

CONTRIBUTORS TO THIS VOLUME

Richard A. Beatty
Arthur L. Colwin
Laura Hunter Colwin
Jean Clark Dan
Leonard Machlis
Thaddeus Mann
Charles B. Metz
Leonard Nelson
Alberto Monroy
Erika Rawitscher-Kunkel
Albert Tyler

FERTILIZATION

*Comparative Morphology,
Biochemistry,
and Immunology*

edited by

CHARLES B. METZ

*Institute of Molecular Evolution
School of Environmental and Planetary Sciences
University of Miami
Coral Gables, Florida*

and

ALBERTO MONROY

*Institute of Comparative Anatomy
University of Palermo, and
Research Unit for Molecular Embryology
National Council of Research
Palermo, Italy*

Volume I

ACADEMIC PRESS

NEW YORK • LONDON 1967



COPYRIGHT © 1967, BY ACADEMIC PRESS INC.

ALL RIGHTS RESERVED.

NO PART OF THIS BOOK MAY BE REPRODUCED IN ANY FORM,
BY PHOTOSTAT, MICROFILM, OR ANY OTHER MEANS, WITHOUT
WRITTEN PERMISSION FROM THE PUBLISHERS.

ACADEMIC PRESS INC.

111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by
ACADEMIC PRESS INC. (LONDON) LTD.
Berkeley Square House, London W.1

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 66-29671

PRINTED IN THE UNITED STATES OF AMERICA

List of Contributors

Numbers in parentheses indicate the pages on which the authors' contributions begin.

Richard A. Beatty, Agricultural Research Council Unit of Animal Genetics, and University of Edinburgh, Edinburgh, Scotland (413)

Arthur L. Colwin, Department of Biology, Queens College of the City University of New York, Flushing, New York (295)

Laura Hunter Colwin, Department of Biology, Queens College of the City University of New York, Flushing, New York (295)

Jean Clark Dan, Department of Biology, Ochanomizu University, Otsuka, Bunkyo-ku, Tokyo, Japan (237)

Leonard Machlis, Department of Botany, University of California, Berkeley, California (117)

Thaddeus Mann, Agricultural Research Council Unit of Reproductive Physiology and Biochemistry, Cambridge University, Cambridge, England (99)

Charles B. Metz, Institute of Molecular Evolution, University of Miami, Coral Gables, Florida (163)

Leonard Nelson, Toledo State College of Medicine, Toledo, Ohio* (27)

Alberto Monroy, Institute of Comparative Anatomy, University of Palermo, and Research Unit for Molecular Embryology, National Council of Research, Palermo, Italy (369)

Erika Rawitscher-Kunkel, Department of Botany, University of California, Berkeley, California (117)

Albert Tyler, Division of Biology, California Institute of Technology, Pasadena, California (1), (369)

* Present address: Program Director, Developmental Biology, National Science Foundation, 1800 G Street, Washington, D. C.

Preface

The research of recent years has disclosed many new facts concerning the physiological mechanisms of fertilization in various forms of life, including microorganisms, and amply supports the view that sexual reproduction is a fundamental property of living systems. Accordingly, it seemed desirable to assemble the available information in a comparative treatise. "Fertilization," then, reviews gamete physiology and fertilization mechanisms in plants, animals, and microorganisms with special emphasis on information obtained with the methods of comparative morphology, biochemistry, and immunology.

This work is not designed as a mere catalogue of facts or as a review of the literature; rather it is intended as a critical evaluation of existing data and ideas, presented in a form that will point up new lines of approach. In addition, it is hoped that it will provide a stimulus for the comparative study of reproductive phenomena in different organisms, from the simplest to the most highly differentiated, covering morphological, physiological, chemical, and physical aspects.

The chapters are written by specialists actively engaged in the particular domain in which they have been asked to contribute. A special effort has been made by the authors to make their presentations appropriate in scope and treatment for biologists, research workers, and teachers alike.

This work is to a considerable extent the outgrowth of an association of the editors and many of the contributors at the Marine Biological Laboratory. This association included a training program in fertilization and gamete physiology made possible by support from the National Institutes of Health, U. S. Public Health Service.

Woods Hole, Massachusetts
December, 1966

CHARLES B. METZ
ALBERTO MONROY

Contents of Volume II

Tentative

Bacteriophages

L. D. Simon and T. F. Anderson

Bacteria

Giuseppe Sermonti

Fungi

E. C. Cantino and E. Horenstein

Algae

L. Wiese

Higher Plants

H. F. Linskens

Paramecium

K. Hiwatashi

Fishes

E. Nakano

Sperm Entry in Mammals

C. R. Austin

Fertilization of the Mammalian Egg *in Vitro*

C. Thibault

Anomalies of Fertilization

J. Piko

Control of Fertility—Mechanisms Affecting Gametogenesis in Mammals

H. Jackson

CHAPTER 1

Introduction: Problems and Procedures of Comparative Gametology and Syngamy^{*}

Albert Tyler

DIVISION OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY,
PASADENA, CALIFORNIA

I. Basic Problems of Gamete Physiology and Fertilization	2
II. Problems of the Quantitative and Qualitative Control of Fertility	5
III. The Comparative Approach	7
IV. The Experimental Method	8
V. Spallanzani and the Philosophy of Fertilization	9
A. The Animality of the Spermatic Worms	11
B. Views of Harvey and Leeuwenhoek on the Generation of Animals	14
C. Demonstration of External Insemination	16
D. Artificial Insemination and Disproof of the <i>Aura Seminalis</i>	16
E. Role of the Spermatozoon in Fertilization	17
VI. Mechanism of Syngamy	19
VII. Patterns of Reproduction	20
A. Basic Features of Reproduction	20
B. Forms of Asexual Reproduction	21
C. Forms of Sexual Reproduction	22
D. Manner of Meeting of Egg and Sperm	24
References	26

The purpose of this chapter is to state some of the basic and practical problems of reproductive physiology, to indicate the directions from which contributions toward their solution have been coming, and to note the further problems that emerge from continued investigation. The relation of these studies to human welfare is also to be considered here, although the surveys that are presented in the succeeding chapters are

^{*} Preparation of this article and original work of the author reported herein was supported by grants from the National Institutes of Health (GM-12777) and the National Science Foundation (GB-28).

intended primarily as studies in basic biology apart from their possible utility in these directions.

I. Basic Problems of Gamete Physiology and Fertilization

The various chapter titles in these volumes indicate in broad terms the basic problems of reproductive physiology. These problems can be stated in a number of ways. Essentially they constitute the principle questions that are asked as one explores the subject. The terms in which the problems are formulated depend upon the current "state of the art" in the specific area and on the form in which the investigator seeks to present the answers. One may group these problems in several categories.

In the first place, there are the series of problems that concern gametogenesis. Among these are the problems associated with chromosomal behavior, for example, the reduction in the number of chromosomes from diploid to haploid, a process entailing a synapsis of homologs and often new association ("crossing-over") of parts. The mechanics and chemistry of these processes are as yet not well understood, but much new knowledge has been obtained by modern techniques, including tracer methodology. Other problems of gametogenesis concern the cellular transformations that the spermatocytes and oocytes must undergo in producing the fully formed spermatozoa and eggs. These transformations appear to be quite different in the two types of gametes. However, essentially the problems for both are those of differentiation, the central features of which may well be the same, not only for the germ cells but for the various kinds of somatic cells as well. The importance of extrinsic factors in this process is illustrated by the many experiments on sex reversal. It is clear that the direction in which the primitive germ cell differentiates is determined not by its own chromosomal constitution but by that of the somatic tissue of the gonad in which it develops.

In the case of the egg, the enormous enlargement undergone by this cell raises special problems concerning the mechanism of the growth process. As the accounts in Chapters I-2* and II-11 indicate, the investigations of this subject have raised additional questions as to the extent to which this growth is accomplished by intrinsic synthetic activity or "parasitically" by incorporation of materials completely manufactured by other tissues.

Gametogenesis also poses problems concerning the mechanism of liberation of the gametes from the gonads and of the timing of this liberation. In large part these problems are now expressed in endocri-

* In these notations roman numerals indicate volume numbers; arabic numerals indicate chapter numbers.

nological terms, as are the earlier problems associated with the ripening of the gonads themselves. In mammals much is now known concerning the operation of environmental factors in these processes through the hypothalamic-pituitary-gonadal feedback circuit.

The life span of the shed gametes, the motility of the sperm, the further maturation of the shed gametes, and the mechanism of transport through the genital ducts are additional areas of basic biological investigation. As the accounts in Chapters I-2,3, II-8,9 and 10 indicate, there is considerable new information pertaining to these areas and providing, in some cases, hypotheses of mechanisms in physicochemical terms.

Questions of specific attractive influences (chemotaxis) between the gametes constitute another group of basic problems. Except for some recent work with certain coelenterates (Miller, 1964, 1966) the phenomenon of chemotaxis itself has not been clearly demonstrated in animals. It does evidently occur between the gametes of opposite sex in ferns and other plants. In the latter there is information available as to the chemical nature of the chemotactic substances, although the mode of action still remains obscure. As the account of the subject in Chapter I-4 indicates, the phenomenon of trap action interferes with the interpretation of experiments to test for the occurrence of chemotaxis in animals. This subject, then, continues to be a challenging one for appropriate investigation.

The specific adherence of sperm to egg raises general problems of recognition on the part of cells. These problems are evident from the species specificity and tissue specificity of fertilization, which is now known to be primarily operative at the stage at which the gametes first come into contact. The questions may be asked in the form, "Why do sperm fertilize only eggs of the same or a closely related species, and why do they not fertilize other tissue cells of the organism?" Some answers to certain aspects of these questions are available in terms of chemical substances (fertilizins and antifertilizins) that serve as the specific receptors. The basic nature of these problems is indicated by the analogies that have been made with reactions between immunological reagents that are designated as antigens and antibodies. The extent of progress and the further questions raised are given in Chapter I-5.

Penetration of the sperm through the extraneous coats of the egg is now largely understood in terms of the presence of enzymic agents in the acrosome of the sperm. The special problems in this area deal with the release of this material from the acrosome, determination of its chemical nature, and its mode of action on the egg coat. It is of additional interest that the active agent in mammalian sperm, hyaluronidase, is found also in bacteria and that it has a similar use; namely, to enable the

bacterial cell to break down the polymeric hyaluronic acid barrier and reach susceptible tissue cells.

Penetration of the egg plasma membrane itself evidently does not involve the use of these enzymic agents or any others as far as is presently known. The problems here concern the fusing of two separate cells into a single one. For the entrance of the sperm there are no openings in the egg plasma membrane large enough to provide admittance. Openings of such size would very probably permit the egg contents to flow out. This problem is emphasized by the fact that the sperm is able to penetrate the egg plasma membrane without subsequently leaving any holes in the membrane. As the account presented primarily in Chapter I-7 indicates, there are two somewhat different initial answers to this question in terms of what is observed by electron microscopy of thin sections. One proposal is an edge-on joining of membranes, with an opening formed at the initial contact point and the sperm membrane finally left as a patch in the zygote membrane. The other is based on a progressive lateral joining of the gamete membranes with early breakdown of the conjoined membranes, so that all of the sperm plasma membrane, along with an equivalent amount of the egg plasma membrane, disappears while engulfment ensues in a pinocytotic or phagocytotic manner. Further consideration of this subject is given below.

The establishment of the block to polyspermy constitutes another set of problems of special interest. This block occurs in most, but not all, species of animals and is set up with considerable rapidity. Explanations have been offered in simple physical terms, whereby egg receptors on microvilli retract below the vitelline membrane and thus become inaccessible to the sperm. However, there are a sufficient number of apparently contradictory situations so that it evidently must be modified if this answer is to have general applicability. Other phenomena, such as the opening up of the cortical vesicles, offer additional bases for interpreting the establishment of the block to polyspermy, but critical experiments have not yet been performed, as the accounts given in Chapters I-5, II-8 and 10 indicate.

Finally, there are a host of problems raised by the phenomenon of activation of the egg, whereby a chain of events is set in motion that transforms egg into adult. These are the problems of the physicochemical nature of the reactions responsible for the initial visible changes upon fertilization and of the ensuing changes that lead to the development of a new individual. Central to these problems is the turning on of protein synthesis and its relation to the turning on of specific gene activity. These are problems for which initial answers are coming at a relatively rapid rate, as indicated in Chapter I-8.

II. Problems of the Quantitative and Qualitative Control of Fertility

Apart from their intrinsic interest and relation to other areas of biology, the results of the investigation of the basic problems of reproductive physiology may be expected to have direct application to two major problems of humanity. One is the problem of too rapid growth of populations. It is recognized that when one expresses resources in terms of available energy, then the nuclear energy that is potentially available (Brown, 1954) could support a very large population. However, the rates at which populations grow in most areas of the world, including in many cases communities in industrially well-developed countries, far exceed the rates at which the energy resources are being made available by the requisite development of industrial technology. This subject has been discussed in many demographic treatises, but reference may be given here to a concise account (McElroy *et al.*, 1963) published by the National Academy of Sciences Committee on Science and Public Policy.

Apart from these considerations, individual families may have other reasons for wishing to control their fertility, and by methods that they consider most suitable and desirable. Conversely, there is the general problem of overcoming sterility. For this purpose it is generally considered that the same kinds of basic investigations of reproductive physiology are needed as may be applied to the reduction of fertility.

It should be noted, too, that problems of fertility control pertain also to organisms other than man, in particular, to those (farm animals, noxious insects, etc.) that affect human welfare or survival. Again it may be expected that the study of the basic problems of reproductive physiology is ultimately more likely to contribute greatly to the solution of these problems than is the more direct empirical approach. This is not to disparage the latter type of investigation, however, which is often needed in order to provide immediately useful solutions.

The other major practical problem is the qualitative control of fertility in man and other organisms. This problem can be stated simply as one of "improving the breed." Although it appears that there are differences of opinion among people as to what may constitute "improvement," it is possible to state in general terms what most people may accept as reasonable. This is that the "improvement" should be in a direction that enables individuals to cope more successfully with the exigencies of the environment and to live "peacefully" with each other.

Until recent years the exploration of this problem was done mainly by "eugenic" methods of selective breeding. These methods, as far as humans are concerned, have not had very wide acceptability; nor have they come under governmental control to any appreciable extent. Presumably this is because of a general suspicion that, although the stand-

ards for such selection may initially be such as to tend to eliminate obviously bad genes from the population, ultimately standards may be set that are potentially dangerous to individuals and to the human race as a whole.

With the rapid advances that are being made in our knowledge of how the characteristics of an organism are determined, it is now possible to discuss problems of qualitative control of fertility in terms of what has been called (Lederberg, 1963) "euphenics" (see Tyler, 1964ab, 1965). Essentially the methods of euphenics apply to modification of the phenotype of an organism by nongenetic means, that is, by methods other than selective breeding. Presumably, however, cell transformations in higher organisms, if accomplished, by administration of preparations of DNA to the fertilized egg, the developing organism, or the adult would be included in this category. There have, in fact, been reports of transformation having been obtained with isolated DNA in higher organisms, but so far these have not proved readily reproducible. The failures cannot, however, be considered to have excluded the possibility of cell transformation, and one may assume, in view of the fact that transformation by DNA has been achieved in many microorganisms, that there are no theoretical hurdles in the way of its achievement in higher organisms. More likely the stumbling blocks may be attributable to technical factors such as the destruction of the DNA by nucleases in the body fluids.

Other kinds of agents, such as specific antibodies, may also be expected to find use ultimately in the control of development. At present, however, it would appear that the mRNA's constitute the most promising tools of euphenics. The mRNA's that are templates for the various proteins in a differentiated tissue cell are represented by many molecules per cell, and, since only a small portion of the genome is active in any one cell, the various specific mRNA's should be more readily obtainable in quantity than the corresponding DNA's. Again there have been a number of reports in the literature of specific differentiation effected by RNA's in cells of higher organisms, but so far none have proved readily reproducible. As noted in Chapter I-8, even though the synthetic RNA polyuridylic acid can stimulate incorporation of amino acid in unfertilized sea urchin eggs, the effect is not specific, or at least does not remain specific very long in the intact cell. Nevertheless, the difficulties here again appear to be more technical than theoretical, and one may anticipate that these problems will be overcome.

Inasmuch as one may construct synthetic mRNA's of various kinds, it seems clear that there are possibilities for the production in cells of many new kinds of agents that permit better functioning and protection against disease and other noxious agents. Thus, one may contemplate, as

the science of euphenics develops, the possibility not only of repairing inherited biochemical deficiencies but of going beyond what is provided by the best available collection of genes.

It should be clear, too, that the prospects for controlling development are greater the earlier the stage of development that is subject to the experimental conditions. Thus, the studies of gamete physiology and fertilization that are discussed in these volumes may be expected to contribute greatly toward the realization of the goals of euphenics.

The practical problems of human reproduction have world-wide significance. It is interesting to note that the World Health Organization has established a section in this area in its Division of Maternal and Child Health, following a report by a scientific group (Caldeyro-Barcia *et al.*, 1964) that treats in concise form many of the pertinent basic features relating to human health and welfare.

III. The Comparative Approach

There are several reasons why the investigation of many diverse kinds of organisms is important to the understanding of biological processes. One is that some organisms may exhibit better than others one or another of the special features of the biological process under investigation. For example, the long time (30–40 minutes) required for sperm entry in *Nereis* offers advantages for studying this phase of fertilization. Another reason that studies with diverse organisms are of value is that conditions for successful experimental manipulation with respect to particular processes may differ, and some organisms may prove more suitable than others. For example, in mammals *in vitro* fertilization has so far been reasonably consistently achieved in rabbits and Syrian hamsters, but not in rats or mice. In fact, in the latter, *in vitro* culture of the egg has not as yet been consistently achieved from the one-cell stage, although it proceeds rather readily when started at the four-cell stage.

A further, and perhaps the most cogent, reason for adopting a comparative approach is that it permits broad generalization. It is clear that the most important features of any biological process are those which are common to diverse organisms. Thus, this must hold for the various biological processes under consideration here, mainly those of gametogenesis, those of the maintenance of functional state of the gametes, those of fertilization, and the component and ensuing processes. For example, one may question whether or not capacitation of sperm, a phenomenon which so far has been found only in mammals (see Chaps. 8 and 9, Vol. II), is of general significance for fertilization unless an analogous phenomenon is found in other groups of animals. This statement does not imply that special adaptive features that have evolved in various

species are not important areas of investigation. However, it does imply that these features must be properly assessed and not permitted to obscure the basic features of the processes of gametogenesis and fertilization.

In the present two volumes the broad scope of the comparative approach is illustrated by the fact that the organisms under consideration not only range widely among various species of multicellular animals and plants but that they also include protista, bacteria, and viruses. As indicated in Chapters II-1,2,3,4, and 6, syngamy, or processes analogous to syngamy, have been described in the latter groups of organisms. Again it is clear that in addition to the intrinsic interest that the study of the "mating behavior" of these organisms may have, knowledge of the extent to which the processes correspond to those in higher organisms provides further basis for the discovery of underlying principles and the formulation of unifying theory.

IV. The Experimental Method

While stressing the importance of the comparative approach, it is well that we recognize that the experimental method represents the most essential procedure for the effective exploration of problems of gamete physiology and fertilization, as it does for all biological problems. It seems desirable, then, that a few words be devoted to this subject here. This discussion appears desirable, too, because of misconceptions that many students seem to have derived as a result of the present increasing application of chemistry to biology. The emphasis that has been placed on this application, currently designated as "molecular biology," has resulted in obscuring the most important element in the truly great discoveries that have been made. This element has been the effective application of the experimental method. The mere use of chemical methods of analyzing biological materials, in itself, is not likely to provide analysis and interpretation of the biological process.

Essentially the experimental method is the performance of a series of tests in which one known component of the system is systematically altered. This procedure does not insure that correct conclusions will be drawn from the study; nevertheless, such tests are essential to successful causal analysis. For further account of the experimental method, some of the statements made by one of its foremost proponents, T. H. Morgan, in the Introduction to his "Experimental Embryology" (1927) are pertinent and are quoted here.

In recent years a good deal has been said about the importance of quantitative methods in biological research. The impression is sometimes given that the value

of a piece of work is commensurate with the number of measurements it contains. No one will deny the need of exact measurement in terms of some objective unit, but the value of a contribution of this kind may bear no relation to the number or the exactness of the measurements that it contains. In themselves, the measurements may be as empty of significance as any other kind of descriptive materials. The statistical answers that can be wrung out of such measurements may have very little meaning if the quantities measured depend on a multiplicity of causes. Statistical treatment may indicate that certain results are significant, but what they signify no man may know, or even surmise. But when the materials measured have been made as uniform as possible, which means that the condition(s) under which the differences were measured have been largely controlled, then the results will give a significant answer. Experience in the physical sciences has shown that reliable data are those obtained by exact measurements. The nature of the material and the use to which these measurements are put is the important feature of the scientific procedure.

The working hypothesis is another indispensable tool in experimental science. Essentially it is an attempt to find an answer to some feature of a complex situation in terms of better understood relations, which, from a mechanical standpoint, means reference to accepted chemical or physical principles. It will be observed without further elaboration that hypotheses, as here interpreted, can have little meaning without reference to the more advanced physical sciences; for, without reference to a better understood background they become mere speculations, however logical their presentation may appear. The justification of their use in the physical sciences is found in their fruitfulness and suggestiveness in the progress of research. This is conceded in physics and chemistry, and enough has been done even in the biological sciences to warrant their employment.

It is repeatedly said that there is no experiment without a control. This slogan, too, may be misunderstood. In any simple physical experiment, where all the conditions are known, experiments are carried out without any control, because the conditions of the experiment are themselves controlled. The need of a control arises in cases where many or all of the conditions are unknown. Then two situations are brought about that differ from each other in one known condition (that may be simple or complex). The different results obtained are attributed to the differences in the two situations, and one or the other condition is said to be the cause of the difference. In preliminary experimental work this is often a valuable and indispensable means of finding out more about the environmental or internal conditions under which certain events do or do not happen. It is often an essential part of discovering the factors present in a complex relation, but when the conditions have become simplified, the control, as such, may be no longer needed. This occurs in the famous *experimentum crucis*. In experimental embryology we are far from having reached this happy consummation, and as the following pages will only too well reveal, many of the "experiments" do little more than describe what happens to the developing egg or embryo under a variety of new conditions imposed on them by the investigator in the hope they will do something that may throw light on some of their properties not revealed under the conditions in which they "normally" develop.

V. Spallanzani and the Philosophy of Fertilization

It is not intended that an extended history of the subject of gamete physiology and fertilization be presented in this Introduction. This

omission does not imply a lack of regard for the importance of studying the origins of a subject, for there is undoubtedly much of value to be gained from examining the background of biological discoveries. Here, however, space considerations preclude any attempt to present the early history systematically. In addition, there are relatively readily available texts (e.g., Cole, 1930; Needham, 1934) in which this subject has been covered rather well.

One historical aspect of the subject does seem to warrant attention here, however. It concerns the misinterpretation, on the part of the most outstanding 18th-century experimental biologist, the Abbé Lazzaro Spallanzani, of experiments that had, in fact, demonstrated that the spermatozoa were the fertilizing agents in seminal fluid. His filtration experiments, in which the spermatozoa-free filtrate was found to be sterile, showed quite clearly the importance of the spermatozoa. By his erroneous interpretation it is very likely that the development of biology, particularly genetics and embryology, was set back a great many years, for the weight of his authority undoubtedly led most of the biologists during the years 1780–1875 to accept his conclusion with practically no reservations. Thus, the correct conclusion, drawn by Prévost and Dumas in 1824 and by Newport in 1851–1853 upon their repetition of the filtration experiments, at the time received very little attention, as did also the observations of Barry in 1840–1847, of Nelson in 1851–1852, of Keber in 1853, and of Meissner in 1854 on the presence of spermatozoa within the membrane and inside the egg. Only after the work of O. Hertwig and Fol in 1875–1879, which showed actual penetration and union of egg and sperm nuclei, was there general acceptance of the view that the spermatozoon initiates and participates in the development of the egg.

This long delay may be attributed, then, to the fact that a contrary view had been expressed by Spallanzani, who was held in high regard because of his many great biological (as well as physical and geological) discoveries. At least five of these discoveries are considered of the first magnitude; namely, (a) disproof of spontaneous generation in infusion animals, (b) discovery of arterio-venous anastomosis, (c) artificial insemination, (d) “chemical” digestion, and (e) tissue respiration. In addition, Spallanzani carried out many beautiful experiments and analyses on the origin of fountains, on the rebounding of a stone from water, on regeneration, on anaerobiosis, on phosphorescence, and in many other areas.

It seems worthwhile, then, and pertinent to the subject of these volumes, to give an account here of some of Spallanzani’s experiments, along with the work of others that served as a basis for his conclusions. Obviously the experimental method had failed in this instance, and at

the hands of an investigator who had used it quite successfully on many occasions. This account will be given largely in the form of excerpts from Spallanzani's own publications.

A. THE ANIMALITY OF THE SPERMATIC WORMS

There was in Spallanzani's time still considerable objection on the part of many biologists to considering the spermatozoa as living objects or as normal constituents of semen. Buffon called them organic molecules, and Linnaeus also regarded them as inanimate. Leeuwenhoek was aware that such doubts would arise, as is evident in his first report of their discovery (letter of 1677 in the 1679 *Phil. Trans.*, Vol. 12; an English translation by Dobell is available in Cole, 1930, pp. 9-12), in which he emphasized that he saw them in semen from a healthy man as well as in the specimen brought to him by Ham, which had come from a luetic individual. His first report was followed immediately in the *Philosophical Transactions* by a skeptical letter from Secretary Aldenburg (dated Jan. 1, 1678) and by a reply by Leeuwenhoek (dated March 18, 1678), in which the latter told of finding the animalcules in other animals than man and submitted figures of those of the dog and rabbit. Within the next five years Leeuwenhoek had described the spermatozoa of nearly all the main groups of animals, but his views concerning their role in generation (see below) and his claim that he found males and females among them without presenting any real evidence as to how they multiplied probably contributed to the fact that the spermatozoa failed to be immediately and generally accepted as normal living constituents of semen.

Spallanzani, by means of great numbers of observations and experiments on characteristics and behavior of spermatozoa in various animals under a variety of conditions, succeeded in gaining more general acceptance of their animality and in distinguishing between them and the infusoria found in putrefying material. Most of this work is reported in his "Opuscoli di fisica animale e vegetabili." The following quotations are from Chapter VI, which is headed, "New observations and experiments of the author which tend to determine particularly the nature of the spermatic animalcules. Questions about the same." Spallanzani reported as follows: "Finding myself to have one day in winter a rich supply of semen, obtained from the spermatic vessels of a cadaver, and wanting to keep the animalcules alive for several hours, I exposed it to the sun in a watch crystal, which I put outside a window. The sun's heat was at 17 degrees, which keeps these beings active for awhile. Returned after an hour to observe the semen, I was surprised to find them almost all immotile."

To test whether this was apparent or real death Spallanzani exposed the animalcules to more heat (near the fire) but to no avail. On the other hand, those left in the shade became fully active when brought near the fire. He repeated the experiment many times the succeeding spring, with the same results; the sun's heat always proved fatal in about an hour, although the temperature never approached that previously shown to cause death. "On the contrary no unfortunate accident happened to the putrefying organisms of the same semen (because the sun's heat did not reach 33 or 34 degrees); which proves still further the essential difference between one and the other."

Spallanzani continued these experiments and showed by using sealed capillary tubes (in which the spermatozoa live when exposed to the sun even when the temperature reaches 32°C) that the death was due to evaporation which occurs in the watch crystals. But he puzzled about why there should be two kinds of heat, "the one, solar, the other that of a room, in the first heat the semen alters in a manner that irreparably kills its guests, in the second it does not proceed thus." He concluded, "I have not made sufficient observations for the solution of this problem."

Having shown that such long duration of motion and of life in hermetically sealed tubes arises by virtue of the vermicelli being guaranteed and excluded from the air, it was easy to believe with the arguments of induction that a similar thing should occur in any season. But the inductive arguments are not of any value for the Philosopher, if they don't get much assistance from the fact, being found too many times fallacious, and the present case can furnish a new example. Our animalcules, then, in cold season lose their motion in tubes closed by hermetic seal in the same time as within watch crystals exposed to the open air. . . . I found them immobilized in 1½ hours, whether in capillary tubes or in open air at the temperature of 6° above freezing.

He later showed that immobility due to cold is simple lethargy and tested the effect of changing repeatedly from heat to cold, etc.

Spallanzani maintained from his earliest years an extensive correspondence with his friend Charles Bonnet, the eminent biologist at Geneva. Bonnet continuously encouraged and praised Spallanzani's work, even when the latter's findings disproved some of his own fond theories. But more important he repeatedly made many valuable suggestions concerning problems worth investigating and experiments to undertake. Spallanzani did in fact follow many of these suggestions and acknowledged them by publishing pertinent letters from Bonnet.

In this work he published a letter from Bonnet (April 20, 1771) in which all kinds of questions about spermatozoa are raised. Spallanzani reduced these to three: (a) "What might be the origin of the spermatic

animalcules?" (b) "How does propagation take place in them?" and (c) "What might be their use?"

"In regard to the first, although Bonnet affirmed nothing, one sees, in short, the propensity that he has for the opinion that the spermatric worms come from the outside. That has already been the opinion of many accredited writers. . . . Thus the cavalier Linnaeus is of the feeling that the worm *taenia* has its natural habitat in the water."

Spallanzani noted that some of the smallest of these worms are found in water and others in some fish, notably the tenches, and that seemed to lend some plausibility to his idea, but he went on to say, "We would need above all to be sure of the identity of the species found in the water, and those that reside in the human body; which certainty it is not yet able to have. We cannot however deny that the most certain observations show that some worms, if not of the human body, at least of that of certain animals may be legitimate offspring of insects from the outside (large) world. Such are those that nest frequently in the straight intestine of the horse, in the frontal sinuses of ewes, of rams, of she-goats, and of the gullet (throat) of the stags, as the clearest and most expert naturalists Vallisneri and Reamur have discovered." Spallanzani did not believe that this observation could possibly apply to the spermatric worms, because in all the pools, stagnant waters, ditches, ordinarily rich in microscopic animals, which he had examined "with every attention" he had not seen a single species that resembled the spermatric worms. In a footnote he noted that Leeuwenhoek, likewise, had failed to see spermatozoa in any of various waters and that Müller, who described 146 species of worms, had seen only one, called "*cercaria*," which resembled the spermatric worms of the ram. But this was described only occasionally, and he considered it to be far from furnishing the sperms (seminal fluids) of various animals their species of spermatozoa. He argued against the possibility of the spermatozoa assuming different forms, and he also presented a negative experiment on propagating and changing them.

"Mr. Bonnet suggested the most ingenious experiment of attempting to keep the spermatozoa alive in infusions, and the infusion animals in sperm (seminal fluid). I had done this already in part, but with the death of the one and the other, in transferring the putrefying organisms from foul semen to fresh semen, and the spermatozoa from the fresh semen to the foul semen." He also employed plant infusions ("in order to satisfy fully the erudite curiosity of my excellent friend") instead of putrid semen, and adjusted the temperature to that of the semen when it is within the animals, and that of the semen to which the infusoria were transferred to that of the atmosphere. But he could not prevent

their death. He argued also that the spermatozoa could not possibly adapt themselves to the kind of nutriment found in the semen if their origin were external, and he gave examples of caterpillars dying when they were changed from one kind of plant to another (e.g., the silkworm removed from a mulberry leaf to some other) or even from one part of a plant to another.

He believed, then, that the spermatozoa originate in the body, and he reported having seen them in the blood of amphibians and mammals. He considered them to be transmitted from the mother to the offspring and believed that the body fluids serve as medium for them until puberty, when they move into their proper nest.

As to their propagation, he noted that he had never seen them divide. He suggested that they multiply by buds or shoots, as do many kinds of polyps.

As to their use, he suggested that they might have the same relation to the body as do other races of larger worms. But their exact role was to him, at that time (1776), a "question transcending the sphere of human knowledge." He cited the opinion of Leeuwenhoek, who considered them the authors of generation, "such that those from man would be just so many homunculi, those of the horse so many colts, those of the bull so many calves. And thus we say of the others; which thinking I cannot deny be ingenious, and it is only a pity that it lacks the truth." Spallanzani considered this view to be disproved by the work of Haller, and by his own, showing that the fetus belongs uniquely to the female and that it entirely exists in her before fecundation. He continued, "The vermicelli excluded from this use, there has not been lack of assigning them some other, some physiologists wanting that they are destined to create in us the venereal pleasure, others that they serve to keep the semen fluid, others that they participate in some other recondite end..."

B. VIEWS OF HARVEY AND LEEUWENHOEK ON THE GENERATION OF ANIMALS

Spallanzani had evidently studied very carefully the works of Harvey and of Leeuwenhoek, which up to his time were undoubtedly considered the most important on the subject. Yet apart from the question of the role of the spermatozoa, which were unknown to Harvey, these two men differed on a highly important question; namely, whether or not the semen reaches the uterus. Harvey denied that it does, while Leeuwenhoek reported that it does and at the same time showed that it is highly improbable that the egg (actually the Graafian follicle, which he accepted as the egg) reaches the uterus. Harvey's views are presented in

his "Anatomical Exercitatione, Concerning the Generation of Living Creatures" (pp. 542-546 of the 1653 English translation of the 1651 Latin text). He concluded that the sperm imparts fecundity to the uterus by a kind of contagion, and that woman is rendered "prolific by no sensible corporeal agent, as the iron touched by the Lodestone, is presently indowed with the virtue of the Lodestone, and doth draw other iron-bodies to it."

Leeuwenhoek reported in 1685 (*Phil. Trans.*, Vol. 15) the finding of living spermatozoa in the uterus of a dog after coition. In a previous article (1683 *Phil. Trans.*, 13, 74-81) he presented his views on the role of the spermatozoa in generation. An excerpt from this article follows.

My opinion therefore of these eggs is, that they are emunctories, or the emptyings of some vessels lying near, such as are often found among the membranes, or adhering to the bowels of animals. But as to generation tho I have formerly been very reserv'd in declaring my thoughts thereof, yet being now further instructed by manifold experience, I dare venture to affirm it rather to come from an animalcule, (such as I find not only in Human seed, but that of all birds, beasts, fishes, and insects) than an egg. And the rather for that I find in the seed of a man, as also of a dog, two different sorts of animalcules, answering the different sexes of male and female.

It may be queried, if one animalcule of seed be sufficient to produce a foetus, why are there so many thousands in one drop of it?

I answer that in an apple tree (enduring an hundred years and bearing every year a great many thousand blossoms, which may a great part of them be apples, having each of them six or eight seeds) each seed being placed in a proper soil, and carefully cultivated, is capable of becoming a tree; yet it may happen that nothing grows from all the apples that fall down; whether thro want of sun, rankness of grass, weeds, or other accidents. So in the womb each animalcule might suffice for a generation, if the place where it comes to be nursed be fit for it; but the womb being so large in comparison of so small a creature, and there being so few vessels and places fit to feed it, and bring it up to a foetus, there cannot be too great a number of adventurers when there is so great a likelihood to miscarry.

It may be asked again, why a woman bringeth forth but one or two children, since if there were but two proper places in a uterus several of the animalcules might there be fed.

I answer, it may happen to these animalcules, as it does to seven or eight seeds put into a small hole of the ground; that seed which puts out the biggest and strongest root, starves all the rest, and becomes a tree.

It may be asked me again, why I make the animalcules found in the seed of several animals to be of such different sizes, comparatively to the animals they belong to viz. in the space of a small sand in the seed of a cock 50000. In the row of a codfish 10000, in the row of a ruff (which fish is a 1000 times less than a codfish) the animalcules as big as the others. Whereas it seems reasonable that the animalcules ought to be in bigness to one another, as the creatures in which they are found; from whence it would follow, that those animalcules which are in insects, would never be capable of being discovered, because of their exceeding smallness.

I answer that we must satisfy our selves in these things as well as we can, for (not to speak of a coconut) a great wall-nut with his green shell weighs down a 1000 apple tree seeds, and yet the proportion between the trees is not so great.

