vesicles (Fig. 5). With cortical reaction, however, the central bodies of discharged cortical vesicles become labeled (Fig. 6). Once in the fertilization space, central bodies fuse with the inner surface of the fertilization coat, transfering the labeling to this latter structure (Fig. 7). Cells incubated without hydrogen peroxide (Figs. 8 and 9) or incubated in complete medium after having been prefixed (Figs. 10 and 11) do not present any peroxidase activity.

DISCUSSION

The extracellular materials of starfish eggs have been morphologically characterized (Holland, 1980; Longo *et al.*, 1982; Cameron and Holland, 1983), but only a few studies have made reference to the chemical composition of cortical vesicle materials (Crawford and Abed, 1986; Sousa and Azevedo, 1987, 1988, 1989).

In the present paper we demonstrate that in the starfish *M. glacialis*, discharged cortical vesicles contain alkaline phosphatase in the matrix and peroxidase in the central body. As both activities are only detected after the cortical reaction is initiated, it is not possible to ascertain

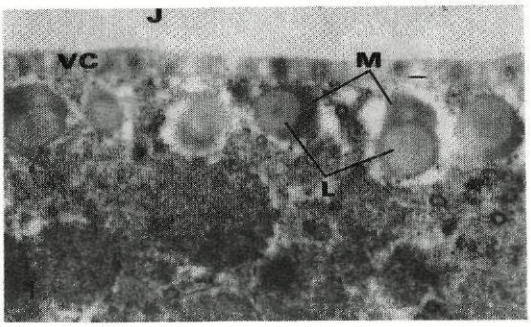
FIGURE 1 In the unfertilized oocyte no alkaline phosphatase activity is seen in the matrix (M) or central bodies (L) of cortical vesicles. J: jelly coat; VC: vitelline coat. × 18,400.

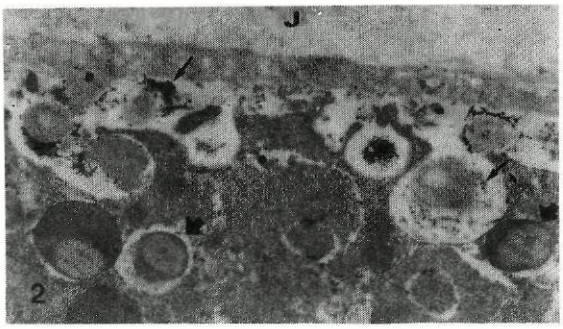
FIGURES 2 and 3 Alkaline phosphatase activity is observed in the matrix of exocytosed (arrows) but not of unreacted (large arrows) cortical vesicles (Fig. 2). Later, scattered lead deposits (double arrows) appear in the fertilization coat (FC) as cortical vesicles materials fuse with it (Fig. 3). Fig. 2: × 13,300; Fig. 3: × 15,000.

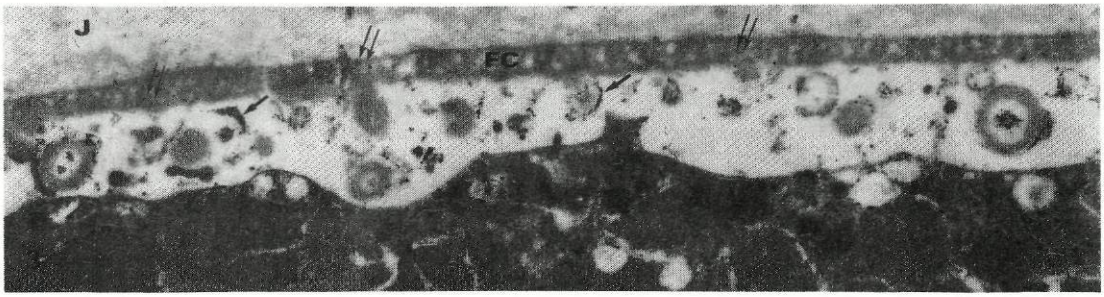
FIGURE 4 No alkaline phosphatase activity is observed when the substrate was omitted from the medium. × 23,500.

FIGURE 5 In the unfertilized oocyte no peroxidase activity is observed in the matrix (M) or central body (L) of cortical vesicles. × 21,600.

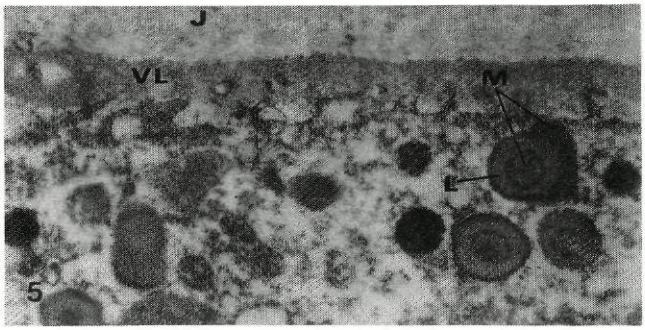
FIGURE 6 At the beginning of cortical reaction, peroxidase activity is seen in the exocytosed central bodies (arrows). × 17,300.

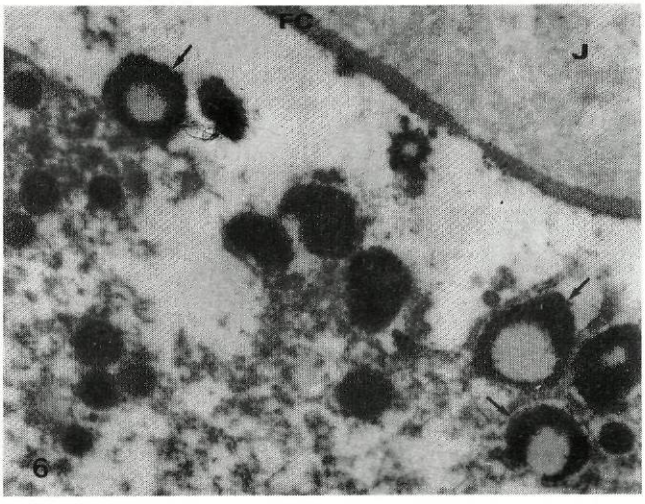

J
VC
M
L
1

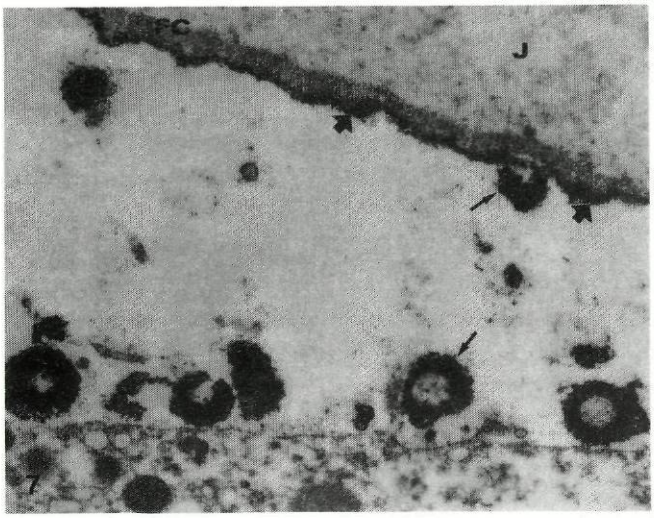

J
2

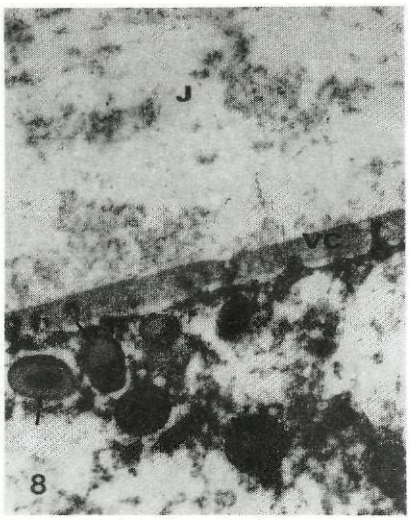

J
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3

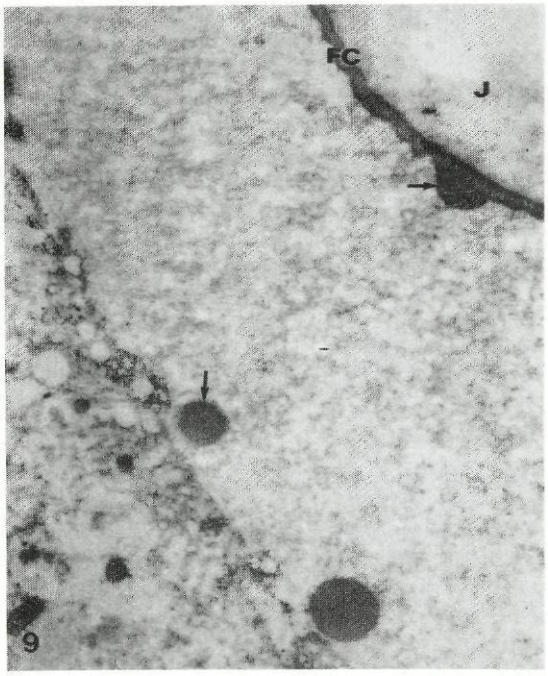

FC
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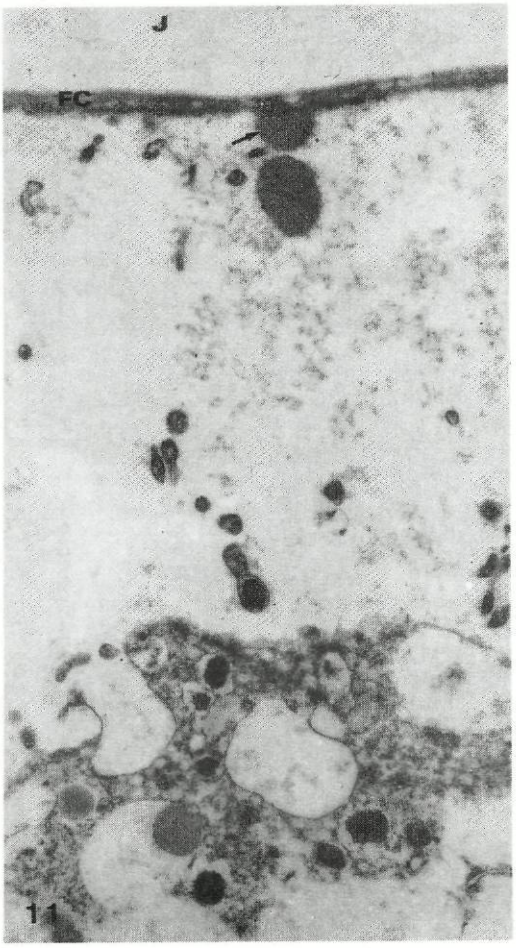

J
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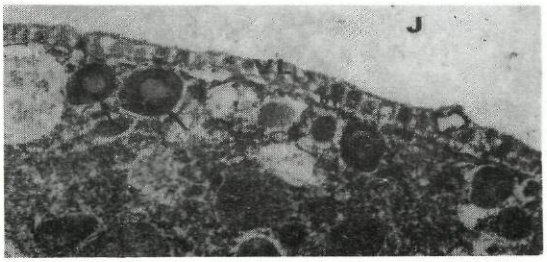

FC
J
6


FC
J
7


J
VC
8


FC
J
9


J
FC
11


J

whether the enzymes become activated with egg activation or if the unreacted oocyte is impermeant to incubation reagents. Unreacted oocytes, however, may not be impermeant to substrates because AcPase (using Na-β-glycerophosphate) and peroxidase activities have been histochemically detected in starfish and sea urchin intact cortical vesicles and yolk (Klebanoff *et al.*, 1979; Ferrand, 1984; Sousa and Azevedo, 1989). Further, in the case of AcPase activity, this enzyme was also shown to be activated during exocytosis (Anderson, 1968; Sousa and Azevedo, 1989). So, our results suggest that in *M. glacialis*, cortical vesicles contain two latent enzymes, AlPase and peroxidase, that become activated following the cortical reaction.

Release of ovoperoxidase from sea urchin eggs is known to harden the fertilization envelope with tyrosine crosslinks, blocking the entry of additional sperm (Foerder and Shapiro, 1977). Concerning peroxidase localization in the fertilization coat, our results differ from those in sea urchins, as in *M. glacialis* peroxidase activity appears restricted to the inner layer of the fertilization coat, while in the sea urchin the entire fertilization coat acquires that staining characteristic (Klebanoff *et al.*, 1979).

In spite of having been detected in discharged cortical vesicle materials, the role of non-specific phosphatases has not yet been determined. In *M. glacialis*, the presence of these enzymatic activities in the cortical vesicle components that fuse with the fertilization coat, suggests their involvement in the transformation of this latter structure.

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Figure 7 As the central bodies fuse with the fertilization coat (FC), peroxidase activity is transfered to its inner layer (large arrows). × 17,300.

Figures 8 to 11 Unfertilized or reacted eggs incubated without hydrogen peroxide (Figs. 8 and 9) or incubated after fixation (Figs. 10 and 11) show no peroxidase activity in the central body of cortical vesicles (arrows) or in the fertilization coat (FC). Fig. 8: × 15,500; Fig. 9: × 13,500; Fig. 10: × 10,600; Fig. 11: × 15,800.

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Ultrastructural Localization of Calcium in the Acrosome and Jelly Coat of Starfish Gametes

(Asteroidea/sperm acrosome/oocyte jelly/calcium)

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The potassium pyroantimonate technique was employed to localize calcium ultrastructurally on both male and female starfish gamete regions that first interact at fertilization. In the spermatozoon of *Marthasterias glacialis*, antimonate precipitates in the peripheral dense component of the acrosomal vesicle, while in the oocyte it precipitates in the jelly coat and beneath the oolemma. Calcium was identified in the precipitates by testing the chelator-sensitivity and by X-ray microanalysis of the precipitates.

Introduction

Calcium ions are known to mediate sperm and egg activation (5, 24). Ultrastructurally, calcium has been localized on the plasma and acrosomal membranes of mammalian sperm (2), and on the vitelline coat, plasma membrane and cortical organelles of echinoderm eggs (3, 16, 17).

Since calcium is the main ion involved in gamete activation at fertilization, the present study was undertaken to examine, by means of antimonate precipitation, the distribution of calcium in those starfish gamete regions which first interact at fertilization, i.e., the sperm acrosome and the oocyte jelly coat.

Materials and Methods

Specimens of *Marthasterias glacialis* (Echinodermata, Asteroidea) were collected at the Portuguese intertidal North Atlantic coast. Small gonad pieces were fixed in unbuffered 2% glutaraldehyde containing 2% K-pyroantimonate in 0.01 N K-acetate, pH 7.8, prepared as in Klein et al. (9), for 1 h at 4°C. After several washings in 0.01 N K-acetate, the material was postfixed in 1% OsO_4-2% K-pyroantimonate in 0.01 N K-acetate for 1 h at 4°C, and then washed in 0.05 N K-acetate, pH 7.8. All K-pyroantimonate solutions were filtered through 0.2 μm millipore filters prior to use (2). Control specimens were fixed in the same solutions without K-pyroantimonate. Dehydration and embedding were carried out by standard techniques (18). Some ultrathin sections collected on copper grids were floated on a solution of 6 mM EGTA or EDTA, pH 8, for 1 h at 60°C and control grids were floated on deionized water in the same conditions (26). Sections were examined without counterstaining in a JEOL 100CXII TEM at 60 KV. For microanalysis, sections 2 μm thick were collected on carbon-coated formvar film on copper grids and examined in a JEOL 35C SEM equipped with a Tracor TN 2000 microanalyser at the Engineering Faculty. The analysis was made with an accelerating voltage of 20 kV and emission was collected over a detecting time of about 400 s. The peak for calcium was resolved by fitting the Sb spectrum generated by a piece of antimony to the sample spectrum using XML from Tracor (filtered minimum square fit technique) and then subtracting the fitted Sb spectrum to the sample spectrum.

Results

In spermatozoa, antimonate precipitates in the peripheral dense component of acrosomal vesicles (Fig. 1). Treatment of sections with deionized water slightly decreases the staining intensity (Fig. 2), while treatment with either chelators or fixation without K-pyroantimonate cause the absence of all precipitates (Figs. 3 and 4). Microanalysis of sperm acrosomes shows X-ray spectra with calcium-antimonate peaks (Fig. 5).

In oocytes, the pyroantimonate fixation procedure preserved the extracellular coats well but not the cytoplasmic organella. Precipitates appear concentrated at the outer jelly region and along the inner surface of the oolemma (Figs. 6 and 7). Stained jelly fibrils were seen to pass through the unstained vitelline coat and end over the oocyte

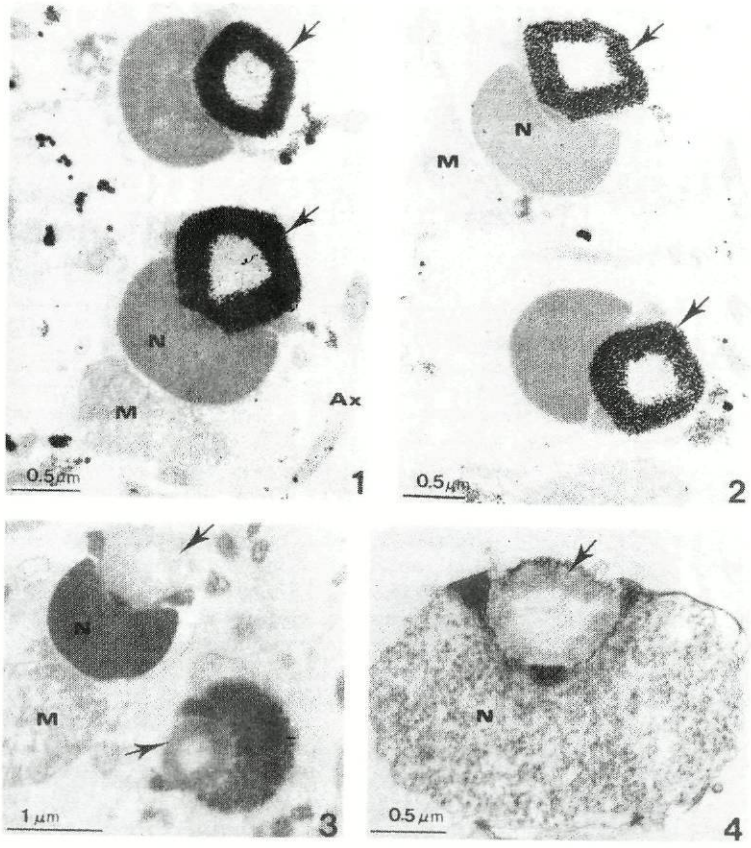


Fig. 1 Antimonate deposits are observed in the peripheral dense component of acrosomal vesicles (arrows). N, nucleus; M, mitochondrion; Ax, axoneme.

Figs. 2–4 Treatment of sections with deionized water slightly decreases acrosome (arrows) staining intensity (Fig. 2), while treatment with EGTA (Fig. 3) or fixation without K-pyroantimonate (Fig. 4) abolishes all deposits.

plasma membrane (Fig. 7). Treatment of sections with deionized water sligthly decreased the staining intensity (Fig. 8), while treatment with either chelators or fixation without K-pyroantimonate caused the absence of all deposits (Figs. 9 and 10).

Discussion

In sperm heads, calcium has been localized in the outer acrosomal and plasma membranes (2), together with calmodulin and several enzymes (1, 19, 20, 23, 25). All these membrane associated components were suggested to be involved in sperm activation (4, 6, 7, 12–14, 24, 27). Although pyroantimonate can combine not only with calcium but also with other cations, the reaction conditions used were selective for calcium localization (26), and both the chelator sensitivity of precipitates and the X-ray microanalysis results indicate that our precipitates contain calcium. To our knowledge, this is the first report describing the localization of calcium in sperm acrosomal vesicles. We do not know, however, if this calcium is to be mobilized for the acrosomal reaction or if it is restricted to the Ca^{2+}-Mg^{2+}-ATPase which is known to exits in that same peripheral dense component of starfish acrosomal vesicle (11, 19).