C. DEMONSTRATION OF EXTERNAL INSEMINATION

In an extensive series of observations and experiments on several species of amphibians, Spallanzani succeeded in disproving the earlier views of Linnaeus and others that insemination must always be internal. The work is reported mainly in Dissertation I of Volume II of his "Dissertazioni di fisica animale e vegetabile" which includes the famous breeches experiment.

The Abbé Nollet, alluding in a letter dated nine years ago, to the hint in my prospectus, of my discovery of the existence of the tadpole before fecondation, expresses himself in this manner: "What you say of the existence of the tadpole before we can perceive any act of fecondation, has particularly struck me. About thirty years ago, Mr. Reaumur and myself made many researches relative to this subject. We attended for weeks very diligently and patiently, to what passed while the male embraced the female. I remember putting breeches of waxed taffety on the male, and watching a long time without perceiving any appearance that denoted an act of fecondation."

I will not here stop to enquire by what fatality it happened, that two naturalists, so intelligent and attentive, failed in their enquiries. The idea of the breeches, however whimsical and ridiculous it may appear, did not displease me, and I resolved to put it in practice. The males, notwithstanding this incumbrance, seek the females with equal eagerness and perform, as well as they can, the act of generation; but the event is such as may be expected: the eggs are never prolific, for want of having been bedewed with semen, which sometimes may be seen in the breeches in the form of drops. That these drops are real seed, appeared clearly from the artificial fecondation that was obtained by means of them.

D. ARTIFICIAL INSEMINATION AND DISPROOF OF THE *Aura Seminalis*

The establishment of the fact of external insemination in amphibians led Spallanzani directly to attempt to inseminate eggs of these species artificially. In this experiment, as is well known, he succeeded admirably. He was thus encouraged to repeat Malpighi's attempt on the silkworm and to try also the dog, and with both he was again successful. The work demonstrated clearly that contact with semen is essential for fertilization, and he made numerous tests which showed the minuteness of the amount of semen required, its passage through the gelatinous coat of the egg, its effectiveness at any point on the surface, etc.

In the experiments with frogs, he estimated the amount of semen needed for successful insemination and found it to be exceedingly small. The following quotation from Chapter IV of Dissertation II of Volume II of his "Dissertazioni di fisica animale e vegetabile" gives his estimate:

"How small a portion of the prolific liquor will fall to the share of each tadpole? . . . I have found a globule one fiftieth of a line in diameter taken out of a mixture of three grains of seed, with eighteen ounces of water, was often capable of fecundating a tadpole (CXLIV). Desirous of knowing the proportion which the tadpole (that of a frog is two-thirds of a line in diameter) bears to the particles of seed diffused in a drop of this dimension, I have found on calculation that it is as 106,477,777;7:1."

Strangely enough, Spallanzani considered this result as "apparently favorable to the seminal aura, which, in the general opinion is nothing but the vapor of the seed exceedingly rarefied." He therefore performed an extensive series of careful experiments which convincingly disproved the prevalent notion that any such vapor might be the effective agent in fertilization.

E. ROLE OF THE SPERMATOZOON IN FERTILIZATION

Spallanzani thought that he had established the ovist's theory of preformation, and therefore he was prejudiced against assigning any role to the spermatozoa that would involve their participation in development. Nevertheless, he performed a number of experiments to test their dispensability. The following are excerpted from Chapters I and IV of Dissertation II.

CXXII . . . while I was engaged in these experiments, I was eye-witness of an accident that deserves to be mentioned. Having often observed the seminal liquor of the toad, I found it very full of spermatic worms, which, like those of the frog, have an oblong shape, and writhe their body as they move. Upon two occasions I have been greatly surprised at finding this fluid totally destitute of inhabitants. I was induced to try, whether it is also destitute of fecundating virtue, but I found that it was just as effectual in this respect, as that which most abounds with these diminutive animals. . . .

Let us take a short view of the question. Those who consider the spermatic worms as the immediate authors of generation must, of necessity, suppose that they exist in the seed whenever fecundation takes place. It is therefore obvious, that according to this system the seminal fluid will be unprolific, when it is totally destitute of these inhabitants. And indeed its advocates admit this. But it does not agree with facts, though in the seed of the animals so often mentioned vermiculae may be generally observed, yet in that of two toads they were entirely wanting; but it impregnated tadpoles, just as well as the seminal fluid of other individuals, however well peopled, that might be (CXXII). In the next place, when the seed of the frog or the toad is mixed in equal quantity with human urine or vinegar, the worms are all destroyed.

The seed, however, as I observed in the proper place (CXXXIV), did not lose its prolific power. It retained this power when a few grains were mixed with twelve ounces of water, and even with eighteen, though I could not distinguish a single spermatic worm, so thinly were they dispersed through that large body of water. It

ought likewise to be observed, that in seed long kept, which yet answered the end just as well, I often found the worms dead, and floating upon the fluid of the vesicles, and likewise upon the juice of the testicles. Lastly, I could easily bathe tadpoles with seminal liquor abounding in worms and yet prevent them from having the least concern in fecundation. When a drop is put upon the object glass of the microscope, every part of it is full of worms. But when a little evaporation has taken place, they begin to quit the circumference, and assemble towards the center, as the animalcules of infusions usually do. As evaporation advances, they continue to fly towards the center, though many are caught, and die in the dried part of the drop. At this time I could take up with the point of a needle, at the edge of the liquor, many drops that were entirely without worms. That I might be quite certain, I placed what I took upon another object glass; and when I found that it was quite free of worms, I touched several tadpoles, and found that they came to perfection. My long experience in the world of microscopical animals, whether belonging to man or animals, will, I hope, vouch for me that I was not deceived in this delicate investigation. These facts prove then that the system of Leeuwenhoek and his followers is false.

This work evidently convinced Spallanzani quite thoroughly that spermatozoa are not the effective agents. Later he performed the filtration experiments, which are reported (Cole, 1930) as follows: "If spermated water be filtered through cotton materials it loses much of its fecundating power, and it loses it entirely if it be filtered through several thicknesses of blotting paper. Filtration through two papers produces a partially sterile filtrate, and the sterilization becomes more complete the more papers are used, until a filtrate which has passed through six or seven papers is absolutely sterile. If now the filter paper itself be expressed into water which contains non-fecundated eggs, they at once proceed to develop."

There is no attempt to reconcile these results with Spallanzani's previous ones, and he did not alter his view. Several factors may be responsible for this. He was perhaps strongly influenced by Vallisneri who was against the idea that the spermatozoa enter the egg, because the process was without analogy in the insect world and because no one had seen spermatozoa within an ovum or a pore through which it could have entered; in addition, he argued that if there are such pores through which the sperm could enter, the yolk would run out of the egg. Another factor was Spallanzani's belief that he had demonstrated preformation in the egg.

Another factor was very likely the minuteness of the amount of semen required for insemination, which to Spallanzani could mean only that semen acts in the manner of a chemical stimulant, such as the drugs known to stimulate the heart.

One factor certainly was clear; namely, that he considered his earlier experiments completely demonstrative and was evidently averse to chang-

ing his published views. This factor is certainly not unique to Spallanzani, nor do we lack examples in the present day. It might come about either because one has ignored the conflicting evidence—one's own as well as that of others—or because one has indulged in mental gymnastics in order to explain away such evidence and thus maintain some pet theory or point of view. Such bullheadedness is, of course, of particularly great concern when indulged in by investigators who have acquired a position of some authority in a particular field.

While it is not to be expected that this presentation will serve to obviate the dangers of experimental errors and misinterpretations which continue to be made in this difficult field, it may be hoped that it will contribute to lowering their frequency as well as their impact upon the development of biology.

VI. Mechanism of Syngamy

Statements as to what constitutes the most critical step in the series of processes that constitute sexual reproduction are generally not very meaningful. In fact, one may conceive of ways by which the perpetuation of the species can be accomplished with the omission of one or another, if not all (as by the asexual methods of many organisms), of the various steps. However, attention does focus on the actual fusion of the gametes as being a central feature of reproduction in most animals and plants.

The exact mechanism of this union is still poorly understood. As noted earlier, and as discussed in Chapter 5, there is some basis for an understanding of the initial specific adherence of the gametes to each other in terms of specific receptor substances (fertilizins and antifertilizins) on the plasma membrane of the gametes. Also, for the mechanism of penetration of extraneous coats of the egg the principal features seem now almost self-evident in terms of enzymes (vitelline membrane lysins) present in the sperm's acrosome and the opening up of the latter upon contact with the egg, as discussed in Chapters 5 and 6 and as will be considered in Chapter 8 of Volume II. The further problems have thus become increasingly susceptible of expression and resolution in physicochemical terms.

For the actual processes of syngamy itself, there is still much that needs to be learned on a morphological level, although such information is now being provided for many species of animals, primarily by electron microscopic studies, amplified in some instances by special methods of light microscopy. For these processes there is as yet no complete accord among various investigators as to interpretations of the available evidence concerning the general manner in which the respective mem-

branes join. In the account given in Chapter 7, the favored interpretation is that whereby the plasma membrane of the sperm is finally left as a patch in the zygote membrane, the initial fusion presumably being an edge-on joining of the two plasma membranes following their initial contact. In the account to be given in Chapter 8 of Volume II, and by the present author (Tyler, 1965), the evidence is interpreted in terms of a progressive lateral joining of the two plasma membranes, with subsequent breakdown of the fused membranes, so that the egg plasma membrane finally engulfs the contents of the sperm, while all of the sperm plasma membrane disappears with an equivalent amount of that of the egg (cf. Tyler and Tyler, 1966).

The latter interpretation provides a mechanism for moving the material of the sperm into the egg, is consistent with what has been observed in pinocytosis or phagocytosis, and incorporates the feature of specific interaction between the receptors (fertilizin and antifertilizin) of the respective membranes. However, while it appears to many of us that the bulk of the evidence favors the latter interpretation, it is clear that there has been sufficient information of an apparently contrary nature to convince some investigators that the former interpretation is the correct one. Obviously the answer will come from further investigation by more unequivocal methods. Obviously, too, the search for the correct answer here is important, not for any decision as to which particular investigators were correct but for the further experiments that are suggested by the primary answer and for the further control that it provides over the processes of sexual reproduction in all organisms.

VII. Patterns of Reproduction

A. BASIC FEATURES OF REPRODUCTION

As noted earlier, most species of higher organisms reproduce by sexual processes. However, there are many that indulge in one or another asexual form of reproduction, either exclusively or alternatively. While these volumes are concerned primarily with the gametes and their interactions, it is desirable for purposes of perspective to indicate here, and to define in elementary fashion, the various forms of reproduction. No attempt will be made here to discuss the evolutionary questions concerning the possible modes of origin of the various forms of reproduction, nor the question of natural selection. The wide distribution of sexual forms of reproduction testifies to their selective value. In principle, too, the basis for the selective value is now largely understood in terms of the increased genetic variation that the sexual processes provide in the form of varied types of individuals on which natural selection may operate.

In both asexual and sexual reproduction the new individual is derived from one or more cells of the parent or parents. The origin of a new living organism from material other than preexisting living cells has not yet been demonstrated, although many biologists consider this achievement to be not too remote. From one direction this problem is being attacked by many kinds of experiments in which organic substances characteristic of living material are produced from simpler substances under relatively mild conditions (see Fox, 1965, for a current review). From another direction interesting leads may be provided by experiments in which the infection of *Bacillus subtilis* with the free DNA of a virus, such as polyoma or vaccinia, results in the formation of complete virus of the type corresponding to the infecting DNA (see Bayreuther and Romig, 1964).

Basically it appears that the essential feature of the development of a new individual in the reproductive process consists in the expression of the information coded in the DNA. Except for that of certain plant and bacterial viruses, RNA's do not appear to have replicative capacity, although they are capable of serving as messengers between DNA and protein. So far, for the development of a new individual the evidence indicates a unidirectional transfer of information from DNA to RNA to protein. There does not, however, seem to be any theoretical basis as yet for excluding the possibility of information transfer in the reverse direction. In *in vitro* systems at least, RNA-primed DNA synthesis has been described, and this work gives some encouragement to the exploration of the possibility of the natural occurrence of instances of reverse information transfer.

B. FORMS OF ASEQUAL REPRODUCTION

Asexual processes of reproduction are generally classified as binary fission, multiple-fission fragmentation, budding, and polyembryony.

In "binary fission" there is an equal or nearly equal longitudinal or transverse splitting of the body of the parent and growth of each of the two fragments to parental size and form. The protists commonly reproduce by this method. Among the metazoans the sea anemones provide an example of longitudinal fission and the planarians of transverse fission.

In multiple fission, schizogony, or sporulation there are several new individuals formed. The Sporozoa provide examples of this, a common form of which is the formation of a cyst by a series of nuclear divisions of the original cell, followed later by division of the cytoplasm.

Fragmentation occurs in some metazoans, particularly the platyhelminths, the nemerteans, and the annelids. In this process there are a

number of fragments produced by the parent worm, and the new animals are produced from each by regeneration of missing parts. This process is also illustrated in certain starfish (e.g., *Linckia*), in which a single arm can regenerate a complete animal.

The process of budding is characterized by the formation of a small mass of cells on the parental body. In internal budding this mass assumes parental form after separation from the parental body, while in external budding the parental form is assumed before separation. The former occurs among bryozoans and in freshwater sponges, in which the internal buds, termed gemmules, are groups of primitive cells within a dense capsule that is drought and temperature resistant. The analogous buds of the bryozoans are termed statoblasts. External budding occurs commonly in sponges, coelenterates, bryozoans, flatworms, and tunicates. Certain of the coelenterates and cestodes illustrate an alternation of generation (metagenesis), in which an asexual form buds off sexual individuals (medusas in the case of the hydroids, or the proglottids in the case of the cestodes). The temporary association of the buds is referred to as strobilization. Where the buds remain more permanently associated, as in many species of sponges, coelenterates, bryozoans, and tunicates, an organic colony results, often with specialization of individual members for particular functions.

The production of two or more organisms from one individual in an early stage of development is termed polyembryony, and examples of this process are found throughout the animal kingdom. In mammals it is a consistent feature of the development of armadillos. Thus in *Dasypus novemcinctus*, the nine-banded armadillo, the four offspring produced at a single birth are identical quadruplets, resulting from polyembryony occurring during the late blastocyst stage of development. In humans about one-third of twin births are monozygotic, and the individuals have thus been produced by the occurrence of polyembryony, presumably also at a late blastocyst stage.

C. FORMS OF SEXUAL REPRODUCTION

While fertilization (syngamy) represents the principal form of sexual reproduction in animals, there are three additional types that are designated conjugation, autogamy, and parthenogenesis. All forms involve the occurrence of certain special nuclear changes, termed reductional, or meiotic, prior to the production of the new individual.

Ciliate protozoa typically exhibit conjugation, which is a temporary union of two individuals, during which each receives a micronucleus from the other. The details of the process may differ in different species, and will be described in Volume II. Here it may suffice to emphasize

that within clones (organisms derived from a single individual by mitotic divisions) conjugation does not ordinarily occur. Conjugating individuals belong to strains that are termed mating types, rather than males or females, inasmuch as they do not possess distinctive morphological traits correlated with ability to mate and because there may be, within a species, several interfertile mating types, which would mean more than two sexes.

Autogamy also occurs in ciliate protozoa and can be considered the analog of parthenogenesis. In this process the nuclear changes preparatory to conjugation occur, but there is no mating; hence there is no transfer of micronuclei, but a reunion of the prospective migratory micronucleus with the stationary one.

Fertilization in animals is defined as a series of events in which two cells, the nuclei of which had previously undergone meiotic division, unite to form a single cell, the zygote. The gametes of metazoans are of two morphologically distinct types, spermatozoa (microgametes) and ova (macrogametes). The fusion involves the union of the two nuclei, each of which has at the time the haploid number of chromosomes; namely, one representative of each pair of chromosomes present in a somatic cell of the species. In most species of animals the reduction in chromosome number is not completed by the egg until after fertilization, and this situation has some interesting implications and consequences that are discussed in Chapters I-8,9 and II-10.

The two kinds of gametes are ordinarily produced by two kinds of individuals, termed males and females. However, consistently in some species and occasionally in others, both may be produced by a single individual, termed an hermaphrodite. In certain hermaphroditic species, e.g., the tunicate *Ciona intestinalis*, the eggs of one individual cannot be fertilized, or can be fertilized only with difficulty, by sperm of the same individual, although they can be readily fertilized by sperm of almost any other individual. This situation is an illustration of the high degree of interaction specificity that may be exhibited by the gametes.

There are some species of flagellate protista (e.g., *Polytoma*) in which the mating gametes are not visibly different from each other or from the parental strain of cells, and these gametes are termed isogametes. Other species are anisogametic, with differences in some cases (e.g., *Chlamydomonas braunii*) in size and in others (*C. coccifera*) in the fact that one gamete is motile and the other immotile. The properties and interactions of the gametes in such organisms will be considered in Volume II, Chapter 4.

In some organisms (e.g., the axolotl, certain tunicates) sexual reproduction may occur in larval stages, a condition termed neoteny. The

analogous production by a larva, as in the gallfly *Miaster*, of eggs that develop parthenogenetically is termed paedogenesis. Evidently the differentiation of the gametes is not dependent upon the organism attaining its full morphological and physiological development.

Parthenogenesis is considered a form of sexual reproduction, inasmuch as it involves development from a gamete; namely, the egg. This form of development, without participation of the spermatozoon, occurs naturally in many species of animals, particularly among the insects, crustaceans, and rotifers. Among the birds, the eggs of turkeys commonly initiate development spontaneously and, while most are abnormal and die early, some have been reared to adults. In many animals parthenogenesis may alternate with fertilization, as in aphids that produce parthenogenetic females for part of the year, then males as winter approaches, whereupon fertilization occurs. The "winter eggs" hatch out into females the following spring. The gypsy moth *Lymantria* illustrates the occurrence of natural parthenogenesis in response to scarcity of males. Control of the reproductive process is further illustrated by the classic example of the honeybee, in which the queen is inseminated only once, during the nuptial flight. The sperm are stored for as long as five years, and the female controls their use in order to permit the eggs that she lays to be fertilized and develop into workers and queen or to develop parthenogenetically into the drones, which are haploid males.

D. MANNER OF MEETING OF EGG AND SPERM

As a conclusion to these introductory remarks brief reference should be made to the adaptations developed by animals to insure that sperm and egg encounter one another. The principle methods consist of either the liberation of the eggs and sperm near each other in the surrounding water or the introduction of the sperm into the genital tract of the female. There are, however, many groups of animals that deviate from these two patterns. Again, it is from a consideration of unusual types of reproduction that the basic elements of the process may be more clearly revealed. A comprehensive treatise on sex and sexuality throughout the animal kingdom was published by Meisenheimer (1921). A concise account of the behavior of four groups of animals that exhibit features of special interest was presented by Rothschild (1956) on the basis of more recent investigations. These four include the sponges, the bedbugs, the spiders, and the leeches.

In the sponge *Sycon raphanus* and other species the sperm is drawn in through the pores in its body wall, in the course of the normal process of water intake, and is expelled through the osculum by action of the flagella of the choanocytes. The sperm do not, however, directly reach

the eggs, but, rather, first enter a choanocyte. There the sperm is encapsulated in a "spermiokyst," where it loses its tail and enlarges to about three times the original size of the head and midpiece. Only a choanocyte near an egg is entered. The choanocyte loses its collar and flagellum and attaches itself to the surface of the egg, where the spermatozoon is transferred. Prior to this transfer the egg moves away temporarily and engulfs two "nurse cells."

In bedbugs the male transfers the sperm into a structure called Ribaga's organ, on the right side of the female's body. They must pass through the cells lining this organ into the body cavity and then up the walls of the female's reproductive tract until they reach the ovaries. Phagocytic cells in Ribaga's organ and along the way destroy many of the sperm but enough get through to accomplish fertilization.

In spiders the male uses its second pair of legs, the palps, for transfer of sperm. The semen is deposited in a special web spun by the male, and he dips his palps repeatedly into the semen drops. Then, following nuptial dances, the palps are inserted into the genital orifice of the female and the semen expressed therein by muscular action on the body fluid of the male.

A variation in many groups of animals makes use of spermatophores for transfer of sperm. In cephalopod mollusks one of the arms, termed "hectocotyl," is modified for this purpose and in some species may separate and proceed independently, with its packets of sperm, to find the female. In many species of salamanders a preliminary copulatory behavior is followed by deposition of spermatophores on twigs or other objects, from which the female later removes them by the lips of her cloaca. Leeches also form spermatophores which are attached, hypodermically, on the dorsal surface of the recipient. From there the sperm must pass through tissues of the body wall, into the body fluid, and through the walls of the ovary to fertilize the eggs. Again, as in the bedbug, phagocytic cells destroy many of the spermatozoa en route. The spermatophore contains, at the attachment end, enzymic material, which, with the aid of pressure exerted by its walls, helps the process of penetration through the body wall. A similar process occurs in many other worms, rotifers, and arthropods.

While these special adaptations, along with other traits of the animals, must evidently, in sum, provide selective advantage, the nature of this advantage is by no means clear, and no attempt will be made to discuss this question here. However, it is clear that organisms possessing unusual reproductive features such as these provide important additional experimental material for unraveling problems of gamete physiology and fertilization.

REFERENCES

- Bayreuther, K. E., and Romig, W. R. (1964). Polyoma virus: production in *Bacillus subtilis*. *Science* **146**, 778-779.
- Brown, Harrison (1954). "The Challenge of Man's Future." Viking, New York.
- Caldeyro-Barcia, R., Dorfman, R. I., Ferin, J., Greep, R. V., Harris, G. W., Huggett, A. St. G., Kar, A. B., Kobayashi, T., Mardachev, S. R., Parkes, A. S., and Tyler, A. (1964). "Biology of Human Reproduction," pp. 1-30. Report of a WHO Scientific Group. *World Health Organ. Tech. Rept. Ser.* 280, Geneva, 1964.
- Cole, F. J. (1930). "Early Theories of Sexual Generation." Oxford Univ. Press, London and New York.
- Fox, Sidney, W. (1965). A theory of macromolecular and cellular origins. *Nature* **205**, 328-339.
- Lederberg, J. (1963). The biological future of man. In "Man and His Future" (G. Wolstenholme, ed.), pp. 263-273. Little, Brown, Boston, Massachusetts.
- McElroy, W. D., Allen, W., Berelson, B., Coale, A., Dorn, H., Markert, C. L., Nelson, W., and Tyler, A. (1963). "The Growth of World Population: Analysis of the Problems and Recommendations for Research and Training." Publication 1091. Natl. Acad. Sci.—Natl. Res. Council, Washington, D.C.
- Meisenheimer, J. (1921). "Geschlecht und Geschlechter im Tierreiche. I. Die Natürlichen Beziehungen." Fischer, Jena or Stuttgart.
- Meyer, A. W. (1939). "The Rise of Embryology." Stanford Univ. Press, Stanford, California.
- Miller, R. L. (1964). Isolation of the chemotactant of *Campanularia* (Laomedea) calceolifera. *Biol. Bull.* **127**, 381.
- Miller, R. L. (1966). Chemotaxis during fertilization in the hydroid *Campanularia*. *J. Exptl. Zool.* **162**, 23-44.
- Morgan, T. H. (1927). "Experimental Embryology." Columbia Univ. Press, New York.
- Needham, J. (1934). "A History of Embryology." Cambridge Univ. Press, London and New York.
- Rothschild, Lord (1956). Unorthodox methods of sperm transfer. *Sci. Am.* **195**, No. 5, 121-131.
- Spallanzani, Lazzaro (1776). "Opuscoli di fisica animale e vegetabile," Modena; (1826). In "Opere di Spallanzani" Vol. 3, Milan; (1932). "Opere di Spallanzani," Vol. 2, Hoepli edit.
- Spallanzani, Lazzaro (1780). "Dissertazioni di fisica animale e vegetabile," Vol. 2. Modena; English translation, (1784). London.
- Spallanzani, Lazzaro (1803). English translation by John Graham Dalyell, Esq. "Tracts on the Natural History of Animals and Vegetables, 2nd ed." Edinburgh. Printed for William Creech and Archd. Constable, Edinburgh and T. N. Longman and G. Rees, London.
- Tyler, A. (1964a). Survey of some investigations of problems of reproduction by immunologic and protein-biosynthetic methods. *Proc. VIIth Intern. Conf. on Planned Parenthood: Excerpta Med. Intern. Congr. Ser.* 72, pp. 527-538.
- Tyler, A. (1964b). Immunologic aspects of fertility control. *Clin. Obstet. Gynecol.* **7**, 829-843.
- Tyler, A. (1965). The biology and chemistry of fertilization. *Am. Naturalist* **99**, 309-334.
- Tyler, A., and Tyler, Betty S. (1966). Physiology of fertilization and early development. In "Physiology of Echinodermata," (R. A. Boolootian, ed.), Chapter 28. Wiley (Interscience), New York.

CHAPTER 2

Sperm Motility^{*}

Leonard Nelson[†]

TOLEDO STATE COLLEGE OF MEDICINE, TOLEDO, OHIO

I. Introduction	27
II. Morphology of Cilia and Flagella	31
A. Ultrastructure of Cilia and Flagella	31
B. Cytodifferentiation	39
III. Nature of the Flagellar Wave	41
A. Physical Aspects	42
B. Chemistry of Flagella and Flagellation	44
IV. Contraction-Relaxation Cycles	51
A. Contractile Proteins	51
B. Relaxation Phenomena	56
C. Coordination of the Flagellar Wave	59
V. Extraneous Chemical Factors	60
A. Dilution Effects: O ₂ , CO ₂ , pH	61
B. Cations	62
C. Osmotic Effects	66
D. Body Fluids, Extracts, Antibodies	68
E. Hormones, Antibiotics, Vitamins	71
F. Inhibitors: Metabolic, Sulfhydryl, Interaction, Surfactant	73
VI. Extraneous Physical Factors	77
A. Temperature	77
B. Geotropism	78
C. Galvanotropism	79
D. Radiation: Visible, Ultraviolet	79
E. Viscosity	81
VII. Motility in Male and Female Reproductive Tracts	82
VIII. Conclusion	83
References	85

I. Introduction

Recently Bishop (1962a) noted, "since the critical analyses of ciliary and flagellar activity by Sir James Gray some thirty years ago, no sur-

^{*} This work has been supported by Career Development Award 2 K3-GM-15, 193-06 and by Research Grant GM 06815-06 of the National Institutes of Health. The author also wishes to acknowledge prior support from the Commonwealth Fund in the form of a travel grant.

[†] Present address: Program Director, Developmental Biology, National Science Foundation, 1800 G Street, Washington, D. C.

vey of the phenomenon of sperm motility has appeared, although during the intervening years the process has been extensively investigated on a number of significant fronts." In addition to that review, Bishop has edited a symposium on "Spermatozoan Motility" (1962b) and has written a chapter in Young's "Sex and Internal Secretions" in the "Biology of Spermatozoa" (1961); Fauré-Fremiet has also written a review, "Cils vibratiles et flagelles" (1961); Salisbury and Lodge (1962) discussed the metabolic aspects of sperm motility; Mann's revised and expanded second edition of "The Biochemistry of Semen and of the Male Reproductive Tract" has appeared (1964), and Burnasheva *et al.* have examined "Structural Organization and Biochemical Bases of the Movement of Cilia and Flagella" (1963), while the third edition of Marshall's "Physiology of Reproduction" contains a chapter by M. W. H. Bishop and Walton on "Metabolism and Motility of Mammalian Spermatozoa" (1960). Other reviews have been concerned primarily with fine structural aspects of cilia and flagella (e.g., Fawcett, 1961). Several monographs have also appeared: "The Biology of Cilia and Flagella" by Sleight (1962); "Cilia, Ciliated Epithelium and Ciliary Activity" by Rivera (1962); "Physiologie (Bewegung) der Spermien" (Symposia Biologica Hungarica, 1964).

This increase in reviewing activity undoubtedly reflects the interest attaching to a field in which the submicroscopic morphology of a cell highly adapted for motion offers a tempting challenge to speculation upon physiological mechanisms. Moreover, these specialized systems have apparently attracted the attention of both those whose interests have been primarily oriented to problems of reproductive biology and those concerned with the biology of cell movement. To the problems have been applied a diversity of technological devices and methods, not the least of which have been those elaborated by the practitioners of muscle biochemistry and ultrastructure. The "spermatologists" have been cautioned repeatedly against a "tendency to follow the lead of the muscle investigators too slavishly, with a consequent stifling of the possibility to develop new methods and approaches" (Hayashi, 1962). This point is well taken, as far as it goes, but sperm investigators should not feel diffident about applying any and all methods, novel or well established, which may shed light on an increasingly complex problem.

The sperm cell is peculiar, if not unique, in its form, function, habits, properties, and destiny. The "mature" sperm cell is a terminal cell, the end product of a complex developmental process, that has virtually completed its differentiation, and so its biosynthetic activities appear to be limited, except as noted by Abraham and Bhargava (1963a; 1963b), who claim to have detected RNA metabolism in association with acro-

somal protein synthesis. The processes of biosynthesis in this cell form thus appear to be directed primarily to maintenance of the energetic demands of its motile activity, although Gray (1962) suggests that the accuracy of estimates of useful propulsive effort "might well depend on whether the metabolic activity necessary to maintain mechanical effort is large or small compared with that necessary to maintain other active processes within the cell which are not directly connected with movement." At least 70 % of the total energy dissipated by the moving mammalian sperm serves to provide energy for the "active" contractile component of the tail wave (Rikmenspoel, 1965b). The streamlining of the sperm cell's economy may be reflected in the jettisoning of most excess cellular material (including the endoplasmic reticulum and the ribosomes), the last of which to be lost, the kinoplasmic droplet, slipping away as a final stage in spermatogenesis. During maturation of the mammalian spermatozoon, the kinoplasmic droplet was found on cytochemical analysis to contain Golgi material, phospholipids, and proteins, including RNA protein, mitochondrial lipoproteins, and other residual inclusions of spermatogenic cell cytoplasm. Guraya (1963) suggests that the droplets "probably constitute the endogenous source of energy for the movement of the spermatozoa through the accessory ducts."

One concomitant of this maturation phenomenon is the fact that in epididymal sperm of the rat and the bull at least, the portion of the flagellum, usually most of the midpiece, anterior to the droplet remains quite rigid, while regions posterior to the droplet exhibit active bending couples which are capable of propelling the sperm. Thus, sperm cells from the caput epididymidis of the white rat swim leisurely counterclockwise in a circular motion, with the head retroflected and a bend in the neck piece, while sperm from the cauda swim actively, crisscrossing rapidly in the microscope field. Of these, the cauda sperm fertilize 93 % of ova when inseminated into cornua of females in heat, while only 8 % of ova are fertilized by caput sperm. The latter apparently have not attained the capability of passing the uterotubal junction and ascending the oviducts (Blandau and Rumery, 1964). Some implications of this stiff-necked attitude of the not quite mature sperm cell are that propulsive wave propagation is an "intrinsic" property of all longitudinal segments of the conductile-contractile elements arranged throughout the entire length of the flagellum, and that while these two physiological attributes, conduction and contraction, are interdependent, conduction may occur in circumstances when, and through regions where, contraction is blocked. This, of course, assumes that the impetus for the flagellar activity derives from a site close to the proximal centriolar region near the articulation of the flagellum in the base of the sperm head. (Howe and Black

[1963a] had previously reported that the uterotubal junction in the oestrous rat does not select against the passage of *immotile* rat and foreign sperm. However, only small numbers of sperm were recovered, up to five per oviduct, and after intervals of from 1½ to 10¼ hours following introduction into the uteri. Probably the revolving sperm of Blandau and Rumery [1964] effectively resist the antidromic movements of the uterotubal junction.)

Actively motile sperm of a large proportion of animal and some plant organisms are built on the filiform pattern, with minor or major variations on this theme in regard to relative dimensions and shape of head, middle piece, and principal piece and presence of accessory structures such as undulating membranes and spurs. The motility of a system organized in this fashion involves physical interactions with the medium and near neighbors and must also overcome restraints imposed by its own structural members, as well as entail chemical exchanges through its surface components with the environment. Any analysis of the requirements for the motility of such a system must thus consider questions of the transduction of chemical to mechanical energy from morphological as well as chemical points of view.

For example: What is the nature of the contractile protein or proteins? How are they related functionally and spatially to each other and to other flagellar structures? What is the immediate energy source? Is it stored? If not, how is it supplied? Does it recycle to be replenished, and by what enzymic processes? How is the site of production related to the site of utilization? Can intermediates or substrates be absorbed directly from the medium or must they be transferred or transported from some distant site in the sperm?

How is the contractile wave propagated: via physical activation, as by mechanical stretching of adjacent or opposing elements? by chemical mediation via a conductile impulse system? How are the waves coordinated and how are these processes related to the capability of the sperm of certain species momentarily to reverse their direction? In other words, what are the control mechanisms which govern flagellar movement? Can the contractile process be separated from the conductile process? If so, what are the consequences?

What mechanisms come into play when the sperm is shifted from one set of environmental conditions to another? The mammalian sperm cell, typically quiescent in the epididymis, rapidly becomes activated manifold on mixing with seminal plasma and ejaculation and then is transferred into the secretions of the female reproductive tract (or experimentally may be washed and resuspended and remain motile in a variety of diluents); while the marine invertebrate spermatozoon is

quiescent in the gonad, to become metabolically active on spawning and exposure to a dilute and oxygenated environment. The highly dramatic shift from a low-keyed existence to a condition of rapid depletion of substance makes some unusual demands on the cell, which is adapted not only to withstand the strains and rigors of these sudden transitions but also to carry its mission to completion. From most evidence at hand, it appears that it is only the motile sperm (at least in species with motile sperm) that fertilizes the egg.

To quote Mitchison (1961), "This list of questions is, of course, a personal one and it is obviously incomplete and biased towards the problems I have worked on." To it may be added some questions posed to spermatozoa by Rothschild (1961), some of which he has attempted to answer, others merely to clarify: "Which is your skeleton? Why nine? Do you need twice this number when you are unusually thick? What are the two middle ones for? Are you by any chance nine bacteria? Are you twisted once? Do you react when you are stretched, or when you get a message from headquarters? To whom and by whom is the message delivered? Do you roll for sartorial reasons or by accident?"

II. Morphology of Cilia and Flagella

A. ULTRASTRUCTURE OF CILIA AND FLAGELLA

A vast amount of attention has been directed to the intriguing ultrastructure of the filiform spermatozoon and cilia, of which the flagellum apparently is more than merely a complex, special case. It is increasingly evident that fine structural analysis, while essential, is not sufficient of itself for interpretation of the physiological mechanisms which it subserves. The submicroscopic morphology establishes the boundary conditions and the framework within and upon which depend the motile activity.