Echinoderm oocyte jelly is known to trap calcium because of its richness in sulphated glycoconjugates. The ultrastructural localization of calcium in the jelly coat, however, has not been reported before. Our results show that calcium appears concentrated at the outer jelly region. This particular jelly region has also been recently demonstrated to concentrate acid polysaccharides (21). The fine deposits which accompany the inner jelly fibrils also made possible to demonstrate that some of them pass through the vitelline coat and end over the oolemma, a finding not yet

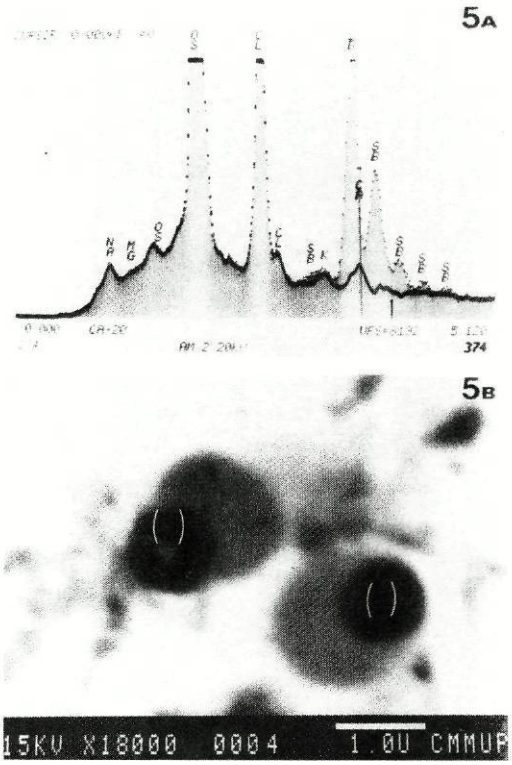


Fig. 5 A. X-ray spectra from acrosomal precipitates showing the Sb(L)-Ca region (dotted line); the Ca (K) peak is resolved after subtracting the standard Sb spectrum from that generated from acrosomes (full line). B. Electron micrograph of a spermatozoon in a 2 μm thick section. The X-ray analysis was concentrated in the area between brackets.

shown in other species.

In spite of our staining technique not designed to study the calcium distribution in the oocyte cytoplasm, our finding of an extensive submembranar calcium deposit suggest that, at least in *M. glacialis*, this store might be one of the major intracellular systems to be released at fertilization. An increase in the level of free calcium in the fertilized echinoderm egg is known to trigger the activation of the egg (3, 5, 17, 22), but the structures involved in the sequestration and intracellular release of calcium remain ill-defined. Some claim that calcium is stored in the cortical endoplasmic reticulum (8, 10), while others argue that is not the case (15, 16). As no study on this problem has been dedicated to starfishes, we are presently investigating by electron microscopy which calcium stores in starfish eggs change upon fertilization.

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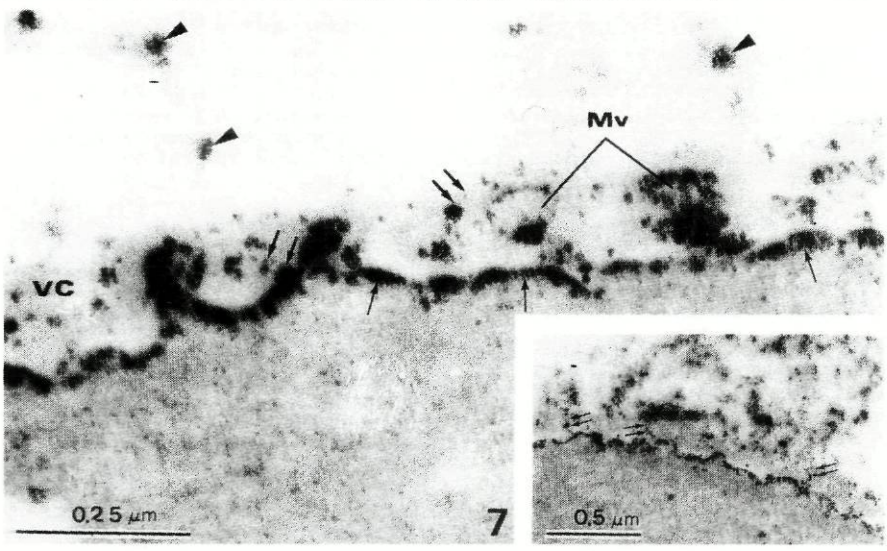
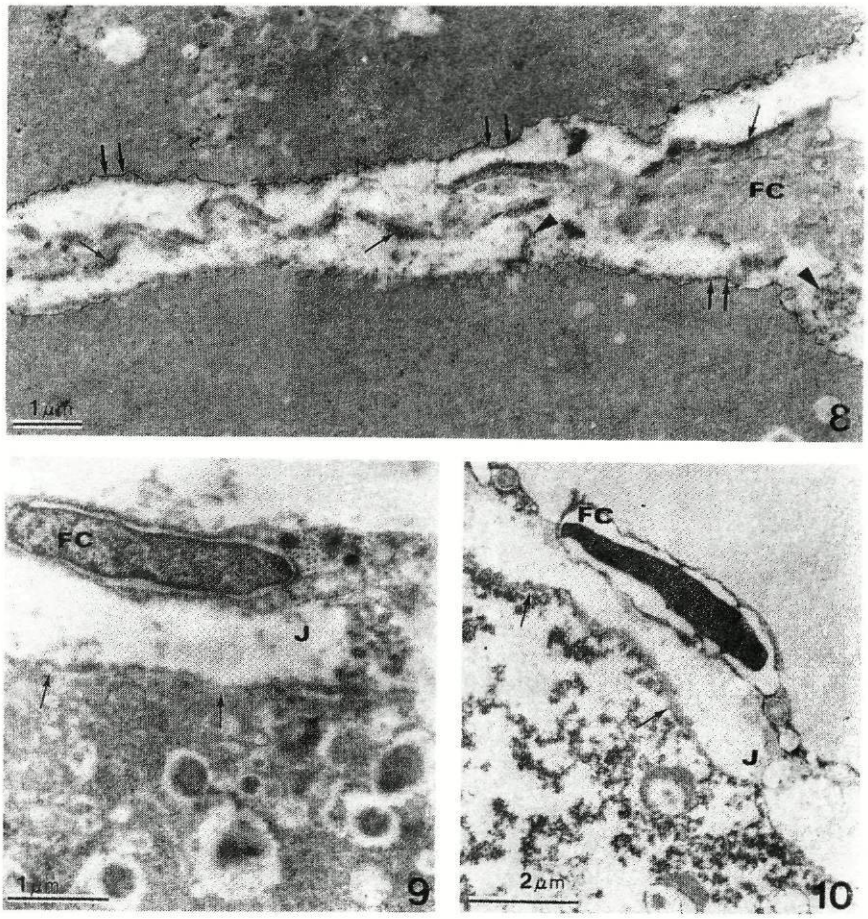


Fig. 6 Antimonate deposits are observed on the outer jelly region (arrows), jelly fibrils (arrowheads), and under the oolemma (double arrows), FC, follicular cell; VC, vitelline coat.

Fig. 7 Jelly fibrils are covered with fine antimonate deposits separated at intervals by denser accumulations of precipitates (arrowheads). Jelly fibrils (double arrows) pass through the vitelline coat (VC) and end over the oolemma (see also inset). The oolemma inner surface is coated by precipitates (arrows). Mv, microvilli.



Figs. 8–10. Treatment of sections with deionized water slightly decreases staining intensity at the outer jelly region (arrows), jelly fibrils (arrowheads), and under the oolemma (double arrows) (Fig. 8), while treatment with EGTA (Fig. 9) or fixation without K-pyroantimonate (Fig. 10) abolishes all deposits.

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Histochemical Studies of Gametes of the Starfish *Marthasterias glacialis* (Echinodermata, Asteroidea) Before and After Fertilization, With Special Reference to Jelly Coat

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Abstract. The jelly coat (JC) of the starfish *M. glacialis* was characterized by means of combined histochemical, cytochemical and electrophoretical methods, both before and after fertilization. Light microscopy revealed neutral (the main component) and acidic (in the form of a fibrillar network) mucosubstances in the JC, with carboxyl and sulphated groups in close proximity to vicinal hydroxyls. Ultrastructural cytochemistry showed that the JC is subdivided into large amorphous aggregates (neutral polysaccharides) and a fibrillar and branched component (acidic polysaccharides). After cellulose acetate electrophoresis, the JC separates into two moving and negatively charged bands (each comprise about 25% of total JC) and one band that remained at the origin (50% of total JC). All bands stained with alcian blue but PAS and protein reactivity was restricted to the band that remained at the origin. These components were subjected to the alcian blue critical electrolytical concentration staining as well to different enzymatic treatments. Glycosaminoglycans were also extracted from the JC (about 20% of total JC) and presented as a main band with a similar Rf to standard hyaluronic acid. This study reports the first histochemical study on the macromolecular structure of the JC in starfishes.

Introduction

Increasing evidence points to the importance of extracellular complex glycoconjugates in cell-cell interaction and cell regulation. Complex carbohydrates have been implicated in sperm-egg recognition, binding and activation in both vertebrates and invertebrates (Miller and Ax, 1990).

The chemical nature of the jelly coat of echinoderms is well documented. In the sea urchin, about 20% of the jelly coat is protein and about 80% are polysaccharides, and these components work together to chemically attract and activate the spermatozoon at fertilization (Shapiro and Eddy, 1980; Schuel, 1984).

The polysaccharide component of the jelly coat of sea urchins was studied since many years ago and was demonstrated to contain a poly-fucose sulphate or a poly-galactose, but no hexosamines (Vasseur, 1948, 1950; Minganti and Vasseur, 1959; Ishihara *et al.*, 1973). Several studies then offered evidence for the presence of different sialic acids in the jelly coat (Immers, 1968; Isaka *et al.*, 1969, 1970; Hotta *et al.*, 1970a, 1970b, 1973). Lorenzi and Hedrick (1973) then demonstrated by cellulose acetate electrophoresis and immunological methods that the jelly coat could contain 2-5 macromolecules, of which two were negatively charged. The work of SeGall and Lennarz (1979) finally demonstrated that the jelly coat was constituted by a high molecular weight glycoprotein or proteoglycan rich in sialic acid (90%), which was named sialoprotein and that comprises 20% of the jelly coat, and a fucose sulphate polysaccharide of high molecular weight, which comprises 80% of the jelly coat and is responsible for inducing the acrosomal reaction of the spermatozoon.

More recently, the fucose sulphate polysaccharide was shown to induce the acrosomal reaction by means of its proteinaceous core with the help of small peptides (SAP) (Shimizu *et al.*, 1990; Mikami-Takei *et al.*, 1991). The SAP are another component of the jelly coat, and they also attract and activate the motility and the metabolism of the spermatozoon (Vacquier, 1986; Garbers, 1989; Suzuki, 1990).

In the starfish, the jelly coat was demonstrated to contain a large sulphated glycoprotein (ARIS) and a group of steroidal saponins (Co-ARIS), which induce the acrosomal reaction, an oligopeptide (SAP) that participates in the induction of the acrosomal reaction and that activates the spermatozoon, and a high mannose glycoprotein (Uno and Hoshi, 1978; Ikadai and Hoshi, 1981a, 1981b; Matsui *et al.*, 1986a, 1986b; Endo *et al.*, 1987; Nishiyama *et al.*, 1987a, 1987b; Hoshi *et al.*, 1990a, 1990b).

The jelly coat represents the macromolecular structure against which the starfish spermatozoon specifically attaches and reacts during fertilization (Sousa and Azevedo, 1985). Despite the intensive biochemical characterization of the jelly coat, there is a lack of morphological information concerning the specific differentiation of the different components of the jelly coat. In the present study, the jelly coat of the starfish *M. glacialis* was characterized by means of combined histochemical, cytochemical and electrophoretical methods, both before and after fertilization.

Materials and Methods

Several specimens of *M. glacialis* were collected at the Portuguese intertidal North Atlantic coast, 30 Km North from Oporto, and

maintained in the laboratory in well-aerated sea water.

For histochemical studies of jelly coat, mature ovaries were fixed in Bouin's fluid for 24 h , processed through alcohol and benzene, and embedded in paraffin (Ganter and Jollès, 1969). Sections were cut at 7 µm. The following histochemical methods were employed, all without counterstaining: (1) 1% Alcian blue 8GX (Sigma) at pH 0.5 (Ab pH 0.5) according to Lev and Spicer to demonstrate sulphate groups (Lev and Spicer, 1964; Ganter and Jollès, 1969, 1970) and at pH 2.5 (Ab pH 2.5) according to Wagner and Shapiro to demonstrate those acid groups which remain ionized at and above this pH (Ganter and Jollès, 1969, 1970); (2) Methylation to modify functional groups (carboxyl and sulphate) of polysaccharides (Ganter and Jollès, 1969, 1970). Sections were collodionized and then immersed in 1% HCl in absolute methanol at 37°C (mild methylation) or at 60°C (active methylation) for 4-12 h. Some of the sections were then submitted to saponification to reverse the methylation of carboxylic acids (1% KOH in 70% ethanol at room temperature for 30 min). After methylation and saponification, sections were stained with Ab pH 0.5 and Ab pH 2.5. Methylation was also performed as in Pearse (1968), with 0.1 N HCl-absolute methanol for 8-96 h at 37°C (mild) or at 60°C (drastic). (3) Alcian blue critical electrolyte concentration technique (Ab-CEC) to identify the different negatively charged acidic groups (Scott and Dorling, 1965; Pearse, 1968). (4) Metachromatic reaction with 0.02% Azure A at different pH levels (pH 0.5 to pH 5) to demonstrate acid mucopolysaccharides with sulphate and carboxyl groups. Sections were examined in the solvent in which they were stained prior to dehydration and mounting (Pearse, 1968). (5) The standard periodic

acid-Schiff (PAS) reaction for vicinal hydroxyl groups of mucopolysaccharides according to McManus (Pearse, 1968; Ganter and Jollès, 1969, 1970). The PA was made fresh and, as with the Schiff reagent (Merck), was protected from light. (6) Acid hydrolysis to split off sialic acid (Ganter and Jollès, 1969, 1970; Jondeung and Czihak, 1982). Collodionized sections were incubated in 0.85% NaCl (control) or in 0.02 N Na-acetate/HCl solution at pH 2.5 (mild hydrolysis) for 4 h at 60°C, or in 0.1 N sulfuric acid (strong hydrolysis) for 1 h at 60°C. After washing in water, sections were stained by Azure A pH 4, Ab pH 2.5 and PAS. (7) Diamine methods were carried out by using N,N'-dimethyl-m-phenylenediamine dihydrochloride and N,N'-dimethyl-p-phenylenediamine dihydrochloride (Sigma) (Spicer, 1965; Pearse, 1968; Ganter and Jollès, 1969, 1970). The following procedures were used: (a) periodic acid-paradiamine (PA-pD) method to visualize neutral mucopolysaccharides; (b) low iron diamine (LID) to localize sialo and weakly acidic polysaccharides; (c) high iron diamine (HID) to demonstrate sulphate groups; (d) metadiamine staining (mD) and blockage of basophilia by periodate-metadiamine sequence (PA-mD). In all cases, sections were observed without further staining and after staining with Ab pH 2.5. For LID and HID, some sections were also pretreated with periodic acid. Abolition or reduction of basophilia of Ab indicates the presence of vicinal hydroxyls in close proximity to acid groups, while the sequence HID-Ab pH 2.5 demonstrates sulphated and nonsulphated acid polysaccharides. (8) Aldehyde fuchsin (AF) according to Halmi and Davies (Pearse, 1968; Ganter and Jollès, 1969, 1970) to stain carboxyl and sulphated groups by using basic fuchsin and paraldehyde. (9) The sequential

staining procedures of Ab pH 0.5 - PAS and Ab pH 2.5 - PAS to differentiate neutral and acidic mucopolysaccharides, and AF-Ab pH 2.5 to distinguish sulphated from weakly acidic mucopolysaccharides (Spicer, 1965; Pearse, 1968; Ganter and Jollès, 1969, 1970). (10) Mercury-bromophenol blue (Hg-BPB) to detect total protein, Ninhydrin-Schiff for protein bound amino groups, Millon reaction (Baker modification) for tyrosine-containing proteins (for enhancing contrast, after boiling, sections were immediately introduced in water instead of allowing them to come to room temperature in the solution), and the DMAB-nitrite reaction for tryptophan-containing proteins (Pearse, 1968; Ganter and Jollès, 1969, 1970).

For electron microscopy studies of complex polysaccharides, mature male gonads were cut, squeezed through gauze, and the obtained semen was stored at 4°C as dry sperm. Mature female gonads were cut in 0.2 µm Millipore filtered sea water (FSW) at pH 8, and the liberated oocytes were then left in FSW until their spontaneous maturation which is characterized by loss of follicular cells and germinal vesicle breakdown (Meijer *et al.*, 1984). Sperm suspensions were prepared by the addition of 10 ml FSW to 0.1 ml dry sperm. Insemination was carried out by adding 2 ml of sperm suspensions to 2 ml of oocytes collected from the bottom of goblets where they had sedimented. Small pieces of ovaries and testis, free oocytes and fertilized eggs were processed for: (1) Ruthenium red staining (RR) staining according to Anderson (1968); (2) Alcian blue at pH 6.9, with the presence of $MgCl_2$, and at pH 1, using the staining method of Crawford and Abed (1986); (3) High iron diamine-thiocarbohydrazide-silver proteinate (HID-TCH-SP) sequence according to Spicer *et al.*

(1978) and Sannes *et al.* (1979). Briefly, tissues and cells were fixed with 2.5% glutaraldehyde in 0.2 M Na-cacodylate pH 7.2 - FSW (1:3) for 2 h at 4°C, washed in the same buffer, and incubated in the staining solution for 20 h. For controls, the material was incubated in an HID solution without N,N'-dimethyl-phenylenediamine or methylated for 18 h at 60°C with 0.1 N HCl in methanol before incubation in the staining solution; and (4) Cationic Cacodylate Iron Colloid (CCIC) as in Seno *et al.* (1983, 1985). Briefly, tissues and cells were fixed as above for HID, washed, and stained at pHs 1.8, 4 and 7, firstly for 1 h at room temperature followed by 8 additional hours in an ice-bath. In all cases, the material was dehydrated in ethanol series to propylene oxide and embedded in Epon. Ultrathin sections were cut with a diamond knife (Dupont) in a LKB ultratome (Nova) and observed without counterstaining in a JEOL 100 CX II transmission electron microscope (TEM) operated at 60 kV.

For histochemical studies *on grid*, the material was fixed in glutaraldehyde made in FSW - Na cacodylate (3:1) pH 7.2, washed, dehydrated and embedded in Epon. The following staining techniques for complex polysaccharides were applied to ultrathin Epon sections collected on gold grids: (a) 5% aqueous tannic acid (20 min), pH 2.6 - 3% aqueous uranyl acetate (10 min) sequence (**TA-UA**) (Sannes *et al.*, 1978). For controls each step was alternatively omitted; (b) phosphotungstic acid (PTA) staining in various solutions at different pHs for 20 min: at pH 0.5 with 1% PTA in 10% aqueous chromic acid (**PTA-CrA**) (Rambourg *et al.*, 1969) and 1-5% PTA in 1N HCl (**PTA-HCl**) (Weinstock and Leblond, 1971; Kudo, 1982), and at pH 1.6 with 1-5% aqueous PTA (PTA-aq) (Krimer and Esponda, 1980; Kudo, 1982) and 1-5%

PTA in 10% acetone (PTA-acetone); (c) periodic acid-thiocarbohydrazide-silver proteinate (PA-TCH-SP) sequence (Thiéry, 1967) for 72 h. For controls, sections were not oxidized or treated without TCH; (d) periodic acid-silver methenamine (PA-SM) sequence (Rambourg, 1967) and the PA-CrA-SM sequence (Dawson and Filipe, 1976). In both methods, just one staining step was performed (20-25 min), with 10 ml of staining solution in covered glass Petri dishes with 5 cm in diameter. For controls, the PA step was omitted; (e) the CCIC staining solution at pHs 1.8, 4, and 7 for 45-90 min; (f) the HID staining solution, for 18 h.

For scanning electron microscopy (SEM), the fixed oocytes were washed in 0.2 M Na-cacodylate pH 7.2, postfixed with 1% osmium tetroxide in buffer for 2h at 4°C, dehydrated in ethanol series, critically point dried in carbon dioxide, mounted on specimen stubs with double-coat tape, coated with gold, and examined in a JEOL 35 C SEM (at the Engineering Faculty).

The oocyte jelly coat was isolated as crude jelly coat by the acid method of Matsui *et al.* (1986). The resulting supernatant was dialysed against distilled water (Spectra/Por, MW cutoff x 3,500) and then lyophilized (SeGall and Lennarz, 1979). Protein concentrations were determined by the method of Lowry *et al.* (1951).

For cellulose acetate electrophoresis (CAE), Cellogel sheets, 5.7 x 14 cm, were previously equilibrated in electrophoresis buffer (0.05 M calcium acetate pH 7.2). Samples (10-20 µl/lane) were applied with a LRE applicator (Medizin Technik). Standards were from Sigma and consisted of 1 mg/ml water of dermatan sulfate, heparan sulfate, chondroitan sulfate, hyaluronic acid and heparin. Electrophoresis was

performed in an electrophoresis apparatus (EP-166 Medizin Technik) at a constant voltage of 200 V for 1 h (14 V/cm). Sheets were then stained with Coomassie blue (Cb), PAS or Ab-CEC. For Ab-CEC (Scott, 1985) gels were stained overnight and then washed and stored in the same CEC solution without Ab. For general Ab staining, we used a 0.5% solution in 7% acetic acid. For the PAS reaction, acetic acid fixed gels were washed in 3% acetic acid for 15 min, oxidized for 1 h at 4°C with fresh 1% periodic acid in 3% acetic acid, washed 3 x 5 min with water, stained overnight at 4°C in the Merck Schiff reagent, and stored in water or in the reagent at 4°C. After being photographed, sheets were made transparent with methanol:acetic acid (86:14) for 1 min, followed by methanol for 1.5 min, and then dried at 37°C for 15 min. Transparent sheets were scanned with a densitometer (2500/2510 Transidyne, General Corporation, Ann Arbor, Michigan) at 650 nm, using a slit of 5 x 0.5 mm and with the zero at the minimum.