To simplify this discussion I shall identify the inner ring of nine doublet fibers, the α -, or primary, fibers, since these are the most constant components; the central pair, the β -, or secondary, fibers, and the outer ring of nine coarse longitudinal fibers, the γ -, or tertiary, fibers (see Fig. 1). The characteristic "classical" $9 + 2$ and $9 + 9 + 2$ composition of cilia and flagella has yielded to the occasional appearance of as many as five supernumerary coarse outer longitudinal fibrous elements, or even the absence of one or two of these coarse filaments, as well as variation in the number of the oversize coarse filaments (usually nos. 1, 5, and 6 in the human, rabbit, rat, and bull; now no. 9 is included as well) among the usual 9. Since these deviations from the normal occur in a minute fraction of the population and are detectable only in the electron

microscope, they cannot yet be regarded as of great consequence in an account of factors governing sperm movement, nor is there at the moment any indication whether the deviant fibers affect or even permit movement (Bawa, 1963; Afzelius, 1963). The presence of additional fibers does not usually occasion any detectable decrease in the size of any of the standard fibers. In general, the ring of nine coarse outer γ -fibers occurs in all mammalian spermatozoa examined, as well as in some birds, some mollusks, some insects, and snakes. The outer set, the γ -fibers,

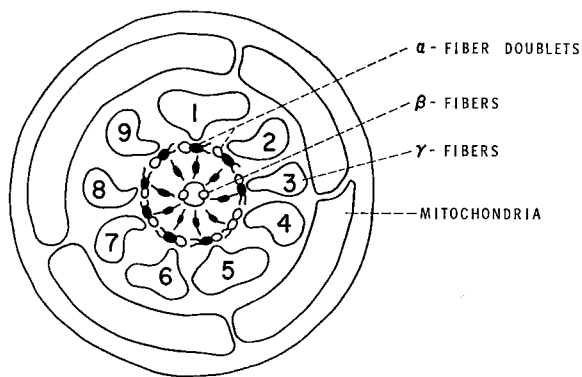


FIG. 1. Schematized representation of a cross section of a mammalian sperm midpiece illustrating the distribution and relative size of the longitudinal fibers. α -fiber doublets, with less dense and more dense component (depending on fixation), bearing paired arms, are the most consistent fibrous components and are present in most cilia and flagella examined in the electron microscope. They may have a contractile function in cilia or simple $9 + 2$ flagella. β -fibers, the central pair, are present in most cilia and flagella, although lacking in some. The most labile of the filamentous components, they are thought by some workers to have conductile properties. γ -fibers, large, coarse, outer longitudinal fibers, are characteristic of mammalian and some avian sperm and are cytochemically identified with contractile proteins. They are absent in cilia and marine invertebrate sperm flagella. Note the extra large size of fibers 1, 5, and 6. In some animals, e.g., the bat, fiber 9 is of the same dimensions as 1, 5, and 6.

appear somewhat rudimentary in the snail, squid, snake, and rooster sperm, suggesting a transitional stage on the way to the mammalian form. If these larger fibers have arisen adaptively, providing the thicker mammalian sperm with an effective propulsive system supplanting the function of the original ring of nine doublets, then it would appear unfruitful to look for too close an identity between the motile mechanisms of these two extremes and ciliary or flagellar action in cells with soma-based basal granules. It appears that the filiform sperm of marine invertebrates and of freshwater and marine fishes not only lacks this

accessory set of fibers but also has not acquired the coarse fibrous sheath which envelops the midpiece and extends down to the tail endpiece of mammalian and other sperm. Rothschild (1961) has already suggested that the double array of fibers may be needed when the sperm are unusually thick, that is, endowed with the fibrous sheath.

Perhaps it would be instructive to seek a possible correlation between the complexity of flagellar fine structure and the nature of the media through which the spermatozoa normally swim. Sperm cells with the more complex $9 + 9 + 2$ flagella occur predominantly in those animals practicing internal fertilization, a process which necessitates movement through seminal plasma and female reproductive tract fluids (e.g., cervical mucus). Such fluids are presumably more viscous than the media encountered by the free-swimming $9 + 2$ spermatozoa of organisms spawning broadcast into an external fresh- or saltwater environment. The acquisition of the accessory γ -fibers may thus be related to their adaptive value in meeting the added viscous load.

Among the deviations from the standard pattern of the filamentous components, the absence of the β -fibers, as in *Scalpellum*, a barnacle, does not hamper flagellar movement. The fact that these spermatozoa can swim in either direction, headfirst or tailfirst, and even show a reversal of flagellar waves has led Afzelius (1962) to conclude that the role of the β -fibers appears to be one of directing the beat rather than bending the flagellum. This evidence alone may not be sufficient for such a broad generalization, for some forms with undulating membranes endowed with a set of β -fibers are also capable of reversing their direction. In an ingenious piece of work, Randall *et al.* (1964) used proflavine, 100 mg/l, to induce mutation in the flagellate *Chlamydomonas reinhardtii*; the mutants sink to the bottom of the liquid culture media. While their flagella appear approximately normal in length, the whole organisms are unable to move, even though some of the flagella exhibit slight uncoordinated twitching. Electron micrographs show the nine doublet plus central two array with moderately densely staining subsidiary fibers and radial lamellae in the wild-type. The mutants' flagella do not show well-defined projections or the sidearms characteristic of the α -fibers; there appears to be less organized material in the matrix; and the central pair, the β -fibers, appear highly disorganized. The mutants apparently lack the ability to complete the organization of some of their submicroscopic flagellar elements. Although these investigators equate the nonmotility of the mutant with a structural defect, the lack of formed β -fibers, they may be overlooking the possibility that, since some uncoordinated twitching does occur, these fibers may be concerned more with conduction and coordination than, as a contractile system, directly

with motility per se. Other workers have observed that the β -fibers are probably the most highly labile components of flagella. From an engineering point of view, the central pair of fibers would probably be the least effective and the most highly disadvantaged to serve as propulsive elements. Moreover, when ATPase has been demonstrated in situ, it has usually not been associated with the central two fibers. Examination of the ATPase activity and its cytological distribution in the mutants could possibly afford some clues relating the lesion to the paralysis.

The α -fiber doublets are composed of two subunits. In appropriately fixed specimens of a number of species, one member of the pair appears to be a hollow tubular structure which resembles, to Afzelius (1962), the sarcotubules in the helical smooth muscle of the leech; while the other subunit has a compact appearance, and it is from this subunit that the sidearms usually project laterally toward the adjacent doublet. Spokes extend radially inward from the α -fibers (usually from the compact subunits) toward the central β -fibers and may traverse the central sheath. Gibbons (1963) claims, on the basis of their solubility in media of high ionic strength (0.5–0.6), that the arms are the contractile elements in *Tetrahymena* cilia. In mammalian sperm, under appropriate conditions, the entire axial filament complex, α -fibers, β -fibers, spokes, and arms, dissolve in 0.1 M KCl! No claims have yet been made for the spokes.

To the central pair or β -fibers have been ascribed, although very tentatively, roles as skeletal or supporting elements, presumably lending rigidity to the cilium or flagellum, and as conductile elements. They certainly appear to be the most labile structures of the filamentous system, being readily lost from frozen-dried preparations and from KMnO_4 -fixed tissue. In fact, Bawa (1963) reported that the whole axial filament complex, consisting of the α - and β -fibers, is lost after permanganate treatment. In frozen-dried or glutaraldehyde-fixed cells postfixation with osmium or staining with metal salts renders the axial filament more readily visible. These facts suggest a chemical distinction between the coarse outer γ -fibers and the axial filament set. This conclusion is substantiated by the work of Nagano (1963) on fine structural changes in the spermatid flagellum following experimental cryptorchidism in the rat. Not only were the axial filaments disrupted and disoriented, with the β -fibers becoming obscure, but the tubular subunit of the α -fibers appeared more sensitive than the dense, compact subunit. Only the coarse α -fibers retained their "normal" appearance after up to 26 days of confinement in the rat abdominal cavity. However, even after frozen-dried rat sperm had been extracted with Guba-Straub solution (buffered

KCl-phosphate, ionic strength 0.6), long enough to extract ATPase and to remove material reactive with myosin antibodies, the coarse γ -fibers retained their morphological identity in the electron microscope (Nelson, 1962). This points to a composite, multichemical structure for the γ -fibers.

The fine structure of the individual α - and β -fibers of human (André, 1963), rat (Pease, 1963), and bull sperm (Nelson, unpublished) axial filament has been examined in the electron microscope using negative staining methods. Each of the tubular fibers contains up to 10 protofibrils, or longitudinally oriented filaments, with a beaded appearance. This whole system also resists extraction with Guba-Straub solution. Moreover, the periodicity and dimensions of these filaments makes it unlikely to identify them with actin or myosin, although Pease surmised that these filaments possess great mechanical strength on the grounds that they withstand the physical agitation to expose them. Even if additional evidence concerning their supposed strength could be brought to bear, physical considerations make it unlikely that the β -fibers could contribute much to the rigidity of the flagellum, because of the mechanically unfavorable position of these fine structural elements at the axis of the flagellum.

Mammalian spermatozoa generally are characterized by the presence of the mitochondrial sheath, which is formed and transformed during spermiogenesis in relation to the distal centriole. By the time of completion of spermiogenesis, the inner fine structure of the midpiece mitochondria has adopted a considerably more condensed appearance than the mitochondria of the ordinary somatic cells. This may be of particular significance in terms of the peculiar metabolic requirements of the maturing and mature sperm cell. The mitochondrial sheath also shows up as a major factor in the approximately 25% taper of the flagellum from the midpiece to the tip of the tail (Bahr and Zeitler, 1964) and probably contributes heavily to the internal resistance to bending in the moving sperm cell. The complement of coarse γ -fibers, the axial filament complex, and the matrix in which they are embedded are all encompassed within the confines of the mitochondrial sheath. There is as yet no information available concerning the nature, composition, or functional significance of the matrix (i.e., whether it serves as the physical substratum of the glycolytic enzyme system normally found in the supernatant fraction prepared from other cell-type homogenates or as part of the active contractile system), nor for the relatively thin layer of cytoplasm which lies between the mitochondrial surfaces and the plasma membrane. The plasma membrane itself is apparently quite friable. Excessive manipulation, such as repeated centrifugation and resuspension

or cold shock, seem irreversibly to increase the permeability of the flagellar surface, since molecules the size of the cytochromes appear in the medium. Furthermore, we have observed (Austin and Nelson, unpublished) that the mitochondrial sheath of nonmotile flagella, obtained from the semen of a bull which ejaculates naturally decapitated sperm, lacks the covering plasma membrane, and the sheath frequently becomes unraveled, usually from the anterior end. The mitochondrial sheath terminates at the so-called "ring centriole," which thereby delineates the posterior limits of the midpiece of the mammalian sperm. (In the so-called "primitive" sperm of many fish and marine invertebrates, the mitochondria form a limited number of spherical clusters at the base of the head and do not appear to contribute to any extensive sheathlike structure overlying the flagellum. For the most part, these "primitive" sperm also lack the coarse γ -fibers.)

Anatomically, the mitochondrial sheath and the enlarged γ -fibers typify the mammalian sperm midpiece. Functionally, however, the fiber complex is an integral part of the entire flagellum (the principal piece should be regarded, therefore, as originating at the caudal end of the neck piece). Even though the γ -fibers generally taper gently through the so-called "middle piece" to the so-called "principal piece," the chemical and mechanical events directly involved in flagellation are fundamental attributes of the filament complex proper of the *entire* flagellum. The chemistry and physics of the mitochondria in the midpiece sheath are peculiar to these mitochondria, as distinct from the filament complex of the flagella, and perhaps as well, as distinct from "somatic" mitochondria. One may not justifiably attribute, then, the biochemistry of the midpiece exclusively to either the mitochondrial or the filamentous components, unless one either physically or cytochemically cleanly separates these two sets of elements into rigorously identifiable systems. This may be achieved with difficulty, by ultrasonic disruption, at the risk of great damage to the components and possibly with very small recovery. Following differential centrifugation, the preparation should be monitored for "purity" in the electron microscope.

The principal piece of spermatozoa in general is surrounded by a microtubular fibrous sheath which varies in appearance from that of a flattened helix to irregularly shaped circumferential ribs oriented perpendicular to the long axis of the tail. The sheath in several species may also contain two lateral ridges or longitudinal columns which run down to the end piece and are in the same plane of cross section as a line joining the pair of β -fibers of the axial filament complex. One of the striking features of the principal piece is the manner in which the outer longitudinal γ -fibers undergo both a reduction in size and a systematic

reduction in number until they finally disappear altogether as such (see Fig. 2). First, fibers no. 3 and 8 phase out, next fibers 4 and 7, then 2 and 9, and finally 1, 5, and 6 (see also Lindahl and Drevious, 1964). Fibers 3 and 8, and 4 and 7 move into close proximity, with an inward projection from the longitudinal columns, and further work may show actual fusion with, or incorporation into, the fibrous sheath. Perhaps the

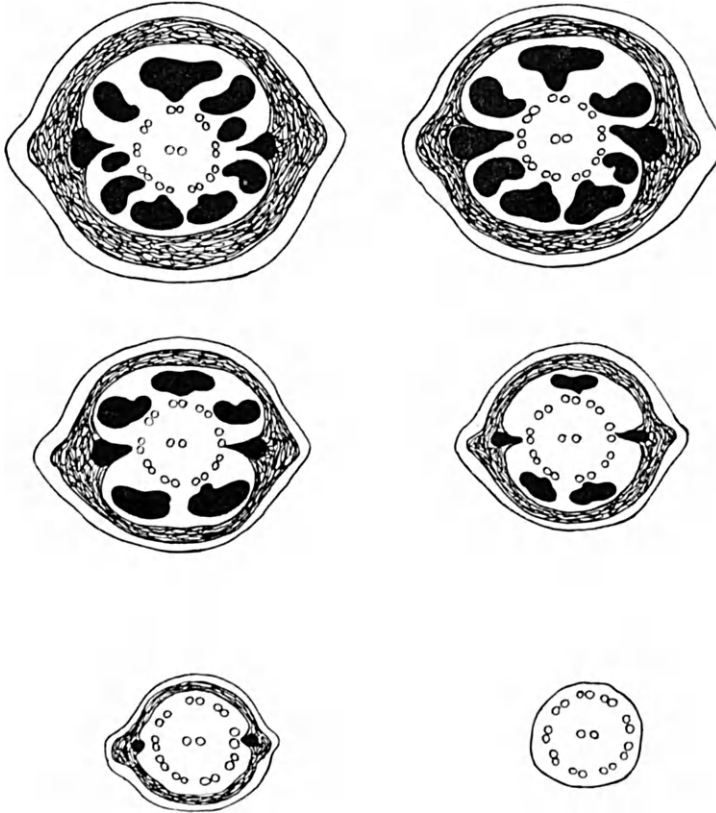


FIG. 2. Cross sections through principal piece and endpiece of mammalian sperm tail showing apparent incorporation of γ -fibers with inward projections of longitudinal columns, starting with fibers 3 and 8, then 4 and 7, 2 and 9, etc. (Modified from Fawcett, 1961.)

sperm cell may reply to Rothschild's question, "I have an exoskeleton!" This sort of an organization could perhaps underlie the perpendicular asymmetrical major wave in one plane and minor wave in the biplanar action exhibited by "rotating" sperm recorded cinemicrophotographically by Rikmenspoel (1964). The movement of the bull sperm flagellum is apparently the sum of two vibrations, the amplitude in the plane of the

head becoming increasingly greater than the amplitude perpendicular to the head as the wave approaches the end of the tail (Rikmenspoel, illustrated in Fig. 4). Since the largest γ -fibers, Nos. 1, 5, 6, and perhaps 9, extend further distally than the others, they presumably contribute most to this increase in amplitude.

Among the most interesting recent contributions to the burgeoning literature on submicroscopic spermatozoology have been those concerned with the neck and centriolar region and the relations between the proximal centriole and the origins of the γ -fibers (see Fig. 3). The latter appear to arise as segmented or cross-banded structures, two pairs of which are fused to form what Nicander and Bane (1962b) call "im-

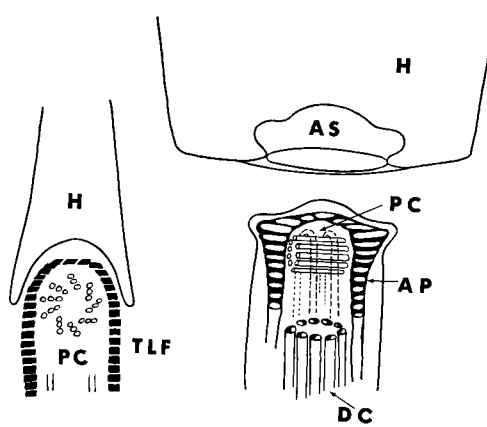


FIG. 3. Schematic diagrams illustrating the attachment of the neck to the head of a mammalian spermatozoon. (H) Head. (AS) Articulation socket in head. (PC) Proximal centriole. (DC) Distal centriole. (AP) Articulation plate. (TLF) Thin laminated fibers. (Modified from Saacke and Almquist, 1964.)

plantation plates." (Since this term has the unfortunate connotation that during spermatid differentiation the flagellum becomes embedded or implanted into the nucleus, rather than secondarily the nucleus moves down to surround the anterior end of the flagellum, it is perhaps preferable to use a more descriptive and less prejudicial term such as "articulation.") The proximal centriole is oriented perpendicular to the flagellum, with its long axis parallel to the flattened surface of the head in mammalian sperm. Austin (1965) found that in reptilian spermatozoa some of the γ -fibers appear to encircle the proximal centriole completely. The γ -fibers then may be firmly anchored anteriorly in this fashion or in the segmented articulation plates which flare out like inverted wedges, with their bases in close proximity to the nuclear membrane.

Saacke and Almquist (1964), in a highly detailed study of the neck and tail of normal ejaculated bull sperm fixed in KMnO_4 or OsO_4 , described the striated appearance of the broadened origins of the γ -fibers consisting of 13–15 highly electron-dense lamina per fiber. The neck is encompassed in a recess or articulation socket at the base of the head. There are two lateral articulation plates, each composed of two fused fibers (also shown by Rothschild, 1962), plus at least four other thin laminated fibers close to each other and thinner than the articulation plates. These all appear to be embedded in an expanded homogeneous matrix which forms a common base for articulation of the fibers in the head recess. The lipped socket seems to hold this system in place. The complex of laminated fibers and segmented articulation plates appear to form an arch or hood over the proximal centriole. Saacke and Almquist (1964) suggest that these articulation plates may undergo alternate contractions and expansions in this region, which may then initiate a planar or two-dimensional flagellar wave. However, the articulation plates, confined to the neck region, are quite distinct in appearance from the more complex periodicity characteristic of striated muscle, and the only present indication for such an involvement in wave production is this rather superficial resemblance to the muscle. Moreover, the biophysical evidence of Rikmenspoel (1964) indicates that the flagellar wave may be the summation of several planar waves originating in planes perpendicular to each other.

B. CYTODIFFERENTIATION

One of the fine structural fields in need of fuller exploration is that correlating the acquisition of motility with the development of the flagellar components during spermiogenesis.

Further knowledge of the more intimate details relating fine structural components to the initiation and production of the flagellar wave depends on the thorough exploration of the differentiation of the flagellum and the development of its capacity for independent movement. Leblond and Clermont (1952), on the basis of periodic acid-Schiff staining reactions, divided the "cycle of the seminiferous epithelium" of the rat into 4 phases comprising 19 stages; these authors later described 14 similar stages in spermiogenesis of the monkey (Clermont and Leblond, 1955). Austin and Sapsford (1952) described the emergence of a fine filament from one of the centrioles of the rat spermatid during the first, second, and third stages of spermiogenesis. They reported that the filament, which is often longer than the diameter of the cell, exhibited vigorous undulating movements even before they could detect the extension of the filament beyond the cell body. Movements of the axial fila-

ment seemed to cause quite distinct changes in the position of the centrioles in the cytoplasm. Sometimes the motility was very vigorous and appeared to bring about rapid deformations of the cell surface each time it was struck from within by the filament. (However, there has been some suggestion that the filament has already emerged from the cell.) Clermont (1964) essentially confirmed this observation in describing the slow and wavy flagellar motion in stages 3–5, which then continues throughout spermiogenesis. Meanwhile, at stages 7–9, a unidirectional undulation of the cytoplasmic membrane extends over one-third of the cell surface at the caudal pole, the site of emergence of the flagellum. During stages 10–13 the cytoplasm elongates and the paddle-shaped caudal pole shows a rhythmic beat, including a partial lateral twist. Subsequently, cytoplasmic motility stops while the flagellum continues undulation. Of particular interest is the fact that the mitochondria do not begin to congregate around the flagellar filaments until late in spermiogenesis, well after the undulations have begun. Moreover, during the mitochondrial transformation and partial coalescence into a continuous, or even discontinuous, helical sheath in mammalian spermatids, a further internal modification, a condensation, has been taking place. The effects of this intense lamination on the biochemical capacities of the mitochondria and their role in the economy of the mature sperm have yet to be determined (see André, 1963).

Most of the studies on spermateliosis at the electron microscope level have concentrated on the terminal stages, so that little has so far been learned of the origins and formation of the γ -fibers. This is most unfortunate, since these fibers probably carry the main burden of flagellation in the mammalian and avian sperm. Obviously, the α -fiber in mammalian spermatids and in the sperm of organisms lacking the γ -fibers must be endowed with motile ability, as indicated in the reports cited above and innumerable others. In a paper on "Cilia and Flagella That Do Not Conform to the $9 + 2$ Pattern," Afzelius (1963) pointed out that when higher numbers of supernumerary fibers occur (more than 10 altogether in the cross-sectional ring) a new shell of filaments is formed. He suggested that filaments may be built up of "vesicular" material through centriolar induction in the abnormal cilia and flagella. The mechanism of formation of basal bodies and flagella in the fungus *Allomyces arbuscula* (Renaud, 1964; Renaud and Swift, 1964) may shed some light on this general problem of the differentiating structures. During differentiation into gametangia, the centrioles occur in pairs, one member of which, increasing threefold in length, serves as a basal body for flagellar growth. "Primary" cytoplasmic vesicles appear to pinch off from the cell membrane, come into contact with the basal body, and

flagellar formation occurs in association with this. Tenuous fibers extend from the basal body, invaginating into the primary vesicle. The flagellum then continues to grow by lengthening of the fibers. Regeneration in the flagellates *Astasia longa* and *Ochromonas danica* following mechanical amputation of the flagella starts after a short lag and proceeds at a rate decreasing with time. Incorporation of tritiated acetate and leucine occurs more rapidly in regenerating than in nonregenerating flagella. Rosenbaum and Child (1964) claim that this signifies that some of the flagellar components are not present in the cell prior to the amputation, but must be synthesized from low molecular weight precursors obtained from the medium. If such is the case, either the transfer of information or of soluble substances from the cell body, or both, must be limited in part by the rate at which these factors may diffuse or be transmitted down the flagellum. (The rate of diffusion of any substances down sperm flagella, including ATP, is a matter of consequence in understanding the factors involved in providing energetic material to support the flagellar wave.)

In bulls, boars, dogs, cats, rabbits, and rats, the distal centriole gives rise to the tail primordium of the young spermatid. The cell membrane invaginates to form a deep pocket, from which the proximal part of the tail primordium emerges. At the same time, the longitudinal columns begin to develop, becoming broader and longer, and apparently consisting of fine branching and interconnecting fibers. These fibers run a circular course and ultimately form the fibrous sheath of the principal piece of the tail (Nicander, 1962). But the fibrous sheath never seems to extend to the invaginated part of the axial filament.

The relationship of the differentiation of the flagellar components to the development and distribution of associated enzymes and the acquisition of motility still remains to be investigated systematically. It appears, on admittedly limited evidence, that motility is an "intrinsic," perhaps even "myogenic," property of the flagellar anlage, since the tail rudiment exhibits movement almost from the moment that the structure itself is detectable. (Austin and Sapsford, 1952; Clermont, 1964.) It is not clear whether these initial movements are spasmodic or regular, but their character appears to become modified with maturation; this may be related to the supply of energetic material and to differences in physical environment when the flagellum is enclosed within, or is in the process of being extruded from, the spermatid.

III. Nature of the Flagellar Wave

In the mature and nearly mature spermatozoon, the physical as well as the chemical climate markedly affect the flagellar wave. Since Bishop's

(1962b) extensive review only a modicum of information concerning the nature of the flagellar wave has appeared.

A. PHYSICAL ASPECTS

Rikmenspoel (1962, 1965b) examined living bull sperm cells, washed and suspended in egg yolk citrate buffer, and found that they fall into two distinct classes, those which move in fairly straight paths while rotating along their longitudinal axes, and cells which swim in small, almost closed circular paths without any rotation. In the nonrotating cells, which Rikmenspoel regards as pathological (immature?), swimming in a two-dimensional circular path (see also Blandau and Rumery, 1964), "each point of the tail executes an almost purely sinusoidal vibration around a median position." The amplitude increases while the velocity of the tail wave decreases toward the distal end. The "normal" cells rotate, exhibiting a helical wave with unequal amplitudes in two perpendicular components—one in the plane of the head, the other at right angles to the head plane. Rikmenspoel (1965b) expressed the tail motion as the summation of two sinusoidal functions of unequal amplitude, with a main (slower) component and a lesser (faster) component (Fig. 4). The forward velocity for six nonrotating cells averaged 70 μ /second; tail frequency, 24 cps; amplitude, 6.6 μ . For seven rotating cells, the forward velocity averaged 100 μ /second; frequency of the main component (greater amplitude), 13 cps; faster component (lesser amplitude), 30.5 cps; and frequency of rotation, 8.8 cps. Brokaw and Wright (1963) claim that the bending waves of the posterior flagellum of *Ceratium*, a marine dinoflagellate, are not true sine waves but rather circular arcs joined by short unbent segments. The bending waves maintain a constant amplitude; therefore, presumably, periodic active bending must occur all along the flagellum.

Brokaw (1963) also reported that the flagella of the alga *Polytoma uvella* exhibit coordinated active bending along their entire length, producing a propulsive effect. The bending waves pass distally with little if any decrement in velocity or amplitude in intact glycerinated cells as well as in isolated flagella. The isolated flagella resemble the intact in that no obvious damping in amplitude occurs as the wave passes distally, although both frequency and amplitude are somewhat less in the isolated than in the normal flagella. Removal of Ca^{++} from the medium increases the beat frequency from 10–20/second up to 50–60/second. Decrease in frequency to 1–2/second may result from a decrease in ATP or from an increase in viscosity by addition of methyl cellulose to the medium; in the methyl cellulose, the wave patterns do not obviously

differ from the normal, whereas the tenfold decrease in ATP concentration necessary to lower the frequency to a similar value causes many flagella to beat abnormally. With further lowering of the ATP concentration, the flagellar rhythm may become irregular, with pauses in either extreme lateral position. In the methyl cellulose, lateral bending occurs only near the tip, suggesting that, in this form, the plane of bending twists somewhat as the waves pass along the flagellum.

The difference in wave patterns induced by low ATP in comparison to

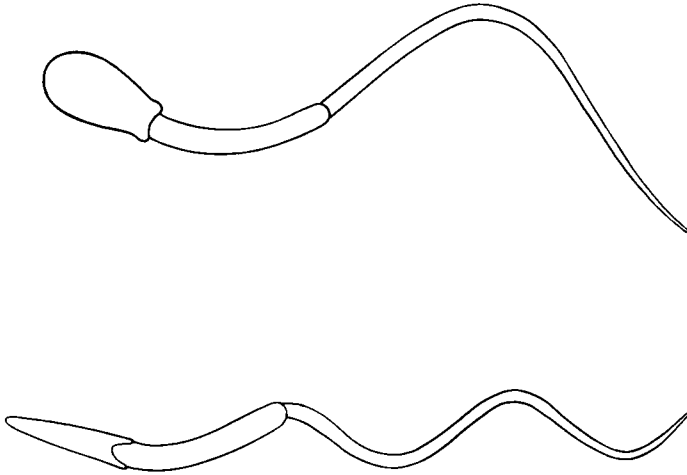


FIG. 4. Illustration of biplanar waves in bull sperm. The top wave, in the plane of the head, has larger amplitude, slower frequency; the bottom wave, in the plane perpendicular to the head, is the faster component (as described by Rikmenspoel, 1965b), resulting in three-dimensional undulation.

the mere slowing down in highly viscous media is not inconsistent with Bishop's suggestion (1962b) that at least one effect of ATP on beat frequency results from its plasticizing action, which purportedly decreases the *internal* viscosity of the flagellum. Brokaw (1963) suggests, rather, that in the presence of insufficient ATP "the mechanism responsible for producing normal nearly sinusoidal bending waves functions imperfectly." Thus, the bending elements may not be entirely independent, although other evidence suggests that while distal elements may be directly or indirectly dependent on the action of the proximal elements, still situations arise in which distal elements exhibit bending waves in the apparent absence of proximal activity. However, Brokaw feels that the basic similarity in behavior among various kinds of flagella

indicates that flagellar waves are generated by similar mechanisms involving proximal- to distal-coordinated activity of bending elements distributed along the flagellum. McKeen (1962) concludes from his observations on zoospores of *Phytophthora*, *Saprolegnia*, *Allomyces*, and *Achlya* that in these organisms also flagellar movement results from the expenditure of chemical energy within the flagellum itself rather than from any pulls originating within the cell and acting rhythmically on the flagellar base.

According to Rikmenspoel's model calculations (1965b), the stiffness of the bull sperm tail originates in the fibrous sheath, which has a Young's modulus of 3×10^7 dynes/cm². To overcome this structural resistance, as well as damping of the tail wave by the viscous drag of the medium, active contractile elements must be distributed continuously along the tail. The energy dissipated by the active contractions must equal at least 70 % of the total energy dissipation. If the contractile elements are within the γ -fibers, the maximum tension developed by each of these fibers must amount to 4×10^6 dynes/cm², which is within the range of $1-5 \times 10^6$ dynes/cm² for skeletal muscle. Rikmenspoel (1965c) showed that the motility of bull sperm is dependent on a continuous supply of energy; he found that amytal causes a simultaneous decrease in both respiration and motility. He further regarded ATP as the immediate source of the contractile energy.

B. CHEMISTRY OF FLAGELLA AND FLAGELLATION

1. Energy Sources

ATP has long been implicated as a source of energy for flagellar movement. In 1945, Lardy and Phillips found an acid-soluble organic phosphate in bull spermatozoa, which yielded inorganic (ortho-) phosphate on 7-minute hydrolysis in hot 1 N HCl. The same year Mann (1945) identified the compound by chemical analysis as ATP, and Engelhardt (1945) described the work of his student, Burnasheva, in preparing spermosin, a myosinlike substance with ATPase activity, from bull sperm homogenates. The flagella of fragmented bull sperm hydrolyze ATP; the specific activity of the tail fraction is equivalent to from three to six times that of the midpiece fraction (Nelson, 1954). Burnasheva (1960) showed by paper chromatography that bull sperm extracts contain GTP and GDP, as well as AMP, ADP, and ATP. Adenylic acid comprises about 40 % and inosinic acid about 25 % of the total nucleotides, but only ATP, which makes up from 10-27 %, supports motility. Regulation of the ATP level is maintained by respiration and

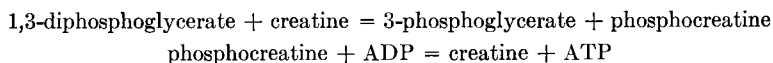
glycolysis. According to Burnasheva (1960), sperm cell motility continues until the ATP pool is exhausted in suspensions in which respiration and glycolysis are blocked simultaneously. Addition of glucose leads to renewal of metabolic processes, resynthesis of ATP, and restoration of motility. Thus the energy derived from oxidative processes may not directly contribute to the mechanical activity of the cells but rather to the synthesis of high energy phosphate, as ATP, which Burnasheva states is the actual energy source for the motor function of the cells. Gonsse (1962) confirmed this concept in view of the fact that mammalian spermatozoa use glycolysis preferentially even in the presence of oxygen. The energy provided via oxidative phosphorylation may be stored, while that derived from glycolysis is readily available for movement. Schindler and Nevo (1962) also substantiated these observations in showing that the motility of fowl and bull sperm is aerobic obligatory in the absence of reducing sugars in the medium. Not only does carbohydrate breakdown result in ATP production but it also spares endogenous lipid reserves, and Turner (1962) claims that at the same time carbohydrate utilization also serves in the active synthesis of lipids. In bull sperm, the total lipid (about 75% of which is phospholipid) comprises about 12% of the cell and about 1.35% of the seminal plasma (Komarek *et al.*, 1964). Turner (1962) suggests that ATP may also be utilized in reactions contributing to the maintenance of some of the sperm cell constituents. In this connection, Scott and co-workers (1963) propose that in the ram epididymis, "even if, as is generally assumed, the spermatozoa are in a quiescent state, at least some basal metabolism might be expected in order to maintain the integrity of the cells." They point out that while reducing sugars may be absent and short-chain fatty acids in low concentration, the large amount of lactic acid could serve as the substrate for maintenance metabolism of the epididymal sperm. (Lactate in the female reproductive tract could also serve in this fashion.) In boar epididymal sperm, the ATP level in actively moving cells invariably exceeded that of ADP and AMP, but was almost undetectable in "resting" cells (Tsuchiya, 1962). During incubation at 37°C for 3 hours, the total amount of acid-soluble nucleotides decreased appreciably with a concomitant increase in nucleotide metabolites, e.g., uric acid. Similarly the ATP content of fresh undiluted sea urchin sperm was lower than that of ADP and AMP in experiments reported by Taguchi *et al.* (1963). A significant decrease in both ATP and ADP occurred following extensive exhaustion of endogenous phospholipids (highly diluted semen, and with continuous removal of CO₂); but even after prolonged incubation, neither ADP nor ATP showed marked changes when low utilization of

the endogenous substrates occurred, that is, when both motility and oxygen consumption were relatively low, so that the supply and utilization of energy-rich phosphates were well balanced. On the other hand, the low motility, even over a considerable range of dilution, of the spermatozoa of the barnacle *Balanus balanus* has been correlated with a low content of ATP and other phosphorylated intermediates (Barnes, 1962). In another example of the intimate association of ATP and motility, addition of ATP increased both the motility and half-life of human spermatozoa from 28.2 to 46.3 hours at 20°C (Iiuzuka *et al.*, 1959). These workers also claim a marked increase in both sperm motility and metabolic activity after the semen had been frozen in glycerol containing ATP.

Quantitative estimates of the nucleotide content of spermatozoa reveal that initially the ATP concentration per sperm cell probably suffices to meet the needs of the motile apparatus and support the premise that biosynthetic reactions may maintain the ATP level for considerable periods of time. Brackett *et al.* (1963) used the firefly assay method for determining the ATP content of *washed* spermatozoa: rabbit semen contained 30–50 p μ mols* per sperm cell, rooster semen contained 37 p μ mols, while guinea pig epididymal sperm contained 114 p μ mols per cell (1 p μ mol equals 10⁻¹⁸ mols). Newton and Rothschild (1961) found by Dowex-1 chromatography that freshly ejaculated bull semen contains a mean of 129 p μ mols of ATP per sperm cell, 120 p μ mols of ADP, and 271 p μ mols of AMP plus inosine (in approximately equal concentrations). During anaerobic incubation, the heat production declined rapidly to a low level and impedance change frequency fell to zero in less than 2 hours, while there was only a relatively small decrease in ATP, ADP, and AMP plus inosine over a 4-hour period. In the 2–4-hour interval, anaerobic heat production remained low and fairly constant, but then abruptly dropped to zero, accompanied by a sharp decrease in ATP and ADP, with a corresponding increase in AMP and inosine. The lack of correlation between changes in nucleotide concentration, impedance change frequency, and anaerobic heat production led to a reinvestigation of the possible role of phosphagens in sperm metabolism, since previous reports on the presence of phosphagens in semen have been inconsistent, not to say contradictory. Of 250–300 p μ mols of highly labile, non-nucleotide organic phosphate, about 50 p μ mols assayed as creatine phosphate; the rest may be acetyl phosphate or 1,3-diphosphoglyceric acid. The latter substance can contribute to the enzymic phosphorylation

* p μ mols stands for picamicromoles.

of creatine (see Cori *et al.*, 1958) and ultimately to the regeneration of ATP via the reactions:



Mammalian seminal plasma contains an extremely high amount of creatine phosphokinase (Lehmann and Griffiths, 1963). Newton and Rothschild (1961) also suggest that it may be misleading to attribute the ATP:ADP ratio of the bull spermatozoon to the cell as a whole, since the ATP:ADP ratio in the mitochondria may differ from that in non-mitochondrial constituents of the sperm cell; this could lead to erroneous conclusions concerning the sites and pathways of ATP resynthesis. This, in turn, could lead to misconceptions about the role and interactions of the various other sperm cell organelles in the mechanics of ATP transport to the loci of utilization.

Detailed accounts of the chemical constitution of the various structural components of spermatozoa may be found in the reviews by Bishop (1961, 1962a) and Mann (1964; Chap. 3 this volume). As far as can be conclusively determined, the head of the spermatozoon contributes very little, if anything, directly or indirectly, to the initiation, production, regulation, or control of the flagellar wave or to the energetic processes and substrates underlying flagellar movement. At least, independent substantiation of the reported presence in the sperm heads of such enzymes or substrates appears to be lacking. For the most part, the head of the completely mature sperm cell may well be described as a package, the contents and wrappings of which appear to remain inert or dormant until delivered to the destination. On the other hand, isolated flagella "models" may be activated by ATP in appropriate media, and the semen of some bulls with an unspecified congenital defect contains swimming, naturally decapitated spermatozoa. Jones (1962) considers this defect to arise from the inheritance of a sex-linked trait in Guernsey bulls; seven siblings ejaculated semen containing detached sperm with motile tails. One such Guernsey bull, maintained at the A.R.C. Unit of Reproductive Physiology and Biochemistry, Cambridge, England, ejaculates 100% decapitated spermatozoa. Large numbers of the flagella exhibit active coordinated motility, so that low magnification ($\times 40$ in the light microscope) reveals mass wave-front formation in the semen. Masaki and Hartree (1962) investigated the distribution of metabolic activity, phospholipid, and hyaluronidase after separating this semen by differential centrifugation into a supernatant fraction of motile flagella and two sediment fractions each containing heads as well as immotile flagella.

They claim that the motile flagella fraction accounts for 75% of the respiratory activity, most of the fructolytic activity (although, according to their calculations, the "heads" have about 25% of the fructolytic activity both of the swimming tail fraction and of normal semen), about 70% of the plasmalogen and of the total phospholipid, but much less of the hyaluronidase than the so-called "head" fraction. The immotile flagella, according to the interpretations of these workers, have only negligible activity, and so therefore the activity of the head-immotile flagella fractions supposedly reflects the activity of the heads alone. Since Masaki and Hartree did not investigate the properties of either the heads or immotile flagella alone, this claim is not at all justified. In fact, semen obtained from the same bull was later successfully separated into three relatively clean fractions: motile flagella, immotile flagella, and heads, and assayed for acetylcholinesterase and adenylypyrophosphatase (Nelson, 1966a). These enzymes appeared exclusively in the flagellar fractions. In preliminary tests, head fractions contaminated with immotile flagella gave results similar to those obtained with mechanically disrupted bull sperm. Even though Masaki and Hartree studied the distribution of different substances and activities from Nelson, the implication is clear, as they apparently recognize, namely, that "it is surprising that the heads, being essentially cell nuclei, should exhibit a marked respiratory activity" (Masaki and Hartree, 1962). Tibbs (1962) reported very low acetylcholinesterase activity, about 0.02 and 0.14 mg/gm/hour at 37°C, in the spermatozoa of trout and perch, and this almost entirely in the heads. However, in sonically disrupted bull sperm, the head fraction has less than 20% of the tail acetylcholinesterase activity, which ranges from 170 to 350 mg acetylcholine hydrolyzed per gram of protein per hour at 25°C (Nelson, 1964); while in naturally decapitated sperm the purified head fraction activity was nil, the specific activity of motile flagella was about 180 mg/gm/hour, and that of non-motile flagella over 300 mg/gm/hour (Nelson, 1966a). Cytochemical evidence supports the finding that the heads lack cholinesterase activity (Nelson, unpublished).

Similarly, the heads lack adenylypyrophosphatase, while the specific activity of the motile flagella amounts to about 300 mg ATP hydrolyzed per gram of protein per hour; and of the immotile flagella, a little over 100 mg/gm/hour (Nelson, 1966a). This also confirms earlier electron microscopic cytochemical observations (Nelson, 1962). Mohri (1964b) was able to extract an enzyme from the sperm tails of the sea urchin and the sea robin (teleost) which split only the terminal phosphate from ATP; although he did not indicate whether any of this enzyme could be extracted from the heads, this seems highly unlikely.

The use of cytochemical methods for studying the distribution of

enzymes has proved quite informative (see Fig. 5). Edwards and Valentine (1963), with the electron microscope, and Young and Edwards (1963), with the ultraviolet microscope, used the tetrazolium method to detect succinic dehydrogenase in the middle piece (mitochondria) of rabbit sperm. In neither case did the heads or principal pieces appear to contain formazan deposits. Tetrazolium tended to slow down the movement of the spermatozoa when the concentration of this salt was sufficiently high to produce formazan deposits in the presence of succinate in the medium. Balogh and Cohen (1964) reported that they found di- and triphosphopyridine nucleotide diaphorases, succinic dehydrogenase, lactic dehydrogenase, and malic dehydrogenase and slight amounts of isocitric and glucose 6-phosphate dehydrogenases in air-dried sperm smears. The presence of the enzyme was revealed as finely granular diformazan deposits limited to the sperm midpieces after treatment with nitro blue tetrazolium and the appropriate substrates and cofactors. Since Hrudka (1965) also observed formazan deposits in mammalian sperm midpieces when the nitro blue tetrazolium acted only on endogenous substrates, caution must be exercised in these claims concerning the presence of specific dehydrogenases. It thus appears that many of the enzymes and systems involved in intermediary metabolism may be found in the mammalian sperm midpiece. Mohri and co-workers (1964), in an investigation of the biochemical properties of bull sperm mitochondria, isolated the middle pieces by sonic disintegration and differential centrifugation. The midpieces oxidized succinate and glycerol 1-phosphate at P:O ratios of 2 and near 3 with pyridine-nucleotide-linked substrates; the rate of oxidation was increased upon addition of cytochrome c. Krebs cycle intermediates were also oxidized. In this respect, the sperm midpieces biochemically resemble the mitochondria of "conventional" somatic cells, even though microscopically the sperm mitochondria have undergone extensive morphological modification. However, since the axial filament had not been removed from the midpieces, the fibrous elements cannot rigorously be excluded from possible involvement in biochemical events associated with ATP synthesis. In frozen-dried rat epididymal sperm a number of enzymes appear to occupy the γ -fibers (see Fig. 5). These include (ATPase, inorganic pyrophosphatase, and succinic dehydrogenase (Nelson, 1962). Succinic dehydrogenase appears in the mitochondria of bull sperm (Nelson, unpublished), confirming the observations of Edwards and Valentine (1963), Balogh and Cohen (1964), and Hrudka (1965). Nagano (1964) incubated glutaraldehyde-fixed rat epididymal sperm in ATP at neutral pH, but since he post-fixed his material in osmium tetroxide, he obtained granular deposits at virtually all interfaces in his cross sections. This result may indicate that if the reaction products originally had precipitated at the sites of enzyme

action they would probably have redissolved and redeposited at the various interfaces following osmication. This is an unfortunate limitation of electron microscopic cytochemistry: namely, those procedures which most informatively demonstrate the sites of enzyme action do not yield highly detailed ultrastructural refinements, and those fixation and staining procedures which most beautifully preserve the submicroscopic morphology frequently cause secondary diffusion and redeposition artifacts, making the results cytochemically suspect, if not invalid.

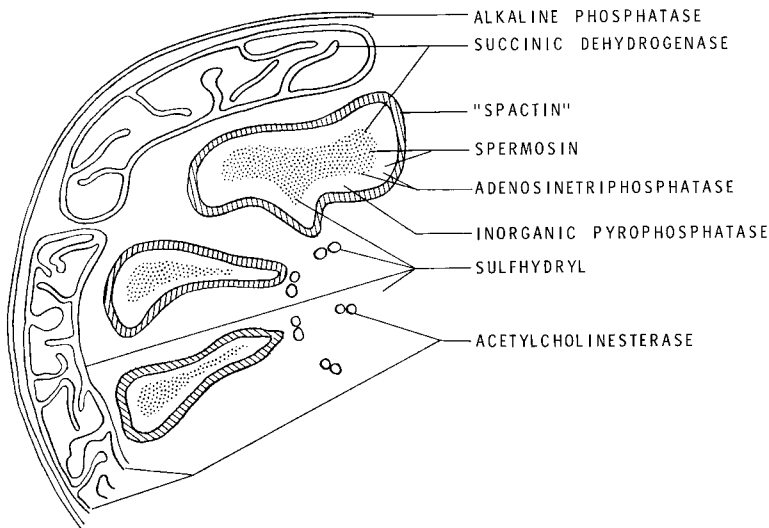


FIG. 5. Summary illustration of cytochemical distribution of biologically important substances in a cross-sectional segment of a mammalian sperm cell.

2. Transport of Energetic Material

The whole problem of the transport of energetic material seems particularly crucial, since the rate of ATP utilization exceeds the rate of ATP diffusion through the principal piece by at least a factor of 3 in mammalian sperm and possibly more in invertebrate-type sperm (by reason of the relatively greater distances from the mitochondria to the distal end of the flagellating system). The calculations, based on the free diffusion of ATP through a column of pure water, may represent an upper limit, since such ideal conditions are not likely to be encountered within the confines of the flagellum. And yet, diffusion is most probably involved; over what distances and from what source, through what interfaces and in what structures may not so readily be resolved. Thus far, ATP seems to be the substrate most immediately associated in the mechanochemical coupling process, and also serves as the cardinal link between spermatozoan motility and the various metabolic processes. The

bulk of the ATP present initially in mammalian sperm is probably formed during spermatogenesis in the testes. Posalaky's (1965) findings "point to a process of functional differentiation evolving at the same time in the middle-pieces, and resulting in the activation within the mitochondria, of the intermediary enzymes of glycolysis and fructolysis." "The content of ATP and with it the motility of ejaculated spermatozoa both depend on the normal metabolism of fructose" (Mann, 1964). Lactic acid may also serve as a substrate under appropriate circumstances, i.e., before access to seminal fructose and after leaving the seminal plasma in the female reproductive tract. A highly significant correlation between the amount of lactate dehydrogenase per sperm and the percentage of motile sperm occurs in fowl semen (Hammond *et al.* 1965). The fructose and the lactic acid, produced exogenously, must, of course, pass through the cell membrane. But so far the cytochemical evidence indicates that the ATP may be regenerated in the same γ -fibers close to where it is utilized in the production of the flagellar wave.

The entrance of hexoses into the bull spermatozoon apparently requires the expenditure of energy. Fructose transport is sensitive to incubation temperature, cold shock, and metabolic inhibitors such as cyanide, iodoacetate, fluoride, and p-chloromercuribenzoate; transport can occur under anaerobic conditions, anoxia stimulating the process (Flipse, 1962a). Flipse has also made use of galactose as an indicator of hexose transport, since even though galactose is not completely metabolically inert, in a short-term experiment the utilization is sufficiently low to permit the measurement of the rate of transport. This transport requires the expenditure of some energy, since it also is sensitive to iodoacetate and mercuric chloride, but remains unaffected by insulin. However, some transport is apparently passive and not energy-dependent (Flipse, 1962b). While these experiments deal nicely with the passage of substrate from the environment into the sperm cell, unfortunately they provide no information about transport within the flagellum, and this problem may have to await the completion of electron microscopic cytochemistry to define the sites of lactic, glycerophosphate and glucose 6-phosphate dehydrogenase activity, currently in process in the author's laboratory. However, Flipse's work may merely indicate that hexose transport is linked to hexose utilization.

IV. Contraction-Relaxation Cycles

A. CONTRACTILE PROTEINS

Suspensions of *Mytilus* sperm tails in seawater or isotonic KCl (0.53 M) readily precipitate out on five- to twentyfold dilution with distilled water, or 10^{-4} M MgCl_2 (Nelson, 1954). ATP slows the rate of precipita-

tion. [On the other hand, ATP hastens the precipitation of diluted KI extracts of starfish sperm flagella, Nelson, 1965b).] Tibbs (1962) indirectly confirmed this fact when he observed that in fresh isolated perch sperm tails, water uptake depends on hydrolysis of ATP. Fluoride, iodoacetate, and pyrophosphate inhibit the uptake of water at concentrations at which these substances become effective as enzyme inhibitors. ADP may substitute for ATP, but ITP, adenylic acid, and sodium phosphate and pyrophosphate will not. Aged sperm tails, while retaining their ATPase activity, do not swell, indicating that the water uptake depends on the structural integrity of the flagellum as well as on the breakdown of ATP. According to Tibbs, there may be a relation between sperm motility and the swelling effect of isolated tails. This relation presumably depends on deformation or configurational changes in ATP-hydrolysis-mediated contractile proteins of the flagella. ATP also causes an immediate decrease in the turbidity of a suspension of isolated *Tetrahymena* cilia. However, destruction of the surrounding membranes by digitonin increases the response to ATP, showing that the ATP acts directly on the (contractile?) protein (Gibbons, 1964). As in the case of the isolated fish sperm flagella, ADP was effective, while AMP, ITP, and pyrophosphate had no effect. In neither instance, and this seems to apply to flagellar systems in general, have the investigators ruled out the intervention of a myokinase-like system which would mediate the reaction:



In fact, Kinoshita (1959) demonstrated the presence of this enzyme in starfish and sea urchin sperm, and Bishop and Hoffmann-Berling (1959) in bull sperm. Moreover, Mohri (1964b) applied the digitonin treatment and alkaline 0.6 M KCl to the sperm tails of sea urchin and sea robin (teleost) and obtained highly active extracts that split only the terminal phosphate from ATP, supporting the view that a myokinase may have been present in Tibbs' and Gibbons' preparations. Incidentally, further extraction of this ATPase with 5% cold perchloric acid yielded a substance (ATP?) with an ultraviolet absorption maximum at 258 m μ . This finding clearly indicates that the extractable contractile protein of the sperm flagella may actually be a complex similar in some respects to the actin-myosin system obtainable from muscle, since ATP-binding is an attribute of the actin moiety of the actomyosin complex. Kelly and Pautard (1965) report that they isolated an ATPase from trout and perch sperm flagella which has the appearance of short rods in the electron microscope. They consider that in the "simple" 9 + 2 flagella of fish sperm the ATPase is probably present in the active free state in the

form of a filament of molecular dimensions, but that the activity, availability, and shape of the molecule may depend on the proportions of nucleotides present. This may serve as the molecular basis for a deformable protein complex configurationally similar to actomyosin.

Brokaw (1965) estimates that the inactive regions of a cilium or flagellum must be very stiff, with an average elastic modulus of 5×10^8 dynes/cm² or more, but that this stiffness must be greatly reduced during active bending. He thinks that the structures responsible for the stiffness may be distributed throughout the flagellar or ciliary cross section. Should such a high degree of stiffness actually exist, passively propagated waves in the inactive regions could not serve as the activating stimulus for active bending, as proposed by Machin (1963). However, Rikmenspoel (1965a,b) calculated the stiffness of the much bulkier bull sperm flagellum to be at least an order of magnitude *less* and to be an attribute primarily of the fibrous sheath. In order to overcome this stiffness (see Nelson, 1962; Fig. 4, p. 177), as well as the viscous drag of the medium, it is necessary to assume active contractile elements in the flagellum which dissipate approximately 70% of the total energy of the system (Rikmenspoel, 1965b).

If the contractile elements of the mammalian sperm are identified with the γ -fibers, the maximum tension developed is within the range of tension developed by skeletal muscle. This, of course, does not imply a direct morphological or chemical identity of the flagellar fibers with the interdigitating filaments of skeletal muscle. It may not be necessary to invoke a sliding filament mechanism for flagellar activity, for even in some striated muscles contraction different from the "classical" model of Hanson and Huxley has been demonstrated. For example, in the horseshoe crab, striated muscles undergo a form of contraction which involves a homogeneous shortening of the A-band (Stephens, 1965a), and while the striated adductor muscle of the scallop *Pecten irradians* contains thick and thin filaments, the A-band shortens from a length of 2.3 to 0.8 μ as the sarcomere shortens from 3.2 to 1.2 μ . (Sanger and Szent-Györgyi, 1965).

The fact that brief trypsinization enables rabbit psoas myofibrils to duplicate the feat of A-band shortening which occurs in glycerinated invertebrate muscle contracted in the presence of ATP does not vitiate the significance of the *occurrence* of A-band shortening (Stephens, 1965b). It may merely shift the emphasis of these physical events to the level of the potential for structural deformability inherent in the molecular members of the contractile system. On the other hand, Satir (1964) claims, on the basis of electron micrographs of lamellibranch gill cilia, that since he can see no filament shorter than others, this should support

a sliding filament model of ciliary movement involving filament-matrix interaction. But since only 3% shortening is required to produce a wave of appropriate radius of curvature for sperm propulsion, this could easily escape notice in electron micrographs.

Burnasheva (1958) has made one of the most significant and dramatic observations on the nature of the contractile protein in sperm flagella. Using conventional means for the extraction of myosin and actomyosin from muscle, she prepared "spermosin" from bull sperm homogenates. Not only does spermosin possess high ATPase activity, but this protein undergoes a reversible interaction with rabbit muscle actin. The resulting synthetic actospermosin exhibits a configurational change in the presence of ATP virtually identical to the sharp viscosity drops induced by ATP in actomyosin sols. The viscosity then reverts to the initial value upon hydrolysis of the ATP by the ATPase activity of the preparation. Burnasheva concluded that since the flagella, even after prolonged extraction (24 hours), yielded only spermosin, which by itself exhibited no viscosity change on addition of ATP, therefore the flagella must lack any actinlike component. However, the important attribute of the spermosin discovered by Burnasheva, its capability of forming a reactive dual protein complex, clearly indicates a structural specificity very similar to the complementary combining sites of myosin for actin.

The γ -fibers of rat sperm flagella have been found to consist of a multicomplex organization of enzymes and "contractile" proteins by means of cytochemical and immunocytochemical procedures. Preliminary observations indicate that the core portion of each of the γ -fibers contains a myosinlike protein, while the cortical region consists of material which appears to be reactive with skeletal muscle actin antibodies (Nelson and Plowman, 1963) (see Fig. 5). Should the contractile mechanism in flagella depend on an actin-myosin type of interaction, contraction could occur as a reversible syneresis or conformational change, since shortening of the order of 3% would suffice to accommodate the radius of curvature of the propagated flagellar wave. In this respect, at least, the flagellar proteins, while necessarily deformable to a degree under specified conditions, need not undergo so extensive configurational changes as those exhibited by actomyosin sols or threads or the proteins comprising the A-bands of invertebrate striated muscle.

Plowman and Nelson (1962) obtained an actinlike component from starfish sperm flagella by conventional extraction procedures. While this protein polymerizes in 0.1 M KCl, contains bound nucleotide, and combines with rabbit myosin, the specific activity of the "flactospermosin" complex as an ATPase did not exceed that of the rabbit myosin alone. This response differs from skeletal muscle proteins in which combination

with actin greatly enhances the ATPase activity of myosin. The isolation and purification of sperm actin is currently being reinvestigated. Pautard (1962) claimed that he was able to obtain from fish sperm flagella an unusual "actinlike" gel that had ATPase activity and contracted rhythmically on addition of ATP. X-ray diffraction patterns of these strange preparations, however, do not seem to resemble those of skeletal muscle actin, nor do actin gels customarily exhibit contractility or ATPase activity. The nature of the contractile protein or protein complex will remain an open question until more direct methods of approaching it become available. The use of digitonin to break down the barriers to solubilization and extraction of these proteins should prove helpful (see Mohri, 1964b). Apparently frozen-dried specimens respond well, since solutions of ionic strengths of 0.6 remove the antimyosin reactive material from rat sperm so treated (Nelson, 1962).

Mohri's work has since been extended. The ATPase isolated from digitonized *Arbacia* sperm flagella hydrolyzes only 1 mole of phosphate per moles of ATP in the presence of a large excess of enzyme. Although intact flagella had ATPase activity, none could be extracted by Weber-Edsall solution without prior treatment with digitonin or freeze-thaw disruption of its combination with flagellar structures. The extract was maximally activated by 1–5 mM Mg^{++} or Ca^{++} at pH 8.6, with the Ca^{++} activation amounting to only 50–70% that of Mg^{++} . The ultraviolet absorption spectrum had a peak at 270 $m\mu$, resembling that of actomyosin. Glycerinated flagella showed three to four times the ATPase activity of intact flagella (Claybrook, 1965). Extracts of starfish sperm flagella isolated by sonic disruption had similar ATPase activity. Digitonin-treated *Asterias* sperm flagella yielded an extract which also exhibited ATP sensitivity, undergoing a sharp drop in viscosity in the presence of 0.2 mM ATP ascorbate and returning to the initial viscosity on hydrolysis of the ATP. The spermosin could be removed by denaturation with alcohol. Dialysis to remove the alcohol yielded a faintly opalescent solution, which sedimented into a clear pellet at high-speed centrifugation. The pellet was soluble in 0.6 M KI, in 0.1 M KCl, and in 10 μM ATP ascorbate. These solutions rapidly salted out, forming a fine colloidal suspension. If the pellet were dissolved in deionized water, the aggregation into a colloidal suspension was delayed, becoming cloudy only after about 30 minutes. These KCl, deionized water, and ATP solutions underwent a marked drop in viscosity in the presence of 0.6 M KI, which could be reversed on dialysis against the ATP ascorbate in a manner that resembled depolymerization and repolymerization, or G-to F-actin transformation. This substance, "flactin," lacked both ATPase activity and ATP sensitivity. The reduced viscosity had a value of just

under 0.2 at infinite dilution, indicating that the protein units within the colloidal aggregates had an axial ratio of about 1:4 (Nelson, 1965b; 1966b). The spermosin, flactin, and flactospermosin were obtained by operational methods developed for the extraction of contractile proteins from muscle. Some of the properties thus far elucidated formally resemble their muscle counterparts. However, in other properties they differ somewhat from their muscle counterparts, including their relation to the fine fibrous structures with which they appear to be associated in the respective cell types. This is not unexpected, since the nature of the flagellar wave and the degree of conformational change which the flagellar fibers must undergo also differ considerably from the situation occurring in the filaments involved in muscle contraction. If an interaction between two proteins mediated by ATP and divalent cations does indeed underlie flagellar movement, it may be more in the form analogous to the bending of a heated bimetallic strip thermoregulator than the interdigitating slide thought to underlie muscle contraction. Until the nature of the interacting components in flagella is further clarified, it is pointless to become exercised over the degree to which a formal similarity to muscle actually exists.

In contrast to the situation in flagella, protozoan cilia yield a single protein with Mg^{++} - and Ca^{++} -activated ATPase activity. Gibbons and Rowe (1965) believe this consists of a 14 S globular fraction, which may form 30 S polymers, and that it is derived from the arms projecting laterally from the 9 α -doublet fibers of the *Tetrahymena* cilia. They also indicate, without citing much pertinent evidence, that this protein, which they call 'dynein,' shows little resemblance to the contractile proteins of muscle, but it closely resembles myxomyosin associated with streaming in slime molds and has some similarity to the protein of the mitotic apparatus. These observations suggest that the molecular basis of ciliary activity may differ considerably from that of flagellar activity in spite of the apparent similarity in their submicroscopic morphology.

B. RELAXATION PHENOMENA

The status of relaxation as an integral element of the contractile cycle underlying the propagated flagellar wave is perhaps even less clear-cut than the contractile protein situation. The occurrence of relaxation phenomena in sperm has already been alluded to above in the work of Kinoshita (1959) and Bishop and Hoffmann-Berling (1959). These workers demonstrated that sperm flagellar models are sensitive to the Marsh-Bendall relaxing factor, and Kinoshita isolated a motility-inducing factor from sperm using the Bendall procedures; Kinoshita (1965) re-

cently found that this factor differs in some of its properties from the relaxation factor. The extracted sperm models may be reactivated in the presence of ATP and Mg^{++} ; but in the presence of the relaxing factor, Ca^{++} (which otherwise has no effect) is required to cause the models to twitch repeatedly. Apparently it is not sufficient to restore this factor to the medium in order to induce the models to swim forward, since the extracted flagella have lost the capacity to coordinate and propagate the undulatory wave, even though repetitive twitching occurs in many spatially disconnected segments. Bishop and Hoffmann-Berling (1959) proposed that during the flagellation cycle, Ca^{++} and Mg^{++} may shuttle back and forth between contraction and relaxation systems. The Mg^{++} is presumed to activate the contractile system and Ca^{++} the relaxation system, as in muscle. On the basis of the observation that Mg^{++} is a strong activator of sperm ATPase (more so than Ca^{++}), and also that inorganic pyrophosphatase has an absolute Mg^{++} requirement in sperm flagella while pyrophosphate may competitively inhibit flagellar ATPase, Nelson (1959) suggested that Mg^{++} alone may serve as a common activating ion alternately switching on the contraction and relaxation phases (see Fig. 6). Pyrophosphate is one of a number of substances which acts as a plasticizer or relaxing agent in glycerinated muscle and could serve to relax the contracted flagellar fibers. The fiber could return to the active state through formation of a contractile protein-Mg-ATP complex, the Mg^{++} having been liberated from the relaxed fiber-Mg-pyrophosphate complex through the enzymic intervention of the Mg-dependent inorganic pyrophosphatase. Electron microscopic cytochemistry has revealed that inorganic pyrophosphatase and ATPase occupy closely associated regions within the flagellar γ -fibers (see Fig. 5), and that treatment with 50% glycerol extracts the inorganic pyrophosphatase without affecting the ATPase (Nelson, 1962). However, as suggested by Bishop (1962b), the pyrophosphate-Mg-pyrophosphatase complex may not by itself constitute the complete relaxation system.

The *Mytilus* sperm pyrophosphatase referred to above exhibited bimodal pH optima at neutrality and about pH 8.6, is Mg^{++} dependent, is inhibited by sulfhydryl reagents, and has a Michaelis constant of 4.3×10^{-5} (Nelson, 1959). Sperm pyrophosphatase of several mammalian species, including human, bull, and ram, has a pH optimum in acetate buffer at 5.6, is not influenced by the absence of Ca^{++} or Mg^{++} , is not affected by $10^{-3} M$ EDTA, is partially inhibited by $10^{-3} M$ mercuric acetate, and has a Michaelis constant of only 4×10^{-3} . This enzyme is concentrated in the sperm cells, but also occurs in the seminal plasma, according to Buruiana and Hadarag (1963). The latter investigators

conclude that osmotic work plays the principal role as the energy source for the rhythmic movement of the male gametes. They feel that the various phosphatases (acid, alkaline, and pyro-) maintain the ionic gradient necessary for this sort of action. Unfortunately, they do not elaborate or offer any substantiating evidence for such a scheme.

The possibility that Mg^{++} ions may exercise a dual role in the relaxation and contraction phases of the cycle is strengthened by the observations of Watanabe and co-workers (1964) that Mg^{++} has a triphasic effect in glycerinated muscle fibers and myofibril suspensions. With in-

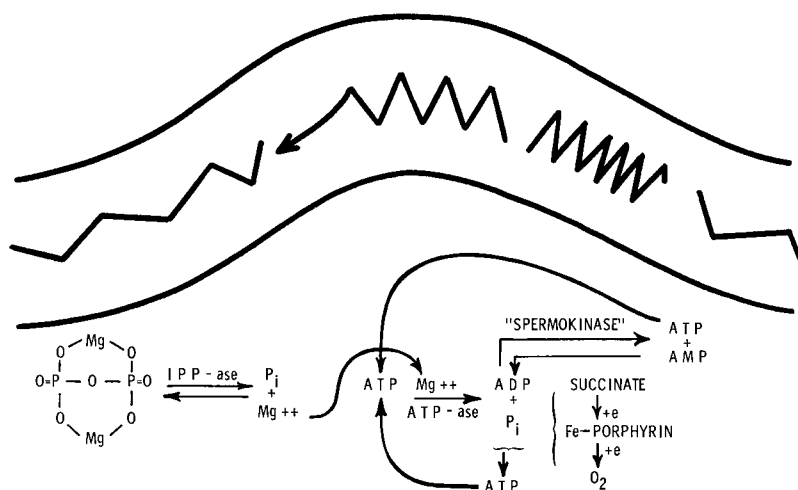


FIG. 6. Scheme of contraction-relaxation cycle in one γ -fiber of mammalian sperm flagellum. From left to right: Mg-pyrophosphate in the relaxed segment going into the active state as inorganic pyrophosphatase dephosphorylates pyrophosphate and freeing bound Mg^{++} , which, in center, serves to activate spermosin ATPase. ATP hydrolysis is associated with a configurational change in contractile protein. "Spermokinase" may cause dismutation of 2 ADP mols to form 1 ATP mol plus 1 AMP mol. ATP also could be regenerated via oxidative phosphorylation in γ -fibers. The presence of large amounts of creatine phosphokinase in seminal plasma (not illustrated) could also serve to regenerate ATP, or resynthesis of Mg-pyrophosphate could initiate the relaxation phase and begin the cycle.

creasing Mg^{++} concentrations up to $25 \mu M$ there occurs an increase in tension; between 25 and $1000 \mu M$ Mg^{++} tension decreases. The third phase, another increase in tension above $1 mM$ Mg^{++} , is attributed to the effect of the small amount of Ca^{++} which is usually present as a contaminant in commercially prepared Mg salts. Watanabe *et al.* (1964) regard the first and second phases as due to a complex of Mg and ATP. Thus reversible complex formation between Mg and ATP or pyrophosphate

may depend on critical concentration levels of the components, and only minor, short-range translocation of the divalent cation could serve to shift the flagellar system from activation of contraction to activation of relaxation phases. Whether enzymic dephosphorylation of the adenylyl- and inorganic pyrophosphate at the critical Mg^{++} -level constitutes a self-contained and self-limiting regulatory system has not been determined; nor in fact has the intracellular Mg^{++} concentration been determined.

C. COORDINATION OF THE FLAGELLAR WAVE

Uncoordinated or random twitching of reactivated sperm models does not lead to effective propulsion, indicating that coordination must be superimposed upon, or intrinsically incorporated in, the periodic cycling of alternate contractions and relaxations. Speculation centered on the presence and possible role of a conductile system in sperm has received added impetus from observations on the submicroscopic morphology of flagella. Sets of fibers remain to which no function based on physical or chemical procedures has been ascribed, although Bradfield (1955) suggested that the β -fibers, the inner pair, may function in a conductile system in cilia, while the γ -fibers could have evolved a conductile function in mammalian sperm flagella, conveying impulses arising from the proximal centriole. Bradfield based some of these intriguing conclusions on the stability, relative extractability, and spatial relationships of the fibrous components; these may still serve as a rational base from which to approach the problems of flagellar contraction and conduction. Conduction, coordination, and even automaticity have traditionally been associated with neurohumors, related substances, and associated enzymes.

Bulbring and co-workers (1953), reporting the effects of acetylcholine, eserine, atropine, curare, and adrenaline on ciliary movement in the gill plate of *Mytilus edulis*, concluded that acetylcholine, as well as serving as a humoral transmitter of nerve impulses, may also function to control rhythmic activity in various tissues, for the performance of which it is produced and destroyed locally. Sekine (1951) found that boar spermatozoa had cholinesterase activity nearly equal to that of brain tissue, although Tibbs (1960) found only small amounts of acetylcholinesterase in fish sperm, and that primarily in the heads. On the other hand, Applegate and Nelson (1962), studying *Mytilus* sperm, observed that $5 \times 10^{-5} M$ eserine increased the rate of flagellation and that a fairly high acetylcholinesterase activity occurred in the tail fraction, with little in the heads. Bull sperm flagella were also highly active enzymically and preferentially hydrolyzed acetylcholine over benzoyl and butyryl choline (Nelson, 1964). Electron microscopy has revealed

that the acetylcholinesterase (thiolacetic acid used for the cytochemical assay; [Nelson, unpublished]) is almost exclusively flagellar in its distribution, occurring just below the plasma membrane in both middle piece and principal piece in bull sperm (see Fig. 5). The proximal centriole appears to be negative, although the central pair of β -fibers occasionally show some slight cytochemical reactivity. The heads are negative, a fact which is substantiated by *in vitro* assays on heads obtained from naturally decapitated bull sperm (Nelson, 1966a). The significance of these findings in terms of conductile mechanisms can be judged only by extrapolation from the role of acetylcholinesterase in neural tissue. Further investigation on the effects of acetylcholine and cholinesterase inhibitors on sperm motility is required.

V. Extraneous Chemical Factors

It is apparent, however, that if intrinsic mechanisms within sperm and their flagella regulate the motile activity, these physical-chemical mechanisms are capable of adjustment or response to extrinsic physical and chemical factors. An often-repeated observation indicates that near-neighbor sperm cells will assume a synchronous rhythm under appropriate conditions. Machin (1963) postulated that waves of bending will arise spontaneously if, in a flagellum, changes in the length of its contractile elements cause changes in tension *after a delay*. The properties of the contractile elements must be nonlinear if the amplitude of these waves is to remain finite. According to Machin, this nonlinearity has two consequences: (a) control of the frequency and direction of propagation of the waves can be exercised from the proximal end of the flagellum; and (b) two nearby flagella will synchronize, beating with a common frequency and wavelength. Periodic active bending, however, must occur all along the flagellum in order to maintain a constant amplitude of the bending waves.

The synchrony of flagellar action extends to more than just one neighbor, to the degree that wave formation described by Reynolds and Rothschild (1963) leads to stirring of the medium such that "augmented diffusion" of oxygen occurs. This short-range order occurs even at comparatively low sperm densities (in bull sperm at dilutions of 1/8–1/20), producing group sperm velocities greater than those of isolated spermatozoa. "Viscous interactions which predominate in a sperm suspension take place over greater distances than inertial interactions, which is another reason why, at these sperm densities, spermatozoa may be expected to influence each other's motions and not be distributed at random" (Reynolds and Rothschild, 1963). However, a reduction in temperature or further reduction in sperm density decreases the sperm order.

Sea urchin sperm, on the other hand, rarely seem disposed to move in unison with their neighbors and at high population density tend to remain quiescent.

A. DILUTION EFFECTS: O₂, CO₂, pH

Oxygen uptake and rate of motility of sea urchin sperm increase on dilution, but viability decreases. This so-called "dilution effect" has been the subject of much study and speculation, summarized by Rothschild (1961) as possibly involving O₂ saturation, trace metals in seawater and their putative uncoupling effects, chelating agents, and the effects of pH change on sperm activity. He concluded that of these pH of the medium may be most important, since a small change may cause a marked increase in O₂ uptake. A protracted lack of oxygen inhibits movement. Furthermore, the CO₂ inhibition of sea urchin sperm motility parallels the pCO₂, and, according to Mohri and Yasumasu (1963), any level of CO₂ above 1%, both in oxygen and in air, inhibits O₂ uptake. Introduction of oxygen or air completely reverses the inhibitions. The inhibitory effect of CO₂ far exceeds that caused by a drop in pH when the pH of the seawater is stabilized between 8.50 and 6.75 with histidine buffer. The fertilizing capacity of the *Arbacia* sperm, however, may be extended by suppression of motility with increased CO₂ or lowered pH (Branham, 1964a). Vertebrate spermatozoa also became reversibly inactivated under anaerobic conditions. Fowl sperm agglutinate on inactivation and disperse on reactivation, and bull sperm resume motility immediately on aeration. Schindler and Nevo (1962) reported that inactivation takes from 2–30 minutes, depending on "vigor" and dilution, and is inversely related to the degree of motility and directly related to the oxygen and sugar content of the medium. In the absence of reducing sugar, motility is aerobic obligatory. Motility declines sharply and rapidly when bull semen is gassed with CO₂ at 37°C; recovery occurs with O₂ gassing, although the extent of immobilization and recovery depends on the medium, that is whether the sperm cells are suspended in seminal plasma, the nondialyzable fraction of seminal plasma, or saline (Vandemark and Koyama, 1963). On the other hand, while bubbling undiluted boar semen with CO₂ reversibly inactivates the sperm cells, treatment with egg yolk citrate increases the number of progressively motile cells (Liess and Grove, 1963). Bull sperm in phosphate-buffered saline had initially higher rates of motility at pH 8.5 than at pH 6.5, although the rate dropped more rapidly at higher than at lower pH.