Isolation of glycosaminoglycans (GAGs) from the crude jelly coat (0.7 mg/ml water) was done according to Whiteman (1972, 1973) as modified by Whiteman and Henderson (1977). The GAG-Alcian blue complex was left to equilibrate for 2 h at room temperature. Centrifugations were done at 5,000 rpm for 15 min at 4°C. The precipitate was dried overnight at room temperature, then dissolved in 50 µl water, centrifuged and the supernatant was used for CAE.

Glycosaminoglycan concentration was determined by the method of Whiteman (1972, 1973). Precipitation was done for 4 h at room temperature and the precipitate was dissociated with 1 ml of 7.5% aqueous SDS by vortex mixing. After 30 min at room temperature solutions were read at 678 nm in a Pye-Unicam SP6-550

spectrophotometer. Concentrations were determined in triplicate with 25 µl and 50 µl of a crude jelly coat solution at 0.7 mg/ml water. The standard calibration curve was done with a starting solution of chondroitin-4-sulfate, 200 µg/ml water, over the range of 0-10 µg/ml.

For enzymatic treatments, crude jelly coat was submitted to the following digestions: **chondroitinase ABC** (Sigma, from *Proteus vulgaris*, 0.55 U/mg) was used at 0.25 U and 0.75 U/mg sample for 3 h and 24 h at 37°C in 0.1 M Tris - HCl - 0.1 M Na-acetate, pH 7.3 (Yanagishita *et al.*, 1979; Heinegard and Sommarin, 1987); **hyaluronidase** (Sigma, from sheep testes, type V, 2,000 U/mg) was used at 50 U, 100 U and 200 U/mg sample for 3 h and 24 h at 37°C in 0.15M NaCl, 0.1 M Na-acetate, 0.001 M disodium EDTA, pH 5 (Cowman *et al.*, 1981; Turner and Cowman, 1985; Min and Cowman, 1986); **neuraminidase** (Sigma, type V, from *Clostridium perfringens*, 1.7 U/mg) was used at 25 U and 100 U/mg sample for 24 h at 37°C in 0.1 M Na-acetate pH 5.3 with 0.04 M CaCl (Kudo, 1982; Delgado and Zoller, 1987); **trypsin** (Sigma, type III, from bovine pancreas, 11,680 U/mg) was used at 10 µg and 0.3 mg/mg sample, **protease** (Sigma, type VI, from *Streptomyces griseus*, 5 U/mg) and **pronase E** (Merck, from *Streptomyces griseus*, 95,000 PUK/g) were used at 2 mg/mg sample, all three for 3 h and 24 h at 37°C in 0.1 M Tris-HCl, 0.1 M Na-acetate, pH 7.3 (Yanagishita *et al.*, 1979; Grimek and Ax, 1982; Heinegard and Sommarin, 1987; Huey *et al.*, 1990). Digestions were terminated by boiling the samples for 5-10 min. Samples were then stored at -20°C until used.

Results

Light microscopy

A fibrillar-like component of the mature oocyte jelly coat reacted with Ab pH 2.5 but with Ab pH 0.5 a more intense staining was observed, suggesting both the presence of uronic acid groups and of sulphated groups in jelly polysaccharides (Figs. 1-3). To confirm this finding, sections were submitted to mild and drastic methylation and then stained with Ab pH 2.5 and Ab pH 0.5. Methylation abolished staining with Ab at both pHs but only those sections that were mild methylated could be partly restained with Ab pH 2.5 and pH 0.5 after saponification with KOH. The absence of alcianophilia at pH 2.5 with demethylation after the drastic treatment, suggests that with this method even carboxyl groups are blocked or destroied. The absence of alcianophilia at both pHs after mild methylation and the prompt restaining after KOH treatment implies, however, that in our conditions the staining with Ab pH 0.5 is not specific for sulphated polysaccharides alone. This could explain the stronger staining with Ab pH 0.5 than with Ab pH 2.5 as refered above. To further substantiate this finding, the Ab-CEC was performed (Figs. 4-12). The presence of $MgCl_2$ intensifies the staining when compared to the control (Fig. 4). The specific staining gradually increases untill 0.4 M $MgCl_2$ (Figs. 5-8) and afterthat decreases. At 0.6-0.8 M $MgCl_2$ no staining is noted besides a very delicate staining (Figs. 9-12). Again, these results suggest that the jelly coat is not highly sulphated and that carboxyl groups predominate. When increasing quantities of $MgCl_2$ are added to Ab-acetic acid the pHs of the

solutions gradually decrease (from 2.6 to 1). The simultaneous decrease in the staining intensity, however, is not dependent on the masking action of proteins, because the staining with Ab at pH 1 or even at pH 0.5 is not affected if $MgCl_2$ is omitted.

We then studied the presence of methacromasia with Azure A at pH 0.5 to pH 5, and observed that jelly coat exhibited β-methacromasia (purple) which increased with pH. Critical points were as follows: (+) at pH 0.5, (++) at pH 1.5, (+++) at pH 2. (++++) at pH 3, and (+++++) at pH 5. After dehydration all methacromasia was removed. It is known that methacromasia signifies the presence of free electronegative surface charges of a certain minimum density, that β-methacromasia is due to the presence of the weaker acidic groups (PO4, COOH) in high local concentration, and that bellow pH 2 only sulphated polysaccharides retain the dye (Ganter and Jollès, 1969, 1970; Pearse, 1969). So, the results again suggest that jelly coat has a lower content in sulphated groups.

When sections were stained by the PAS technique an intense pink staining was observed (Fig. 13). This reaction clearly stains more jelly coat material than with Ab. The PAS reaction stains polysaccharides containing hexoses, fucose and sialic acid but not those containing hexoxamines and hexuronic acids, as is the case of GAGs (Rambourg, 1971). When a combined Ab pH 2.5-PAS reaction is performed (Fig. 14), a large purple jelly coat is observed. These results then suggest that the jelly coat possesses a mixture of non-acidic and acidic polysaccharides, with the predominance of the former.

After mild hydrolysis, staining with Azure A pH 4 and Ab pH 2.5 was

Legends

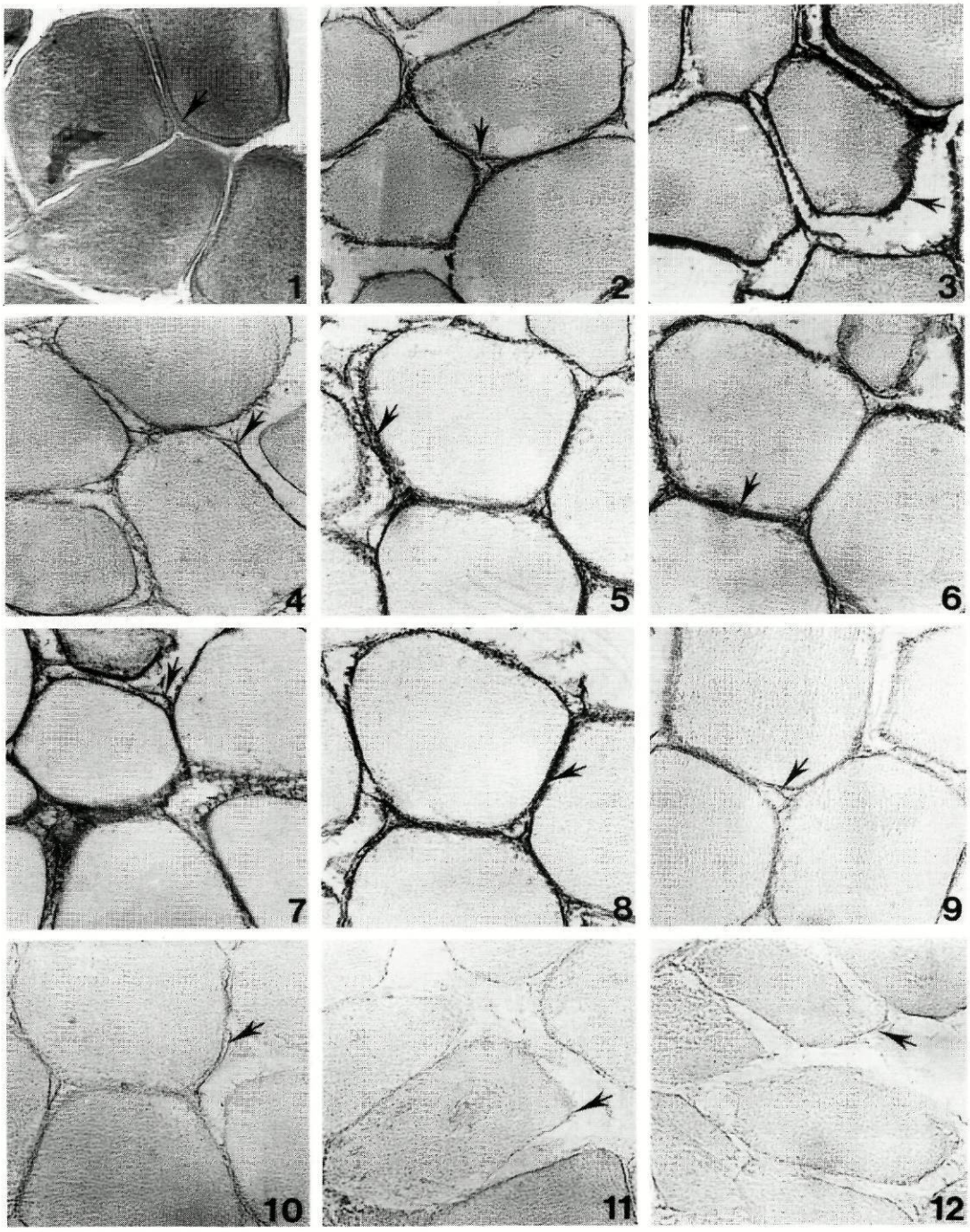
Figs. 1-36. Histochemical staining (arrows) of mature oocytes in the ovary of *M. glacialis*. x 315.

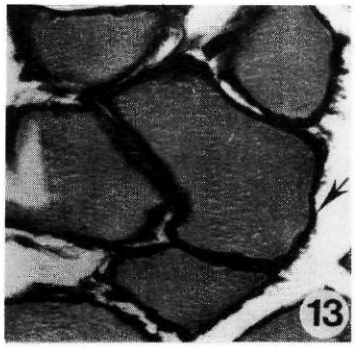
Fig. 1. Hemalumen-Eosin.
Fig. 2. Ab pH 2.5.
Fig. 3. Ab pH 0.5.

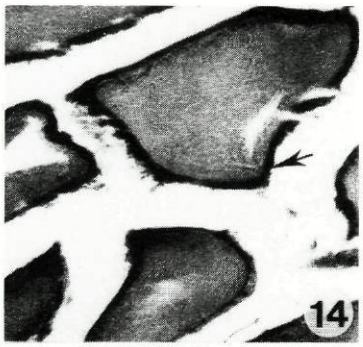
Fig. 4-12. Ab-CEC (M refers to $MgCl_2$ concentrations).

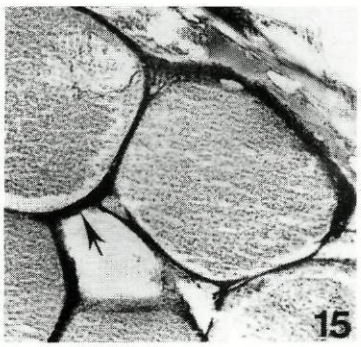
Fig. 4. 0.0 M.
Fig. 5. 0.05 M.
Fig. 6. 0.1 M.
Fig. 7. 0.2 M.
Fig. 8. 0.4 M.
Fig. 9. 0.6 M.
Fig. 10. 0.8 M.
Fig. 11. 1.2 M.
Fig. 12. 1.5 M.

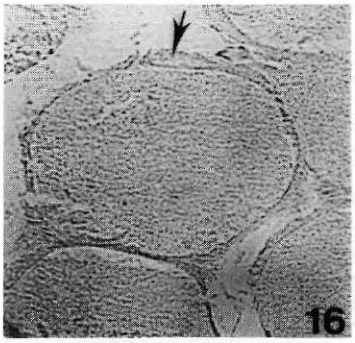
Fig. 13. PAS.
Fig. 14. Ab pH 2.5-PAS.
Fig. 15. PA-pD.
Fig. 16. mD.
Fig. 17. mD-Ab pH 2.5.
Fig. 18. PA-mD.
Fig. 19. PA-mD-Ab pH 2.5.
Fig. 20. LID (unpreserved jelly coat).
Fig. 21. LID (preserved jelly coat).
Fig. 22. LID-Ab pH 2.5 (unpreserved jelly coat).
Fig. 23. LID-Ab pH 2.5 (preserved jelly coat).
Fig. 24. PA-LID.
Fig. 25. PA-LID-Ab pH 2.5.
Fig. 26. HID (unpreserved jelly coat).
Fig. 27. HID (preserved jelly coat).
Fig. 28. HID-Ab pH 2.5.
Fig. 29. PA-HID.
Fig. 30. PA-HID-Ab pH 2.5.
Fig. 31. FA.
Fig. 32. FA-Ab pH 2.5.
Fig. 33. Hg-BPB.
Fig. 34. Ninhydrin-Schiff reaction.
Fig. 35. Millon reaction.
Fig. 36. DMAB-Nitrite reaction.

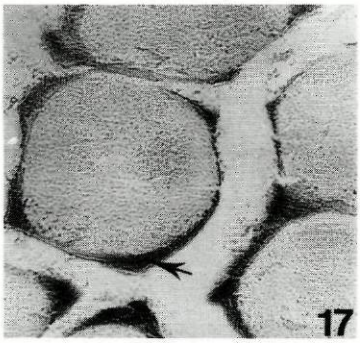

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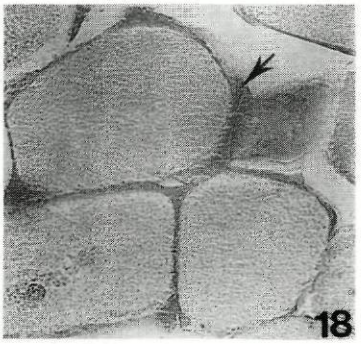

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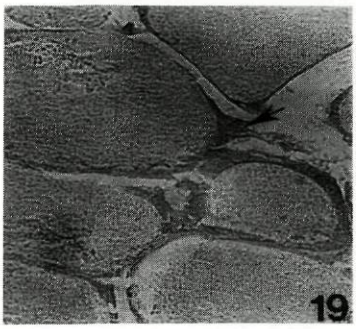

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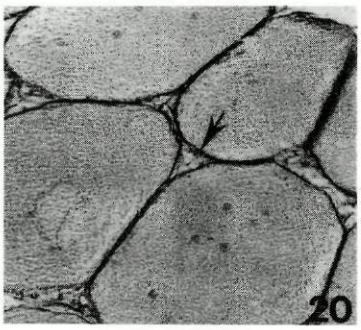

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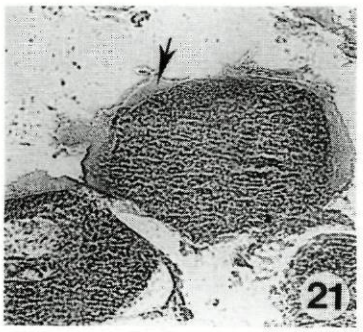

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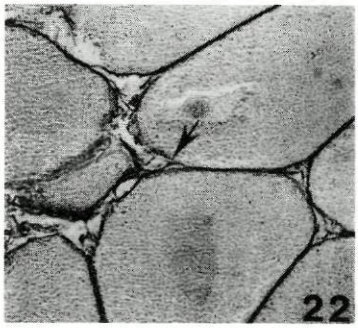

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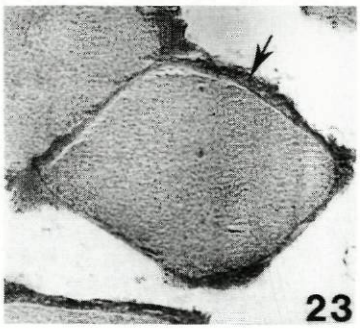

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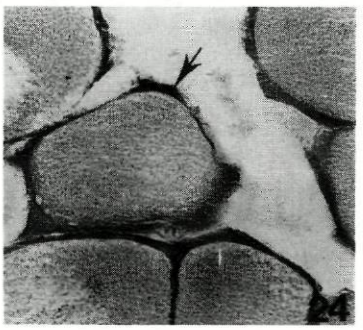

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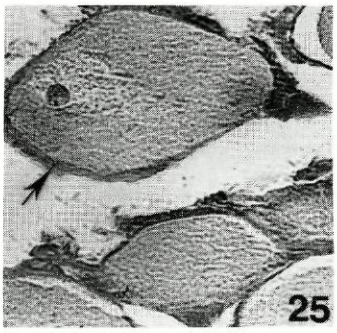

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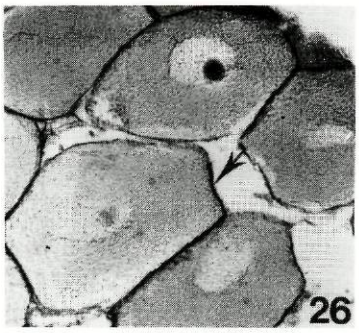

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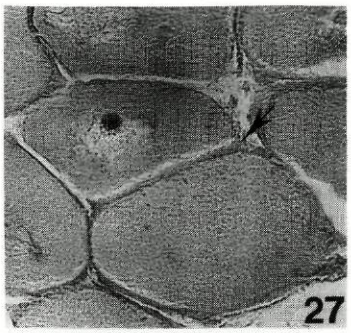

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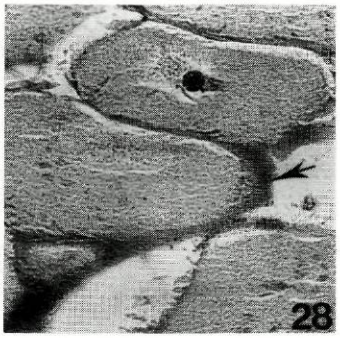

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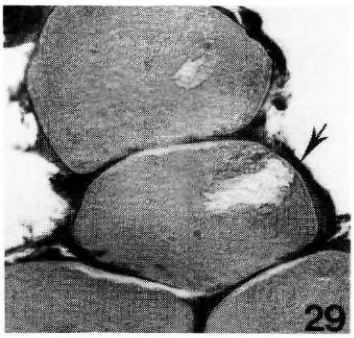

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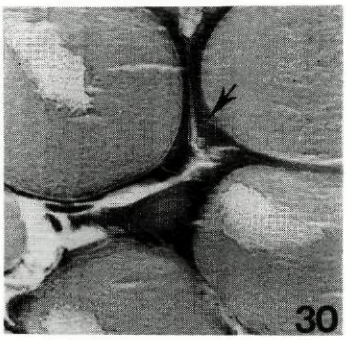

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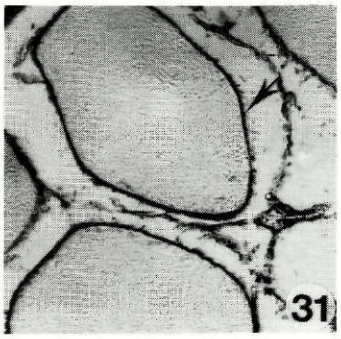

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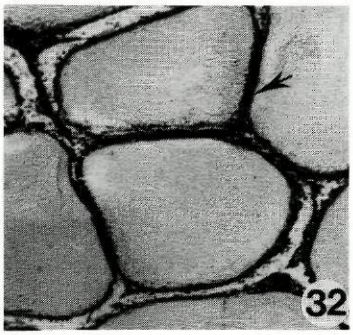

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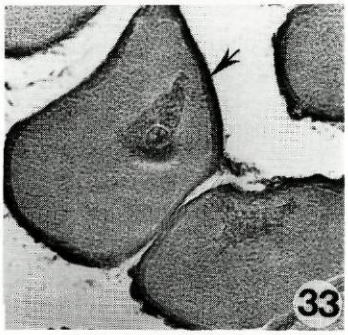

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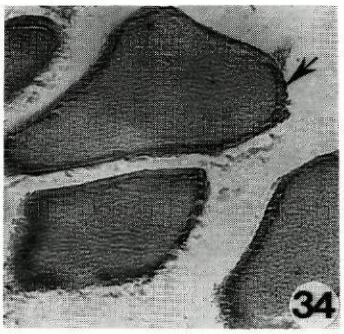

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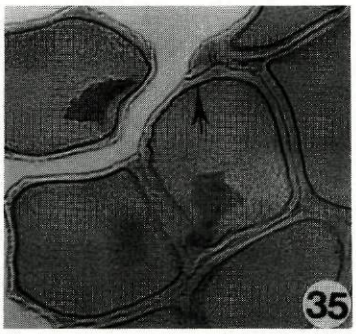

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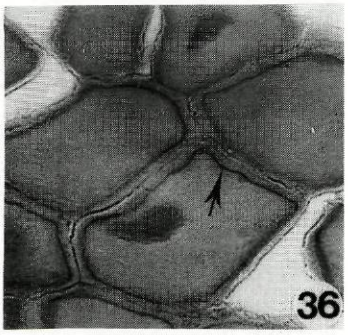

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slightly diminuished (++++), while no differences were noted with PAS (+++++). After strong hydrolysis, however, both the methacromasia and the alcianophilia of jelly coat were strongly reduced (++). Despite this, the PAS staining remained unchanged (+++++). These results then suggest the presence of sialic acid in jelly coat, and that the PAS staining must be notably dependent on the presence of hexoses and fucose but not on sialic acid.