At pH 8.5 after 1 hour, most sperm cells were twitching or circling rather than swimming in a straight line, although in other buffers and saline all motile cells swam in straight lines. In uterine cervical mucus at pH 8.4, the sperm swam at 60 μ /second; at this rate the sperm should

arrive in the neutral pH uterus in about 30 minutes. Fowl sperm maintained *in vitro* under anaerobic conditions in a pH-stat initially have a high acid production, which gradually declines to a constant rate over a $1\frac{1}{2}$ -2-hour period. This coincides with a drop in motility which subsequently remains constant for 40-50 hours, after which the motility drops to zero in 5-10 hours, while metabolic activity continues for at least 20 hours after motility has ceased. While the metabolic rate decreased to one-quarter, motility decreased to one-half. Assuming that total physical activity (number of motile cells multiplied by average mechanical energy expended by moving sperm) decreases by one-half, Nevo *et al.* (1963) inferred that a constant fraction of metabolic energy is converted to mechanical energy.

The cow cervical mucus seems to afford some protection from harmful effects of dilution which occur even with seminal plasma as diluent or with balanced salt solutions and the supernatant from a saline suspension of unwashed sperm cells. The cow cervical mucus, which dead sperm cannot penetrate, may afford some protection from the usually harmful effects of dilution due to a physical constraint restricting dispersion of the sperm cells and thereby confining them to narrow strands (Tampion and Gibbons, 1963b). These workers also reported that 20,000 sperm cells per cubic millimeter seemed to be a limiting density, since at higher concentrations the sperm jostle each other, thereby slowing down linear progression; at lower concentrations the dilution effects become apparent with decrease in swim rate and increase in percent of nonmotile cells. Wales and White (1963) found that the viability of diluted dog sperm depends on the presence of proteins (bovine globulin, casein, egg albumin) and alanine, and that in the presence of these substances, which prevent the harmful dilution effect, the motility is as good as in concentrated suspensions. Accessory gland secretions (e.g., prostatic fluid), seminal plasma, or "overnight sperm water" improved the viability of diluted rabbit, ram, human, cock, and bull sperm. Since the unrelated high molecular weight substances seem to protect the diluted cells from the detrimental effects of dilution, Wales and White (1963) concluded that this effect is due to dilution of the seminal plasma as well as loss of substances from the cells, suggesting a physical rather than a chemical protective action. The loss of K^+ and Mg^{++} by washing and dilution may be retarded by casein, acacia, alanine, and serine, while prostatic fluid, bovine albumin and globulin, and egg yolk are ineffective if no K^+ or Mg^{++} are added. The high molecular weight substances may also prevent inactivation of enzymes by dilution.

B. CATIONS

The role of K^+ in sperm motility has so far not yet been completely

and unequivocally elucidated. Salisbury and Graves (1963) prepared "epididymal-like" bull sperm, "free of substrate," by massive dilution during collection, in a citrate-bicarbonate buffer medium with a K:Na ratio of 3:1; this ratio is similar to the K:Na ratio occurring in the testes. Under these conditions the spermatozoa remain immotile as long as they are kept anaerobic in a 100% CO₂ atmosphere. The authors believe that the high K⁺ content of this diluent inhibits motility by decreasing fructose uptake and thereby inhibiting ATP synthesis, with resultant loss of motility.* Dott and White (1964), however, observed that *removal* of potassium also causes a decrease in motility and impedance change frequency in ram spermatozoa. Unless the phosphate or bicarbonate diluents contained at least 0.004 M K⁺ in semen diluted 1:100, the ram spermatozoa became immotile within 3 or 4 hours at 37°C. The greater the degree of dilution, the greater the need for K⁺ in the diluting fluid to preserve the motility of the spermatozoa. Assuming that sperm obtain the energy for motility in any of three ways: anaerobic breakdown of fructose to lactate; aerobic oxidation of lactate via the Krebs cycle; and aerobic breakdown of fatty acids formed from endogenous plasmalogen, potassium depletion must critically affect the ability of ram spermatozoa to produce or make use of energy from the oxidation of exogenous substrate. Unless K⁺ were present, the impedance change frequency of samples in substrate (acetate, fructose, or lactate) fell below that of the sample without substrate, but when K⁺ was present, fructose, lactate, and acetate maintained good activity for prolonged periods. Salisbury and Graves (1963) further suggested that the high K⁺ content of their diluent inhibits motility through its inhibitory action on spermatozoan ATPase, citing as evidence Tibbs' (1959) work on perch sperm flagellar ATPase. Unfortunately, this is an overstatement of the case, since treatment of the perch sperm flagella with 1 M KCl reduced the Mg⁺⁺-activated ATPase activity from about 224 μg P liberated/ml flagellar suspension/hour to 144 μg P/ml/hour and the Ca⁺⁺-activated ATPase from about 64 μg P/ml/hour to 56 μg P/ml/hour. But, "In neither case was it anywhere near abolished" (Tibbs, 1959). To complicate matters still further, O'Shea and Wales (1964) reported that potassium (16-64 mM) adversely affects ram sperm motility during cooling from 30°C down to 5°C, while magnesium is beneficial, and that as the sodium content and the Na:K ratio of the diluent increased, the depression caused by high K⁺ slightly improved sperm motility during cooling, but there was an interaction between potassium and phosphate which resulted in an increase in the detrimental effect of potassium with the

* Salisbury and Graves (1963) neglect the fact that CO₂ abolishes mammalian sperm motility. The relevance of K⁺ to the inhibition of sperm motility may be open to question.

increase in phosphate. When Tris was substituted for phosphate buffer, the potassium effect was virtually eliminated; veronal buffer restored the deleterious potassium effect. The multifarious phenomena suggest that high intracellular K^+ levels are responsible for the adverse effects caused by the presence of K^+ in the medium. Since Tris penetrates human red cell membranes (Omachi *et al.*, 1961) and also competitively interferes with K^+ uptake by bacteria (Macleod and Onofrey, 1954), O'Shea and Wales (1964) interpret their observations as indicating a possible decrease in K^+ uptake induced by Tris, which decreases the adverse K^+ effect during chilling. They further suggest that miscellaneous high molecular weight substances protect the spermatozoa against the detrimental effects of excessive dilution and washing (see above) by the physical action of preventing the loss of intracellular materials such as potassium and magnesium. From all this it is evident that the intracellular content of potassium must be maintained at some critical level for optimum sperm motility and that increase or decrease in this level beyond certain undefined limits directly or indirectly exerts an adverse effect on sperm motility. The mechanism of action of K^+ sensitivity and the specific K^+ -dependent system, possibly an enzyme or a structural protein, remain to be worked out. In this connection it is worth emphasizing that not all species exhibit identical K^+ responsiveness. While *Mytilus* sperm washed in filtered seawater and resuspended in isotonic (0.53 M) KCl not only show no loss of ATPase activity but the intact sperm continue to swim unperturbedly for at least 30 minutes or longer (Nelson, 1955), *Arbacia* sperm cells rapidly become inactivated in isotonic KCl (Steinbach, 1964, personal communication). Furthermore, $\frac{1}{2}$ M and 1 M dimethyl formamide and acetamide in seawater markedly and reversibly accelerate *Arbacia* sperm motility and O_2 consumption for several (up to 7) hours. The same treatment markedly and rapidly reversibly decelerates *Mytilus* sperm. Preliminary experiments indicate that potassium loss may accompany this exposure to higher concentrations of the formamide and acetamide (Steinbach and Ortiz, personal communication). Watanabe and Maruyama (1964) reported that formamide and dimethyl formamide, as well as acetone, caused reversible relaxation in glycerinated rabbit psoas muscle fibers that had been subjected to Mg-ATP-induced contraction. Formamide was also very effective in "clearing" actomyosin suspensions, a phenomenon which purportedly corresponds to relaxation of glycerinated muscle fibers. The "clearing" effect and relaxation depend on the presence of divalent cations. Both inorganic pyrophosphate and ITP can substitute for ATP, and Ca^{++} can substitute for Mg^{++} when the organic solvents are used to induce relaxation. The fact that ITP can substitute for ATP in this relaxation suggests that the carboxyl group of the organic solvents acts by bringing about a

local conformation change on the contractile protein rather than acting on the amino group of the ATP. Moreover, the effective concentration of Mg^{++} also depends on the concentration of KCl. Thus, even model systems which are presumably free of permeability barriers are influenced by the presence of potassium. One of the first steps to be taken in the elucidation of the role of potassium in sperm motility is to measure the K^+ distribution and its possible involvement in ionic gradients. For most cells ionic gradients of K^+ internal $>$ K^+ external and Na^+ external $>$ Na^+ internal exist (Steinbach, 1952). Sperm of *Arbacia*, *Mytilus*, and *Phascolosoma* resemble other cells in maintaining, relative to their seawater environment, a high internal K^+ and low Na^+ and Cl^- (Steinbach and Dunham, 1961). The head-midpiece fraction of these invertebrate sperm retains the ionic selection properties of the whole sperm, while the isolated tails appear freely diffusible to all constituents. With the use of radioactive tracers, Steinbach and Dunham showed that the Na^+ and K^+ of the sperm are freely exchangeable with the environment and could find no significant amount of K^+ sequestered within the sperm or otherwise made inaccessible to free exchange. While ionic gradients have generally been associated with cell irritability and impulse conduction, "it has been very difficult to decide just what physiological function causes the gradients and which functions depend on them" (Steinbach and Dunham, 1962). They suggest, however, that the contractile organelle of the sperm cell is dependent for its presumed ion gradients on ion-concentrating mechanisms which may be localized in the midpiece mitochondria. Thus it seems possible that the normal coordinated undulatory activity may depend on ionic gradients imposed on intact flagella by the metabolic activity of the midpiece (Steinbach and Dunham, 1961), while in glycerinated sperm models such control is lacking. It would be interesting and possibly highly informative if it were possible to measure membrane potentials and their changes in single sperm cells.

Sodium occupies the role of the major bulk cation in maintaining favorable osmotic conditions in seminal plasma in mammals (and, of course, in the seawater in which the sperm of marine invertebrates maneuver). No similar amount of information appears to be available concerning the role of sodium ions per se in sperm motility except for the general and frequently repeated observation that media with Na:K ratios higher than 1 more favorably support motility than those in which the situation is reversed. In fact, isotonic NaCl appears to support sperm motility satisfactorily over fairly prolonged exposures for some species, while for others the absence of K^+ renders such a solution toxic. The high K^+ content of accessory gland secretions seems to suppress motility, or at least to conserve the energy resources of the spermatozoon by maintaining the cells in a quiescent state (see Salisbury, 1962; Mann,

1964). Mg^{++} serves as an essential cation in the ATP reactivation of glycerinated models of sperm from species ranging from locust, sea urchin, starfish, and squid to rabbit, rat, guinea pig, mouse, bull, and man (Hoffmann-Berling, 1959; Kinoshita, 1959; Bishop and Hoffmann-Berling, 1959). Mg^{++} shares with Ca^{++} and in some cases Mn^{++} and Zn^{++} the role of activator of flagellar ATPase. Higher concentrations of Zn^{++} may block sperm ATPase. Zinc has also been described as an *in vivo* inhibitor and regulator of starfish sperm motility in a series of papers emanating from Fujii's laboratory. The affinity of zinc for spermatozoa has recently been stressed by Rice (1964), who found pronounced morphological changes in human sperm tails following treatment with dialkyldithiocarbamates. This potent spermicidal effect may involve the formation of crystalline Zn dialkyldithiocarbamate lipoprotein complexes. Chelating agents such as EDTA inhibit the formation of these crystalline complexes. However, Zn^{++} has not so far been implicated as a controlling factor in the motility of human or other mammalian sperm.

Calcium and magnesium and other divalent cations play an unusual role in flagellar movement of *Chlamydomonas snowiae*. These ions reverse and prevent the inhibition of motility induced by ATP and dinitrophenol. The inhibition may accompany coiling and detachment of flagella from the algae. Salyrgan also causes flagellar detachment, however, in a rigid extended state. Marcus and Mayer (1963) report further that ADP and AMP in the same concentrations as the ATP have no effect on flagellar movement. Restoration to the normal medium permits the algae to regenerate flagella.

C. OSMOTIC EFFECTS

Coiling or spiralization also occurs in mammalian sperm flagella, but in hypotonic media. Drevis (1963) reports that up to 80% of bull sperm cells undergo flagellar spiralization when placed in calcium-free Ringer solution diluted with four volumes of distilled water. Initially the treatment causes very intense swimming but abolishes rotation around the longitudinal axes, which is then followed by progressive curving and bending of the tails. If within 30–45 minutes the sperm are restored to isotonic Ringer solution, despiralization occurs and "good" swimming results. Swelling alone is not the major factor, since spiralization occurs in the 20% Ringer solution to which polyvinyl pyrrolidone or dextran was added to maintain isotonicity or even hypertonicity. Repeated washing, cold shock, ageing, and storing prevent spiralization, as does pretreatment with millimolar salyrgan or *p*-chloromercuribenzoate. Moreover, the sulfhydryl reagents cause despiralization in hypotonic media, while cysteine abolishes the effects of these agents. Isotonic Ringer

solution containing 15–20% glycerol evokes bending and curving, but without the tight coiling. Drevius (1963) supposes that spiralization caused by hypotonicity is a result of a flux of ions or other low molecular weight substances from the flagella into the medium, causing disturbances in contraction-relaxation mechanisms. However, instead of testing the validity and details of this hypothesis, Lindahl and Drevius (1964) extended their observations in an attempt to establish a relation between the coiling and motility mechanisms of normal sperm. Unfortunately, the unphysiological conditions needed to achieve these interesting effects do not seem to provide a sound basis for the elucidation of such an apparently sensitive physiological process. The “primary” bending in the proximal part of the hypotonic bull sperm flagellum toward the side with large γ -fiber no. 1 appears in about two-thirds of the identifiable fixed cells, with only one-third bending toward fibers 5 and 6. This signifies to Lindahl that the plane of bending toward fiber 1 in normal swimming occupies a period of time about twice as long as toward fibers 5 and 6. In fact, this would mean that the greater pull would be in the direction of the hemisphere containing only three γ -fibers (9, 1, 2) and away from the hemisphere containing four γ -fibers (4, 5, 6, 7), the plane of bending passing through both β -fibers and roughly through γ -fibers 3 and 8. This strains, among other things, the capabilities of the flagellar components. The only substantiating evidence to support these interpretations of these somewhat bizarre events is the not unequivocal observation, based on this same material, that the last γ -fibers to terminate in the most distal part of the tail are 5, 6, 9, and 1, in that order. The distance over which this effort must be transmitted could easily vitiate the presumed advantage ascribed to the longest fibers, 9 and 1. Lindahl and Drevius (1964) also regard the presence of two limbs of the bent flagellum within a single encompassing set of membranes as a fixation artifact. While this may very well be the case, a number of investigators have also observed that marine invertebrate sperm osmotically shocked in hypotonic seawater exhibit tight coiling of the axial filament complex within the swollen but uncoiled outer membranous sheath. This entire phenomenon deserves careful reexamination in light of the effects of media made hypertonic with glycerol (Drevius, 1963), dimethyl formamide, and acetamide, mentioned previously. Moreover, present-day technology for “extending,” chilling, and freezing mammalian and domestic fowl semen for storage and artificial insemination includes in the protocols the addition of such empirically protective substances as glycerol, dimethyl sulfoxide, and ethylene glycol. (For the history of these developments see “Biological Effects of Freezing and Supercooling,” [Smith, 1961], as well as numerous agricultural, dairy, and poultry journals.)

D. BODY FLUIDS, EXTRACTS, ANTIBODIES

In addition to chemically identifiable inorganic as well as organic substances, a heterogeneous assortment of natural biological fluids and extracts affect sperm motility. In 1913, F. R. Lillie observed that egg extracts activated the motility of marine invertebrate spermatozoa as well as first causing their agglutination. (See "Chemistry and Physiology of Fertilization [Monroy, 1965] and Chap. 5 of this volume for critical reviews and evaluations of the fertilizin concept.) Fresh *Arbacia* sperm placed in *Arbacia* egg water from which particulate matter had been removed by centrifugation agglutinate, and after 2-3 minutes the clumps disperse. The middle piece undergoes an "explosive expansion. . . . The dense packing and ordered arrangement of membranes within the mitochondrion are destroyed, leaving only membranous remnants" (Bernstein, 1962). These spermatozoa, which were moderately active in seawater, became intensely motile when mixed with egg water (jelly substance, or "fertilizin"), water from jellyless eggs, and acid-alcohol extracts of the egg (Hathaway, 1963). Hathaway states that these substances and cysteine in concentrations which had no effect on motility stimulate respiration of the spermatozoa. Bernstein (1962) attributes these effects to a surface-active ingredient in the egg water. If Bernstein and Hathaway are dealing with the same substances, it is rather remarkable that motility should increase following destruction of the mitochondrion, assuming, of course, that the mitochondrion in the sperm is the source of all good, as it is reputed to be in more conventional cell types.

The spermatozoa of a number of fishes appear to be attracted and stimulated to increased activity around the egg micropyle of bitterlings and fat minnows. The sperm-stimulating factor is not species specific, but activates heterologous sperm from fishes of different genera and even of different families (Suzuki, 1958, 1959). On the contrary, a stimulating substance isolated from the female gonangium of *Campanularia calceolifera* activates and attracts homologous sperm only, and is without effect on the sperm of *C. flexuosa* (Miller, 1964). Whether these substances are truly chemotactic or primarily trapping agents remains to be more clearly established.

The seminal plasma also contains a variety of substances which may or may not contribute to the maintenance of sperm motility. Part of this fact may be attributed to the effect of dilution of the densely packed and relatively quiescent epididymal sperm and part to the individual constituents contributed by the male accessory glands (see Mann, 1964). Clinical studies on semen samples from a limited number of oligospermic patients have shown that their seminal plasma is inhibitory to

normal sperm, while normal seminal plasma increases the motility of oligospermic sperm (Rozin, 1960). Blood plasma and serum also stimulated the motility of oligospermic sperm when removed from its seminal plasma (Rozin and Rozin, 1962). Rottenstein and co-workers (1960) claim that heating bull seminal plasma to 80°C improved its capacity to maintain motility, and that seminal plasma alone or mixed 50/50 with a citrate-glucose diluent is a better extender than the citrate-glucose alone. They also claim that leakage of unspecified substances from the bull sperm cells improves the "quality" of the seminal plasma in supporting motility of fresh sperm. The significance of such observations could perhaps be enhanced if some of the unspecified factors were identified, the conditions of the experiments standardized, and the mechanisms of their action determined. The use of serum or plasma as a sperm diluent has a fairly long history, which has recently been reviewed by Tyler and Bishop in Chapter VIII of Hartman's "Mechanisms Concerned with Conception" (1963). Sera of many species of animals contain autoagglutinins and heteroagglutinins which secondarily may immobilize sperm cells. In a few circumstances, primary immobilization appears to occur (Edwards, 1962), though this may be a prelude to the death of the cell. In Chang's experience (1947), an unstable, thermolabile, nondialyzable substance, similar to complement, present in fresh sera of a number of mammals killed most of the exposed sperm. He found a stoichiometric relation between the amount of serum substance and the number of sperm affected. All of the substance could be absorbed from the serum, so that spermatozoa subsequently added to the supernatant remained motile and apparently unaffected. Moreover, the material would not readily desorb from the immobilized sperm, suggesting a virtual covalent type of interaction with a structural component of the spermatozoon. On the other hand, Edwards (1960) could not find immobilizing antibodies in normal rabbit sera, but after injection of rabbit semen plus adjuvant he obtained high titers in the sera of two out of five treated rabbits and none in the remaining three. While epididymal spermatozoa were immobilized more slowly than unwashed ejaculated sperm, the final titers were similar. This behavior was somewhat unexpected, since Weil (1960) found that rabbit epididymal spermatozoa lack antigenic material present in seminal plasma. Presumably the sperm cells can take up and firmly bind the antigenic seminal plasma material which originated in the accessory gland secretions.

Pernot (1956) tested the antigenicity of sonically disintegrated guinea pig sperm tails and found seven components, six in common with seminal plasma antigens plus one specific guinea pig sperm component

which may also occur in the heads. The latter antigen was highly tissue specific for the guinea pig, although no mention is made of possible relations of the antigens or the antibodies to sperm motility. Katsh and Katsh (1964) almost invariably obtained seven distinct precipitin bands in rabbit antisera to guinea pig spermatozoa when tested against sonically disrupted guinea pig sperm suspensions. Willson and Katsh (1964a,b) attempted to localize the antigenic sites by using labeled antibodies. Ferritin-labeled antibodies adhered externally to the head, midpiece, and tail plasma membranes as homogeneously electron-dense material in unequal distribution along the cell surface, but apparently did not penetrate the cell membrane. Fluorescein isothiocyanate-labeled antibodies stained the acrosomes brilliantly and the midpiece and principal piece less intensely. Small clumps of fluorescent material adhered to the cell membranes of the head, midpiece, and tail, including the kinoplasmic droplets. Unfortunately, in none of these experiments, either, was observation made of effects on sperm motility. Barth and Russell (1964) were unable to demonstrate sperm-agglutinating or immobilizing activity in sera obtained from male and female mice immunized with mouse sperm, even in the presence of guinea pig serum; the mouse sera contained acrosome-specific antibodies.

Other types of flagella or cilia possess antigenic substances which induce immobilization antibodies. In the case of *Paramecium*, immobilization appears to occur as a consequence of ciliary agglutination. According to Preer (1959), Antigen 51 A, a large asymmetric protein of about 240,000 molecular weight is a major constituent of the cilia. He theorized that the immunization antigens are fibrous proteins associated with ciliary function. The work of van Wagtenonk and Vloedman (1951) lends substance to this notion, since they extracted a protein from paramecia with antigenic reactivity for 51 A antiserum and which splits the terminal phosphate from ATP. Isolated cilia were not used for this extraction, which leaves the origin of this protein a little obscure. Nevertheless, it seems unlikely that a ciliary fibrous protein ATPase would of itself exhibit the strain specificity attributed to the *Paramecium* antigens. As in the case of Chang's (1947) spermatozoa, during the period of immobilization the paramecia removed antibody from solution; they also accumulated lumps of gelatinous material, especially at the ciliary tips. However, the proximal region of the cilia are free and actively moving, so that the adherence at the ciliary tips mechanically interferes with effective activity of the cilia, thus resulting in the immobilization. Recovery is possible provided the serum treatment has not been too severe (Beale, 1957). The cilia and other surface structures appear able to shed the antigen-antibody complex and resume normal activity.

Tetrahymena and *Colpoda* reportedly exhibit an even more dramatic escape from an antigen-antibody shell.

In general, marked species differences occur in the antigenicity of spermatozoa. So far, however, the sperm-immobilizing antibodies have not yielded much information concerning motility mechanisms. Metz and his associates (personal communication) described several components in rabbit anti-sea urchin sperm sera which correspond to subsurface as well as surface antigens in the spermatozoa. Unfortunately, a preliminary attempt to identify some of these antigens with a sperm extract possessing ATPase activity has proved inconclusive (see Chap. 5 of this volume for a more detailed account of gamete immune mechanisms). The simplest explanation for this apparent multiplicity of effects is that antigens covering a critical amount of surface area may absorb enough antibody to mechanically block and prevent free interchange of essential substances between the cell and the environment. Perhaps a critical test for immobilization as an immunochemical phenomenon distinct from agglutination or cell death would be to reverse the antibody-induced cessation of motility, or reversibly to block the process by means of a sperm extract specifically acting on the immobilizin. This could possibly be achieved by the Coons-Coombs procedures using the immobilizin itself as an antigen for the production of an antiimmobilizing antibody. Mittal *et al.* (1963) studied the metabolic effects of rabbit anti-bull semen sera and found very marked depression of such parameters as oxygen uptake, fructose utilization, and lactate production, along with virtually complete inactivation of motility and reduction of numbers of living sperm from 61.5% to 3.1%. Total metabolic activity decreases in antiserum-treated cells, apparently because of the death of the cells, following strong agglutination between the spermatozoa and the antiserum antibodies (Mittal *et al.*, 1965). It is quite apparent that the contractile components of living sperm are no more readily accessible to inward diffusion of the antibodies than those of living isolated muscle. Isolated frog sartorii continue to contract in both preimmune rabbit sera and in rabbit anti-frog actomyosin sera after exposure for 30 minutes to 12 hours (Nelson, unpublished). On the other hand, as mentioned above, frozen-dried rat sperm react immunocytochemically with anti-rat actin and anti-rat myosin sera. It would also be well to test immobilizin for inhibitory effects on specific enzymes associated with sperm motile mechanisms.

E. HORMONES, ANTIBIOTICS, VITAMINS

A number of hormones tested on spermatozoa of a variety of species appear to exert their primary effects on the metabolism and secondarily

on motility, if at all. Scott *et al.* (1963) found that while ram, bull, dog, and cock semen oxidize between 5–10% of the testosterone in the medium to androstenedione, the testosterone had no significant effect on oxygen uptake or glucose oxidation to CO_2 . Mounib (1964) found, to the contrary, that steroid hormones, especially progesterone and testosterone, suppressed bull sperm oxygen consumption, but led to a rise in glucose and pyruvate utilization and lactate production. This led Mounib to the interesting conclusion that the steroid hormones may play a direct part in regulating the spermatozoon metabolic differentiation associated with the shift from the epididymal to the ejaculated and ultimately the capacitated pattern. Since mammalian spermatozoa undergo a series of morphological, permeability, and motility changes during their progression from the testis through the epididymis, the hormonal interactions governing metabolic shifts take on particular significance. For instance, if parts of the rabbit epididymis are ligated for a week and a half, the capacity for *in vitro* motility increases, eosinophilia decreases, and the kinoplasmic droplet migrates and may become swollen and fragmented. Moreover, the proportion of sperm cells with coiled tails increases. After two weeks, progressive degeneration sets in. During this period, fluid accumulates anterior to the ligature, and so the altered environment could be largely responsible for the motility and morphology changes (Gaddum and Glover, 1965). The composition of the accumulated fluid should be examined for clues to the responsible agents, whether hormonal or otherwise.

Perez y Perez (1959) claims that relatively low concentrations of dienestrol (500 U/4 ml semen) caused an increase in bull sperm motility lasting 10–48 hours after addition to the semen. By 60 hours the motility dropped below that of the control sample. He suggests that the dienestrol mobilizes stored reserves, increasing motile activity, but ultimately exhausting the stores, reducing *in vitro* survival. Recently Conti and Cresseri (1963) claimed that at alkaline pH both chorionic and pregnant mare serum gonadotropins increase the motility of amphibian sperm. This effect apparently partially reflects the enhancement of the biological activity of these hormones (increased weight of rat uteri) at high pH (see Umberger and Gass, 1959; Persson and Melander, 1960). However, in an earlier paper, Cresseri and Conti (1962) reported that frog sperm motility was sustained longest in half isotonic NaCl at neutral and slightly alkaline pH. Examination of the data suggests alternatively that since the hormones at 100 U/ml had no significant effect at pH 5.5 and 7.0, they may have somewhat of a nonspecific protective action at pH 8.5 compared with the buffered und unfortified saline alone.

Antibiotics, which must be added to suppress bacterial growth during

metabolic and other studies, likewise may or may not affect the motility of spermatozoa. Antimycin A, which is known to block electron transport at the cytochrome level, begins to inhibit dog sperm motility at a concentration ($2 \times 10^{-8} M$) which has no detectable effect on respiration (Gonse, 1962). Bacitracin and spiramycin reduce human sperm motility, while penicillin, streptomycin, Ledermycin, and terramycin (0.05 mg in 0.1 ml saline) had little effect (Schirren, 1963). According to Wales and White (1960) seminal plasma reduces the adverse effects exerted by certain antibiotics on the motility of fowl spermatozoa.

Recent studies have shown that the coenzyme Q group of vitamins maintain rooster sperm motility four times longer than control samples. Page *et al.* (1961) suggest that the vitamin may be involved in oxidative synthesis of ATP or in oxidative phosphorylation or may prevent the accumulation of inhibitory lipid peroxides, since it does not depress motility, fructolysis, or oxygen consumption. The 6-chromanol of hexahydrocoenzyme Q_4 and α -tocopherol maintained the motility of stored sperm cells for more than 2 weeks, while the water-soluble vitamins and related compounds were essentially inactive (Page *et al.* 1963). Diphenyl p-phenylenediamine, which can act as an electron acceptor and also has vitamin E-like activity, was highly active, too. The rooster sperm were maintained in a phosphate-albumen medium containing oxytetracycline and dihydrostreptomycin sulfate. Coenzyme Q is involved in the succinoxidase system and so ultimately with motility.

F. INHIBITORS: METABOLIC, SULFHYDRYL, INTERACTION, SURFACTANT

The relation of succinic dehydrogenase to sperm motility has been further emphasized by the fact that nitro blue tetrazolium reduces the motility of rabbit spermatozoa in the presence of sodium succinate after formazan deposits appear in the mitochondria. Omission of the succinate or inclusion of malonate reduces the amount of formazan produced (Edwards and Valentine, 1963). These results are not quite consonant with those reported by Bishop and Mathews (1952), who found that the inhibition of motility by triphenyltetrazolium could not be countered by malonate or succinate, fumarate, and malate, but could readily be reversed by glucose and mannose, less readily by fructose and lactose, while sucrose was least effective and ribose had no effect. Moreover, in these experiments on rabbit, bull, and human sperm, motility was immediately impaired on addition of the tetrazolium or the corresponding photoreduced formazan. In $M/100$ glycolyzable sugar, up to $M/50$ tetrazolium had no effect, but $M/10,000$ tetrazolium inhibited motility in the absence of sugar.

Sodium fluoride has proved an interesting tool in studies on sperm metabolism and motility. Apparently this agent exerts its inhibitory effect on enzymes by combining with metal ions, especially Mg^{++} , and with Mg-protein complexes, causing inactivation by formation of Mg-fluoride, Mg-fluorophosphate, or Mg-fluoroprotein complexes. The solubility of fluoride drops from the sodium salt value of somewhat over 1 M in water to the magnesium salt value of 1 mM. Among the enzymes affected, some are involved in energy metabolism, for example, enolase in the glycolytic system and succinic dehydrogenase in the Krebs cycle; some are included among the hydrolyases and catalases. Moreover, muscle ATPase is also sensitive to this inhibitor, so that both ATP-synthesizing and ATP-utilizing systems may be blocked. If the fluoride concentration is not too great, inhibition may be reversed by washing or removal of the inhibitor.

Ram semen treated with 0.02 M NaF respire at one-half the control rate, while fructose utilization is almost completely blocked, and most of the sperm have become immobilized at the end of 2 hours (Mann and Lutwak-Mann, 1948). While Na fluoride at low concentrations inhibits enolase, at 10 mM, fluoride blocks perch sperm tail ATPase (the $MgCl_2$ content of the incubation medium equaled 2.5 mM at the outset of the experiment). Since all the Mg was probably bound by the fluoride at 10 mM, increasing the fluoride concentration to 100 mM did not cause any greater inhibition, but at this level, NaCl and NaCN were also quite inhibitory, even though at 10 mM and lower they potentiated the ATPase activity (NaCN may have acted to maintain the reduced state of the sulfhydryl groups, which are essential for optimum ATPase activity) (see Tibbs, 1962).

Fluoride also reversibly inhibits the motility of bull sperm; according to Lindahl and Wedin (1963), this effect occurs through interference with an enzyme, inside the permeability barriers of the cell, involved in motile mechanisms. However, these workers point out that mathematically their results may also be interpreted as due to blockage of an ATP-synthesizing system.

Gonse (1959) found that the motility of *Spisula* spermatozoa is more sensitive to uncoupling of oxidative phosphorylation than to inhibitory reduction of oxygen consumption. Fifty mM dinitrophenol increases the endogenous respiration of the *Spisula* sperm six- to ninefold, while 1–3 mM sodium azide causes a twofold rise in respiration, and both substances at these respective concentrations completely inhibit motility. On the other hand, 5 mM amytal only slightly lowers motility while causing a 60% decrease in respiration in *Spisula* sperm. Both bull and dog spermatozoa undergo progressive inhibition of motility with a parallel

effect on respiration as the amytal concentration is raised from 1 to 10 mM. Exogenous succinate reverses amytal inhibition of bull sperm motility, while dog sperm responds only to glucose addition (Gonse, 1962).

Sulfhydryl reagents affect metabolism and motility of a wide variety of spermatozoa and flagellates and block ATP reactivation of glycerinated sperm models. These reagents may be classified as mild oxidizing agents (iodosobenzoate), mercaptide-forming agents (heavy metals, *p*-chloromercuribenzoate, and organic arsenicals), and alkylating agents (iodoacetate, iodoacetamide, and *N*-ethylmaleimide). They may act on soluble sulfhydryl regulatory compounds present in the cell, such as cysteine and glutathione, or on the sulfhydryl groups of enzymes (Barron *et al.* 1948; MacLeod, 1951; Nelson, 1960). Abe (1963) found that *p*-chloromercuribenzoate (PCMB) inhibits ameboid movement, gliding movement in *Oscillatoria*, and flagellar movement of *Pandorina morum*. One mM PCMB caused cessation of the flagellation in several seconds, and sometimes the flagella became detached at their basal ends; 100 μ M caused cessation in 2 minutes and 10 μ M had no visible effect. A 10 mM NaCl wash did not restore beating unless 10 mM cysteine was included, while 5 mM cysteine prevented the inhibitory action of 500 μ M PCMB. No correlation was made with possible inhibition of ATPase activity, although myosin ATPase and actin-myosin interaction may be blocked by sulfhydryl poisons; PCMB and *N*-ethylmaleimide inhibit *Mytilus* sperm ATPase and inorganic pyrophosphatase (Nelson, 1959). In low concentration, sulfhydryl reagents potentiate Ca^{++} -activated myosin ATPase, while in excess these agents inhibit the enzyme. According to Kominz (1965), both ATP and pyrophosphate can reduce or block this inhibition, evidently by preventing a conformational change in the myosin, induced by the sulfhydryl agents, which alters the active site of the enzyme.

Of considerable interest have been Brokaw's (1964) observations on the effects of thiourea on flagellar activity of *Lytechinus*, *Ciona*, and *Chaetopterus* sperm and glycerinated sperm models. He noted that 0.15 M thiourea in seawater reduces the beat frequency by 50% in both living sperm and in the models. The effect on reactivated models is indistinguishable from the effect of lowering the ATP concentration, except that the thiourea effect is linearly related to thiourea concentration while the ATP effect is exponential. In testing the combined effects of thiourea and increased viscosity of the medium (with methyl cellulose), Brokaw found different effects on wavelength, amplitude, and frequency and pattern of swimming in the sperm of the three different species, attesting to the versatility and complexity of the systems which

generate flagellar bending waves. When Brokaw (1963) compared the effects of increased viscosity with those of decreased ATP on *Polytoma* flagella, he found that lowered ATP caused abnormal patterns.

Ruegg (1961) and Ruegg *et al.* (1963) introduced the use of thiourea to studies on contractile systems. In the anterior byssus retractor muscle (ABRM) of *Mytilus edulis*, 0.6 M thiourea inhibits the contractile response presumably by direct action on the contractile proteins (not due to inhibition of membrane excitation or to osmotic inhibition). Not only does thiourea inhibit the ATP contraction of glycerinated ABRM but it also induces a complete and reversible relaxation of artificial ABRM actomyosin threads. With isolated actomyosin systems the thiourea must be at least 0.5 M and the ATP at least 1 mM in order to produce relaxation. The effect may therefore be attributed to inhibition of the interaction between actin and myosin.

In the absence of any further details concerning the nature of thiourea-protein interactions in sperm this evidence of itself may not be conclusive, but the similarity of the contractile system response to thiourea in muscle and flagella is striking and highly suggestive. Such an experimental approach merits further application in the determination of the presence of a contractile multiprotein complex in sperm.

Local anesthetics probably inhibit the contractile responses of directly stimulated muscle by blocking excitation at the membrane level and thereby preventing depolarization. Dinitrophenol and azide uncouple the excitation from the contraction mechanism without affecting membrane potential changes and without affecting the isolated contractile mechanism (glycerinated models, actomyosin threads, and myofibril suspensions). On the other hand interaction inhibitors, in the presence of ATP, inhibit the enzymic interaction between actin and myosin, converting the Mg-activated actomyosin ATPase reversibly to Ca-activated myosin ATPase. These include the Marsh-Bendall relaxing factor, some polycations, polyethenesulfonate, EDTA, and thiocyanate in low concentrations (Barany and Jaisle, 1960; Barany *et al.* 1960). Thiourea is highly specific in this respect; it does not appear to act as a Ca^{++} chelator, nor is it metabolized and it does not inhibit the energy supply of the cell, since it has no effect on the ATP store.

Spermatozoa subjected to sublethal concentrations of spermicidal surface active agents show a reduction in speed of movement. However, this loss of motility probably stems from altered permeability, which results in leakage of cell contents (Harvey and Stuckey, 1962a). The appreciable oil solubility and good surface-tension-lowering properties of nonionic and anionic surfactants make them effective agents, while the cationic detergents are active in spite of their low fat solubility. In

bacteria, cation active agents presumably act first by adsorption on the cell wall, followed by disintegration of the cell and release of "vital" constituents (Harvey and Stuckey, 1962b). Mann and Schmid (1961) found that commercial detergents containing dodecylbenzenesulfonate inhibit trout sperm motility in concentrations of from 5 to 100 mg/l.

VI. Extraneous Physical Factors

Modification of some extraneous physical factors of the environment may also provide insights into fundamental aspects of sperm motility mechanisms. These would fall into two categories, those evoking direct responses and those whose actions may be regarded as indirect.

A. TEMPERATURE

Of physical factors influencing sperm motility, temperature, at least rapid cold, resembles the effect of the surfactants. Cold shock causes an irreversible decline in ATP content, loss of motility (Mann and Lutwak-Mann, 1955), an apparent swelling of the lipoprotein surface membranes, with consequent leakage of potassium and cytochrome c, and decrease in fructolysis and oxygen consumption; ram and bull sperm are apparently much more susceptible than human or fowl sperm (De Silva, 1963). Various proteins of unrelated character partially protect the sperm, evidently exerting a physical, rather than a chemical, effect. The proteins' protective action against cold shock (Choong and Wales, 1962) is enhanced by addition of lecithin to the medium, stabilizing the cell membrane and preventing the loss of intracellular substance. Reference has already been made to low temperature storage of spermatozoa and the inclusion of substances such as glycerol, dimethyl sulfoxide, etc., in the diluents. Here the effect of glycerol has been attributed to its presumed prevention of lipoprotein denaturation with cold exposure.

At the other end of the spectrum, high temperatures presumably exert their detrimental effects via enzyme denaturation, as is the case in other cell types. Since the mature spermatozoon presumably lacks the protein-synthetic machinery to regenerate heat-sensitive components, the cell is not equipped to recover and survive. For example, rams exposed to a high ambient temperature (40.5°C) exhibit deterioration of semen "quality." The sperm tails become coiled and even detached under these conditions (Moule and Waites, 1963). Below the inactivation temperature, the rate of sperm motility increases as the temperature increases, ranging in the cockroach, *Periplaneta americana*, from 8.5 μ /second at 15°C to about 100 μ /second at 39°C. Cold inactivation occurs at 10–11°C (Richards, 1963a). At 10°C most sperm were completely inactive,

but a small percentage moved their tails feebly without accomplishing any forward movement. The inactive sperm recover motility with elevation of the temperature. When the data are plotted as logarithm of rate against reciprocal of absolute temperature (Arrhenius plot) over the 15–25°C range, an activation energy of $-13,000$ cal/degree/mole may be calculated, giving Q_{10} of about 2. This is similar to that for heartbeat frequency, running speed, oxygen consumption, and muscle succinoxidase activity of the cockroach and also to *Mytilus* sperm tail ATPase (Richards, 1963b; Nelson, 1959), but higher than wing beat frequency ($Q_{10} = 1.35$), higher than acetylcholinesterase ($Q_{10} = 1.4$), and considerably lower than the Mg-activated cockroach muscle apyrase ($Q_{10} = 3.3$) (Richards, 1956; Davison and Richards, 1954).

It would obviously be misleading to attempt to extrapolate from such data, especially comparing temperature coefficients of the physiological activities of intact cells, organs, or organisms with those of partially purified enzymes, in the search for “master” reactions. Information about rate-limiting processes based on reaction kinetics of *in situ* systems could nevertheless be very instructive. Temperature is certainly an important factor controlling the speed and frequency of ciliary and flagellar beat. Since the activity of other organelles and processes is likewise temperature sensitive, one may safely generalize on this basis merely with respect to the existence, though not necessarily the specific nature or identity, of underlying chemical and physical processes.

B. GEOTROPISM

The motile activity of living sperm under normal conditions usually assures a uniform distribution of the cells in a suspension, except that interactions between adjacent cells may result in ordered “wave-front” formation. Reduction in temperature decreases this sperm order (Reynolds and Rothschild, 1963). The uniformity of distribution may also be altered, of course, if the sperm suspension is subjected to an increase in gravitational field. At forces from 1000 *g* up to 2500 *g*, most marine invertebrate spermatozoa sediment, although some (strongly swimming?) cells may remain in suspension. Normally both mammalian and marine invertebrate sperm may be harvested or concentrated at 1000–1500 *g* for 10 minutes. Usually the investigator discards the presumably harder sperm remaining in the supernatant rather than risk damage to the bulk of the cells in the sediment. Branham (1964a) in a brief note reported that *Arbacia* sperm may be positively geotropic, that is, when centrifuged at 200 *g* for 20 minutes living sperm sedimented faster than sperm from the same suspension killed with 0.02% formalin, or living

sperm immobilized at pH 6.5 or by high CO₂. Normally on the basis of common experience, one would expect the dead or nonmotile sperm to sediment more rapidly, as appears to be the case in sperm suspensions examined on a microscopic slide. Branham suggests that perhaps the sperm become oriented with their heads in the centrifugal direction, in which case swimming sperm would arrive at the bottom of the centrifuge tube before nonswimming sperm. If one assumes an average velocity of about 200 μ /second, then it may be calculated that oriented sperm should reach the bottom of the 10-cm tube in an average time of about 8.5 minutes if not impeded by the crowds of neighboring sperm. Perhaps the "hardier" supernatant sperm cells are really just slow movers; subjectively, they usually appear to be quite vigorous (Branham, 1966).

C. GALVANOTROPISM

Orientation may also be imposed on spermatozoa by exposing them to a d-c electric field (Bangham, 1961; this paper contains a critical appraisal of the problems inherent in attempts at electrophoretic separation of spermatozoa into distinct phenotypic groups). At 25°C, in low ionic-strength media (10 mM NaCl, 277 mM fructose), two types of sperm appeared with orientation depending on whether the head or tail region carries a net negative charge. "Head-anode" sperm swim rapidly toward the anode, but decelerate immediately when the field is turned off. If the field strength is high enough, "tail-anode" sperm move backward, but resume forward swimming when the field is switched off. The proportions of the two types of sperm vary with salt concentration at constant pH, and with pH at constant salt concentration. Complete immobilization at 8°C results in uniform electrophoretic migration toward the anode, tail first at all pH's above 3.5. Apparently, at least one of the factors is the relative distribution of the net charge density at the sperm cell surface, although the factors which may contribute to a differential distribution of these charges have not yet been ascertained.

D. RADIATION: VISIBLE ULTRAVIOLET

The sperm cell exhibits radiation and photosensitivity. For instance, increasing the normal day length, from about 12 to 16 hours of illumination of the intact fowl, reportedly initiates a complex chain of events, including improvement of the "quality" of poultry semen, i.e., improvement of the motility as well as increase in the number of living sperm (Bajpai, 1963). A more direct effect of visible radiation is shown by exposure of bull sperm in coconut milk extender to 1400-foot candles from a white fluorescent light source. Twenty-four- to 72-hour exposure rendered the sperm nonmotile, while the dark controls retained fair

motility over the same period. Catalase added to the diluent (10 mg %) afforded protection, as did increasing the sperm concentration thirteen- to twentyfold (Norman and Goldberg, 1959). When the intensity of radiation was raised to 8500-foot candles, bull sperm motility declined from 75 % to 25 % after $2\frac{1}{2}$ hours and to 0 % motility after 4.5 hours. Over this same period, the dark controls dropped only to 70 % motility. Catalase, nitrogen, and 10^{-4} M cysteine each increased the time for photoimmobilization to 12 hours, as opposed to 7 hours with oxygen in the gas space over the specimens. Photooxidation presumably produces peroxides in the medium. An action spectrum exhibited two sharp peaks at 440–480 $m\mu$ and a shallow broad peak at about 665 $m\mu$. Absorption spectra of densely packed bull sperm showed peaks in the 420–450- $m\mu$ range, with a small peak at 540–560 $m\mu$, leading Norman and co-workers (1962) to the conclusion that a hemeprotein might be implicated. Aged sperm show a significantly increased susceptibility to visible radiation. While brief exposures to light suffice to initiate photooxidation, which continues in the dark, one is tempted to conclude that primary effects depend on the presence of oxidizable substances in the medium which secondarily sensitize or further damage the spermatozoon surface, causing leakage of cell constituents from already dilution-damaged sperm. Even mild mishandling and ageing of sperm cells result in loss of cytochromes (hemeproteins) and other substances to the medium (Mann, 1964). Apparently oxidation of essential sulfhydryl groups also occurs.

Wales and Choong (1963) found that ultraviolet radiation also depressed ram and bull sperm motility. Irradiation at 366 $m\mu$ affected motility more than at 254 $m\mu$. Addition of catalase, cysteine, or glutathione offset the effect here, too. With a synthetic diluent, the "slight detrimental effect" was thought due to photochemical changes within the spermatozoa themselves. When Mounib and Chang (1964) exposed bull semen to 10^3 – 10^5 r of Cobalt-60 γ -rays, there was a drop in metabolism and motility. Here again, buffered egg yolk included in the diluent provided a protective effect. Similarly, the inactivation rate of *Lytechinus* sperm (measured as the increased number of sperm required to fertilize 50 % of a standard number of eggs) increases greatly with the extent of dilution when irradiated. "Dry" sperm, while more radio-resistant than diluted sperm, are nevertheless also fairly sensitive. Care must be exercised, then, first in manipulating the sperm cells, and second in distinguishing between protective action in and by the medium from the effects of ionizing radiations and radiation-sensitive systems within the cells. It therefore would be most advantageous in such investigations to establish uniform standard media, such as Rikmenspoel's (1957) buffered egg yolk, which has been clarified by ultracentrifugation and dialysis

and which affords a fairly long-lived system. It would also be advisable to subject this preparation to careful analysis so as to identify the effective constituents of the medium and permit the preparation of satisfactory and reproducible chemically defined media.

E. VISCOSITY

The use of chemically "inert" substances to increase the viscosity of the swimming medium has provided the means of separating a purely physical effect from a chemical process in the analysis of flagellar movement. Brokaw (1963) has shown that in methyl cellulose a tenfold decrease in the frequency of flagellation does not alter the wave pattern, whereas a similar decrease in frequency brought about by a tenfold reduction in ATP concentration in reactivated glycerinated *Polytoma* causes many flagella to beat abnormally. Speed of bull sperm is inversely related to the viscosity, according to Rikmenspoel. From an analysis of the effects of viscosity and hydrodynamic considerations (Carlson, 1959), it is apparent that the sperm head contributes little to the viscous drag, while most of the energy expended by the sperm must be utilized to overcome the tangential drag of the tail (arising from the exceedingly large surface-to-volume ratio of the tail). Gray (1928) refers to the fact that the rate at which cilia perform work increases with increasing load until a maximum is reached. He interprets this to mean that with increasing load (e.g., increasing viscosity) the rate at which cells expend energy increases. For sea urchin sperm, the power output depending on the viscous drag of the medium comes to about 3×10^{-7} erg/sperm/second (Carlson, 1959); while for bull sperm, applying similar assumptions, this amounts to 3×10^{-8} erg/sperm/second (Nelson, 1958). The latter value has been challenged by Rothschild (1961), who arrived at a figure of 2.1×10^{-7} erg/sperm/second (1961). This sevenfold difference stems from the assignment of somewhat different values to the parameters inserted into the equations. Since the rate of free energy liberation associated with the hydrolysis of ATP is approximately 2×10^{-6} erg/sperm/second, this provides a safety factor of about 10 and is probably a "reasonable" value (Rothschild, 1961).

Assuming that the γ -fibers generate the motive force for mammalian sperm (and the evidence presently available appears to support this contention), and noting the relatively small cross-sectional area that they occupy, and the fact that these fibers contain other enzymes and structural proteins, it is surprising, but perhaps not fortuitous, that the individual fibers can develop a calculated tension of 4×10^{-5} dynes each. This is nearly equivalent, on a weight basis, to the tension developed by striated muscle: 4×10^6 dynes/cm² for sperm fibers compared to 1–5

$\times 10^6$ dynes/cm² for muscle (Rikmenspoel, 1965). Furthermore, it may be calculated that Rothschild's bull sperm expends about 2×10^{-5} dyne-cm (erg) while it traverses 1 cm.

VII. Motility in Male and Female Reproductive Tracts

Perhaps not enough attention has been paid to the fact that the sperm cell is highly versatile and adaptable to the physical changes it normally encounters in its environment (see Bishop, 1961, 1962a). Note has been made of variations both in environmental electrolyte and non-electrolyte composition as the sperm is transported from more to less fluid regions in the epididymal ducts, gets mixed with accessory gland secretions, and in many species with internal fertilization, leaves the seminal plasma to enter the cervical mucus, further to become diluted in uterine and tubal fluids, and occasionally in peritoneal fluids. Obviously the mammalian and avian sperm cell is equally capable of adjusting to the demands of viscosity changes and to metabolic upheavals. Marine material need not undergo quite such a multiplicity of changes and manages successfully, although usually on a much-abbreviated time scale. Dilution effects under experimental conditions in artificial media usually contribute to the rapid expenditure of reserves and deterioration of the sperm. The functional life (motility frequently outlasts fertilizing capacity) of the sperm lasts longer in some segments of the female reproductive tract than others; and the sperm numbers undergo attrition during their migration without apparent effect on their motility (Noyes and Thibault, 1962), although the *in vitro* motility of comparably diluted sperm is much shorter. Moreover, Perloff and Steinberger (1964) found that sperm survived and were actively moving up to 7 days after *in vitro* "insemination" in human cervical mucus. Estrogen administration increases the profuseness and clarity of cervical mucus as well as the ease of penetration by the spermatozoa. According to Moghissi and co-workers (1964), semen contains proteolytic enzymes which facilitate sperm penetration in cervical mucus, but since sperm cells are susceptible to pH changes of the mucus (immobilized in acidic mucus and movement enhanced in alkaline mucus), these factors must be carefully sorted out. In humans, Sobrero and Macleod (1962) reported that sperm retain greater motility in cervical mucus than *in vitro* and that sperm motility itself is essential to bring about entrance into and penetration of cervical mucus, since natural insemination directly into the cervical canal does not occur. In clinical cases of necropermia, even though plenty of sperm cells may be recovered from the vaginal pool, none may be found in the cervical mucus. Sperm survive in vaginal fluids for up to 10–12 hours at the most, while cervical mucus may sustain motility for as

long as 96 hours (Horne and Thibault, 1962a). Sperm distribution becomes uniform in the cervical mucus and much more dispersed than in vaginal semen (Sobrero and MacLeod, 1962). The cervix, the uterotubal junction, and the motility of the spermatozoa themselves are together responsible for the maintenance of a continuous progression of the sperm from the cervix to the site of fertilization (fallopian tubes in the ewe [Mattner, 1963]). Ciliary action may merely disperse the sperm more evenly over the lining of the tubes, but there seems to be increased activity and speed of sperm propulsion in follicular, tubal, and even peritoneal fluids in the human female (Horne and Thibault, 1962a,b). The ascent of sperm through the hamster oviduct appears to be more rapid when females are mated after ovulation than before ovulation (Yanigamachi and Chang, 1963). It is not clear whether such modifications in apparent rates of sperm transport depend primarily on the sperm motility or on the endocrine state of the female reproductive tract, although Howe and Black (1963b) state that in artificially inseminated calves 29–80 days of age, motile sperm were found in the oviducts 13 minutes after vaginal insemination, both in controls and in animals treated with pregnant mare serum and human chorionic gonadotropin. The condition of the reproductive tracts in these calves with respect to muscular activity was apparently not checked.

VIII. Conclusion

It is still difficult to assess quantitatively the dependence of the reproductive processes culminating in fertilization on sperm motility. Generalization is virtually impossible. Rule of thumb, however, correlates nonmotility with nonfertilizing capacity, at least in the majority of fili-form sperm types. Motility may cease literally within seconds after spawning, especially in freshwater species. Under laboratory conditions biological fluids seem to exert a beneficial effect. For example, lake trout sperm "activated" by coelomic fluid and Ringer solution (including Ca^{++} -free) retain their motility and fertilizing capacity longer than in water. But after losing their fertilizing capacity in these solutions, some sperm regain motility on addition of water (Ginzburg, 1963). In domestic fowl, fertilization occurs after ovulation, and for this to occur over long periods of time following a single insemination, physiological conditions in the female reproductive tract must be such as to maintain the spermatozoa in a functional state (Bobr *et al.*, 1964). The sperm stored in uterovaginal glands may be released in appropriate numbers at ovulation. Once released from these glands, the sperm which appear morphologically normal and motile have a relatively short life span (Bobr *et al.*, 1964).

This chapter started with the premise that the spermatozoon is a terminal cell, the end-product of a complicated differentiation process which can undergo no cell division, no further differentiation, and having divested itself of the ribonucleoprotein apparatus essential for protein synthesis, cannot repair or regenerate worn-out components. Its biosynthetic capacities are directed principally to maintenance of an ATP level adequate to supply its mechanical requirements for a finite period beyond which irreversible senescence sets in. Nevo *et al.* (1963) found that fresh sperm added to the pH-stat after the initial batch had ceased its activity remained motile for an additional 30 hours, so that accumulation of toxic products could probably be ruled out; and lactate added to fresh preparations permitted motility to continue for 40 more hours. Thus, the spermatozoon seemingly lacks the "immortality" possible to somatic cells, including ciliated cells, and to flagellates. This is an interesting concomitant of the acquisition of motile activity, for in the quiescent, nonmotile state numerous investigations attest to the longevity of spermatozoa, whether in the male reproductive tract or in the female tract of bats or of domestic fowl or in cold storage. Perhaps in the immature condition the mitochondria function to maintain the structural integrity of the spermatid (as well as in the torpid sperm cell), but after having undergone their own peculiar morphogenesis associated with flagellar formation, the mitochondria abdicate their biological responsibilities while retaining some of their *in vitro* biochemical attributes. A critical application of straightforward biochemical procedures on carefully isolated components may elucidate this aspect of a most intriguing problem. Certainly the methodology developed by the muscle men has yielded valuable insights pointing up the divergencies from, as well as formal similarities to, muscle. Studies underway indicate that spermatozoa of a number of species contain a myosinlike protein, an actinlike protein, and even a tropomyosinlike protein (Szent-Györgyi and Kammer, 1963).^{*} The functional and spatial relationships of these proteins to each other and to flagellar structural units remain to be clarified. Up to a certain point, a number of substances can be provided through the incubation medium to the substrate-depleted nonmotile sperm and thus restore motility. How and where these substances may be synthesized and replenished with utilization and how they are transported within the flagellum to the utmost reaches must still be worked out. The dependence on coordination of a progressively motile spermatozoon may find an analogy in the automaticity of smooth muscle; a highly active acetylcholinesterase may unquestionably assume such a role. The func-

^{*} Electron micrographs of fibrous protein extracted from bull sperm flagella strikingly resemble corresponding muscle substances, viz., flactin and actin, spermiosin and myosin, flactospermiosin and actomysin (Young and Nelson, 1966).

tion of the central pair, the β -fibers, and the physiological fate of the α -fibers in spermatozoa which possess well-defined γ -fibers remain to be assessed, as well as the mechanism whereby simple flagella possessing the α -fibers but lacking the γ -fibers function. Cytochemical studies on transitional forms with "rudimentary" γ -fibers such as occur in gastropod, squid, snake, and some avian sperm could prove fruitful in this regard. Perhaps in mammalian sperm each of the γ -fibers sequentially propagates a shortening wave about 11% out of phase with its neighbor, thereby giving rise to a three-dimensional undulation as visualized in Fig. 7. The

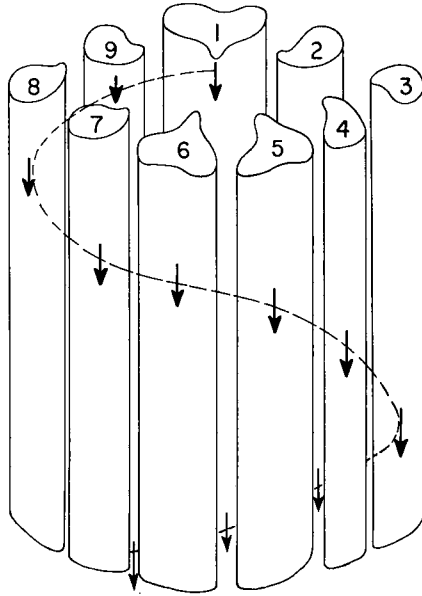


FIG. 7. Sequential contraction, with neighboring γ -fibers about 11% out of phase with each other, resulting in propagation of a contractile wave.

sperm of lower organisms, generally lacking the γ -fibers, appear to propel themselves by means of a two-dimensional wave, if sea urchin sperm may be regarded as "typical." Obviously many answers remain unspoken; perhaps the key questions remain to be asked. New methods and attacks may have to be devised; the old techniques developed in other fields have not yet been fully exploited. Some tools of the spermatologists may even prove useful in other fields. The tail may yet dog the wags.

REFERENCES

- Abe, S. (1963). The effect of p-chloromercuribenzoate on ameboid movement, flagellar movement and gliding movement. *Biol. Bull.* **124**, 107-114.
 Abraham, K. A., and Bhargava, P. M. (1963a). Nucleic acid metabolism of mammalian spermatozoa. *Biochem. J.* **86**, 298-307.

- Abraham, K. A., and Bhargava, P. M. (1963b). The uptake of radioactive amino acids by spermatozoa. The intracellular site of incorporation into proteins. *Biochem. J.* **86**, 308-313.
- Afzelius, B. A. (1962). The contractile apparatus in some invertebrate muscles and spermatozoa. *5th Intern. Congr. Electron Microscopy, Philadelphia, 1962* (S. S. Breese, Jr., ed.), **2**, M-1. Academic Press, New York.
- Afzelius, B. A. (1963). Cilia and flagella that do not conform to the $9 + 2$ pattern. I. Aberrant members within normal populations. *J. Ultrastruct. Res.* **9**, 381-392.
- Anderson, W. A. (1964). Ultrastructure of the flagellum and pellicle of *Trypanosoma lewisi*. *J. Cell Biol.* **23**, 5A.
- André, J. (1963). Some aspects of specialization in sperm. In "General Physiology of Cell Specialization" (D. Mazia and A. Tyler, eds.), Chap. 7, pp. 91-115. McGraw-Hill, New York.
- Applegate, A., and Nelson, L. (1962). Acetylcholinesterase in *Mytilus* spermatozoa. *Biol. Bull.* **123**, 475.
- Austin, C. R. (1965). "Fertilization." Prentice-Hall, Inc. Englewood Cliffs, New Jersey.
- Austin, C. R., and Sapsford, C. S. (1952). The development of the rat spermatid. *J. Roy. Microscop. Soc.* **71**, 397-405.
- Bahr, G. F., and Zeitler, E. (1964). Study of bull spermatozoa. Quantitative electron microscopy. *J. Cell Biol.* **21**, 175-189.
- Bajpai, P. K. (1963). The effect of photoperiodicity on the semen characteristics of poultry. *Poultry Sci.* **42**, 462-465.
- Balogh, K., Jr., and Cohen, R. B. (1964). A cytochemical technic for studying oxidative enzyme systems in mammalian spermatozoa in semen smears. *Fertility Sterility* **15**, 35-39.
- Bangham, A. D. (1961). Electrophoretic characteristics of ram and rabbit spermatozoa. *Proc. Roy. Soc. (London), Ser. B* **155**, 292-305.
- Barany, M., Barany, K., and Trautwein, W. (1960). Die Hemmung der Aktin-1-Myosin Interaktion in lebenden und extrahierten Muskeln durch Urea. *Biochim. Biophys. Acta* **45**, 317-335.
- Barany, M., and Jaisle, F. (1960). Kontraktions Zyklus und Interaktion zwischen Aktin und L-Myosin unter der Wirkung spezifischer Interaktions Inhibitoren. *Biochem. Biophys. Acta* **41**, 192-203.
- Barnes, H. (1962). The O_2 uptake and metabolism of *Balanus balanus* spermatozoa. *J. Exptl. Biol.* **39**, 353-358.
- Barron, E. S. G., Nelson, L., and Ardao, M. I. (1948). Regulatory mechanisms of cellular respiration II. The role of soluble sulfhydryl groups as shown by the effect of sulfhydryl reagents on the respiration of sea urchin sperm. *J. Gen. Physiol.* **32**, 179-190.
- Barth, R. F., and Russell, P. S. (1964). The antigenic specificity of spermatozoa. I. An immunofluorescent study of histocompatibility antigens of mouse sperm. *J. Immunol.* **93**, 13-19.
- Bawa, S. R. (1962). Fine structure of human sperm tail. *5th Intern. Congr. Electron Microscopy, Philadelphia, 1962* (S. S. Breese, Jr., ed.), **2**, M-3. Academic Press, New York.
- Bawa, S. R. (1963). Outer coarse fibers of the mammalian sperm tail: an electron microscope study. *J. Ultrastruct. Res.* **9**, 475-483.
- Beale, G. H. (1957). The antigen system of *Paramecium aurelia*. *Intern. Rev. Cytol.* **6**, 1-23.

- Bernstein, M. H. (1962). Normal and reactive morphology of sea urchin spermatozoa. *Exptl. Cell Res.* **27**, 197–207.
- Bishop, D. W. (1961). Biology of spermatozoa. In "Sex and Internal Secretions" (W. C. Young, ed.), Vol. II, pp. 707–796. Williams & Wilkins, Baltimore, Maryland.
- Bishop, D. W. (1962a). Sperm motility. *Physiol. Rev.* **42**, 1–59.
- Bishop, D. W. (1962b). "Spermatozoan Motility." Am. Assoc. Advance. Sci., Washington, D.C.
- Bishop, D. W., and Hoffmann-Berling, H. (1959). Extracted mammalian sperm models. I. Preparation and reactivation with adenosine-triphosphate. *J. Cellular Comp. Physiol.* **53**, 445–466.
- Bishop, D. W., and Mathews, H. P. (1952). Inhibition of sperm motility by tetrazolium salts. *Science* **115**, 211–212.
- Bishop, M. W. H., and Walton, H. (1960). Metabolism and motility of mammalian spermatozoa. In "Marshall's Physiology of Reproduction" (A. S. Parkes, ed.), Vol. 1, Pt. 2, pp. 264–295. Green and Co., Ltd., London.
- Blackshaw, A. W. (1964). Enzymatic activity of bull testis and epididymal spermatozoa. *Australian J. Biol. Sci.* **17**, 489–498.
- Blandau, R. J., and Rumery, R. E. (1964). The relationship of swimming movements of epididymal spermatozoa to their fertilizing capacity. *Fertility Sterility* **15**, 571–579.
- Bobr, L. W., Lorenz, F. W., and Ogasawara, F. X. (1964). Distribution of spermatozoa in fowl oviducts. I. Residence sites of spermatozoa in fowl oviducts. *J. Reprod. Fertility* **8**, 39–47.
- Bobr, L. W., Ogasawara, F. X., and Lorenz, F. W. (1964). Distribution of spermatozoa in the oviduct and fertility in domestic birds. II. Transport of spermatozoa in the fowl oviduct. *J. Reprod. Fertility* **8**, 49–58.
- Brackett, B. G., Weinman, D. E., and Williams, W. L. (1963). ATP content and effect of light on sperm respiration. *Federation Proc.* **22**, 569.
- Bradfield, J. R. G. (1955). Fibre patterns in animal flagella and cilia. In "Fibrous Proteins and Their Biological Significance," Vol. 9, pp. 306–334. Symp. Soc. Exptl. Biol. Academic Press, New York.
- Branham, J. M. (1964a). A method of rating sea urchin sperm motility. *Biol. Bull.* **127**, 363.
- Branham, J. M. (1964b). Senescence of *Arbacia* sperm. *Biol. Bull.* **127**, 363–364.
- Branham, J. M. (1966). Motility and aging of *Arbacia* sperm. *Biol. Bull.* **131**, 251–260.
- Brokaw, C. J. (1963). Movement of flagella of *Polytoma uvella*. *J. Exptl. Biol.* **40**, 149–156.
- Brokaw, C. J. (1964). Thiourea-induced alterations of flagellar wave forms. *J. Cell Biol.* **23**, 15A.
- Brokaw, C. J. (1965). The stiffness of flagella and cilia. *Abstr. Biophys. Soc. 9th Ann. Meeting*, San Francisco, 1965. p. 148.
- Brokaw, C. J., and Wright, L. (1963). Bending waves of the posterior flagellum of *Ceratum*. *Science* **142**, 1169–1170.
- Bulbring, E., Burn, J. H., and Shelley, H. J. (1953). Acetylcholine and ciliary movement in the gill plates of *Mytilus*. *Proc. Roy. Soc. (London)*, Ser. B **141**, 445–466.
- Burnasheva, S. A. (1958). Properties of spermosin, a contractile protein in sperm cells. *Biokhimiya* **23**, 558–563.
- Burnasheva, S. A. (1960). Role of phosphorylation processes in the motor function

- of sperm cells. *Trans. Inst. Eksperim. Med. Akad. Med. Nauk SSSR*, pp. 231-242.
- Burnasheva, S. A., Fedorova, L. G., and Lyubimova, M. N. (1963). Structural organization and biochemical bases of movement in cilia and flagella. *Usp. Sovrem. Biol.* **56**, 365-380.
- Buruiana, L. M., and Hadarag, E. I. (1963). Sperm pyrophosphatase. *Naturwissenschaften* **50**, 478-479.
- Carlson, F. D. (1959). The motile power of a swimming spermatozoon. *Proc. 1st Nat. Biophys. Conf. Columbus, Ohio, 1956*, pp. 443-449.
- Chang, M. C. (1947). The effects of serum on spermatozoa. *J. Gen. Physiol.* **30**, 321-335.
- Choong, C. H., and Wales, R. G. (1962). The effect of cold shock on spermatozoa. *Australian J. Biol. Sci.* **15**, 543-551.
- Claybrook, J. R. (1965). Isolation and characterization of *Arbacia* sperm tail ATP-ase. *20th Annual Meeting Soc. Gen. Physiol.*, 1965, Woods Hole, Mass.
- Clermont, Y. (1964). Motility of the flagellum and cytoplasmic membrane of the rat spermatids. *Anat. Record* **148**, 271.
- Clermont, Y., and Leblond, C. P. (1955). Spermiogenesis of man, monkey, ram and other mammals as shown by the "periodic acid-Schiff technique." *Am. J. Anat.* **96**, 229-253.
- Conti, A. M., and Cresseri, A. (1963). Action of gonadotropins on the motility of spermatozoa of *Rana esculenta* L. *Boll. Soc. Ital. Biol. Sper.* **39**, 1055-1058.
- Cori, O., Traverso-Cori, A., Lagarrigue, M., and Marcus, F. (1958). Enzymic phosphorylation of creatine by 1:3 diphosphoglyceric acid. *Biochem. J.* **70**, 633-641.
- Cresseri, A., and Conti, A. M. (1962). Osservazioni sugli effetti di soluzioni a vario pH e a diversa osmolarita sullo motilita degli spermatozoi di *Rana esculenta* L. *Boll. Soc. Ital. Biol. Sper.* **38**, 1564-1566.
- Daems, W. T., Persijn, J. P., and Bates, A. D. (1963). Fine structural localization of ATP-ase activity in mature sperm of *Drosophila melanogaster*. *Exptl. Cell Res.* **32**, 163-167.
- Davison, J. A., and Richards, A. G. (1954). Muscle apyrase activity as a function of temperature in the cockroach, crayfish and minnow. *Arch. Biochem. Biophys.* **48**, 485-486.
- De Silva, P. L. G. (1963). Evidence for absence of an effect of temperature shock on fertilizing ability of fowl spermatozoa. *J. Reprod. Fertility* **6**, 371-374.
- Dott, H. M., and White, I. G. (1964). Effect of potassium on ram spermatozoa studied by a flow dialysis technique. *J. Reprod. Fertility* **7**, 127-138.
- Drevius, L. O. (1963). Spiralization in tails of mammalian spermatozoa in hypotonic media. *Nature* **197**, 1123-1124.
- Edwards, R. G. (1960). Complement fixing activity of normal rabbit serum with rabbit spermatozoa and seminal plasma. *J. Reprod. Fertility* **1**, 268-282.
- Edwards, R. G. (1962). Antibodies against spermatozoa. *J. Reprod. Fertility* **4**, 225-226.
- Edwards, R. G., and Valentine, R. C. (1963). Cytochemical demonstration of succinic dehydrogenase in intact rabbit spermatozoa. *Exptl. Cell Res.* **31**, 508-516.
- Engelhardt, V. A. (1945). Phosphoric acid and cell functions. *Bull. Acad. Sci. USSR, Biol. Ser.* **2**, 182-195.
- Fauré-Fremiet, E. (1961). Cils vibratiles et flagelles. *Biol. Rev.* **36**, 464-536.

- Fawcett, D. (1961). Cilia and flagella. In "The Cell" (J. Brachet and A. E. Mirsky, eds.), Vol. II, p. 217-297. Academic Press, New York.
- Flipse, R. J. (1962a). Metabolism of bovine semen XI. Factors affecting the transport of fructose in bovine spermatozoa. *J. Dairy Sci.* **45**, 917-920.
- Flipse, R. J. (1962b). Metabolism of bovine semen. XII. Galactose as an indicator of hexose transport by bovine spermatozoa. *J. Dairy Sci.* **45**, 1083-1086.
- Gaddum, P., and Glover, T. D. (1965). Some reactions of rabbit spermatozoa to ligation of the epididymis. *J. Reprod. Fertility* **9**, 119-130.
- Gibbons, I. R. (1963). Studies on the protein components of cilia from *Tetrahymena pyriformis*. *Proc. Natl. Acad. Sci. U. S.* **50**, 1002-1010.
- Gibbons, I. R. (1964). An effect of ATP on the light scattered by suspensions of cilia. *J. Cell Biol.* **23**, 35A.
- Gibbons, I. R., and Rowe, A. J. (1965). Dynein: a protein with adenosine triphosphatase activity from cilia. *Science* **149**, 424-426.
- Ginzburg, Anna S. (1963). Sperm-egg association and its relationship to the action of the egg in salmonid fishes. *J. Embryol. Exptl. Morphol.* **11**, 13-33.
- Goldberg, E., Pence, V. W., and Norman, C. (1961). Distribution of free amino acids in cock and bull spermatozoa seminal plasma and spermatozoa. *Am. Zoologist* **1**, 356-357.
- Gonse, P. H. (1959). Respiration and motility in *Spisula* spermatozoa. *Biol. Bull.* **117**, 433-434.
- Gonse, P. H. (1962). Respiration and oxidative phosphorylation in relation to sperm motility. In "Spermatozoan Motility" (D. W. Bishop, ed.), pp. 99-132. Am. Assoc. Advance. Sci., Washington, D.C.
- Gray, J. (1928). "Ciliary Movement." Cambridge Univ. Press, London and New York.
- Gray, J. (1962). Introduction: flagellar propulsion. In "Spermatozoan Motility" (D. W. Bishop, ed.), pp. 1-12. Am. Assoc. Advance. Sci., Washington, D.C.
- Curaya, S. S. (1963). Histochemistry of the cytoplasmic droplet in the mammalian spermatozoon. *Experientia* **19**, 94-95.
- Hammond, M., Boone, M. A., and Barnett, B. D. (1965). Studies of the glucose, electrolytes, enzymes and nitrogen components of fowl seminal plasma. *J. Reprod. Fertility* **10**, 21-28.
- Harris, J. E. (1961). The mechanics of ciliary movement. In "The Cell and the Organism" (J. A. Ramsey and V. B. Wigglesworth, eds.), pp. 22-36. Cambridge Univ. Press, London and New York.
- Hartman, C. G., ed. (1963). "Mechanisms Concerned with Conception." Pergamon Press, Oxford and London.
- Harvey, C., and Stuckey, R. E. (1962a). Spermicidal activity of surface-active agents. *J. Reprod. Fertility* **3**, 124-131.
- Harvey, C., and Stuckey, R. E. (1962b). A new method of comparison of spermicidal activities. *J. Reprod. Fertility* **3**, 132-137.
- Hathaway, R. R. (1963). Activation of respiration in sea urchin spermatozoa by egg water. *Biol. Bull.* **125**, 486-498.
- Hayashi, T. (1962). Muscle research and flagellar movement. In "Spermatozoan Motility" (D. W. Bishop, ed.), pp. 279-283. Am. Assoc. Advance. Sci., Washington, D.C.
- Hoffmann-Berling, H. (1959). The role of cell structures in cell movements. In "Cell, Organism, and Milieu" (D. Rudnick, ed.), pp. 45-62. Ronald Press Co., New York.