With the PA-pD technique, after 7 h of staining an orange-brown colour appeared, suggesting the presence of neutral polysaccharides. However, after 48 h the staining was dark-brown (Fig. 15), clearly evidencing the simultaneous presence of acidic and 1,2-vic glycol groups in close proximity. A similar conclusion could be reached with the mD reaction. Without oxidation a light greyish or no staining was observed (Fig. 16), while after mD-Ab pH 2.5 all jelly coat appeared blue (Fig. 17). However, when sections were previously oxidated the jelly coat appeared orange brown (Fig. 18), but after PA-mD-Ab pH 2.5 no blue colour was noted (Fig. 19). These results then suggest the presence of neutral polysaccharides and of polysaccharides both containing acid and 1,2-vic-glycol groups in close proximity. When sections were treated with LID a two-type reaction was observed. When the jelly coat was not completely preserved a fibrillar-like purple-black staining appeared (Fig. 20), but when the jelly coat was completely preserved a greyish staining was observed (Fig. 21). If LID is followed by Ab pH 2.5 the blue colour then stains all jelly coat thickness but little effect has on the incomplete preserved jelly layers (Figs. 22, 23). The reason why non-oxidized oocytes with complete jelly coats stain less with LID suggest that LID could not

bind to the most acidic polysaccharides or that their staining is masked by the overall greyish staining. With PA-LID, the entire jelly coat appears purple-black, but after PA-LID-Ab pH 2.5 no blue staining appears (Figs. 24, 25). The same holds true for HID, HID-Ab pH 2.5, PA-HID and PA-HID-Ab pH 2.5 techniques, although the purple-black staining (presence of sulphated groups) appeared with much more intensity (Figs. 26-30). The results then suggest that the jelly coat must possess at least three polysaccharide components, namely, neutral (the main component) and acid polysaccharides containing sialic/uronic and sulpho groups. The absence of Ab pH 2.5 staining after PA oxidation again gives evidence for the simultaneous presence of acidic and 1,2-vic-glycol groups in close proximity.

When sections were stained with aldehyde-fuchsin some components of the jelly coat stained purple (Fig. 31), but after the sequence AF-Ab pH 2.5 more jelly coat appeared stained with intense purple and blue colours (Fig. 32), thus suggesting the presence of acidic polysaccharides containing carboxylic and sulphated groups.

Concerning protein staining, the Hg-BPB reaction for total protein gave a strong blue staining of all jelly coat (Fig. 33); the ninhydrin-Schiff reaction for protein-bound NH_2 gave a very weak pink staining, and only with prolongued exposure to ninhydrin (48 h) and Schiff reagent (up to 1 h) a stronger pink staining could be obtained (Fig. 34); the Millon reaction for tyrosine-containing proteins stained the jelly coat pink but a strong pink-brown reaction was seen in the region containing the vitelline layer and cortical vesicles (Fig. 35); with the DMAB-nitrite method for tryptophan-containing proteins a similar reaction occurred: the jelly coat stained blue,

like the cytoplasm, but a strong reaction could only be observed in the vitelline layer-cortical vesicle region (Fig. 36).

Electron microscopy

The normal ultrastructure of *M. glacialis* jelly coat evidences two major macromolecular components: a fine fibrillar component that forms large amorphous aggregates and a coarse fibrillar and branched component that spreads between the amorphous aggregates (Figs. 37, 38).

When stained with RR these two structural components appear better preserved but only the fibrillar component binds RR (Figs. 39, 40). This material delimitates both the outer and inner aspects of the jelly coat besides spreading between the amorphous aggregates (Figs. 39, 40). Ruthenium red binds and precipitates GAGs, acidic polysaccharides and polypeptides, but not neutral polysaccharides (Luft, 1966, 1971). Thus, the amorphous aggregates might correspond to the neutral polysaccharides while the fibrillar component reacts as an acidic polysaccharide. With insemination, the amorphous component dissolves away, or becomes disaggregated, and the jelly coat outer border becomes coated by a new material that heavily stains with RR and that is originated from the extrusion of the acrosomal contents (Fig. 41). The acidic polysaccharide component of the starfish spermatozoon is not detected in unreacted cells and thus appears stained only after the acrosomal reaction. This material coats the base of acrosomal process and appears to attach the spermatozoon to the jelly coat (Fig. 41).

After Ab staining in FSW and at neutral pH the jelly coat appears

greatly preserved. The fibrillar component, which is the only stained element, shows two distinct regions: a large waved outer region, and several thinner and branched fibres that originate from the inner aspect of the outer region and traverse all the jelly coat until touching the vitelline layer (Fig. 42). After insemination the amorphous component disaggregates and the fibrillar component is better seen to form a fibrillar and branched matrix all around the egg (Fig. 43). The unreacted spermatozoon is not stained by Ab, but with insemination the acrosomal vesicle contents appear precipitated (Fig. 44). The spermatozoon may react against the large waved outer border of the jelly coat, but usually reacts against the inner stained fibrillar components (Fig. 44). At pH 1 Ab also stains the fibrillar component as also the periphery of the amorphous aggregates (Fig. 45). The staining with Ab in FSW at neutral pH includes the presence of $MgCl_2$, which means that under these conditions Ab can selectively stain GAGs (Reale et al., 1986)

We than tested for the presence of glycoproteins using several staining techniques which are the counterpart of the PAS technique used in light microscopy. The Thiéry technique (20 min PA - 68h TCH - 30 min SP) stained some globular regions of the fibrillar component as well as several regions at the periphery of the amorphous aggregates to which the former component attaches (Fig. 46). Some focal regions at the periphery of the amorphous aggregates to which the fibrillar component does not seem to attach also appear stained by the Thiéry technique (Fig. 46). In the oocyte cytoplasm, glycogen granules, yolk vesicles and cortical vesicles also appear stained. With the cortical reaction both the spiral lamellae and the peripheral

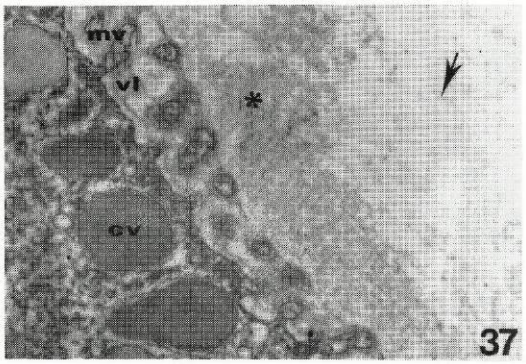
Figs. 37, 38. Ultrastructure of the oocyte cortex of *M. glacialis*. Cortical vesicles (cv) present a spiral lamellae in their centre and a homogeneous and dense peripheral component. The vitelline layer (vl) surrounds the microvilli (mv) and the jelly coat is differentiated into amorphous (*) and fibrillar (arrow) components. With the cortical reaction the contents of cortical vesicles are exocytated (a) and then fuse (b) with the fertilization envelope (fe). Fig. 37, x 22,000; Fig. 38, x 18,000.

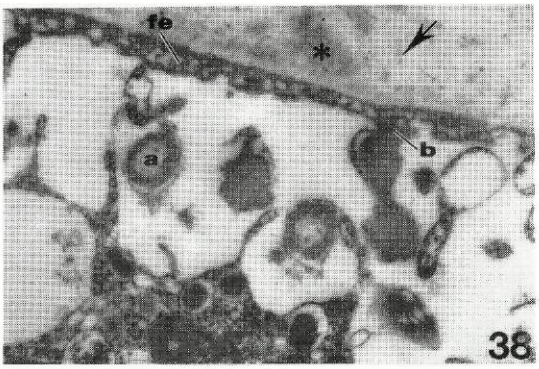
Figs. 39-41. RR staining. Figs. 39, 40. In the unreacted oocyte, RR stains the fibrillar component of the jelly coat, which delimits the outer jelly region (double arrows), spreads between (arrow) the amorphous component (*), and coats the outer face (triple arrows) of the vitelline layer (vl). x 10,000 and x 29,000. Fig. 41. At fertilization the stained component of acrosomal vesicles (double arrows) attaches the spermatozoon to the jelly coat. ap, acrosomal process. x 10,000.

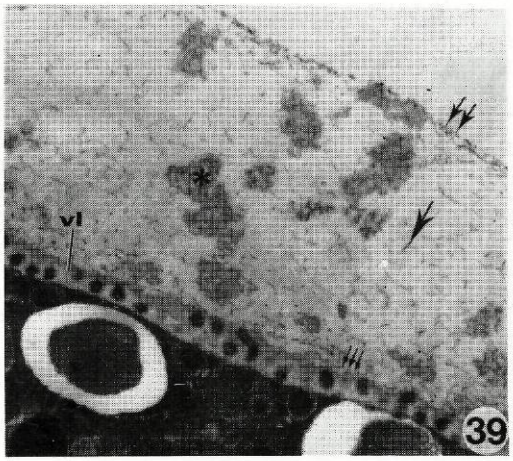
Figs. 42-44. Ab pH 7. Fig. 42. In the unreacted oocyte, Ab stains a large and waved region at the outer border of the jelly (large arrow), and a fibrous component (small arrows) that originates from the former component and traverses the amorphous component (*) to end over the vitelline layer (vl). x 42,000. Fig.43. After insemination, the amorphous component (*) dissolves away, while the fibrillar component remains at the surface of the embryo (large and small arrows). f, flagellum. x 13,000. Fig. 44. The spermatozoon reacts over the Ab stained inner fibres (double arrows) and not at the outer stained region (large arrow). Arrowhead points to the fused acrosomal vesicle and plasma membranes of the spermatozoon; (*), precipitated acrosomal contents. x 21,000.

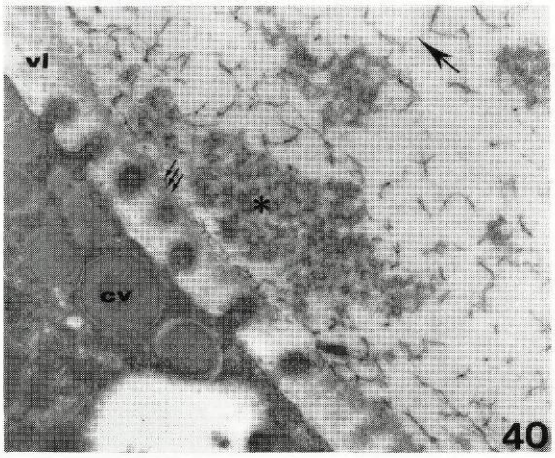
Fig. 45. Ab pH 1. Ab stains the fibrous component (arrow) of the jelly coat and the periphery of the amorphous component (*). x 15,000.

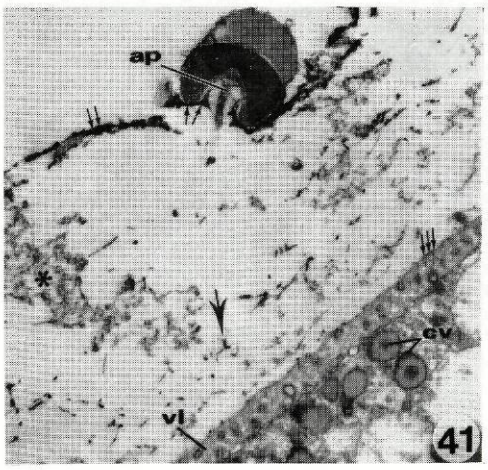
Figs. 46-48. Thiéry technique. Fig. 46. In the unreacted oocyte, staining appears over globular regions of the fibrillar component of the jelly coat (arrows), at the connection between these fibrils and the amorphous component (arrowheads), at some regions of the periphery (double arrows) of the amorphous component (*), in cortical vesicles (cv), and in glycogen granules (g). fc, follicular cells. x 17,000. Fig. 47. After the cortical reaction, stained cortical vesicle contents are exocytated (a) and then fuse (b) with the fertilization envelope (fe), transfering their staining to the inner face (arrow) of this latter structure. (*), jelly coat; mv, microvilli with glycogen granules (g); v, yolk vesicles are also stained. x 22,000. Fig. 48. In the unreacted spermatozoon, glycogen granules (arrow) appear in the mitochondrion (m). f, flagellum; c, centrioles; n, nucleus; av, acrosomal vesicle. x 22,000.

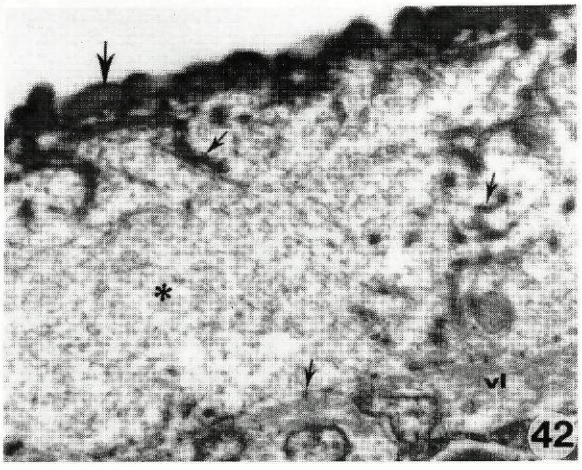

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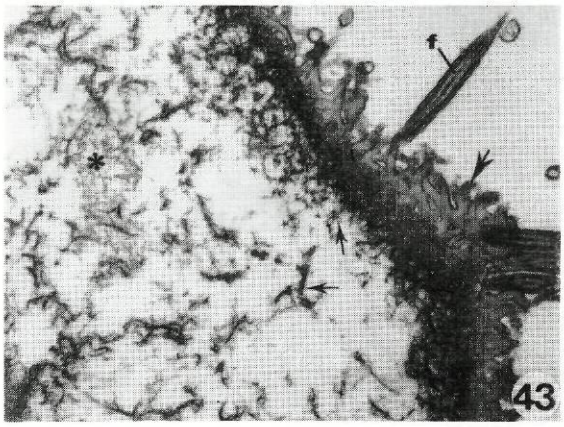

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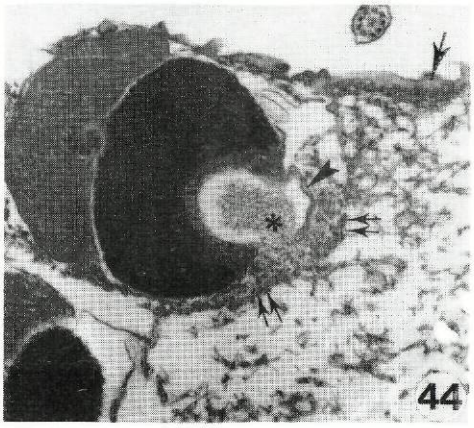

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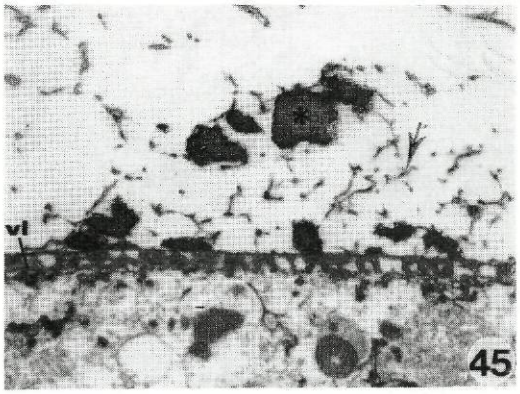

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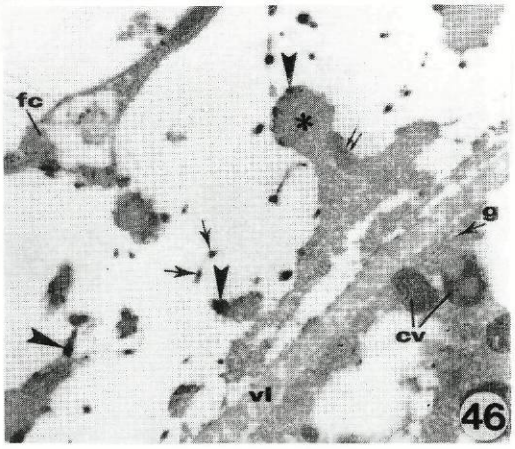

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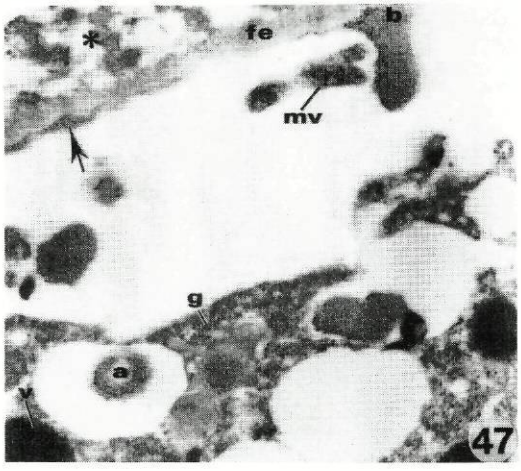

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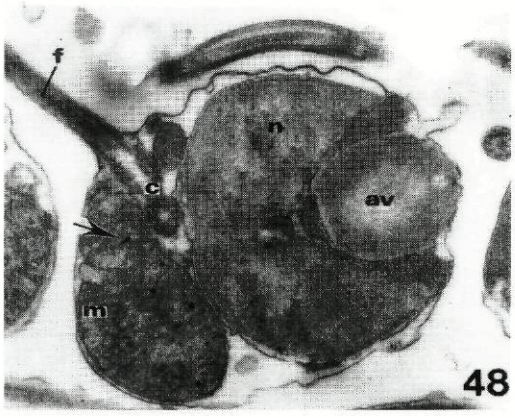

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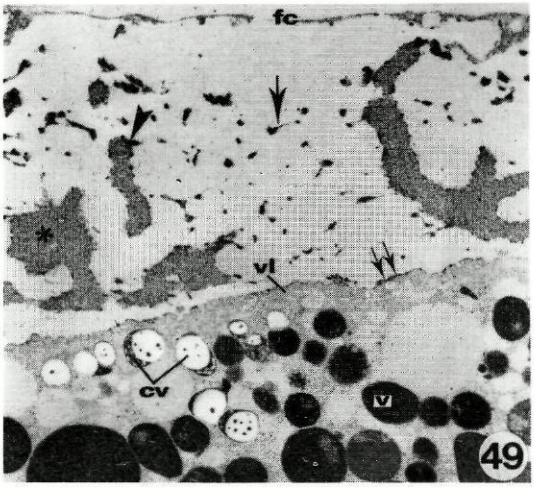

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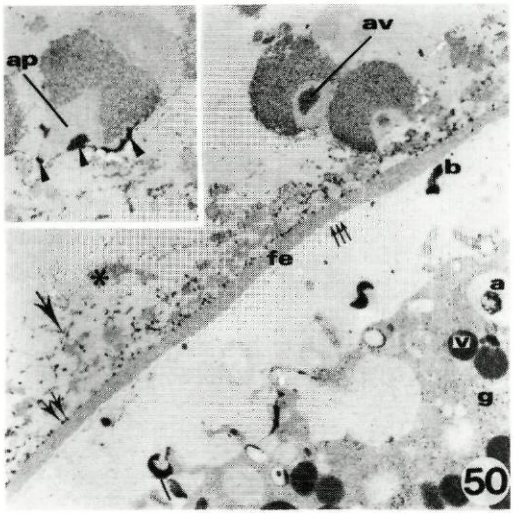