- Holwill, M. E. J., and Burge, R. E. (1963). A hydrodynamic study of the motility of flagellated bacteria. *Arch. Biochem. Biophys.* **101**, 249-260.
- Horne, H. W., Jr., and Thibault, J. P. (1962a). Sperm migration through the human female reproductive tract. *Fertility Sterility* **13**, 135-139.
- Horne, H. W., Jr., and Thibault, J. P. (1962b). Sperm migration through the human female reproductive tract: follow-up of patients studied at laparotomy and at culdoscopy. *Fertility Sterility* **13**, 444-447.
- Howe, G. R., and Black, D. L. (1963a). Migration of rat and foreign spermatozoa through the utero-tubal junction of the oestrous rat. *J. Reprod. Fertility* **5**, 95-100.
- Howe, G. R., and Black, D. L. (1963b). Spermatozoan transport and leucocytic responses in the reproductive tract of calves. *J. Reprod. Fertility* **6**, 305-311.
- Hrudka, F. (1965). Cytochemical demonstration of catabolic activity in spermatozoa by the formazan test. *J. Reprod. Fertility* **10**, 15-20.
- Iiuzuka, R., Sawada, Y., Kuramoto, C., Hara, S., Hirosawa, K., and Okubo, F. (1959). Effects of Na ATP on human spermatozoa and consequent use with frozen semen. *Sogo Igaku* **16**, 273-278.
- Iversen, S. (1964). Surface reflexion interference microscopy of bull spermatozoa. *Quart. J. Microscop. Sci.* **105**, 245-246.
- Jones, Alan W. (1962). Abnormal morphology of the spermatozoa in Guernsey bulls. *Brit. Vet. J.* **118**, 257-261.
- Kamboj, V. P., and Kar, A. B. (1964). Antitesticular effect of metallic and rare earth salts. *J. Reprod. Fertility* **7**, 21-28.
- Katsh, S., and Katsh, G. F. (1964). Immuno-diffusion and immuno-electrophoresis of guinea pig sperm and antispermatogenic factor. *Anat. Record* **148**, 380.
- Kelly, P. G., and Pautard, F. G. E. (1965). The isolation of an ATP-ase from fish sperm flagella. *Abstr. Biophys. Soc. 9th Ann. Meeting, San Francisco, 1965*, p. 130.
- Kinoshita, S. (1959). On the identity of the motility-inducing factor of flagellum and the relaxing factor of muscle. *J. Fac. Sci., Univ. Tokyo, Sect. IB* **8**, 427-437.
- Kinoshita, S. (1965). On the role of the relaxing factor in the flagellar movement of the glycerol-extracted sperm cell models. *Conf. Develop. Biol. and Fertility, Tokyo, Japan, 1965*, pp. 19-20.
- Komarek, R. J., Pickett, B. W., Lanz, R. N., and Jensen, R. G. (1964). Lipid composition of bovine spermatozoa and seminal plasma. *J. Dairy Sci.* **47**, 531-534.
- Kominz, D. R. (1965). Mercurial-induced transformation of myosin prevented by adenosinetriphosphate and pyrophosphate. *Science* **149**, 1374-1375.
- Lardy, H. A., and Phillips, P. H. (1945). Studies of fat and carbohydrate oxidation in mammalian spermatozoa. *Arch. Biochem.* **6**, 53-61.
- Leblond, C. P., and Clermont, Y. (1952). Definition of the stages of the cycle of the seminiferous epithelium in the rat. *Ann. N. Y. Acad. Sci.* **55**, 548-573.
- Lehmann, H., and Griffiths, P. D. (1963). Creatine phosphokinase activity in semen. *Lancet* **2**, 498.
- Liess, J., and Grove, D. (1963). Beitrag zur Inaktivierung von Ejakulates durch Begasung unverdunnten Ejakulates mit Kohlendioxyd. *Tieraerztl. Wochenschriften* **70**, 433-437.
- Lillie, F. R. (1913). Studies on fertilization. V. The behavior of the spermatozoa of *Nereis* and *Arbacia* with special reference to egg extractives. *J. Exptl. Zool.* **14**, 515-574.
- Lindahl, P. E., and Drevious, L. O. (1964). Observations on bull spermatozoa in a

- hypotonic medium related to sperm motility mechanisms. *Exptl. Cell Res.* **36**, 632-646.
- Lindahl, P. E., and Wedin, K. (1963). Spermicidal effects studied with a photographic method. The effect of NaF. *Exptl. Cell Res.* **29**, 242-260.
- Machin, K. E. (1963). The control and synchronization of flagellar movement. *Proc. Roy. Soc. (London), Ser. B* **158**, 88-104.
- McKeen, W. E. (1962). The flagellation, movement and encystment of some phycomycetous zoospores. *Can. J. Microbiol.* **8**, 897-904.
- MacLeod, J. (1951). Sulfhydryl groups in relation to the metabolism and motility of human spermatozoa. *J. Gen. Physiol.* **34**, 705-714.
- Macleod, R. A., and Onofrey, E. (1954). Cation antagonism of the antibacterial action of amines. *J. Biol. Chem.* **210**, 193-201.
- Mann, H., and Schmid, O. J. (1961). Der Einfluss von Detergentien auf Sperma, Befruchtung und Entwicklung bei der Forelle. *Intern. Rev. Gesamten Hydrobiol.* **46**, 419-426.
- Mann, T. (1945). Studies on the metabolism of semen. I. General aspects, occurrence and distribution of cytochrome, certain enzymes and coenzymes. *Biochem. J.* **39**, 451-458.
- Mann, T. (1964). "The Biochemistry of Semen and of the Male Reproductive Tract." Wiley, New York.
- Mann, T., and Lutwak-Mann, C. (1948). Studies on the metabolism of semen. IV. Aerobic and anaerobic utilization of fructose by spermatozoa and seminal vesicles. *Biochem. J.* **43**, 266-270.
- Mann, T., and Lutwak-Mann, C. (1955). Biochemical changes underlying the phenomenon of cold-shock in spermatozoa. *Arch. Sci. Biol. (Bologna)* **39**, 578-588.
- Marcus, M., and Mayer, A. M. (1963). Flagellar movement in *Chlamydomonas snowiae* and its inhibition by ATP and DNP. *Microalgae and Photosynthetic Bacteriol.*, pp. 85-96.
- Masaki, J., and Hartree, E. F. (1962). Distribution of metabolic activity, phospholipid and hyaluronidase between the heads and tails of bull spermatozoa. *Biochem. J.* **84**, 347-353.
- Mattner, P. E. (1963). Spermatozoa in the genital tract of ewes. III. The role of spermatozoan motility and of uterine contraction in transport of spermatozoa. *Australian J. Biol. Sci.* **16**, 877-884.
- Miller, R. (1964). Isolation of the chemotactant of *Campanularia (Laomedea) calceolifera*. *Biol. Bull.* **127**, 381.
- Mitchison, J. M. (1961). Some questions for the cell. In "The Cell and the Organism" (J. A. Ramsay and V. B. Wigglesworth, eds.), pp. 37-59. Cambridge Univ. Press, London and New York.
- Mittal, K. K., Salisbury, G. W., Graves, C. N. (1963). Metabolism of bull semen mixed with anti-semen. *J. Animal Sci.* **22**, 1139.
- Mittal, K. K., Salisbury, G. W., Graves, C. N., and Rasmusen, B. A. (1965). Antigens of bovine semen and the influence of specific rabbit and anti-bull-semen serum on metabolic activity of bull spermatozoa. *J. Reprod. Fertility* **10**, 29-34.
- Moghissi, K. S., Dabich, D., Levine, J., and Newhaus, O. W. (1964). Mechanism of sperm migration. *Fertility Sterility* **15**, 15-23.
- Mohri, H. (1964a). Phospholipid utilization in sea-urchin spermatozoa. *Pubbl. Staz. Zool. Napoli* **34**, 53-58.

- Mohri, H. (1964b). Extraction of ATP-ase from sea urchin and fish sperm tails. *Biol. Bull.* **127**, 381.
- Mohri, H., Mohri, T., and Ernster, L. (1964). Biochemical properties of bull-sperm mitochondria. *Biochem. J.* **92**, 38P.
- Mohri, H., and Yasumasu, I. (1963). The respiration of sea urchin spermatozoa. *J. Exptl. Biol.* **40**, 573-586.
- Monroy, A. (1965). "Chemistry and Physiology of Fertilization." Holt, New York.
- Moule, G. R., and Waites, G. M. H. (1963). Seminal degeneration in the ram and its relation to the temperature of the scrotum. *J. Reprod. Fertility* **5**, 433-446.
- Mounib, M. S. (1964). Effects of certain hormones on the metabolism of bull spermatozoa. *Acta Endocrinol.* **45**, 631-640.
- Mounib, M. S., and Chang, M. S. (1964). Effects of irradiation in the metabolism of spermatozoa. *Radiation Res.* **22**, 144-154.
- Nagano, T. (1962). Observations on the fine structure of the developing spermatozoa in the domestic chicken. *J. Cell Biol.* **14**, 193-205.
- Nagano, T. (1963). Fine structural changes in the flagellum of the spermatid in experimental cryptorchidism of the rat. *J. Cell Biol.* **18**, 337-344.
- Nagano, T. (1964). Electron microscopic observations on the rat sperm tail with special reference to the localization of phosphatase activities. *Symp. Cell Chem.* **14**, 251-260.
- Nashed, N., Mixner, J. P., and Mather, R. E. (1964). Bovine semen metabolism. VI. Comparative effects of initial fructose level and incubation temperature on fructolysis and sperm motility. *J. Dairy Sci.* **47**, 87-89.
- Neilands, J. B., and Stumpf, P. K. (1955). "Outline of Enzyme Chemistry." Wiley, New York.
- Nelson, L. (1954). Enzyme distribution in fragmented bull spermatozoa. I. Adenylpyrophosphatase. *Biochim. Biophys. Acta* **16**, 494-501.
- Nelson, L. (1955). Adenosinetriphosphatase of *Mytilus* spermatozoa. I. Effects of pH, calcium and magnesium, and concentration of enzyme and substrate. *Biol. Bull.* **109**, 295-305.
- Nelson, L. (1959). Adenosinetriphosphatase of *Mytilus* spermatozoa. II. Effects of sulfhydryl reagents, temperature and inorganic pyrophosphate. *Biol. Bull.* **117**, 327-339.
- Nelson, L. (1962). Cytochemical aspects of spermatozoan motility. In "Spermatozoan Motility" (D. W. Bishop, ed.), pp. 171-187. Am. Assoc. Advance. Sci., Washington, D.C.
- Nelson, L. (1964). Acetylcholinesterase in bull spermatozoa. *J. Reprod. Fertility* **7**, 65-71.
- Nelson, L. (1966a). Enzyme distribution in naturally decapitated bull spermatozoa. Acetylcholinesterase and adenylpyrophosphatase (in press).
- Nelson, L. (1965). "Flactin," a contractile protein from starfish sperm flagella. *20th Ann. Meeting Soc. Gen. Physiol., Woods Hole, Massachusetts, 1965.*
- Nelson, L. (1966b). Contractile proteins of marine invertebrate spermatozoa. *Biol. Bull.* **130**, 378-386.
- Nelson, L., and Plowman, K. (1963). Actin in the ultrastructure of the sperm flagellum. *Abstr. Biophys. Soc. 7th Ann. Meeting, New York, 1963*, #MD 4.
- Nevo, A., Caplan, S. R., and Schindler, H. J. (1963). Duration of motility and glycolysis of fowl spermatozoa *in vitro* under anaerobic conditions, constant pH and constant glucose concentration. *J. Reprod. Fertility* **6**, 361-370.
- Newton, A. A., and Rothschild, Lord (1961). Energy-rich phosphate compounds in bull semen: comparison of their metabolism with anaerobic heat production

- and impedance change frequency. *Proc. Roy. Soc. (London)*, Ser. B **155**, 183-194.
- Nicander, L. (1962). Development of the fibrous sheath of the mammalian sperm tail. *5th Intern. Congr. for Electron Microscopy, Philadelphia* (S. S. Breese, Jr., ed.), Vol. 2, p. M-4. Academic Press, New York.
- Nicander, L., and Bane, A. (1962a). New observations on the fine structure of spermatozoa. *Intern. J. Fertility* **7**, 339-344.
- Nicander, L., and Bane, A. (1962b). Fine structure of boar spermatozoa. *Z. Zellforsch.* **57**, 390-344.
- Norman, C., and Goldberg, E. (1959). Effect of light on motility, life span and respiration of bovine spermatozoa. *Science* **130**, 624-625.
- Norman, C., Goldberg, E., and Porterfield, I. D. (1962). The effect of visible radiation on the functional life span of mammalian and avian sperm. *Exptl. Cell Res.* **28**, 69-84.
- Noyes, R. W., and Thibault, C. (1962). Endocrine factors in the survival of spermatozoa in the female reproductive tract. *Fertility Sterility* **13**, 346-365.
- Omachi, A., Macey, R. I., and Waldeck, J. G. (1961). Permeability of cell membranes to amine buffers and their effects on electrolyte transport. *Ann. N. Y. Acad. Sci.* **92**, 478-485.
- O'Shea, T., and Wales, R. G. (1964). Effects of potassium on ram spermatozoa during chilling to and storage at 5°C. *J. Reprod. Fertility* **8**, 121-132.
- Page, A. C., Jr., Smith, M. C., Gale, P. H., Polin, D., and Folkers, K. (1961). Coenzyme Q. XXVIII. Activity of the coenzyme Q group in sperm motility. *Biochem. Biophys. Res. Commun.* **6**, 141.
- Page, A. C., Jr., Smith, M. C., Gale, P. H., Polin, D., and Folkers, K. (1963). Coenzyme Q. XXXII. Coenzyme Q and the maintenance of sperm cells *in vitro*. *Arch. Biochem. Biophys.* **101**, 204-208.
- Pautard, F. G. E. (1962). Biomolecular Aspects of Spermatozoan Motility. In "Spermatozoan Motility" (D. W. Bishop, ed.), pp. 189-232. Am. Assoc. Advancement. Sci., Washington, D.C.
- Pease, D. C. (1963). Ultrastructure of flagellar fibrils. *J. Cell Biol.* **18**, 313-326.
- Perez y Perez, F. (1959). Nuevas aportaciones en torno al efecto del dienestrol en la biología espermatina. *Anales Inst. Invest. Vet. (Madrid)* **9**, 321-337.
- Perloff, W. H., and Steinberger, E. (1964). *In vitro* survival of spermatozoa in cervical mucus. *Am. J. Obstet. Gynecol.* **88**, 439-442.
- Pernot, E. (1956). Recherches sur les constituants antigéniques des spermatozoïdes de cobaye. *Bull. Soc. Chim. Biol.* **38**, 1041-1054.
- Persson, B. H., and Melander, S. E. J. (1960). Effect of pH on biological activity of pregnant mare serum gonadotropin. *Nature* **186**, 163.
- Pikó, L., and Tyler, A. (1963). Quantitative studies on the radiosensitivity of sea urchin spermatozoa. *Am. Zoologist* **3**, 533.
- Plowman, K. M., and Nelson, L. (1962). An actin-like protein isolated from starfish spermatozoa. *Biol. Bull.* **123**, 478.
- Posalaky, Z. (1965). Activity of different dehydrogenases and diaphorases in the spermatogenesis of the rat, and its relation to motility. *Acta Histochem.* **20**, 86-90.
- Preer, J. R., Jr. (1959). Studies on the immobilization antigens of *paramecium*. III. Properties. *J. Immunol.* **83**, 385-391.
- Randall, J., Warr, J. R., Hopkins, J. M., and McVittie, A. (1964). A single-gene mutation of *Chlamydomonas reinhardtii* affecting motility: a genetic and electron microscope study. *Nature* **203**, 912-914.

- Renaud, F. L. (1964). The development of basal bodies and flagella in *Allomyces arbusculus*. *J. Cell Biol.* **23**, 76A.
- Renaud, F. L., and Swift, H. (1964). The development of basal bodies and flagella in *Allomyces arbusculus*. *J. Cell Biol.* **23**, 339-354.
- Reynolds, A. J., and Rothschild, Lord (1963). The stirring of the medium by bull spermatozoa. *Proc. Roy. Soc. (London), Ser. B* **157**, 461-472.
- Rice, E. W. (1964). Morphological changes in human spermatozoa following treatment of semen with certain dialkyldithiocarbamates. *Exptl. Cell Res.* **34**, 186-188.
- Richards, A. G. (1956). Temperature in relation to the activity of single and multiple physiological systems in insects. *Proc. 10th Intern. Congr. Entomol.* **2**, 67-72.
- Richards, A. G. (1963a). The rate of sperm locomotion in the cockroach (*Periplaneta americana*) as a function of temperature. *J. Insect Physiol.* **9**, 545-549.
- Richards, A. G. (1963b). The effect of temperature on heartbeat frequency in the cockroach *Periplaneta americana*. *J. Insect Physiol.* **9**, 597-606.
- Rikmenspoel, R. (1957). An optically clear egg-yolk diluent for bull spermatozoa. *Experientia* **13**, 124.
- Rikmenspoel, R. (1962). Biophysical approaches to the measurement of sperm motility. In "Spermatozoan Motility" (D. W. Bishop, ed.), pp. 31-54. Am. Assoc. Advance. Sci., Washington, D.C.
- Rikmenspoel, R. (1964). Measurements of motility and energy metabolism of bull spermatozoa. *Trans. N. Y. Acad. Sci., Ser. II* **26**, #8 Suppl., 1072-1086.
- Rikmenspoel, R. (1965a). The movement of bull spermatozoa and its relation to elastic constants of the tail. *Abstr. Biophys. Soc. 9th Ann. Meeting, San Francisco, 1965*, p. 149 (#FE8).
- Rikmenspoel, R. (1965b). The tail movement of bull spermatozoa. Observations and model calculations. *Biophys. J.* **5**, 365-391.
- Rikmenspoel, R. (1965c). The inhibition by amytal of respiration and motility of bull spermatozoa. *Exptl. Cell Res.* **37**, 312-326.
- Rivera, J. A. (1962). "Cilia, Ciliated Epithelium and Ciliary Activity." Pergamon Press, Oxford and London.
- Rosenbaum, J. L., and Child, F. M. (1964). Incorporation of tritiated precursors into regenerating flagella of two flagellates. *J. Protozool., Suppl.* **11**, 22.
- Roth, L. E. (1964). Motile systems with continuous filaments. In "Primitive Motile Systems in Cell Biology" (R. D. Allen and N. Kamiya, eds.), pp. 527-548.
- Rothschild, Lord (1959). Anaerobic heat production of bull spermatozoa. II. The effects of changes in the colligative and other properties of the suspending medium. *Proc. Roy. Soc. (London), Ser. B* **151**, 1-22.
- Rothschild, Lord (1961). Sperm energetics. In "The Cell and the Organism" (J. A. Ramsay and V. W. Wigglesworth, eds.), pp. 9-21. Cambridge Univ. Press, London and New York.
- Rothschild, Lord (1962). Sperm movement: Problems and observations. In "Spermatozoan Motility" (D. W. Bishop, ed.), pp. 13-29. Am. Assoc. Advance. Sci., Washington, D.C.
- Rothschild, Lord (1964). Anaerobic fructolysis, lactic acid and heat production of bull spermatozoa. *Proc. Roy. Soc. (London), Ser. B* **159**, 291-296.
- Rottenstein, K., Koefoed-Johnsen, H. H., and Andersen, H. (1960). The effect of spermatozoal extracts on the viability of bovine spermatozoa. *Acta Agr. Scand.* **10**, 280-288.

- Rozin, S. (1960). Studies on seminal plasma: I. The role of seminal plasma in the motility of spermatozoa. *Fertility Sterility* **11**, 277–285.
- Rozin, S., and Rozin, R. (1962). The influence of body fluids on motility of spermatozoa. *Intern. J. Fertility* **7**, 327–337.
- Ruegg, J. C. (1961). On the effect of inhibiting the actin-myosin interaction on the viscous tone of a lamellibranch catch muscle. *Biochem. Biophys. Res. Commun.* **6**, 24–28.
- Ruegg, J. C. (1964). A biochemical approach to the catch problem. In “Biochemistry of Muscle Contraction” (J. Gergely, ed.), pp. 412–421. Little, Brown, Boston, Massachusetts.
- Ruegg, J. C., Straub, R. W., and Twarog, B. M. (1963). Inhibition of contraction in a molluscan smooth muscle by thiourea, an inhibitor of the actomyosin contractile system. *Proc. Roy. Soc. (London), Ser. B* **158**, 156–176.
- Saacke, R. G., and Almquist, J. O. (1964). Ultrastructure of bovine spermatozoa. II. The neck and tail of ejaculated spermatozoa. *Am. J. Anat.* **115**, 163–184.
- Salisbury, G. W., and Graves, C. N. (1963). Substrate-free epididymal-like bovine spermatozoa. *J. Reprod. Fertility* **6**, 351–359.
- Salisbury, G. W., and Lodge, J. R. (1962). Metabolism of spermatozoa. *Advan. Enzymol.* **24**, 35–104.
- Salisbury, G. W., Vandemark, N. L., Lodge, J. R., and Cragle, R. G. (1960). Inhibition of spermatozoon metabolism by $p\text{CO}_2$, pH, K-ion and antibacterial compounds. *Am. J. Physiol.* **198**, 639.
- Sanger, S. W., and Szent-Gyorgyi, A. G. (1965). Band pattern changes in the striated adductor muscle of *Pecten irradians*. *Biol. Bull.* **127**, 391.
- Satir, P. (1964). Filament-matrix interaction during ciliary movement: inferences drawn from electron microscopy of the distal end of the ciliary shaft of lamellibranch gill cilia. *J. Cell Biol.* **23**, 82A.
- Schenkman, J. B., Rickert, D. A., and Westerfeld, W. W. (1964). The α -glycerophosphate dehydrogenase activity of rat spermatozoa. *Federation Proc.* **23**, 433.
- Schindler, H., and Nevo, A. (1962). Reversible inactivation and agglutination of fowl and bull spermatozoa under anaerobic conditions. *J. Reprod. Fertility* **4**, 251–265.
- Schirren, C. (1963). The effects of antibiotics and adrenal steroid hormones on the motility of human spermatozoa. *Arch. Gynaekol.* **198**, 253–260.
- Scott, T. W., Bagett, B., and White, I. G. (1963). Metabolism of testosterone by semen and the effect of testosterone on oxidative metabolism of spermatozoa. *Australian J. Exptl. Biol. Med. Sci.* **41**, 363–368.
- Scott, T. W., Wales, R. G., Wallace, J. C., and White, I. G. (1963). Composition of ram epididymal and testicular fluid and the biosynthesis of glycerylphosphoryl choline by the rabbit epididymis. *J. Reprod. Fertility* **6**, 49–59.
- Sekine, T. (1951). Cholinesterase in pig spermatozoa. *J. Biochem., Tokyo* **38**, 171–179.
- Sleigh, M. A. (1962). “The Biology of Cilia and Flagella.” Pergamon Press, Oxford and London.
- Smith, A. U. (1961). “Biological Effects of Freezing and Supercooling.” Williams & Wilkins, Baltimore, Maryland.
- Sobrero, A. J., and MacLeod, T. M. (1962). The immediate post coital test. *Fertility Sterility* **13**, 184–189.
- Steinbach, H. B. (1952). The sodium and potassium balance of muscle and nerve. In “Modern Trends in Physiology and Biochemistry” (E. S. G. Barron, ed.), pp. 173–192. Academic Press, New York.

- Steinbach, H. B., and Dunham, P. R. (1961). Ionic gradients in some invertebrate sperm. *Biol. Bull.* **120**, 411-419.
- Steinbach, H. B., and Dunham, P. R. (1962). Ionic balance of sperm cells. In "Spermatozoan Motility" (D. W. Bishop, ed.), pp. 55-57. Am. Assoc. Advance. Sci., Washington, D.C.
- Stephens, R. E. (1965a). Muscle contraction departing from the sliding filament model. *Abst. Biophys. Soc. 9th Ann. Meeting, San Francisco, 1965*, p. 129.
- Stephens, R. E. (1965b). Glycerination-induced A-band contraction in invertebrate striated muscle. *Biol. Bull.* **129**, 424.
- Suzuki, R. (1958). Sperm activation and aggregation during fertilization in some fishes. I. Behavior of spermatozoa around the micropyle. *Embryologia (Nagoya)* **4**, 93-102.
- Suzuki, R. (1959). Sperm activation and aggregation during fertilization in some fishes. III. Non-species specificity of stimulating factor. *Annotationes Zool., Japon.* **32**, 105-111.
- Symposia Biologica Hungarica (1964). "Physiologie (Bewegung) der Spermien," Vol. 4. Akademiai Kiado, Budapest.
- Szent-Györgyi, A. G. (1951). A new method for the preparation of actin. *J. Biol. Chem.* **192**, 361-369.
- Szent-Györgyi, A., and Kaminer, B. (1963). Metin and metactomyosin. *Proc. Natl. Acad. Sci. U. S.* **50**, 1033-1036.
- Taguchi, S., Yasumasu, I., and Mohri, H. (1963). Changes in the content of adenine nucleotides upon aerobic incubation of sea urchin spermatozoa. *Exptl. Cell Res.* **30**, 218-223.
- Tampion, D. (1963). Swimming rate of boar spermatozoa. *J. Reprod. Fertility* **6**, 471.
- Tampion, D., and Gibbons, R. A. (1963a). Effect of pH on the swimming rate of bull spermatozoa. *J. Reprod. Fertility* **5**, 249-258.
- Tampion, D., and Gibbons, R. A. (1963b). Swimming rate of bull spermatozoa in various media and the effect of dilution. *J. Reprod. Fertility* **5**, 259-275.
- Terner, C. (1962). Oxidative and biosynthetic reactions in spermatozoa. In "Spermatozoan Motility" (D. W. Bishop, ed.), pp. 89-98. Am. Assoc. Advance. Sci., Washington, D.C.
- Tibbs, J. (1959). The adenosine triphosphatase activity of perch sperm flagella. *Biochim. Biophys. Acta* **33**, 220-226.
- Tibbs, J. (1962). Adenosine triphosphatase and acetylcholinesterase in relation to sperm motility. In "Spermatozoan Motility" (D. W. Bishop, ed.), pp. 233-250. Am. Assoc. Advance. Sci., Washington, D.C.
- Tsuchiya, S. (1962). Metabolism of acid-soluble nucleotides in boar epididymal spermatozoa and its relation to their motility. *Seikagaku* **34**, 561-575.
- Tyler, A., and Bishop, D. W. (1963). Immunological phenomena. In "Mechanisms Concerned with Conception" (C. G. Hartman, ed.), pp. 397-482. Pergamon Press, Oxford and London.
- Umberger, E. J., and Cass, G. H. (1959). Effect of pH on biological activity of chorionic gonadotropin. *Science* **129**, 1738.
- Vandemark, N. L., and Koyama, J. K. (1963). Immobilization and reactivation of bull sperm. *J. Animal Sci.* **22**, 1140.
- Van Duijn, C. (1964). Cytologic details and size frequency distributions in human spermatozoa. *Intern. J. Fertility* **9**, 533-546.
- Van Wagtenonk, W. J., and Vloedman, D. A., Jr. (1951). Evidence for the

- presence of a protein with ATP-ase and antigenic specificity in *Paramecium aurelia*, variety 4, stock 51. *Biochim. Biophys. Acta* **7**, 335–336.
- Wales, R. G., and Choong, C. H. (1963). The effect of ultraviolet radiation on ram and bull spermatozoa. *Australian J. Biol. Sci.* **16**, 885–895.
- Wales, R. G., and White, I. G. (1960). The toxicity of some antibacterials to fowl spermatozoa. *Australian J. Biol. Sci.* **13**, 361–370.
- Wales, R. G., and White, I. G. (1963). Viability of diluted dog spermatozoa *in vitro*. *J. Reprod. Fertility* **5**, 67–76.
- Watanabe, S., and Maruyama, K. (1964). Relaxing effects of formamide on glycerinated muscle fibers and on myosin B suspension. *Am. J. Physiol.* **207**, 809–813.
- Watanabe, S., Sargeant, T., and Angleton, M. (1964). Role of magnesium in contraction of glycerinated muscle fibers. *Am. J. Physiol.* **207**, 800–880.
- Watson, M. R., and Hopkins, J. M. (1962). Isolated cilia from *Tetrahymena pyriformis*. *Exptl. Cell Res.* **28**, 280–295.
- Weil, A. J. (1960). Immunological differentiation of epididymal and seminal spermatozoa of the rabbit. *Science* **131**, 1040–1041.
- Willson, J. T., and Katsh, S. (1964a). Sperm antigen localization by ferritin-labeled antibodies. *Anat. Record* **148**, 411.
- Willson, J. T., and Katsh, S. (1964b). Localization of spermatozoal antigens by fluorescent antibodies. *Anat. Record* **148**, 411.
- Yanigamachi, R., and Chang, M. C. (1963). Sperm ascent through the oviduct of the hamster and rabbit in relation to time of ovulation. *J. Reprod. Fertility* **6**, 413–420.
- Young, L. G., and Nelson, L. (1966). Unpublished observations.
- Young, M. R., and Edwards, R. G. (1963). Ultraviolet microscopy and the detection of formazan deposits. *J. Histochem. Cytochem.* **11**, 444–446.

CHAPTER 3

Sperm Metabolism

Thaddeus Mann

AGRICULTURAL RESEARCH COUNCIL UNIT OF REPRODUCTIVE PHYSIOLOGY AND
BIOCHEMISTRY, CAMBRIDGE UNIVERSITY, CAMBRIDGE, ENGLAND

I. Introduction	99
II. Chemical Characteristics of Semen	101
A. Spermatozoa	101
B. Seminal Plasma	104
III. Anaerobic and Aerobic Glycolysis	105
A. Fructolysis in Whole Semen	105
B. Glycolysis in Washed Sperm Suspensions	106
IV. Respiration	107
A. Endogenous Respiration	107
B. Exogenous Respiration	109
V. Sperm Energetics	111
A. Energy-rich Phosphorus Compounds	111
B. Relationships between Metabolism, Energy Expenditure, and Heat Dissipation by Spermatozoa	112
VI. Significance of Anaerobic and Aerobic Metabolism for Sperm Survival in the Female Tract	114
References	114

I. Introduction

Spermatozoa are endowed with the means of metabolizing a wide range of nutrients, some of endogenous, but most of exogenous, origin. The available intracellular nutrient reserve of the sperm cells consists, so far as is known, mainly of lipids. Among the extracellular substrates, however, are several glycolyzable sugars, as well as a variety of oxidizable fatty acids, amino acids, polyhydroxy alcohols, and other organic compounds. Some are present in the natural environment of spermatozoa, that is, in fluids secreted by the male and female reproductive tract, while others are accessible to the sperm cells only upon the addition of artificial media of the kind that are employed, for example, in the practice of diluting and storing semen for the purpose of artificial insemination.

The two major types of metabolic processes in semen as ejaculated, are respiration and glycolysis. The ability to respire is common to all animal spermatozoa, but with respect to glycolysis, the spermatozoa of different species vary markedly. At one time the view was commonly held that while the spermatozoa of vertebrates are endowed with both glycolytic and respiratory activity, those of invertebrates are capable only of respiration. That belief, however, was based mainly on experiments with the sperm of echinoderms, in which fertilization occurs externally. From more recent studies it would appear that the glycolytic mechanism operates also in the spermatozoa of certain invertebrates with internal fertilization (e.g., some mollusks), while it is absent or only feebly active in certain vertebrates, notably those with external fertilization (e.g., some fishes).

The rate at which spermatozoa are capable of respiration and glycolysis depends mainly on two factors: sperm concentration, or "density," and the intensity of sperm movements, or "motility." Good sperm motility and high metabolic activity are, in fact, so closely correlated that in the practice of semen appraisal measurements of either motility or metabolic rate can be used equally well for the purpose of evaluating the so-called "quality" of semen. It must be continually borne in mind, however, that although good motility and high metabolic activity are often found together in potentially fertile spermatozoa, the level of motility or the rate of metabolism by themselves need not necessarily be indicative of the fertilizing capacity of the spermatozoa. For example, semen which has been irradiated with a large dose of X-rays usually exhibits normal or near normal motility, glycolysis, and respiration, and yet its fertilizing ability may be low, pathologically altered, or absent altogether. Fertilizing ability on the one hand and motility and metabolism on the other are, after all, inherent in two different structural regions of the sperm cell: the genetic material required for fertilization being located in the sperm head, but motility and the catalytic systems required for glycolysis and respiration being associated with the sperm flagellum.

The imposing accumulation of data on the subject of semen metabolism has been reviewed extensively on several occasions (e.g., Salisbury and Lodge, 1962), and recently in the monograph "The Biochemistry of Semen and of the Male Reproductive Tract" (Mann, 1964). Therefore, rather than to refer again to all the numerous topics and individual papers, it is intended here to stress only the broader aspects of sperm metabolism, mainly those which fit closely into the general terms of reference governing the subject of this publication, allowing for a discussion of problems which are of general importance to those engaged in comparative studies of reproduction. It should perhaps be stressed,

however, that of the various environmental conditions which affect sperm metabolism, those that have been given particular attention in recent years are oxygen tension (Lindahl and Thunquist, 1964; Nevo, 1965); carbon dioxide tension (Salisbury and Graves, 1963; Mohri and Yasumasu, 1963); concentration of ions (Lodge *et al.*, 1963; Dott and White, 1964; Wallace and Wales, 1964); and effects of light (Williams and Hamner, 1963).

II. Chemical Characteristics of Semen

Ejaculated whole semen represents a suspension of spermatozoa in the seminal plasma. The spermatozoa originate in one organ only, the testis, but the seminal plasma consists of secretions produced by a number of glands, which, in the higher mammals include the epididymis, vas deferens, ampulla, seminal vesicle, prostate, bulbourethral gland, and certain other accessory organs of reproduction. In recent years considerable progress has been made in chemical studies of both spermatozoa and seminal plasma. The most significant findings are briefly enumerated below, and the main chemical constituents of sperm and seminal plasma are shown in Fig. 1.

A. SPERMATOZOA

Each of the three principal structural components of a spermatozoon, that is, the sperm head, middle piece, and tail, possesses its own chemical peculiarities.