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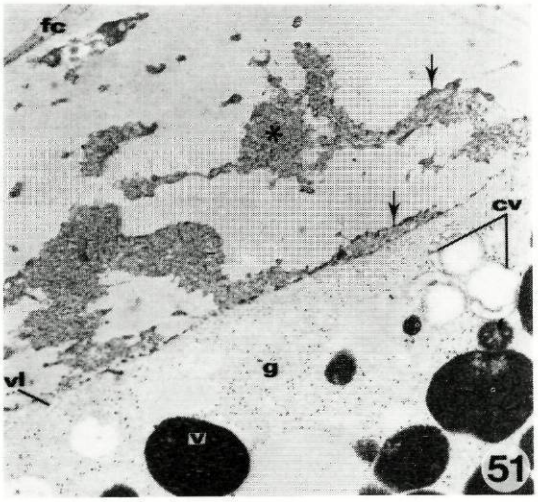
component of cortical vesicles fuse with the fertilization envelope and their staining is then transfered to the undercoating of the elevated fertilization envelope (Fig. 47). The unreacted spermatozoon only shows glycogen granules in the mitochondrial matrix (Fig. 48) and no staining was noted in the acrosomal vesicle contents even after the acrosomal reaction. With the PA-SM technique, the fibrillar component of the jelly coat appears stained while the amorphous aggregates appear less intensely labelled (Fig. 49). Some regions of the peripheral component of the cortical vesicles and the yolk vesicles also appear intensely stained. After the cortical reaction no staining is transfered from cortical vesicles to the elevated fertilization envelope (Fig. 50). The acrosomal vesicle is also stained by PA-SM, and after the acrosomal reaction the stained material attaches the spermatozoon to the jelly coat (Fig. 50). The PA-CrA-SM technique for glycoproteins produces the same effect as the PA-SM but the introduction of the chromic acid step reduces the amount of unspecific staining as it attacks various reducing groups. The PA oxidizes adjacent glycol and α-aminoalcohol groups and transforms them into aldehyde groups which in turn release the silver on reduction of the silver methenamine. The additional chromic acid step also changes the glycols into aldehyde groups. Furthermore, it may produce acidic groups which do not react with the silver and hence the reaction is more specific (Rambourg and Leblond, 1967; Dawson and Filipe, 1976). With this technique the staining of the jelly coat was more homogeneous, the staining intensity of cortical vesicles clearly diminuished, and glycogen now appeared more clearly demonstrated in the oocyte cytoplasm (Figs. 51, 52). The acrosomal vesicle and the

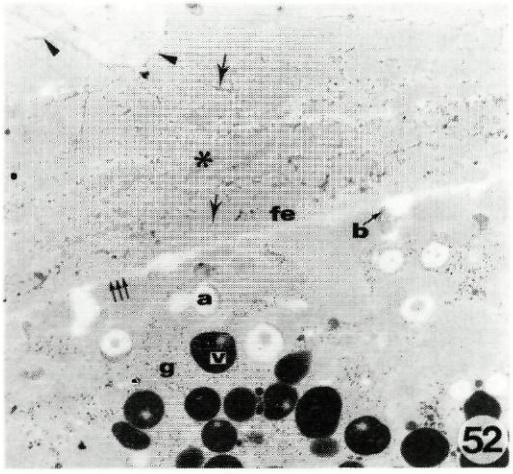
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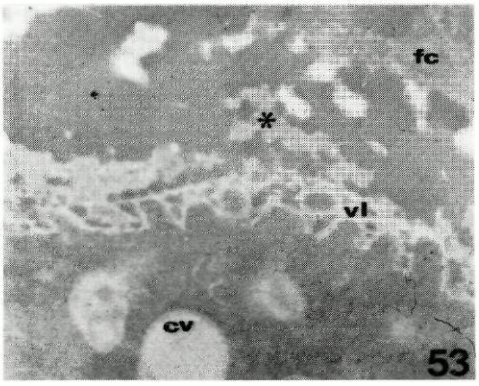
Figs. 49-54. PA-SM (Figs. 49, 50), PA-CrA-SM (Figs. 51, 52), and controls (Figs. 53, 54). Fig. 49. In the unreacted oocyte, PA-SM stains more intensely the fibrillar component of the jelly coat that spreads (arrow) between the less intense stained amorphous component (*) and that attaches to it (arrowhead) and to the vitelline layer (double arrows). Yolk vesicles (v) and some region of the peripheral component of cortical vesicles (cv) are also intensely stained. x 8,000. Fig. 50. At fertilization, staining appears in the acrosomal vesicle (av) contents (arrowheads) which attach the spermatozoon to the jelly coat and coats the acrosomal process (ap). No staining (triple arrows) appears in the fertilization envelope (fe) when exocytated cortical vesicles (a) fuse (b) with it. v, yolk vesicles; g, glycogen granules. x 9,000; inset, x 10,000. Fig. 51. In the unreacted oocyte, PA-CrA-SM stains less intensely the amorphous (*) and the fibrillar (arrows) components of the jelly coat, as also the peripheral component of cortical vesicles (cv). Glycogen granules (g) and yolk vesicles (v) appear intensely stained. x 17,000. Fig. 52. At fertilization, the acrosomal contents of the spermatozoon are not stained (arrowheads). In the perivitelline space exocytated cortical vesicle contents (a) fuse (b) with the fertilization envelope (fe) coating its inner face (triple arrows), but no staining appears in this latter structure. x 9,000. Figs. 53 (unreacted oocyte) and 54 (reacted oocyte). In controls, no staining was found. x 29,000 and x 21,000.

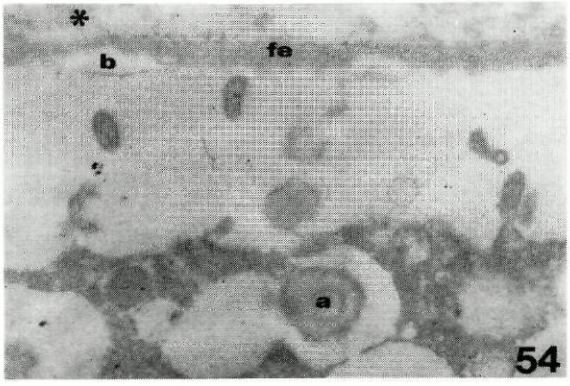

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unspecific nuclear staining of the spermatozoon disappeared (Fig. 52). Unoxidized controls are also shown (Figs. 53, 54). Although with beautiful results, in our hands the SM techniques appeared very difficult to reproduce and obtain convenient results.

The TA method for complex carbohydrates stains all components of the jelly coat and some regions of the peripheral component of cortical vesicles (Fig. 55). No staining was noted on controls (Fig. 56). TA also stained the peripheral electronlucent and the central component of acrosomal vesicles (Fig. 57). After insemination, this component is exocytated and coats the acrosomal process of the spermatozoon (Fig. 58). With the cortical reaction, the stained component of cortical vesicles fuse with the fertilization envelope, but no staining was transfered to this latter structure (Fig. 58).

The PTA staining techniques were applied at pH 0.5 (PTA-HCl and PTA-CrA) (Figs. 59, 60) and at pH 1.6 (aqueous PTA and PTA-acetone) (Figs. 61, 62). PTA at low pH is known to stain the polysaccharides of glycoproteins and not the basic proteins (Rambourg, 1971). The overall results are quite similar to those obtained with TA. However, the amorphous component of the jelly coat was more difficult to stain, while the acrosomal vesicle did not stain with PTA-HCl and PTA-Aq.

All our attempts with the HID and HID-TCH-SP techniques failed to visualize at the ultrastructural level the sulphated complex carbohydrates. We also have tried the direct *on grid* staining of sections, but again no staining was achieved. This result argues against the presence of sulphate groups in the jelly coat, but the absence of staining could also be due to the masking or

inaccessibility of those groups.

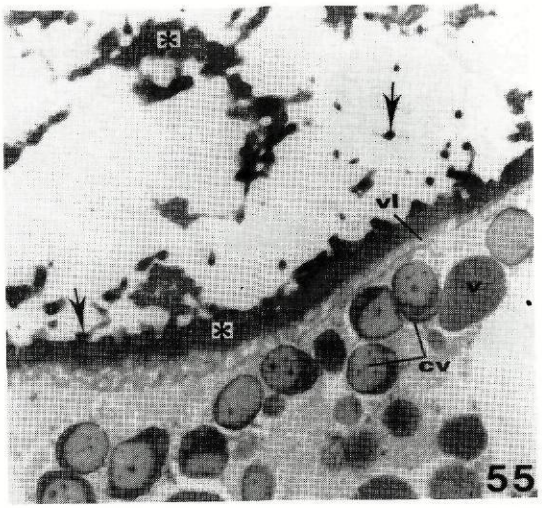
Finally, we have tried to detect the anionic sites on gamete cell surfaces by the CCIC method. No staining was observed at pH 1.4, at pH 4 the labell was very weak, and at pH 7 a stronger staining appeared. Unreacted oocytes did not show any staining (Fig. 63), but after the cortical reaction the peripheral component of cortical vesicles appeared stained (Fig. 64). The fertilization envelope did not acquire any staining after the fusion of the stained component of cortical vesicles with it (Fig. 64). With the direct staining *on grids* we found that the fibrillar component of the jelly coat of unreacted oocytes stained intensely by the CCIC method (Fig. 65). Concerning the spermatozoon, CCIC staining was found in the midpiece at the periphery of microtubules of centrioles and axoneme, as also in the pericentriolar complex (Fig. 66). CCIC staining was also observed in the acrosome, namely, in the apical acrosomal-plasma membrane contact zone, in the baso-lateral periacrosomal material, and in the inner acrosomal vesicle membrane (Fig. 66). After the acrosomal reaction, the periphery of the acrosomal process appeared labelled (Figs. 67, 68).

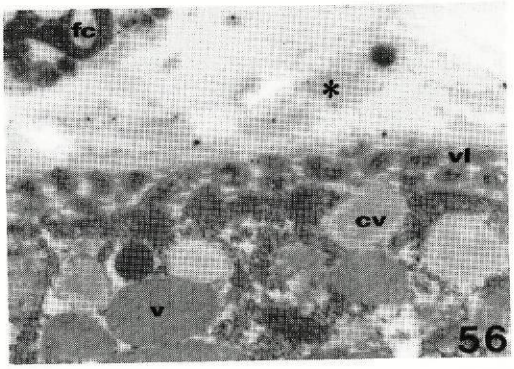
When observed by SEM the jelly coat appears as a complex network of fibrillar material with different thickenesses, which probably correspond to the fibrillar component observed by TEM (Figs. 69, 70). The interstices of this network are probably occupied by the amorphous component, although this last component is not visible either because it was removed during preparation of the material or collapsed against the fibrilar component.

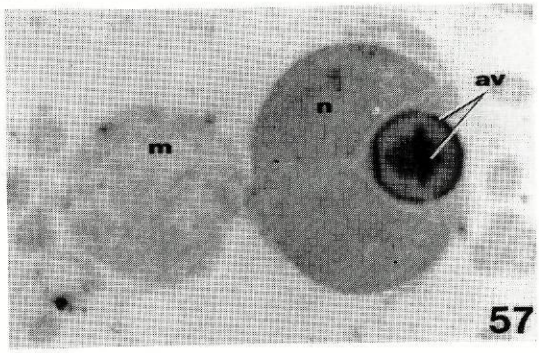
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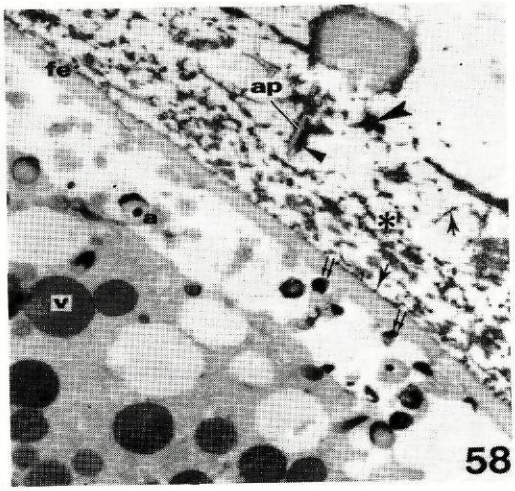
Figs. 55-58. TA staining. Fig. 55. In the unreacted oocyte, TA stains both components of the jelly coat (* and arrows), and some region of the peripheral component of cortical vesicles (cv). The vitelline layer (vl) is not stained. x 16,000. Fig. 56. No staining was noticed in controls. fc, follicle cell; (*), jelly coat; vl, vitelline layer; cv, cortical vesicles; v, yolk granules. x 25,000. Fig. 57. In the unreacted spermatozoon, TA stains the peripheral and central components of the acrosomal vesicle (av). m, mitochondrion; n, nucleus. x 24,000. Fig. 58. At fertilization, the stained acrosomal material (arrowheads) coats the acrosomal process (ap) of the spermatozoon. Exocytated cortical vesicle contents (a) fuse (double arrows) with the fertilization envelope (fe), but this latter structure do not become stained. x 9,000.

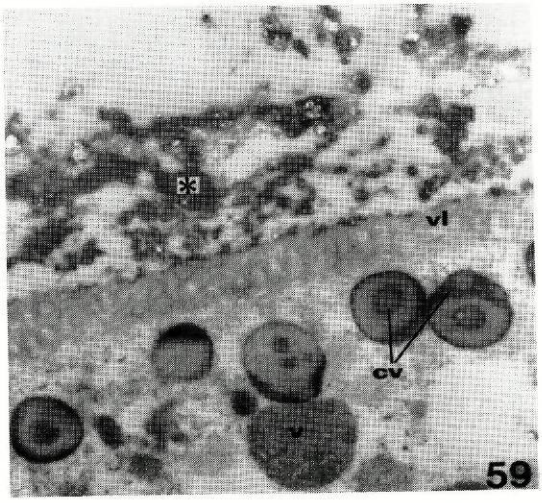
Figs. 59, 60. PTA-CrA staining. Fig. 59. In the unreacted oocyte, PTA-CrA stains the jelly coat (*) and some region of the peripheral component of cortical vesicles (cv). vl, vitelline layer. x 18,000. Fig. 60. At fertilization, the stained component of the acrosomal vesicle (double arrows) coats the acrosomal process (ap) of the spermatozoon. Exocytated cortical vesicle contents (a) fuse (arrowheads) with the fertilization envelope (fe), but this latter structure do not become stained. v, yolk vesicles. x 9,000.

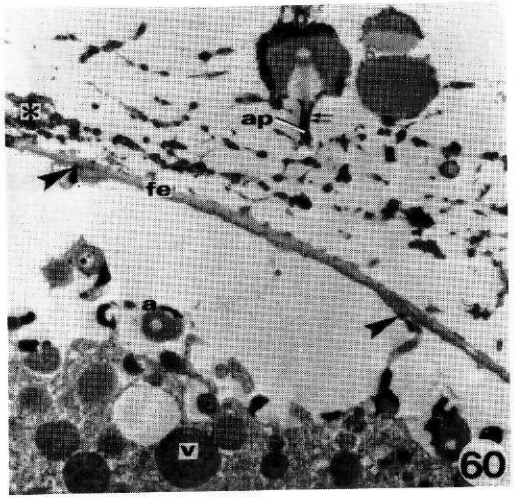

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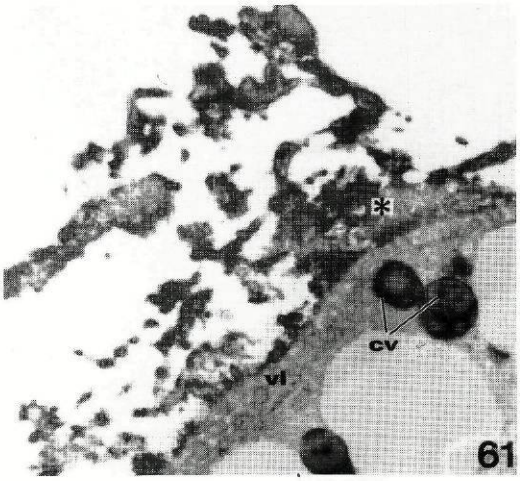

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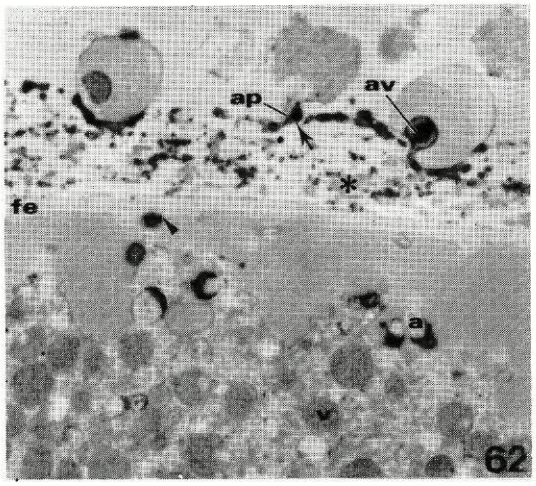

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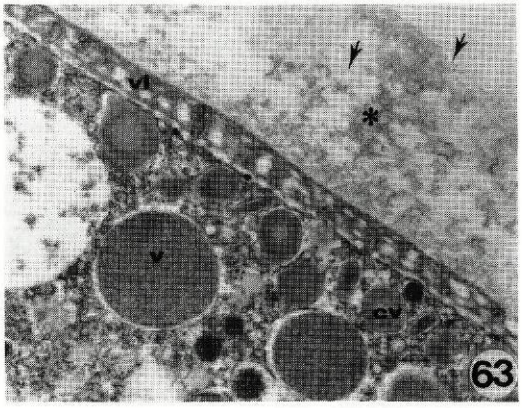
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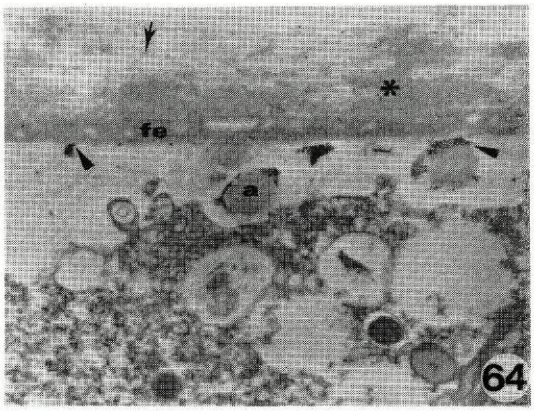
Figs. 61, 62. PTA-Acetone staining. **Fig. 61.** In the unreacted spermatozoon, PTA-Acetone stains all jelly coat (*) and some region of the peripheral component of acrosomal vesicles (av). The vitelline layer is not stained (vl). x 19,000. **Fig. 62.** At fertilization, PTA-Acetone - stained acrosomal vesicle apical and central components coat (arrow) the acrosomal process (ap) of the spermatozoon. Exocytated cortical vesicles (a) fuse (arrowhead) with the fertilization envelope (fe), but no staining is transfered to this structure. v, yolk vesicle. x 9,000.

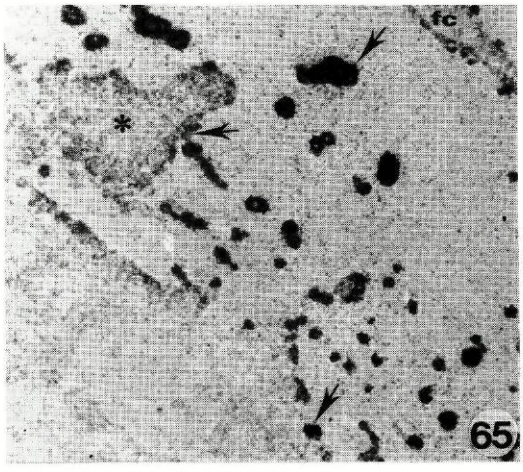
Figs. 63-66. CCIC pH 7. **Fig. 63.** In the unreacted oocyte no staining is noticed in the fibrillar (arrows) and the amorphous (*) components of the jelly coat, as well as in the other oocyte cortex structures. vl, vitelline layer; cv, cortical vesicle; v, yolk vesicle. x 17,000. **Fig. 64.** At fertilization, exocytated cortical vesicle contents (a) are labelled at their peripheral component, that fuse (arrowheads) with the fertilization envelope (fe), but do not transfer their staining to this latter structure. x 16,000. **Fig. 65.** On grid staining. The fibrillar component (arrows) but not the amorphous component (*) of the jelly coat appears stained. x 20,000. **Fig. 66.** In the unreacted spermatozoon, the periphery (arrow) of centrioles (c), the periphery (double arrows) of the pericentriolar complex (pcc), the periphery (triple arrows) of the axoneme (ax), the basolateral component of the periacrosomal material and the apical pole of the acrosomal vesicle (arrowheads) appear labelled. av, acrosomal vesicle; n, nucleus; m, mitochondrion. x 22,000; inset, x 27,000.

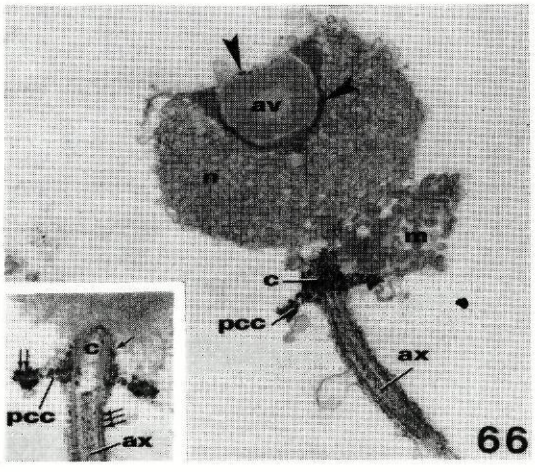

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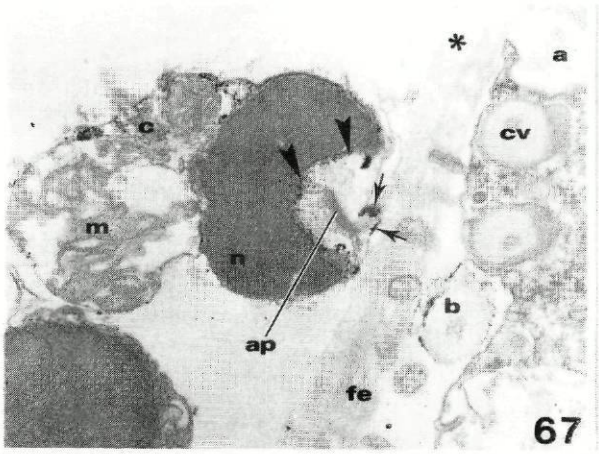

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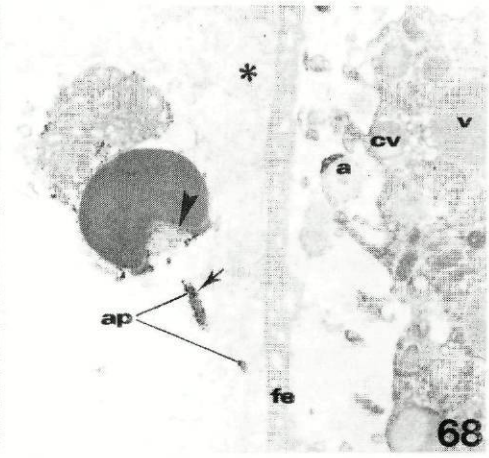

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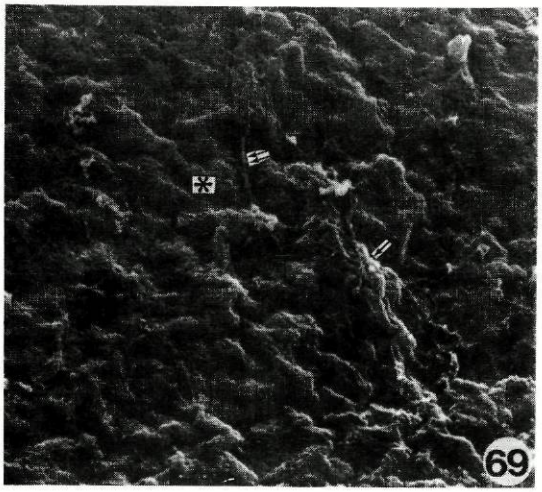
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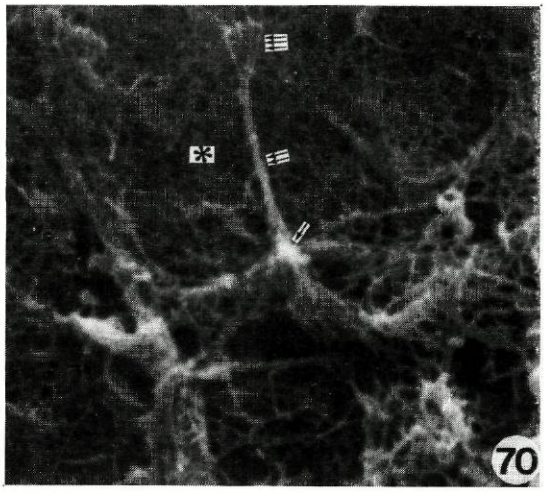
Figs. 67, 68. CCIC pH 7 staining. At fertilization, the labelling is observed in the subacrosomal fossa and over the inner acrosomal vesicle membrane (arrowheads), as well as at the periphery (arrows) of the acrosomal process (ap). Exocytated cortical vesicle contents are labelled (a) and fuse (b) with the fertilization envelope (fe), but no staining is observed in this latter structure. (*), jelly coat; cv, cortical vesicle; v, yolk vesicle. x 23,000 and x 27,000.

Figs. 69, 70. SEM views of the jelly coat in intact and unreacted oocytes. There are large dense fibrous structures (arrow) that progressively branch (double arrows to triple arrows) to give origin to the inner fine fibrillar network (*). x 18,000 and x 41,000.


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70

Cellulose acetate electrophoresis

When crude jelly coat was submitted to CAE and then stained with Ab 3 main bands appeared, of which one remained at the origin (Fig. 71a). With the Ab-CEC method all 3 bands appeared stained between 0.0-0.2 M $MgCl_2$, at 0.4 M $MgCl_2$ the band of intermediary mobility toward the anode disappeared and at 0.6 M $MgCl_2$ the band of faster mobility toward the anode also disappeared, while the band at the origin still remains visible even at 1.5 M $MgCl_2$, although its staining progressively decreased (Fig 71b-h). When gels were stained by Cb and PAS, only the band that remained at the origin appeared stained (Fig. 71i,j). The two migrating bands appeared resistant to chondroitinase ABC digestion (Fig. 72a), while they were partially attacked by neuraminidase (Fig. 72b), hyaluronidase (Fig. 72d) and protease (Fig. 72j). With the Ab-CEC it was possible to demonstrate that hyaluronidase and protease digested the band with intermediary mobility (Fig. 72e,f and k,l). The Ab staining of the band that remained at the origin was slightly diminuished after neuraminidase treatment (Fig. 72b), clearly diminuished after hyaluronidase digestion (Fig. 72d) and completely abolished by the action of protease (Fig. 72j). No changes occurred on this band after chondroitinase digestion (Fig. 72a). The PAS staining of the band that remained at the origin was slightly diminuished after hyaluronidase digestion (Fig. 72i) and abolished with protease treatment (Fig. 72o), while no changes occurred with neuraminidase (Fig. 72c). The Cb staining of this band was only affected by protease treatment (Fig. 72h,n). The densitometric evaluation of the bands revealed by Ab

staining gave the following composition percentages: ± 50% for the band that remains at the origin and ± 25% for each of the two migrating bands (Fig. 73). Protein determination gave a value of 52 µg protein/mg crude jelly coat or about 19% of the total. Glycosaminoglycans were also isolated from crude jelly coat, and after CAE presented as one main band with a similar Rf to standard hyaluronic acid (Fig. 74). However, a minor component remained at the origin (Fig. 74). Spectrophotometric analysis of isolated GAGs showed that they constitute about 20% of the jelly coat. These results thus show that the jelly coat can be subdivided into three main components by CAE. One is neutral and consists of protein and polysaccharides which stain by Ab and PAS, being partially sensitive to hyaluronidase digestion and probably contains GAGs in its composition. The other two components are negatively charged and do not stain with Cb nor PAS. The slower moving band is attacked by protease and hyaluronidase and may contain GAGs in its composition. Concerning sulphate content, the band at the origin was the most rich in that reactive group, being immediately followed by the faster moving band.

Discussion

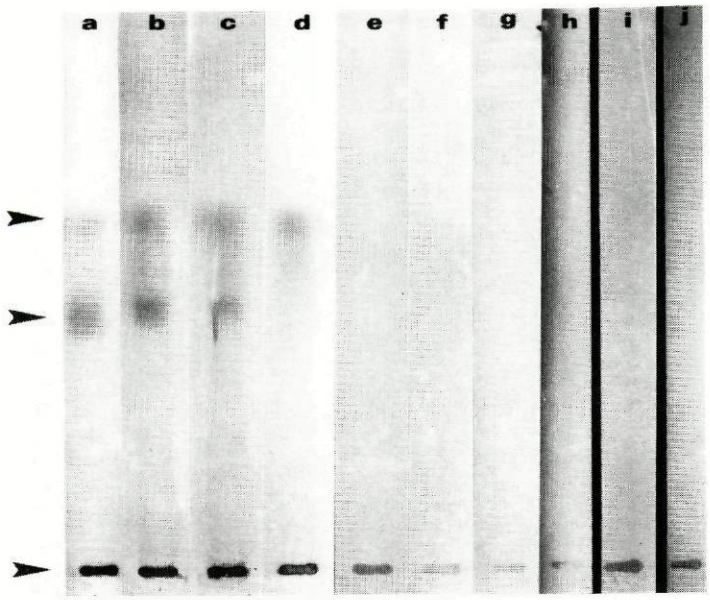
Despite detailed studies on the biochemical components of the jelly coat of echinoderm oocytes, only a few studies have been performed to histochemically characterize the jelly coat.

In the present work, the histochemical characterization of the jelly coat in the starfish *M. glacialis* was done giving special attention to the polysaccharide composition. Our main conclusion is

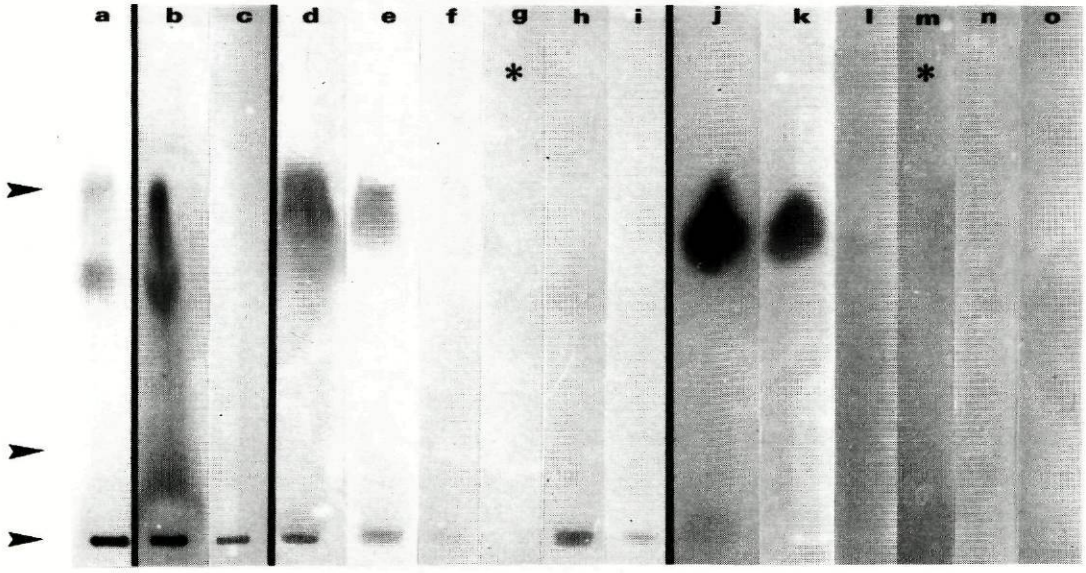
Legends

Fig. 71. Cellulose acetate electrophoresis of crude jelly coat. Ab-CEC staining at different $MgCl_2$ concentrations: 0.0 M (a), 0.1 M (b), 0.2 M (c), 0.4 M (d), 0.6 M (e), 0.8 M (f), 1.0 M (g) and 1.5 M (h). Coomassie blue (i) and PAS (j) stainings. Arrowheads point to the stained bands.

Fig. 72. Cellulose acetate electrophoresis of crude jelly coat after enzymatic digestions. Chondroitinase ABC digestions as stained by Ab (a). Neuraminidase digestions as stained with Ab (b) and PAS (c). Hyaluronidase digestions as stained by Ab-CEC at $MgCl_2$ concentrations of 0.0 M (d), 0.4 M (e) and 0.8 M (f), and as stained by coomassie blue (h) and PAS (i). Protease digestions as stained by the Ab-CEC at $MgCl_2$ concentrations of 0.0 M (j), 0.4 M (k) and 0.8 M (l), and as stained by coomassie blue (n) and PAS (o). (*), point to control strips containing only hyaluronidase (g) or protease (m). Arrowheads point to the stained bands.



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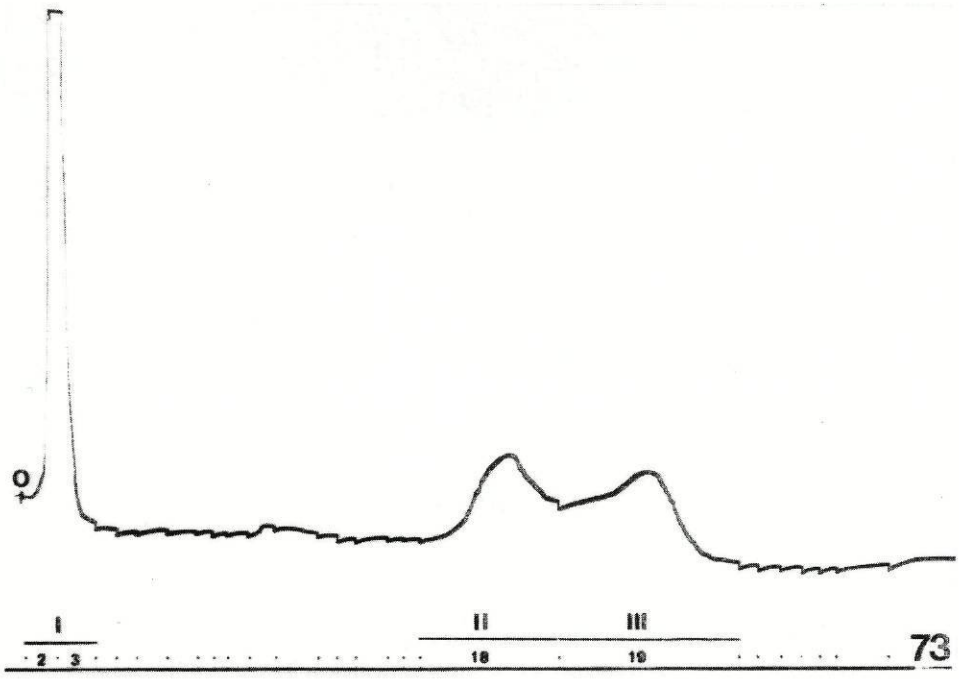


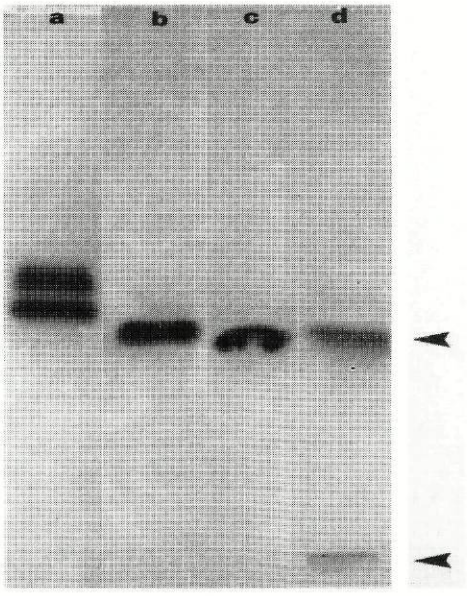
72

Legends

Fig. 73. Densitometric evaluation of Ab stained bands after cellulose acetate electrophoresis of crude jelly coat. Integrated fractions were 2 + 3 (band I, at the origin), 18 (intermediary moving band, II) and 19 (faster moving band, III).

Fig. 74. Cellulose acetate electrophoresis of isolated glycosaminoglycans from crude jelly coat (arrowheads in **d**). Standards consisted of chondroitan sulphate (upper band in **a**), dermatan sulphate (lower band in **a**), heparan sulphate (**b**) and hyaluronic acid (**c**).





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that the jelly coat contains two major polysaccharide substances, neutral and acidic. The acidic substance was found to contain carboxyl (sialic/uronic) and sulphated groups, both in close proximity to vicinal hydroxyls. The acidic substance was observed as a fibrillar network, while the neutral component appears uniformly distributed and occupyes the most part of the jelly coat. Staining for total proteins revealed the presence of a proteinaceous component in the entire jelly coat. Basic amino acids, if present, are in low concentration, while tyrosine and tryptophan were only found at the region that comprises the vitelline layer/cortical vesicles.

When these histochemical results are compared to those in the sea urchin (Jondeung and Czihak, 1982), several differences become apparent. As with the starfish, the jelly coat of the sea urchin shows the presence of neutral and acidic mucosubstances, the latter containing carboxyl and sulphated groups in close proximity to vicinal hydroxyls. On the contrary, however, the sea urchin jelly coat appears much more sulphated. Also, the acidic component could not be distinguished in a morphological basis from the neutral component.

Only a few studies have also been performed in order to ultrastructurally characterize the macromolecular components of the jelly coat. In the present study the reactivity of the jelly coat of the starfish *M. glacialis* was studied with several methods used to detect carbohydrates. Our main finding is that the jelly coat can be subdivided into two components, large amorphous aggregates and a fibrillar and branched component. The amorphous aggregates are the main component of the jelly coat. The fibrillar component delimits the outer border of the jelly coat, spreads between the amorphous

aggregates and binds to them as also to the vitelline layer. After insemination, only the fibrillar componentremains visible in the jelly coat of the embryos. Cytochemical studies suggest that the amorphous aggregates might correspond to the neutral polysaccharides, while the fibrillar component stains as an acidic polysaccharide. It is to this fibrillar component of the jelly coat of the starfish *M. glacialis* that calcium has been localized at the ultrastructural level, further demonstrating that the jelly coat fibres do penetrate the vitelline layer to make contact with the oolemma (Sousa and Azevedo, 1989).

In other echinoderms, only very short ultrastructural cytochemical studies have been reported. In sea urchins, RR was shown to demonstrate an amorphous and a fibrillar component in the jelly coat (Kidd, 1978), while in the starfish, Ab has been shown to stain all the jelly coat (Crawford and Abed, 1986).

We also have visualize the fibrillar component of the jelly coat of the starfish *M. glacialis* by means of SEM. These results support the view offered by Ab staining, as the outer border of the jelly coat shows large and dense fibres which branch to give origin to the inner fibrillar network. A similar micrograph can be found in another starfish in the work of Schroeder and Stricker (1983). More recently, Bonnell and Chandler (1990) have demonstrated in the sea urchin jelly coat a similar pattern of organization by platinum replicas, and could even show that the fibrillar mesh corresponded to the fucose sulphate-rich polysaccharide.

Our cytochemical studies have also shown that polysaccharides could be differentiated in acrosomal and cortical vesicle contents of the starfish *M. glacialis*. In the spermatozoon, RR and Ab stained the

dense peripheral component of the acrosomal vesicle. After the acrosomal reaction this component remains at the outer border of the jelly coat and seems to attach the spermatozoon to the jelly coat. A similar staining with RR has also been demonstrated in the sea urchin (Summers and Hylander, 1974) an in ophiuroids (Hylander and Summers, 1975). Tannic acid, PTA-CrA and PTA-acetone stained the central component of the acrosomal vesicle in *M. glacialis*. After the acrosomal reaction this component coats the acrosomal process. By the CCIC method we could also demonstrate that the surface of the acrosomal process is coated by negative charges. This finding then argues against the proposal that the acrosomal process is coated by positive charged molecules that would facilitate oocyte activation at the momment sperm and oocyte membranes interact (Jaffe, 1990; Longo, 1991). Some portions of the peripheral component of cortical vesicles in *M. glacialis* also appeared stained by RR, Ab, TA and PTA, but this staining was not transfered to the fertilization envelope after the fusion of cortical vesicle contents with that structure during the cortical reaction. This suggest that those stained molecules are modified after the fusion process. However, the staining of cortical vesicles with the Thiéry technique was found in the inner face of the fertilization envelope, although we could not distinguish whose component, the spiral lamellae or the peripheral component, was transfering this staining. In the sea urchin, the spiral lamellae were shown to stain with PTA (Bal, 1970; Schuel, 1978), while Ab stained the peripheral component of the cortical vesicles in the sea urchin (Cameron and Holland, 1985) and in the starfish (Crawford and Abed, 1986).

The jelly coat of *M. glacialis* was isolated and studied by CAE. This technique separated the jelly coat into three components, one with a neutral charge and two negatively charged. All three bands stained with Ab, but PAS and Cb staining were restricted to the band that remained at the origin. With the Ab-CEC technique we showed that the intermediary moving band has a critical electrolytical point at 0.4 M $MgCl_2$ and the faster moving band at 0.6 M $MgCl_2$, while the band at the origin reacts as being highly sulphated. Protease abolished the staining of the intermediary moving band and of the band at the origin. Hyaluronidase digested the intermediary moving band and partially attacked the band at the origin. Neuraminidase seems to partially digest some components of the bands, and chondroitinase ABC do not affect the components of the jelly coat. The jelly coat was also shown to contain about 20% of GAGs, which migrate as a main band with a similar Rf to standard hyaluronic acid, although a minor band remained at the origin. Jelly coat contains about 19% protein, and the band at the origin comprises about 50% of the total jelly coat, while each of the moving bands correspond to about 25% of total jelly coat. Experiments with CAE have also been performed in the sea urchin (SeGall and Lennarz, 1979), with similar results concerning the presence of two negatively charged bands and of one band that remains at the origin. These authors could further demonstrate that the faster moving band corresponded to the fucose sulphate-rich polysaccharide, while the intermediary moving band corresponded to the sialoprotein. The band at the origin was not characterized and the presence of GAGs were also not investigated. Concerning the presence of GAGs in the jelly coat of the starfish *M. glacialis*, if our finding will be

confirmed in the future, then it would be demonstrated for the first time that echinoderms also have GAGs in their jelly coat as happens in mammals (Miller and Ax, 1990).

In conclusion, this work represents the first detailed histochemical characterization of the jelly coat of a starfish. Histochemical and cytochemical experiments showed that the jelly coat contains a fibrillar network of acidic polysaccharides and amorphous aggregates of neutral polysaccharides. The results obtained with CAE, here used for the first time to characterize the jelly coat of starfishes, show three main components, two negatively charged and one with a neutral charge, perhaps containing GAGs in their composition.

Acknowledgments

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SUMÁRIO

O espermatozóide de *Marthasterias glacialis* (Echinodermata, Asteroidea) foi estudado por microscopia de luz e microscopia electrónica. Trata-se de uma célula longa, uniflagelada, de tipo primitivo. A cabeça (± 2 x 2 µm) possui uma forma esférica e contém um núcleo que rodeia um acrosoma esferóide. O acrosoma está contido numa fossa anterior em forma de taça. O acrosoma é formado por uma vesícula acrosómica (d = ± 1 µm) envolvida pelo material periacrosómico. À metade interna da membrana da vesícula acrosómica está aderente um material denso, enquanto que a metade externa contacta intimamente com a membrana citoplasmática apical. O conteúdo da vesícula acrosómica encontra-se diferenciado num estreito componente periférico electronolucente, num componente periférico denso e num componente central claro. O material periacrosómico está dividido em três porções distintas. A porção apical apresenta uma estrutura densa, a porção lateral é menos compacta e a porção basal está contida numa pequena fossa subacrosómica e possui um agregado denso de microfibrilas paralelas ao eixo menor da célula. Na peça intermediária encontra-se uma volumosa mitocôndria anelar (± 2 x 1 µm) que rodeia dois centríolos. O centríolo proximal adere à membrana externa do invólucro nuclear e o centríolo distal fixa-se à membrana citoplasmática por uma série radial de estruturas microfibrilares estriadas, o complexo pericentriolar. O axonema (9 pares periféricos de microtúbulos + dois microtúbulos centrais) origina-se do centríolo distal e, em conjunto com a matriz flagelar, preenche o longo flagelo (± 60 µm). Estes resultados constituem, sobretudo no que diz respeito à organização do

acrosoma, a primeira descrição detalhada da estrutura fina do espermatozóide da estrela do mar (Sousa, M. and Azevedo, C. (1983). *Internat. J. Invert. Reprod.*, 6: 171-180).

Estudou-se a ultrastrutura da espermatogénese na estrela do mar *M. glacialis*, tendo-se observado que as vesículas proacrosómicas são formadas desde a fase de espermatogónia a partir de um dictiosoma. Nestas células também constatámos a presença de corpos densos citoplasmáticos, de estrutura granulo-fibrilar, rodeados por mitocôndrias. Estes corpos densos fragmentam originando corpos densos semelhantes mas de menores dimensões. Durante a fase de espermatócito, quer as vesículas proacrosómicas, quer os corpos densos aumentam de número. Durante a espermiogénese as vesículas proacrosómicas fundem-se entre si para originar uma única vesícula acrosómica por espermatídeo. A futura metade interna da membrana da vesícula acrosómica adquire então um revestimento denso oriundo dos corpos densos, com os quais intimamente se relaciona. De seguida, a base da vesícula acrosómica surge rodeada por uma camada de cisternas de retículo endoplasmático liso, migrando em conjunto do pólo basal para o pólo apical da célula. Subsequentemente, a face anterior do núcleo invagina-se sob o acrosoma para formar uma escavação, a fossa acrosómica, que engloba a vesícula acrosómica e os materiais periacrosómicos que a rodeiam. No acrosoma maduro, as cisternas de retículo endoplasmático liso deixam de ser visíveis. Os corpos densos, para além de contribuírem para a formação do material periacrosómico, foram observados na proximidade de vários outros organelos, nomeadamente os centríolos, o complexo pericentriolar e o revestimento denso das pontes intercelulares dos

espermatídeos. Estes resultados mostram que na estrela do mar *M. glacialis* a acrosomogénese se inicia durante a fase de espermatogónia, completando-se durante a espermiogénese. Os corpos densos citoplasmáticos constituem um novo organelo da linha germinal masculina dos asteróides e a sua função parece corresponder a depósitos primordiais de substâncias necessárias à formação de outros organelos, nomeadamente o material periacrosómico, os centríolos, o complexo pericentriolar e o material denso que reveste as pontes intercelulares dos espermatídeos (Sousa, M. and Azevedo, C. (1988). *Tissue & Cell*, **20**: 621-628).

A espermatogénese de *M. glacialis* foi estudada com a aplicação ultrastrutural do método de coloração pelo nitrato de prata. No núcleo das espermatogónias, a prata marca o nucléolo e regiões focais dispersas ao longo da periferia da cromatina. No citoplasma, a prata marca os corpos densos e os centríolos. Durante a espermiogénese, à medida que a cromatina se condensa os focos argirofílicos dispostos à periferia da cromatina são concentrados nos interstícios nucleares, terminando por desaparecer quando o núcleo finaliza o seu processo de condensação. No citoplasma, os corpos densos argirofílicos foram observados em relação morfológica íntima com as pontes intercelulares, os centríolos, o complexo pericentriolar, o axonema e a base da vesícula acrosómica. Todas as estruturas com as quais os corpos densos se relacionam surgem também marcados pela prata. Estes resultados corroboram os do trabalho anterior, no sentido de que os corpos densos constituem um depósito primordial de substâncias (proteínas argirofílicas ?) necessários à formação de vários organelos do

espermatozóide (Sousa, M. and Azevedo, C. (1988). *Invert. Reprod. Develop.*, 15: 105-108).

Quando o espermatozóide de *M. glacialis* contacta com a geleia do ovócito, adere à sua superfície externa. De imediato, observa-se uma tumefacção da vesícula acrosómica, a que se segue a fusão entre a sua membrana externa e a membrana citoplamática apical. Este fenómeno de exocitose permite ao espermatozóide libertar o conteúdo da vesícula acrosómica sobre a geleia do ovócito. Após a exocitose da vesícula acrosómica, e a partir do material contido na fossa subacrosómica, polimeriza-se um feixe de microfilamentos que adere então à membrana interna da vesícula acrosómica. À medida que aumenta de extensão, este feixe de microfilamentos evagina a membrana interna da vesícula acrosómica, formando um fino (d = ± 0,1 μm) prolongamento celular denominado de perfuratório. O perfuratório penetra então a geleia e depois a camada vitelina da matriz extracelular que rodeia o ovócito, para finalmente se fundir com o oolema. Durante este processo, denominado de reacção acrosómica, o núcleo e a mitocôndria do espermatozóide diminuem de diâmetro e o seu conteúdo densifica-se. O flagelo passa para uma posição lateral, entre o núcleo e a mitocôndria. Estes resultados são os primeiros a mostrar, a nível ultrastrutural, como é que o espermatozóide da estrela do mar reage e se relaciona com o ovócito nas fases precoces da fertilização (Sousa, M. and Azevedo, C. (1985). *Gamete Res.*, 11: 157-167).

Utilizando métodos citoquímicos detectámos, a nível ultrastrutural, uma actividade ATPásica no componente periférico denso da vesícula acrosómica do espermatozóide da estrela do mar *M. glacialis*. Esta

mesma actividade foi também detectada na matriz mitocondrial, nos centríolos e no axonema. Enquanto que no acrosoma a actividade enzimática se mostrou dependente dos iões Ca^{2+} e Mg^{2+}, nos outros organelos ela é apenas dependente do ião Mg^{2+}. Detectámos também uma reduzida actividade de fosfatase ácida na porção apical do componente periférico electronolucente e no componente central da vesícula acrosómica. Durante a fertilização, a actividade da enzima fosfatase ácida torna-se intensa e foi localizada nas vesículas resultantes da fusão entre as membranas citoplasmática e acrosomal externa, bem como ao longo do trajecto aberto pelo perfuratório através da matriz extracelular do ovócito. Em relação à presença de uma ATPase no interior da vesícula acrosómica, desconhece-se qual a sua possível função. Em relação à actividade de fosfatase ácida, estes resultados sugerem que a enzima se encontra inibida no interior da vesícula acrosómica, sendo activada no momento da fecundação. A localização apical e nas vesículas resultantes da exocitose da vesícula acrosómica, sugere que a enzima deverá estar envolvida no processo de fusão entre as membranas citoplasmática e acrosomal externa. Por outro lado, a localização da actividade enzimática no componente central da vesícula acrosómica e no trajecto aberto pelo perfuratório através dos revestimentos externos do ovócito sugere que a enzima poderá também estar envolvida na lise desses materiais do ovócito durante a penetração do espermatozóide. Estes resultados representam a primeira visualização da actividade desta enzima no momento da fecundação (Sousa, M. and Azevedo, C. (1986). *Biol. Cell*, 56: 79-84).

A reacção acrosómica do espermatozóide de *M. glacialis* foi artificialmente induzida com o ionóforo A23187. No sobrenadante das

células reagidas, que contém os materiais libertados a partir das vesículas acrosómicas, detectámos, por doseamento enzimático, uma actividade significativa de fosfatase ácida. Este sobrenadante foi então concentrado e submetido, quer a electroforese em condições desnaturantes em gel de poliacrilamida (SDS-PAGE), quer a electroforese em condições não desnaturantes em gel de amido. A fracção com actividade de fosfatase ácida foi detectada por reacção enzimática com produto fluorescente no gel de amido, tendo sido subsequentemente extraída. Esta fracção foi depois submetida a SDS--PAGE, evidenciando uma única banda com cerca de 63 kDa. Estes resultados confirmam a existência da enzima fosfatase ácida na vesícula acrosómica do espermatozóide e constituem a primeira demonstração directa desta enzima no acrosoma animal (Sousa, M., Moradas Ferreira, P., Amorim, A. and Azevedo, C. (1988). *Biol. Cell*, 63: 101-104).

A reacção acrosómica do espermatozóide de *M. glacialis* foi artificialmente induzida com o ionóforo A23187. No sobrenadante das células reagidas detectámos, por doseamento enzimático, actividades enzimáticas de fosfatase ácida, fosfatase alcalina e ATPase Ca^{2+} e Mg^{2+} dependente. O *p*H óptimo da ATPase é de cerca de 8 e, apesar de hidrolizar vários nucleótidos, o pirofosfato e o β-glicerofosfato de sódio, a enzima apresenta uma nítida afinidade para o ATP. A actividade desta enzima é inibida pelo glutaraldeído, pelo EDTA e pela congelação. O *p*H óptimo da fosfatase ácida é de cerca de 4,5 e o da fosfatase alcalina de 9,5. A actividade da enzima fosfatase alcalina foi também localizada por métodos citoquímicos a nível ultrastrutural.

A actividade enzimática apresenta-se concentrada na porção apical do componente periférico electronolucente bem como no componente central da vesícula acrosómica. Esta localização é sobreposta à da fosfatase ácida, mas a sua actividade é superior. Durante a fecundação, a enzima acompanha a penetração do espermatozóide, mas, comparativamente com a fosfatase ácida, a sua actividade ao longo da matriz extracelular do ovócito é menor. Estes resultados evidenciam por métodos directos a existência na vesícula acrosómica do espermatozóide da estrela do mar *M. glacialis* das enzimas ATPase, fosfatase ácida e fosfatase alcalina. A função da fosfatase alcalina parece ser similar à da fosfatase ácida (ver acima) no que diz respeito, quer à exocitose da vesícula acrosómica, quer durante a penetração do espermatozóide. Este estudo constitui a primeira demonstração de um envolvimento da enzima fosfatase alcalina na reacção acrosómica e na penetração do espermatozóide (Sousa, M. and Azevedo, C. (1988). *Cell Biol. Internat. Rep.*, 12: 1049-1054).

A reacção acrosómica do espermatozóide de *M. glacialis* foi artificialmente induzida com o ionóforo A23187, tendo-se detectado no sobrenadante, por doseamento enzimático, uma actividade esterásica. A actividade enzimática foi observada utilizando o substrato N-benzoyl--L-arginine ethyl ester-HCl (BAEE), específico das proteases tipo tripsina, mas não com o substrato N-acetyl-L-tyrosine ethyl ester (ATEE), específico das proteases tipo quimotripsina. A actividade enzimática mostrou-se sensível a vários inibidores, nomeadamente ao soybean trypsin inhibitor (SBTI), ao N-α-p-tosyl-L-lysine chlorometyl ketone-HCl (TLCK) e ao phenyl methyl sulfonylfluoride (PMSF), todos

específicos para proteases do tipo serine-proteases. Contudo, a actividade enzimática não foi inibida pelo L-1-tosylamido-2--phenylethyl chloromethyl ketone (TPCK), um inibidor das proteases tipo quimotripsina. A presença de uma actividade enzimática proteolítica no acrosoma foi confirmada submetendo o sobrenadante, após concentração, a electroforese em gel parcialmente desnaturante e contendo gelatina (gelatin-SDS-PAGE). Nos geis detectámos duas bandas principais de actividade, uma com baixa actividade proteolítica (com cerca de 144 kDa) e uma banda com elevada actividade proteolítica (com cerca de 21 kDa). Enquanto que os inibidores benzamidina e p--aminobenzamidina inibem ambas as actividades no gel, a congelação provoca a perda da actividade da banda de 21 kDa. Para estudar a acção da protease acrosómica sobre as proteínas da geleia, isolámos por métodos químicos este componente da matriz extracelular do ovócito. Após centrifugações e diálises, a amostra foi liofilizada. Finalmente, incubámos uma solução de geleia com o sobrenadante que contém os produtos acrosomais, sendo depois a amostra precipitada com ácido tricloroacético (TCA), lavada com acetona, desidratada sob vácuo e dissolvida em tampão de amostra para SDS-PAGE. Face aos controles, verificou-se que as bandas glicoproteicas da geleia se apresentavam integralmente digeridas. Estes resultados sugerem a presença na vesícula acrosómica do espermatozóide da estrela do mar *M. glacialis* de um sistema proteolítico complexo, no qual uma pro-enzima (144 kDa) dá origem, por degradação autocatalítica, a uma protease activa (21 kDa). Um sistema semelhante existe nos mamíferos, mas no ouriço do mar tem-se detectado, pelo contrário, uma actividade do tipo quimotripsina. Os nossos resultados, portanto, são os primeiros a

apontar para a possibilidade da existência de um sistema proteolítico superior a nível dos equinodermes (Sousa, M., Moradas Ferreira, P. and Azevedo, C. (1991). *J. Exp. Zool.*, in press).

A região cortical dos ovócitos da estrela do mar *M. glacialis*, bem como a exocitose das vesículas corticais que ocorre após a inseminação, foram estudadas a nível ultrastrutural com a aplicação da coloração pelo nitrato de prata. O córtex do ovócito possui uma camada submembranar de vesículas corticais cujo conteúdo se diferencia em dois componentes, um periférico, denso, e outro central, estruturado na forma de corpo lamelar espiralado. Durante a reacção cortical (exocitose das vesículas corticais), as vesículas corticais começam por sofrer uma tumefacção a que se segue a fusão com a membrana citoplasmática suprajacente e a libertação do seu conteúdo para o espaço perivitelino. À medida que a reacção cortical progride, o espaço perivitelino aumenta de dimensão, afastando da superfície do ovo a camada vitelina. O conteúdo das vesículas corticais funde-se com a camada vitelina elevada. O nitrato de prata marca determinada região do componente periférico das vesículas corticais e, apesar deste se fundir com a camada vitelina durante a reacção cortical, esta não adquire aquela coloração. Estes resultados permitiram, pela primeira vez, estabelecer uma diferenciação química nos componentes das vesículas corticais da estrela do mar e mostrar que o seu componente periférico, apesar de homogéneo em termos morfológicos, é, de facto, quimicamente diferenciado. Durante a reacção cortical constatou-se que os materiais argirofílicos que se fundem com a camada vitelina sofrem uma alteração química, pois as suas propriedades tinturiais não se

transmitem a esta (Sousa, M. and Azevedo, C. (1987). *Canadian J. Zool.*, 65: 2607-2611).

A região cortical e a matriz extracelular do ovócito da estrela do mar *M. glacialis* foram estudadas a nível ultratrutural utilizando a coloração do azul de alciano a *p*H neutro na presença de $MgCl_2$. A matriz extracelular do ovócito encontra-se subdividida em dois componentes principais, a geleia e a camada vitelina. A geleia apresenta uma estrutura fibrilar laxa e é de difícil preservação utilizando os métodos convencionais de preparação de material para microscopia electrónica. É a camada mais extensa e mais externa. A camada vitelina constitui uma estreita porção da matriz extracelular do ovócito e apresenta-se constituída por um material fibrilar denso que delimita e rodeia as pequenas microvilosidades do ovócito. A coloração com o azul de alciano permite preservar a matriz extracelular do ovócito e, na geleia, distinguir dois tipos de componentes, um que marca pelo azul de alciano e outro que não fixa o policatião. O componente da geleia que surge precipitado pelo azul de alciano consiste numa estrutura densa que reveste a superfície externa da geleia. Da sua face interna estendem-se estruturas fibrilares que penetram toda a extensão da geleia para terminarem sobre a camada vitelina. Após a reacção cortical, uma parte do componente periférico das vesículas corticais surge igualmente marcado pelo corante, mas apesar de se fundir com a camada vitelina não lhe transmite essa sua propriedade tinturial. O componente da geleia que não é marcado pelo azul de alciano deixa de ser observado após a fecundação, enquanto que o componente fibrilar alcianofílico mantém a estrutura da geleia. A

coloração com o azul de alciano também nos permitiu demonstrar a formação de uma camada de material, oriundo das vesículas corticais, que fica a revestir a superfície membranar do embrião. Esta camada denomina-se camada hialina, e apresenta um aspecto homogéneo em toda a sua espessura. Pelo contrário, a camada vitelina elevada diferencia-se em várias camadas, tornadas visíveis pela preservação imposta pelo azul de alciano. A camada mais interna deriva da adição dos materiais oriundos das vesículas corticais e apresenta uma estrutura densa e compacta. A camada intermédia retém as características da camada vitelina original, apesar de se mostrar mais compacta. A camada mais externa compõe-se de uma estrutura trilamelar, com duas finas lâminas densas unidas por finas pontes fibrilares. Estes resultados mostram, pela primeira vez, a diferenciação estrutural da geleia do ovócito da estrela do mar, bem como a estrutura e a formação da camada hialina e as transformações morfológicas sofridas pela camada vitelina após a reacção cortical (Sousa, M. and Azevedo, C. (1988). *J. Submicrosc. Cytol. Pathol.*, **20**: 629-633).

As vesículas corticais e a reacção cortical na estrela do mar *M. glacialis* foram estudadas a nível ultrastrutural com a aplicação da técnica para a detecção de polisacarídeos ácidos (vermelho de ruténio) e da actividade de fosfatase ácida. Antes da inseminação o vermelho de ruténio não cora as vesículas corticais, uma vez que este não penetra as membranas celulares íntegras. Imediatamente após a inseminação (2 segundos), mas antes de ocorrer a reacção cortical, as vesículas corticais surgem marcadas por aquele corante. O vermelho de ruténio marca apenas uma região do componente periférico das vesículas

corticais. Após a reacção cortical, este componente funde-se com a camada vitelina elevada mas não transfere para esta a sua coloração. Antes da inseminação detectou-se numa porção do componente periférico das vesículas corticais uma ligeira actividade de fosfatase ácida, aumentando esta actividade dois segundos após a inseminação. Durante a reacção cortical aquele componente funde-se com a camada vitelina elevada mas, nesta, não se detectou qualquer actividade enzimática. Estes resultados sugerem, pela primeira vez, que do contacto entre um espermatozóide e a geleia do ovócito resultam alterações na permeabilidade do oolema, como se demonstra pela entrada do vermelho de ruténio e pela activação da enzima fosfatase ácida. Esta enzima é também pela primeira vez referida como componente das vesículas corticais. Do facto da camada vitelina elevada não adquirir a coloração pelo vermelho de ruténio nem a actividade de fosfatase ácida, conclui-se que as moléculas responsáveis por estas reacções são alteradas após se fundirem com a camada vitelina (Sousa, M. and Azevedo, C. (1989). *Histochemistry*, 90: 353-357).

As vesículas corticais e a reacção cortical na estrela do mar *M. glacialis* foram estudadas a nível ultrastrutural utilizando as técnicas de detecção das actividades enzimáticas de fosfatase alcalina e peroxidase. Antes da reacção cortical não se detecta qualquer actividade de fosfatase alcalina mas, após o seu início, uma porção do componente periférico das vesículas corticais apresenta uma forte reacção enzimática. Quando este componente se funde com a camada vitelina elevada, verifica-se que a actividade enzimática também se passa a detectar sobre esta. Antes da reacção cortical não se detectou

qualquer actividade peroxidática mas, após esta ocorrer, detecta-se uma forte actividade nos componentes centrais das vesículas corticais, bem como na face interna da camada vitelina com a qual aqueles se fundem. Os resultados destas experiências demonstram que ambas as actividades enzimáticas se encontram inibidas no ovócito, sendo activadas com a reacção cortical e transferidas para a camada vitelina elevada. A detecção destas actividades não havia ainda sido feita na classe Asteroidea e a detecção de actividade fosfatase alcalina constitui a primeira detecção e localização nos ovócitos e nos ovos dos animais (Sousa, M. and Azevedo, C. (1990). *J. Submicrosc. Cytol. Pathol.*, 22: 97-102).

A técnica do piroantimonato de potássio, em conjunto com a microanálise por RX dos precipitados, permitiram revelar a presença de depósitos de Ca^{2+} nos gâmetas da estrela do mar *M. glacialis*. No espermatozóide, o Ca^{2+} foi localizado no componente periférico denso da vesícula acrosómica, região onde se localiza a enzima Ca^{2+}-Mg^{2+}--ATPase. No ovócito, esta técnica permitiu localizar o ião cálcio na matriz extracelular, bem como na região submembranar desta célula. Na matriz extracelular do ovócito, o cálcio concentra-se na região mais externa da geleia e ao longo dos componentes fibrilares que daí emergem. Esta coloração permitiu constatar que estes prolongamentos da geleia penetram a camada vitelina, terminando sobre o oolema. Estes resultados demonstram, pela primeira vez, que o ião cálcio é acumulado nos gâmetas, quer na vesícula acrosómica do espermatozóide, quer na geleia e na região submembranar do ovócito. Na geleia, a localização do cálcio na sua parte mais externa poderá desempenhar um papel

relevante na reacção acrosómica, pois é aí que o espermatozóide adere e reage, precisando desse ião para efectuar a exocitose da vesícula acrosómica. A localização do cálcio ao longo dos prolongamentos fibrilares da geleia e a observação de que estes penetram a camada vitelina para estabelecer um contacto directo com o oolema, sugere a possibilidade de existir uma troca informacional directa entre o momento de adesão do espermatozóide à geleia e o oolema. A localização submembranar do cálcio constitui um achado de importância, pois que quando o ovócito reage à maturação induzida pela hormona 1-metiladenina e à penetração do espermatozóide, o seu metabolismo sofre uma activação metabólica dependente do aumento intracelular nos teores do ião cálcio. Assim, aqueles depósitos submembranares de cálcio poderiam corresponder ao cálcio sequestrado que aguarda a activação da célula para ser libertado (Sousa, M. and Azevedo, C. (1989). *Develop. Growth Differ.*, 31: 227-232).

Estudámos por métodos histoquímicos, citoquímicos e bioquímicos a composição em glicoproteínas da matriz extracelular do ovócito de *Marthasterias glacialis*, para tentar fornecer uma descrição morfológica pormenorizada da sua composição macromolecular. Estes estudos foram elaborados no ovário, em ovócitos isolados e em ovócitos inseminados. A nível da microscopia de luz os resultados empregando o azul de alciano (AA) a pH 2,5 e a pH 0,5, a concentração electrolítica crítica com AA, a metilação-AA, a metilação-saponificação-AA, a metacromasia com azur A, a fucsina-aldeído (FA) e a FA-AA mostraram que a geleia não é uma estrutura altamente sulfatada e que os grupos carboxilo parecem predominar. Com o ácido-periódico-Schiff (PAS) e o PAS-AA, evidenciou-se que a geleia contém uma mistura de

polissacarídeos não acídicos e acídicos, com predomínio dos primeiros. A hidrólise do ácido siálico revelou, por outro lado, que a coloração pelo PAS deve ser dependente da presença de hexoses e de fucose mas não de ácido siálico. As colorações com os métodos diamina revelaram que os polissacarídeos neutros parecem constituir o principal componente da geleia, existindo, no entanto, polissacarídeos ácidos com grupos de ácido siálico e sulfúrico. Nos estudos ultrastruturais usámos o AA a pH neutro, o vermelho de ruténio, o ácido periódico (AP)-tiocarbohidrazida-proteinato de prata (Thiéry), o AP-metenamina de prata, o AP-ácido crómico (AC)-metenamina de prata, o ácido tânico, o ácido fosfotúngstico (PTA) aquoso ou em mistura com o ácido clorídrico, AC ou acetona e o ferro coloidal catiónico com cacodilato. Os resultados destes estudos revelaram na geleia dois constituintes principais, um componente fibrilar que forma grandes agregados amorfos e um componente fibrilar ramificado que delimita a geleia, externa e internamente, que se distribui por entre os agregados amorfos e a eles se liga. Estes resultados sugerem que o componente amorfo corresponde aos polissacarídeos neutros enquanto que o componente ramificado reage como um polissacarídeo ácido. Em microscopia electrónica de varrimento, a geleia apresenta também dois tipos fundamentais de componentes macromoleculares. Um, mais externo, é formado por estruturas fibrilares espessas que se ramifica em filamentos cada vez mais finos. O segundo, mais interno, apresenta-se como uma fina rede fibrilar. Pensámos que entre os interstícios desta rede se encontrará o componente amorfo descrito pela microscopia electrónica de transmissão e que não terá sido preservado naquela técnica. Isolámos de seguida a geleia do ovócito e fomos estudá-la

por electroforese em acetato de celulose. A geleia mostrou-se então composta por três componentes, um de carga neutra e dois de carga negativa. O AA cora os três componentes, mas o azul de coomassie e o PAS só coram a banda que permanece na origem. Da análise densitométrica dos geis, constatámos que a banda da origem corresponde a cerca de 50% da geleia, enquanto que a cada uma das outras duas bandas corresponde cerca de 25% da geleia. O estudo pela concentração electrolítica crítica-AA mostrou que a banda de mobilidade intermédia desaparece na concentração de 0,4 M $MgCl_2$, que a banda de maior mobilidade desaparece a 0,6 M $MgCl_2$ e que a banda da origem permanece mesmo na presença de 1,5 M $MgCl_2$, apesar da intensidade da coloração ir diminuindo. A banda de mobilidade intermédia é totalmente digerida pela hialuronidase e pela protease, a banda de maior mobilidade mostrou-se resistente às duas enzimas, enquanto que a banda na origem é totalmente digerida pela protease e apenas parcialmente pela hialuronidase. A neuraminidase parece digerir algum componente das bandas, enquanto que todas as bandas se mostraram resistentes à condroitinase ABC. Finalmente, isolámos da geleia glicosaminoglicanos (GAGs) e o seu doseamento revelou um valor de cerca de 20% em relação à geleia total. Em electroforese os GAGs apresentam-se numa banda principal, apesar de um pequeno componente permanecer na origem. Em comparação com os padrões dermatano-sulfato, condroitano-sulfato, heparano-sulfato e ácido hialurónico, a banda principal dos GAGs migra com um Rf semelhante àquele último. O doseamento das proteínas revelou que aquelas constituem cerca de 19% da geleia.

Estes resultados, no seu conjunto, constituem o primeiro estudo

histoquíco sobre a composição macromolecular da geleia da estrela do mar e revelaram que aquela possui um componente de carga neutra e dois de carga negativa, contendo possivelmente GAGs na sua composição. Se este último resultado se comprovar, demonstraremos assim, pela primeira vez, que a matriz extracelular dos ovócitos dos equinodermes, à semelhança dos mamíferos, também possui GAGs na sua composição (Sousa, M., Pinto, R., Moradas Ferreira, P. and Azevedo, C. (1991). **Submitted to** *Biol. Bull.*).

SUMMARY

The mature spermatozoon of *Marthasterias glacialis* (Echinodermata, Asteroidea) is of the primitive type and is formed by a round head (± 2 x 2 µm), a midpiece (± 2 x 1 µm), and a long flagellum (± 60 µm). In the head there is a round nucleus which is apically invaginated to contain the acrosome. The acrosome contains an acrosomal vesicle (± 1 x 1 µm) and a periacrosomal material. The inner membrane of the acrosomal vesicle is coated by a dense material, while the outer acrosomal vesicle membrane contacts with the apical plasma membrane. The contents of the acrosomal vesicle appear differentiated into an electronlucent peripheral component, a dense peripheral material, and a clear central component. The periacrosomal material is also differentiated into an apical dense region, a lateral less dense component, and a dense aggregate of fibres that is contained in a small subacrosomal nuclear fossa. The midpiece is formed by a large mitochondrion that encircles two centrioles. The proximal centriole is connected to the nuclear envelope, while the distal centriole is linked to the plasma membrane by a pericentriolar complex and gives origin to the axoneme (9 + 2 microtubules). These results are the first to describe the detailed acrosomal structure in a starfish (**Sousa, M. and Azevedo, C. 1983.** *Internat. J. Invert. Reprod.* **6:** 171-180).

During the spermatogenesis of the starfish *M. glacialis*, it was found that the proacrosomal vesicles begin to be formed at the spermatogonial stage by the action of a large dictyosome. In

spermatogonia it was also observed the formation of cytoplasmic dense bodies. During spermiogenesis, proacrosomal vesicles fuse to give origin to the acrosomal vesicle. The inner acrosomal vesicle membrane was then coated with a dense material which is originated from the dense bodies. Afterthat the base of the acrosomal vesicle became surrounded by smooth endoplasmic reticulum cisternae, and then migrates to the apical pole of the cell. In the mature acrosome these cisternae are no longer seen, although they became incorporated in the periacrosomal space. Dense bodies were also observed in close apposition to centrioles, pericentriolar complex, and the dense coat of intercellular bridges. This is the first study on the entire spermatogenetic process in starfishes and demonstrates that the proacrosomal vesicles have their origin from the spermatogonial phase. Dense bodies constitute a newly described organelle, and are supposed to represent primordial storage depots containing the materials necessary to the formation of the periacrosomal material as well as to other cytoskeletal structures of the spermatozoon (**Sousa, M. and Azevedo, C. 1988.** *Tissue & Cell* **20:** 621-628).

When silver staining was applied to the study of the spermatogenesis in *M. glacialis*, it was found that silver stains the nucleolus, some regions at the periphery of chromatin, the centrioles and the dense bodies. During spermiogenesis, as the nucleus condenses, the perichromatin silver staining regions became displaced towards those nuclear zones not yet condensed, and finally disappear. During spermiogenesis, silver stained dense bodies appear to transfer their argyrophilic materials to the base of the acrosomal vesicle as well as

to the centrioles, pericentriolar complex and the dense coat of intercellular bridges. These results support our previous findings suggesting that dense bodies represent primordial storage depots containing the materials which are necessary to the formation of the periacrosomal material and other cytoskeletal organelles of the spermatozoon (**Sousa, M. and Azevedo, C. 1988.** *Invert. Reprod. Develop.* 15: 105-108).

At fertilization the spermatozoon of *M. glacialis* attaches to the outer jelly coat region of the oocyte. Immediatelly thereafter the acrosomal vesicle outer membrane fuses with the plasma membrane to release the acrosomal contents over the oocyte investments. After this event, there is the formation of a bundle of microfilaments that emerge from the subacrosomal material. These microfilaments then evaginate the inner acrosomal vesicle membrane to form a thin acrosomal process that penetrates the oocyte coats to fuse with the oolemma. These results are the first to describe the fine structure of the early events at fertilization in a starfish (**Sousa, M. and Azevedo, C. 1985.** *Gamete Res.* **11:** 157-167).

By cytochemical methods we have shown in the spermatozoon of *M. glacialis* an ATPase activity in the dense peripheral component of the acrosomal vesicle, in the mitochondrion, and in the periphery of microtubules of the centrioles and axoneme. Acid phosphatase activity, on the contrary, was found in the apical and central components of the acrosomal vesicle. At fertilization, acid phosphatase activity was found in the vesicles originated from the fusion between the outer acrosomal vesicle and apical cytoplasmic membranes, as well as along

the path that the acrosomal process makes on the oocyte investments. These results are the first to show an acid phosphatase activity in the acrosome, and suggest that this enzyme might be involved both in the acrosomal reaction and the penetration of the acrosomal process through the oocyte external coats (**Sousa, M. and Azevedo, C. 1986.** *Biol. Cell* 56: 79-84).

The acrosomal reaction of the spermatozoa of *M. glacialis* was induced with the ionophore A23187. Acid phosphatase activity was then found in the supernatants of the reacted sperm suspensions. The concentrated supernatants were submitted to starch gel electrophoresis and the bands with acid phosphatase activity were extracted and then submitted to SDS-PAGE. By this technique we could show that the enzyme has about 63 kDa. These results represent the first direct isolation of an acrosomal enzyme (**Sousa, M., Moradas Ferreira, P., Amorim, A. and Azevedo, C. 1988.** *Biol. Cell* 63: 101--104).

In the supernatants of ionophore A23187 induced acrosomal reacted spermatozoa of the starfish *M. glacialis* we have assayed the activities of several enzymes. ATPase has an optimum pH at about 8, and besides hydrolysing several substrates it was shown to preferentially hydrolyse ATP. The enzyme activity was inhibited by glutaraldehyde, EDTA and freeze-thawing. Acid phosphatase activity has an optimum pH at about 4.5, while that of alkaline phosphatase is around 9.5. Alkaline phosphatase activity was also shown to be present in the apical and central components of the acrosomal vesicle. At fertilization this enzyme activity was found to follow the acrosomal

process through the oocyte investments. These results are the first to show the presence of alkaline phosphatase activity in the acrosome, and suggest that the enzyme may be involved both in the acrosomal reaction and in the penetration of the acrosomal process through the outer coats of the oocyte (**Sousa, M. and Azevedo, C. 1988.** *Cell Biol. Internat. Rep.* **12: 1049-1054).**

Supernatants obtained from spermatozoa suspensions of *M.glacialis* which have undergone the acrosomal reaction by the action of the ionophore A23187 exhibited an esterase activity. Using the substrates BAEE and ATEE, as also the inhibitors SBTI, TLCK and PMSF, we could demonstrate that this enzyme activity is a trypsin-like serine protease. In gelatin-SDS-PAGE experiments, proteolytic activity appeared in two main bands, one of low activity (144 kDa) and another with high proteolytic activity (21 kDa). Benzamidine inhibited both activities in the gel, while freeze-thawing only inhibited the 21 kDa band. Supernatants containing this protease were incubated with isolated jelly coat molecules, and SDS-PAGE analysis showed that the jelly glycoproteins were digested. These results are the first to demonstrate a complex pro-protease-active protease system in the acrosome of echinoderms, and suggest that this system may act on the penetration of the oocyte jelly coat by the acrosomal process **(Sousa, M., Moradas Ferreira, P. and Azevedo, C. 1991.** *J. Exp. Zool.* **in press).**

The cortical reaction of *M. glacialis* oocytes was studied with the application of the silver technique at the ultrastructural level. Cortical vesicles contain two main components, spiral lamellae and a homogeneous and dense peripheral component. Silver stained some

regions of the peripheric component of cortical vesicles. After the cortical reaction, the stained component fuses with the fertilization coat but this latter structure did not acquire that staining pattern. These results are the first to show that the peripheral homogeneous component of cortical vesicles are chemically differentiated, that it contains argyrophilic proteins, and that this stained material is processed after fusion with the fertilization coat (**Sousa, M. and Azevedo, C. 1987.** *Canadian J. Zool.* **65: 2607-2611).**

The oocyte investments and the cortical reaction in *M. glacialis* were studied with the application of alcian blue at neutral pH and in the presence of $MgCl_2$. Ab stained the outermost region of the jelly coat and several fibres that depart from that outer region and traverse the jelly to end over the vitelline layer. Between the fibrous component there are large areas containing loose aggregates of a fine fibrillar material which is not stained by alcian blue. After insemination the loose aggregates dissolve away, while the fibrillar component remains at the outer region of the embryo. After the cortical reaction, some regions of the peripheral component of the cortical vesicles appeared stained by alcian blue. Despite the fusion with the fertilization coat, this latter structure did not become stained by the polycation. Alcian blue preserved the fine structure of the fertilization coat, and it was then possible to distinguished three main regions. The innermost is derived from cortical vesicles, the middle zone corresponds to the former vitelline layer, and the outermost region is formed by two fine lamellae which are connected by fine fibrillar strands. Alcian blue also made possible to preserve and

identify the formation of a large and homogeneous hyaline layer over the oolemma, which is derived from cortical vesicle contents. These results showed for the first time that the jelly coat of echinoderm oocytes can be structurally differentiated. New aspects of the formation and structure of the fertilization coat and hyaline layer were also described (**Sousa, M. and Azevedo, C. 1988.** *J. Submicrosc. Cytol. Pathol.* **20:** 629-633).

The cortical reaction of the eggs of *M. glacialis* was studied by cytochemical techniques to detect acid phosphatase activity and by using ruthenium red to stain acid polysaccharides. Before insemination both reactions were negative, but after insemination and before cortical reaction some zones of the peripheral component of cortical vesicles appear stained. After cortical reaction this component fused with the fertilization coat, but no reaction was detected over the latter structure. These results show for the first time that sperm-jelly coat interaction activates the oocyte even before fusion between gametes has occurred (**Sousa, M. and Azevedo, C. 1989.** *Histochemistry* **90:** 353-357).

The cortical reaction of the starfish *M. glacialis* was studied using cytochemical methods to detect alkaline phosphatase and peroxidase activities. Before cortical reaction both activities were negative, but after the cortical reaction alkaline phosphatase activity was detected in some regions of the peripheral component of cortical vesicles, while peroxidase activity was found in spiral lamellae. Both these components fuse with the fertilization coat, but while alkaline phosphatase activity appears in the middle zone of that

structure, peroxidase acivity is restricted to the innermost aspect of the fertilization coat. These results are the first to detect alkaline phosphatase activity in animal cortical vesicles and peroxidase activity in the cortical vesicles of starfishes. Both activities also appear to be activated only after the cortical reaction (**Sousa, M. and Azevedo, C. 1990.** *J. Submicrosc. Cytol. Pathol.* **22: 97-102).**

Calcium was localized in the dense acrosomal vesicle component of the spermatozoon, in the fibrous component of the jelly coat and under the oolemma of the oocyte of *M. glacialis* by means of the pyroantimonate technique and by RX microanalysis of the precipitates. This technique also enabled us to demonstrate that jelly fibres traverse the vitelline layer to end over the oolemma. In the acrosomal vesicle calcium colocalizes with the ATPase which is calcium and magnesium dependent. The calcium found at the jelly coat may have a role in the acrosomal reaction, while deposits found under the plasma membrane are thought to represent the oocyte stores to be released for egg activation at fertilization. These results are the first to report the ultrastructural localization of calcium in the acrosome and in the jelly coat of animal gametes (**Sousa, M. and Azevedo, C. 1989.** *Develop. Growth Differ.* **31: 227-232).**

Combined histochemical, cytochemical and electrophoretical methods were applied to characterize the polysaccharide macromolecular structure of the jelly coat of *M. glacialis* oocytes. Morphological results show that the jelly coat is subdivided into large amorphous aggregates containing neutral polysaccharides, and a fibrillar network that stains as an acidic polysaccharide. The acid

mucosubstance contains both carboxyl (sialic/uronic) and sulphated groups in close proximity to vicinal hydroxyls. Cellulose acetate electrophoresis (CAE) enabled us to show that the jelly coat contains three main components, of which one is neutral and the other two are negatively charged. PAS and protein reactivity was only found in the band that remained at the origin, while all three stained with alcian blue. Critical electrolytical points were 0.4 M $MgCl_2$ for the slower moving band and 0.6 M $MgCl_2$ for the faster moving band, while the band at the origin remained stained even at 1.5 M $MgCl_2$, showing that it is highly sulphated. The intermediary moving band was completely digested by hyaluronidase and protease, while the band at the origin was totally digested by protease and only partially attacked by hyaluronidase. Neuraminidase appears to digest some components of the bands, but chondroitinase ABC did not affect the bands. Glycosaminoglycans (GAGs) were isolated from the jelly coat and showed to constitute about 20% of the total jelly. GAGs appeared as a main band in CAE and presented with a similar Rf to standard hyaluronic acid, although a minor component remained at the origin. Jelly coat is about 19% protein, the band at the origin constitutes about 50% and each of the two moving bands constitute about 25% of the total jelly. These results are the first to histochemically characterize the jelly coat of the starfish oocyte. We than could differentiate a neutral and two negatively charged components, which may contain GAGs in their composition. This latter finding is for the first time described to occur in the echinoderm oocyte jelly (**Sousa, M., Pinto, R., Moradas Ferreira, P. and Azevedo, C. 1991.** Submitted to *Biol. Bull.*).