The sperm head, which is taken up largely by the sperm nucleus and acrosome, contains DNA as the predominant nuclear constituent, and a lipoglycoprotein complex in the acrosome; in the acrosomal region are also located certain enzymes, e.g., hyaluronidase. The DNA, which in the sperm nucleus is conjugated with protamines and histones, is made up mainly of four deoxyribomononucleotides: adenylic, guanylic, cytidylic, and thymidylic acid. The content of DNA per sperm nucleus and its composition with respect to the ratio between the four mononucleotides are species variable, but they are characteristically constant within a given species, at any rate in "normal sperm," that is, normally shaped, fully motile, and potentially fertile spermatozoa. The acrosomal lipoglycoprotein complex, referred to as acrosomin, probably also differs among species in its composition, for example, with respect to its carbohydrate components, viz. galactose, mannose, fucose, glucosamine, galactosamine, and sialic acid. The lipoglycoprotein complex, at least as obtained from ram sperm acrosomes, is, in addition, endowed with some proteolytic activity, as well as with an ability to disperse cumulus and corona radiata cells around rabbit eggs, and to dissolve the zona pel-

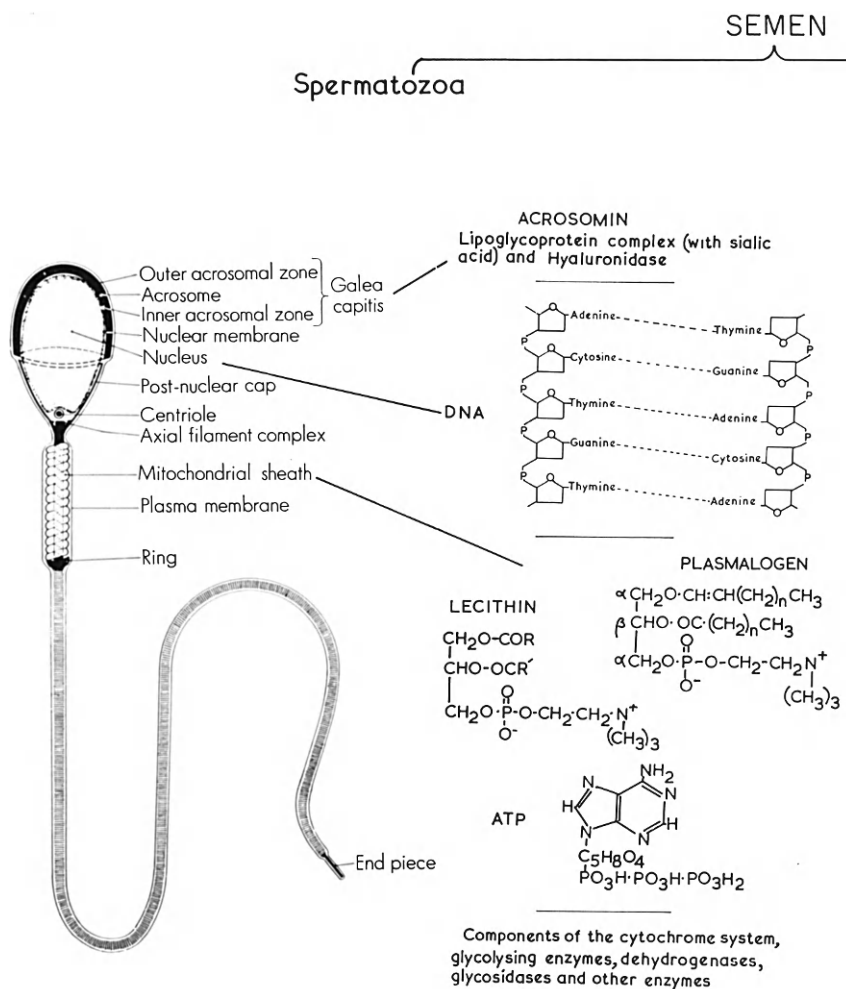


FIG. 1. Diagrammatic representation of the chemical composition of semen.

lucida of these eggs, thus acting as a kind of "zona lysin" (Hartree and Srivastava, 1965; Srivastava *et al.*, 1965).

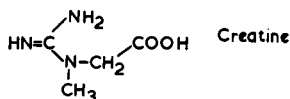
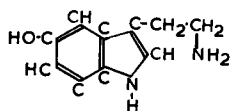
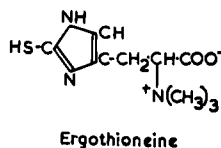
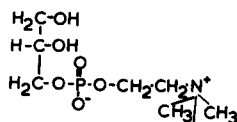
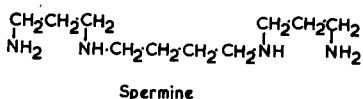
The middle piece and tail, which together make up the sperm flagellum, contain in the center a number of contractile protein fibrils running along the entire length of the flagellum and forming the so-

Seminal plasma

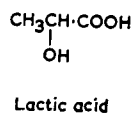
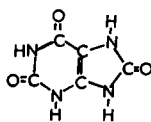
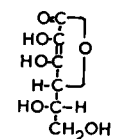
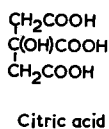
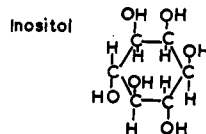
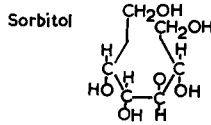
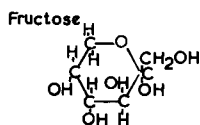
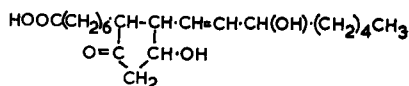
PROTEINS, MUCOPROTEINS and PROTEOLYTIC ENZYMES

e.g. vesiculase, coagulase, fibrinolysin, fibrinogenase, aminopeptidase, pepsinogen, transaminases, deaminases, phosphatases, 5-nucleotidase, ATP-ases, nucleases, nucleosidases, glycosidases

NITROGENOUS BASES



Prostaglandin E



called axial filament complex of the spermatozoon. In the middle piece, but not in the tail, these fibrils are surrounded by the mitochondrial sheath, a lipid-rich structure composed of mitochondria which are wrapped around the axial filament. The most outstanding feature of this structure is the presence in it of the complete cytochrome system. In

addition, the flagellum is the site of various dehydrogenases and the complex of enzymes and coenzymes necessary for glycolysis and respiration. The flagellum is also the locus of ATPase, the enzyme held to be responsible for providing directly the energy for sperm movements, through its action on ATP. The high lipid content of the sperm cell, much of it concentrated in the middle piece, is due chiefly to two phospholipids: lecithin (phosphatidyl choline) and plasmalogen (phosphatidal choline). The latter, an aldehydogenic lipid, differs from lecithin by having in place of one acyl residue (fatty acid) an unsaturated ether grouping (a substituted vinyl ether) from which a fatty aldehyde can be liberated by acid treatment. However, spermatozoa of different species possess different proportions of lecithin, plasmalogen, and related lipids: ram spermatozoa are extraordinarily rich in plasmalogen, but those of the sea urchin carry mostly lecithin and have little plasmalogen.

B. SEMINAL PLASMA

The seminal plasma, i.e., the acellular portion of the ejaculate, differs in many ways from other body fluids. Mammalian seminal plasma quite often contains a remarkably high concentration of several free amino acids, notably glutamic acid, and some unusual nitrogenous bases such as spermine, spermidine, choline, phosphorylcholine, glycerylphosphorylcholine, ergothioneine, and creatine. In many mammals and in man, the seminal plasma contains fructose as the main reducing carbohydrate, quite often in concentrations exceeding the blood glucose level, as well as two closely related polyols, namely, sorbitol and inositol. The seminal plasma of most higher mammals also shows a high concentration of citric acid. Some mammalian species, especially the human, contain in the seminal plasma a group of unusual organic acids derived from cyclopentanone, the so-called "prostaglandins," which are endowed with distinct pharmacodynamic activity upon the blood vessels, uterus, and smooth muscles in general. Another characteristic feature of the mammalian seminal plasma is the presence of a wide range of very active enzymes, including proteolytic ones, of which some play an important part in semen coagulation and liquefaction; various phosphokinases, phospholipases, and phosphatases, the latter group including the powerful 5-nucleotidase, the acid phosphatase, and no less than three different ATP-splitting enzymes; a series of nucleolytic enzymes, ranging from nucleases and nucleotide pyrophosphatase to nucleosidases; and a large group of glycosidases, the most active ones being β -glucuronidase, α -mannosidase, β -galactosidase, and β -acetylglucosaminidase. The presence of these enzymes in the seminal plasma is puzzling, because elsewhere in the animal body one would expect to find them within, rather than

outside, living cells. Examples of such supposedly intracellular enzymes in which the seminal plasma abounds are amino oxidase; transaminase; lactic, malic, and isocitric dehydrogenase; phosphohexose isomerase; and zymohehexase (aldolase).

Systematic chemical analyses of nonmammalian seminal plasma have not as yet been very extensive, but there are clear indications that in birds, frogs, fishes, reptiles, insects, mollusks, and even echinoderms, the seminal plasma as a whole, or certain individual male accessory secretions, also exhibit some peculiarities of composition such as a high content of free amino acids, chiefly glutamic acid, in avian seminal plasma; 5-hydroxytryptamine (serotonin) in the claspersiphon secretion of the spiny dogfish *Squalus acanthias* and in the spermatophoric sac of the common octopus *Octopus vulgaris*; pyrophosphate in the ejaculatory duct of the hawkmoth; fructose, glucose, and trehalose in honeybee semen; and potassium ions in the seminal plasma of many animals, including amphibians, fishes, and echinoderms (see Mann and Lutwak-Mann, 1963; Mann, 1964).

III. Anaerobic and Aerobic Glycolysis

When deprived of oxygen, the spermatozoa can turn to extracellular glycolyzable sugars as their chief source of metabolic energy. In whole semen, fructose presumably is primarily utilized, provided always that sufficient fructose is available in the seminal plasma. Spermatozoa that have been separated from the seminal plasma, for example, by centrifugation and washing, can carry on anaerobically only if the seminal plasma has either been restored to them or substituted by an artificial medium containing a glycolyzable sugar, such as fructose, glucose, or mannose.

A. FRUCTOLYSIS IN WHOLE SEMEN

Anaerobic incubation at body temperature of freshly ejaculated semen of man and those mammals in which fructose is normally present causes a progressive decline of the fructose content and a simultaneous accumulation of lactic acid. In the presence of a suitable buffer, fructolysis progresses often almost linearly, the rate depending on the actual number and motility of the spermatozoa. In normal bull semen, for example, the so-called "index" of fructolysis approaches 2, that is, up to 2 mg fructose are anaerobically metabolized to lactic acid in the presence of 10^9 bovine spermatozoa in 1 hour at 37°C. Man, ram, and bull show approximately equal rates of fructolysis in semen, but in boar semen the rate is much lower. Apart from sperm density and motility, there are other factors capable of influencing the rate of fructolysis, such as

temperature, pH, and the initial fructose content. A poor fructolysis rate has been observed in certain cases of bovine and human subfertility, possibly due to the coincident decrease of sperm motility. On the whole, one expects fructolysis to be related to sperm density and motility and not to the fertilizing power of spermatozoa.

Substances which inhibit sperm fructolysis, e.g., fluoride, heavy metals, iodoacetate, chloromercuribenzoate, iodosobenzoate, hydrogen peroxide, various oxidizing reagents, and surface active agents, all at the same time inhibit motility. In fact, there is a close correspondence between the effects of most inhibitors on fructolysis and motility. Moreover, when fructolysis is inhibited reversibly, e.g., by fluoride or high CO_2 , the effect on sperm motility is equally reversible. Thus these are spermiostatic agents, while substances which abolish the fructolytic ability of spermatozoa irreversibly, and at the same time have also a permanent immobilizing effect on spermatozoa, must be designated as true spermicidal agents.

B. GLYCOLYSIS IN WASHED SPERM SUSPENSIONS

Much of the present knowledge concerning sperm glycolysis is derived from studies of washed sperm suspensions to which sugars were added, and in most of these studies the sperm suspensions were prepared from ejaculated semen by centrifugation and washing with Ringer solution or similar isotonic media. Provided that centrifugation and washing are not excessive, such suspensions usually contain motile and metabolically active spermatozoa.

If glucose or fructose are added singly to a buffered suspension of washed ram or bull spermatozoa and the mixture incubated anaerobically, lactic acid is formed at approximately the same rate from either of the two sugars. However, if the washed spermatozoa are supplied with a 50:50 mixture of glucose and fructose, then in spite of an unchanged rate of total lactic acid production, glucose will be utilized preferentially, the rate of glucose utilization becoming more than 50% and that of fructose less than 50%. The suppression of fructose utilization by glucose probably has its foundation in the behavior of hexokinase, which initiates the process of glycolysis in spermatozoa and is well known to have a higher affinity for glucose than fructose.

The evidence available at present indicates that the intermediary stages of glycolysis in spermatozoa resemble those established in other animal tissues. Glucose or fructose added to a washed sperm suspension enters the glycolytic cycle by way of the hexokinase-catalyzed reaction with ATP to form first monophosphohexoses and subsequently diphosphofructose, phosphotriose, phosphoglyceric acid, phosphopyruvic

acid, pyruvic acid, and lactic acid. There is good evidence in support of the concept that ATP represents the cardinal link between the motility of spermatozoa and glycolysis, and that the content of ATP, and ultimately the survival of spermatozoa, depend, anaerobically at any rate, on the maintenance of sperm glycolysis. As in other tissues, the final stage in glycolysis involves two oxidoreductive processes, the first an oxidation of phosphotriose to phosphoglyceric acid coupled with a reduction of NAD^+ to NADH, and the second a reduction of pyruvic acid to lactic acid coupled with an oxidation of NADH to NAD^+ .

Introduction of oxygen into an anaerobically incubated sample of either whole semen or a sugar-containing suspension of washed spermatozoa causes a diminution in the rate of sugar utilization, owing to the Pasteur effect, and at the same time oxidation of lactic acid sets in. But it is a characteristic of mammalian spermatozoa that the extent of the Pasteur effect is relatively slight. In that respect again, however, spermatozoa of different species differ; moreover, even within a given species, variations are often observed between ejaculated and epididymal spermatozoa.

IV. Respiration

Both types of sperm respiration, endogenous and exogenous, depend on the catalytic activity of the cytochrome system, which, apart from the cytochromes a, a_3 , c, c_1 , and b, includes one more, rather unusual, component, designated by Gonse (1962) as b_d . The content of cytochromes a and a_3 is strikingly high: in bull spermatozoa it represents $0.16 \mu\text{mols}$ and $0.176 \mu\text{mols}/10^9$ sperm, respectively.

Both the endogenous and exogenous respiratory activity of spermatozoa are usually, though by no means always, correlated with sperm concentration and motility. It is customary to express it in terms of Z_{O_2} , a coefficient denoting $\mu\text{l O}_2$ taken up by 10^8 spermatozoa per hour. In bull, ram, rabbit, and cock semen, Z_{O_2} values are of the order of 10–20 at 37°C . For the sea urchin *Echinus esculentus*, a value of 6 at 15°C has been reported. In spermatozoa removed from the spermatophores of the American squid *Loligo pealii*, we found a Z_{O_2} value of about 10 at 20 – 25°C (Austin *et al.*, 1964). In the Crustacean *Balanus balanus*, noted for its negligible sperm motility, oxygen uptake was reported as only $0.18 \mu\text{l O}_2/10^8$ sperm/hour at 10°C (Barnes, 1962).

A. ENDOGENOUS RESPIRATION

Spermatozoa washed free of seminal plasma, and thereby deprived of glycolyzable sugar, retain their ability to consume oxygen to an appreciable extent, but not for as long as in whole semen. In the absence

of extraneous sugar, they appear to utilize mainly the intracellular phospholipids as the source of aerobic energy. In vertebrates and invertebrates alike, it seems fairly certain that phospholipids occupy a pivotal position in the endogenous respiration of spermatozoa. The actual mechanism, however, which controls the aerobic breakdown of intracellular sperm phospholipids is variable. Thus, for example, in washed ram spermatozoa the phospholipids (largely consisting of plasmalogen) are broken down to free fatty acids without, however, a concomitant appearance of any appreciable quantities of acid-soluble glycerylphosphoryl compounds (Hartree and Mann, 1961). In sea urchin spermatozoa, on the other hand, the breakdown of phospholipids (involving almost all fractions, including lecithin, cephalin, sphingomyelin, and the inositol-containing phospholipid) results not only in the liberation of fatty acids but in the appearance of acid-soluble glycerylphosphoryl compounds as well (Mohri, 1961; Mohri and Horiuchi, 1961). In both instances, however, it is the oxidative degradation of the liberated fatty acids which provides the ultimate source of aerobic energy for the endogenously respiring spermatozoa. The concept that intracellular phospholipids constitute the main source of energy for such spermatozoa is further supported by the observations on the respiratory quotient, which in the absence of extraneous nutrients is not far removed from a value of 0.7. The enzymic pathways involved in the actual oxidation of fatty acids still remain to be explored.

Among factors which influence the endogenous respiration of spermatozoa the so-called "dilution effect" deserves special attention, with particular reference to sea urchin semen. When shed into seawater, sea urchin spermatozoa become suddenly intensely motile, and at the same time their respiration increases. The motility, however, is of limited duration and declines progressively in spite of the presence of oxygen. If the initial dilution with seawater is excessive, the decline may follow almost immediately after the onset of activity. It is possible to prolong the viability of such dilute sperm suspensions by various means, for instance, by replacing seawater with egg water, that is, seawater in which sea urchin eggs have been kept for some time; by using a solution of the jelly which normally surrounds sea urchin eggs; by enriching the seawater with certain proteins, peptides, or amino acids, which may act by virtue of either metal-complexing properties or buffering capacity, or simply as a protective colloid.

Up to a point, changes in the respiration and motility caused by dilution resemble those induced either by extensive washing of spermatozoa or, in general, by procedures which accelerate the process of so-called "sperm senescence." In mammalian spermatozoa, which on the

whole respond to dilution, washing, etc., in much the same way as sea urchin sperm, the early stages of senescence are marked by swelling and degeneration of lipoproteins which normally coat the sperm cells. This stage is succeeded by increased cell permeability, leakage of vital intracellular constituents (including some proteins, e.g., cytochrome c), decrease in the content of ATP and other coenzymes, oxidation of intracellular sulfhydryl groups essential for normal sperm motility, an upset in ionic-exchange reactions (especially those concerned with a loss of potassium ions), and occasionally an apparent "activation" of succinate dehydrogenase.

However, even with respect to the response of spermatozoa to the damaging influence of dilution, washing, or aging, great differences have been observed not only among species but also between epididymal and ejaculated spermatozoa belonging to the same species. Similar observations have been made on spermatozoa with respect to the so-called "cold shock," that is, the rapid and largely irreversible loss of metabolic activity, motility, and fertilizing power, which is induced in semen or sperm suspensions of certain mammals by rapid cooling from body temperature to slightly above 0°C. Human spermatozoa are said on the whole to be more resistant to cold shock than those of either bull or ram.

B. EXOGENOUS RESPIRATION

Of the various substrates for exogenous respiration of spermatozoa some, but by no means all, actually occur in the seminal plasma or in the secretions of the female reproductive tract. Fructose, sorbitol, and lactic acid are physiological constituents of mammalian seminal plasma which are capable of sustaining the normal sperm respiration, but glucose and mannose can do so equally well, though normally glucose occurs in semen in trace amounts only, while free mannose is totally absent. Glycerol, another substrate which can be effectively oxidized by spermatozoa, is not present in semen, except possibly in traces, but may arise in the female reproductive tract by the enzymic action of a diesterase on a normal seminal constituent, namely, glycerylphosphorylcholine (White *et al.*, 1963; Wallace *et al.*, 1964). Some of the volatile fatty acids oxidizable by spermatozoa, like acetic acid, have also been found in semen, but because of their low concentration in seminal plasma, it is uncertain whether they play any substantial role in the normal respiration of spermatozoa.

Like other metabolic processes in semen, exogenous sperm respiration exhibits some striking species peculiarities. In mammalian and avian spermatozoa an increased rate of respiration due to the addition of an extracellular substrate is usually associated with an increase in motility,

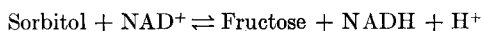
but sea urchin spermatozoa may respond to the addition of certain substances by an increased oxygen consumption which is either not coupled with a change in motility or even results in a marked inhibition of motility. Trout spermatozoa oxidize pyruvate more rapidly than acetate, and glucose only very little (Terner and Korsh, 1963). Ram and bull spermatozoa oxidize acetate in preference to glucose, but with dog spermatozoa the reverse appears to be true. Ram spermatozoa oxidize lower fatty acids containing an even number of carbon atoms better than those with an odd number, but dog and cock spermatozoa oxidize acetate and propionate equally well (Scott *et al.*, 1962). Ram and bull, but not boar or stallion, spermatozoa can oxidize sorbitol (Aalbers *et al.*, 1961; Mann *et al.*, 1963).

The oxidation of sugars such as glucose, fructose, or mannose probably proceeds mainly via lactic acid, that is, after glycolytic conversion.

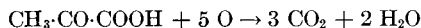


The evidence for the occurrence of a direct pathway of sugar oxidation via phosphopentose is weak, particularly with respect to mammalian spermatozoa. In sea urchin sperm, on the other hand, several enzymes of the phosphopentose cycle have been demonstrated, including phosphohexose-isomerase and phosphoketose-epimerase.

As regards glycerol and sorbitol, the former appears to be oxidized mainly via dihydroxyacetone, while the latter has been definitely shown to undergo oxidation under the influence of a specific enzyme, the sorbitol dehydrogenase (ketose reductase), in the presence of NAD.



Next to lactic acid, pyruvic acid is one of the substrates most readily oxidized by mammalian spermatozoa. Some of it, no doubt, is oxidized directly, according to the following equation:



Another part, however, is probably metabolized by a dismutation process, two molecules of pyruvate interacting so that one is oxidized to acetate and carbon dioxide and the other is simultaneously converted to lactic acid:



Apart from lactic and pyruvic acid, various other organic acids can be oxidized by spermatozoa. Bovine spermatozoa, for example, have been shown to produce $^{14}\text{CO}_2$ from ^{14}C -labeled formate, acetate, butyrate, octanoate, myristate, palmitate, and stearate (Flipse, 1960).

Since sperm respiration depends on the catalytic activity of the cytochrome system, it is understandably sensitive to inhibitors of that system such as cyanide and azide. That applies undoubtedly to the sperm of all those mammals that have been so far studied. It may not, however, always apply to sea urchin spermatozoa, which at least under certain conditions have been reported to respond to azide with an increase, rather than a decrease, in oxygen consumption. For a detailed discussion of substances affecting respiration, and of other related aspects of sperm metabolism, the reader is referred to more recent publications on that subject (Flipse, 1962; Salisbury and Lodge, 1962; Turner, 1962; Mann, 1964).

V. Sperm Energetics

There is much to be said in support of the concept that the prime role of both the anaerobic and aerobic sperm metabolism is to generate and maintain the energy-rich phosphorus compounds and store the chemical energy in the form of these compounds, since they are needed to support sperm motility.

A. ENERGY-RICH PHOSPHORUS COMPOUNDS

The main energy-rich phosphagen present in spermatozoa of both vertebrates and invertebrates is ATP, and the enzyme mainly responsible for making available the energy of ATP-phosphate bonds is in all probability the sperm-ATPase.

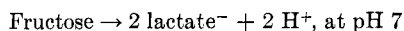
Using new methods of nucleotide analysis, Newton and Rothschild (1961) showed that bull spermatozoa examined immediately after collection of semen contain on the average 129×10^{-12} μ moles ATP, 120×10^{-12} μ moles ADP, and 271×10^{-12} μ moles adenylic acid and inosine per single spermatozoon, and that the content of easily hydrolyzable phosphorus, ATP, and ADP decreases during anaerobic incubation of semen. According to these authors, there is also present in bovine spermatozoa quite an appreciable quantity of phosphagens other than ATP and ADP, one-fifth of the highly labile phosphate groups being accounted for as phosphocreatine. A slightly lower level of phosphocreatine was reported earlier by Yanagisawa (1959) in sea urchin spermatozoa (*Hemicentrotus pulcherrimus* and *Pseudocentrotus depressus*). The results obtained by Taguchi *et al.* (1963) in *Pseudocentrotus depressus* indicate that both ATP and ADP decrease markedly when incubation experiments are made with suspensions of low sperm density and in the presence of KOH as CO₂-trapping reagent, but not in concentrated sperm suspensions and in the absence of KOH, indicating the involvement of CO₂ as a nucleotide-stabilizing agent.

Much speculation, but not nearly enough experimental evidence, backs up the present ideas concerning how, in fact, the nucleotides transmit their chemical energy. According to the most recent view concerning the molecular basis of flagellar activity in general (Silvester and Holwill, 1965), the contractile capacity of the flagellum is a property of "contractile units" in the axial filament, identical in distribution with the "structural subunits" of the individual protein fibrils. The contractile units are associated with bound adenosine phosphate molecules for most of the period of the flagellar beat. The proposed contraction-relaxation cycle begins when a mechanical deformation, conveyed passively along the beating flagellum, stimulates a particular unit to split the bound ATP to the bound ADP, and in doing so provides it with enough energy from dephosphorylation to perform work on the flagellar system. Contraction follows quickly after stimulation, and then the unit "relaxes." Now, the bound ADP is exchanged with free ATP in the local environment, and in that way enough bound ATP is formed to initiate the next contraction cycle. According to that concept, the frequency of flagellar beat should depend foremost on the rate with which bound ATP is replaced by free ATP.

B. RELATIONSHIPS BETWEEN METABOLISM, ENERGY EXPENDITURE, AND HEAT DISSIPATION BY SPERMATOOZOA

According to recent studies, chiefly by Rothschild (1961, 1962), the actual correlation between sperm metabolism *in toto*, the metabolic energy made available by ATP splitting, the mechanical energy expended by spermatozoa during their movements, and the heat dissipated by the spermatozoa can be expressed as follows.

In anaerobically fructolyzing semen the chief source of metabolic energy is the enzymic process of fructose conversion to lactic acid, a process which, ideally, should make available to the spermatozoa an amount of energy equal to the free energy change of the reaction:



namely, -47.3 kcal, or $-47.3 \times 4.184 \times 10^{10}$ ergs/mol fructose.

In that case, 10^9 bull spermatozoa, which, as mentioned earlier, can metabolize fructose at a rate of 2 mg sugar/hour at 37°C , should be capable of transferring free energy at a rate of 525 mcal/hour, or,

$$\Delta F = -6.10 \times 10^{-6} \text{ erg/spermatozoon/second.}$$

However, as previously pointed out, it is not fructolysis as a whole, but the enzymic hydrolysis of ATP itself which is supposed to act as the direct source of energy for the spermatozoa, and the free energy change

associated with that reaction is only about -6.9 kcal/mol (at pH 7, 25°C , and in the presence of Mg^{++}). Assuming, then, that bull semen contains on the average 129×10^{-12} μmols ATP/spermatozoon, and that the hydrolysis and resynthesis of ATP in semen keep perfectly in step with each other, we may conclude that a bull spermatozoon should be able to draw for its requirements on the free energy made available by the reaction

$$\Delta F = 1.8 \times 10^{-6} \text{ erg/spermatozoon/second.}$$

In fact, as Rothschild pointed out, one-ninth of the energy associated with ATP splitting should be ample to cover the total energy expended by a spermatozoon during its actual movements, namely,

$$2.11 \times 10^{-7} \text{ erg/spermatozoon/second.}$$

His estimate is based on the following considerations.

When a sperm tail moves, each of its elements, δS , elicits a reaction which can be resolved into a longitudinal component, δH , and a transverse component, δT . Both of these components depend on the forward velocity, $\bar{V}$ (for example, $91 \mu/\text{second}$), and the velocity, relative to the sperm head, of waves along the tail, the energy expended in unit time by the tail element equaling δT multiplied by its transverse velocity, V_t . The total energy dissipated per second, W , can be calculated from the equation,

$$W = \frac{\bar{V}^2 \eta l \pi b^2 k^2}{[\ln(2\lambda/a) - 0.5][1 + \frac{1}{4}\pi b k]} \left\{ 2 \left(\frac{V_w}{\bar{V}} \right)^2 - \left(\frac{V_w}{\bar{V}} \right) + \frac{3}{4} b^2 k^2 \right\},$$

where η = viscosity of the medium, 0.01 poise; l = length of the sperm flagellum, for example, 60μ ; b = amplitude of the bending waves of motion, 7.5μ ; λ = length of the same waves, 40μ ; a = tail radius, 0.3μ ; $k = 2\pi/\lambda$; $V_w = v\lambda$ = velocity of tail wave relative to the sperm head; and v = tail beat frequency, $9/\text{second}$.

If an anaerobically metabolizing suspension of bull spermatozoa is placed in an isothermal calorimeter, the heat produced is the heat of combustion, or enthalpy change, ΔH , associated with the breakdown of fructose to lactic acid. Experiments in which the anaerobic heat production of bull spermatozoa has been measured simultaneously with fructolysis are consistent with a value ΔH (fructolysis) = -27.7 kcal/mol fructose. In accordance with that value, 10^9 bull spermatozoa, which break down fructose at a rate of 2 mg/hour at 37°C , should be able to produce as heat up to 300 mcal/hour at 37°C .

$$\Delta H = 3.5 \times 10^{-6} \text{ erg/spermatozoon/second.}$$

Introduction of oxygen into a metabolizing sperm suspension would, of course, result in a manifold increase of heat production, since the enthalpy change associated with a total oxidation of a molecule of fructose to CO_2 and H_2O is -670 kcal, that is, 24 times more than that of anaerobic fructolysis (-27.7 kcal).

VI. Significance of Anaerobic and Aerobic Metabolism for Sperm Survival in the Female Tract

Anaerobic sperm metabolism, i.e., fructolysis, is essential in animals with internal fertilization under conditions of oxygen scarcity, such as arise presumably in freshly ejaculated semen of high density, with closely packed spermatozoa, following deposition in the female reproductive tract. However, anaerobic fructolysis would cease concomitantly with the fall of fructose supply, when a switch to an alternative source of metabolic energy would become imperative. By that time, the process of sperm dilution, dispersal, and ascent in the female reproductive tract would be in progress, and the spermatozoa should thus be capable of relying for their metabolic needs on oxygen available in the uterus, partly to oxidize the lactic acid which they had been producing themselves from extracellular glycolyzable carbohydrate (exogenous respiration), but partly also to utilize oxidatively their own intracellular lipid reserve (endogenous respiration). There is little doubt that the partial pressure of oxygen within the female reproductive tract is ample to satisfy these requirements, the more so since spermatozoa possess a highly efficient cytochrome system, and consequently are capable of maintaining an optimal rate of respiration at very low concentrations of oxygen (less than 1%).

What, however, still remains to be elucidated is the relative importance to the spermatozoa of anaerobic glycolysis, aerobic glycolysis, exogenous respiration, and endogenous respiration, not merely during their ascent along the reproductive tract but also at the site of fertilization. Further study is also needed on the relations between sperm metabolism, motility, and the energy changes associated with alterations in the adenine nucleotide pool of spermatozoa, which are associated with their activity, and, ultimately, senescence.

REFERENCES

- Aalbers, J. G., Mann, T., and Polge, C. (1961). Metabolism of boar semen in relation to sperm motility and survival. *J. Reprod. Fertility* **2**, 42.
Austin, C. R., Lutwak-Mann, C., and Mann, T. (1964). Spermatophores and spermatozoa of the squid *Loligo pealii*. *Proc. Roy. Soc. (London)*, Ser. B **161**, 143.
Barnes, H. (1962). The oxygen uptake and metabolism of *Balanus balanus* spermatozoa. *J. Exptl. Biol.* **39**, 353.

- Dott, H. M., and White, I. G. (1964). The effect of potassium on ram spermatozoa studied by a flow dialysis technique. *J. Reprod. Fertility* **7**, 127.
- Flipse, R. J. (1960). Metabolism of bovine semen. IX. Glutamic-oxaloacetic and glutamic-pyruvic transaminase activities. *J. Dairy Sci.* **43**, 773.
- Flipse, R. J. (1962). Comments on certain aspects of the metabolism of spermatozoa. In "Spermatozoan Motility" (D. W. Bishop, ed.), p. 133. Am. Assoc. Advance. Sci., Washington, D.C.
- Gonse, P. H. (1962). Respiration and oxidative phosphorylation in relation to sperm motility. In "Spermatozoan Motility" (D. W. Bishop, ed.), p. 99. Am. Assoc. Advance. Sci., Washington, D.C.
- Hartree, E. F., and Mann, T. (1961). Phospholipids in ram semen: metabolism of plasmalogen and fatty acids. *Biochem. J.* **80**, 464.
- Hartree, E. F., and Srivastava, P. N. (1965). Chemical composition of the acrosomes of ram spermatozoa. *J. Reprod. Fertility* **9**, 47.
- Lindahl, P. E., and Thunquist, L. O. (1964). Effects of low oxygen pressures on the oxygen consumption of bull spermatozoa. *Arkiv Zool.* **16**, 367.
- Lodge, J. R., Salisbury, G. W., Schmidt, R. P., and Graves, C. N. (1963). Effect of phosphate and of related co-factors on the metabolism of bovine spermatozoa. *J. Dairy Sci.* **46**, 473.
- Mann, T. (1964). "Biochemistry of Semen and of the Male Reproductive Tract." Methuen, London.
- Mann, T., and Lutwak-Mann, C. (1963). Comparative biochemical aspects of animal reproduction. *Bull. Acad. Roy. Med. Belg.* **3**, 565.
- Mann, T., Minotakis, C. S., and Polge, C. (1963). Semen composition and metabolism in the stallion and jackass. *J. Reprod. Fertility* **5**, 109.
- Mohri, H. (1961). Column chromatographic separation of phospholipids in sea-urchin spermatozoa. *Sci. Papers Coll. Gen. Educ., Univ. Tokyo* **11**, 109.
- Mohri, H., and Horiuchi, K. (1961). Studies on the respiration of sea-urchin spermatozoa. III. Respiratory quotient. *J. Exptl. Biol.* **38**, 249.
- Mohri, H., and Yasumasu, I. (1963). Studies on the respiration of sea-urchin spermatozoa. V. The effect of P_{CO_2} . *J. Exptl. Biol.* **40**, 573.
- Nevo, A. C. (1965). Dependence of sperm motility and respiration on oxygen concentration. *J. Reprod. Fertility* **9**, 103.
- Newton, A. A., and Rothschild, Lord (1961). Energy-rich phosphate compounds in bull semen: comparison of their metabolism with anaerobic heat production and impedance change frequency. *Proc. Roy. Soc. (London), Ser. B* **155**, 183.
- Rothschild, Lord (1961). Sperm energetics. An account of work in progress. In "The Cell and the Organism" (J. A. Ramsay and V. B. Wigglesworth, eds.). Cambridge Univ. Press, London and New York.
- Rothschild, Lord (1962). Anaerobic heat production and fructolysis of bull spermatozoa at different temperatures. *J. Exptl. Biol.* **39**, 387.
- Salisbury, G. W., and Graves, C. N. (1963). Substrate-free epididymal-like bovine spermatozoa. *J. Reprod. Fertility* **6**, 351.
- Salisbury, G. W., and Lodge, J. R. (1962). Metabolism of spermatozoa. In "Advances in Enzymology and Related Subjects of Biochemistry" (F. F. Nord, ed.). Wiley (Interscience), New York.
- Scott, T. W., White, I. G., and Annison, E. F. (1962). Glucose and acetate metabolism by ram, bull, dog and fowl spermatozoa. *Biochem. J.* **82**, 398.
- Silvester, N. L., and Holwill, M. E. J. (1965). Molecular hypothesis of flagellar activity. *Nature* **205**, 665.

- Srivastava, P. N., Adams, C. E., and Hartree, E. F. (1965). Enzymatic action of lipoglycoprotein preparations from sperm-acrosomes on rabbit ova. *Nature* **205**, 498.
- Taguchi, S., Yasumasu, I., and Mohri, H. (1963). Changes in the content of adenine nucleotides upon aerobic incubation of sea-urchin spermatozoa. *Exptl. Cell Res.* **30**, 218.
- Terner, C. (1962). Oxidative and biosynthetic reactions in spermatozoa. In "Spermatozoan Motility" (D. W. Bishop, ed.), p. 89. Am. Assoc. Advance. Sci., Washington, D.C.
- Terner, C., and Korsh, G. (1963). The oxidative metabolism of pyruvate, acetate and glucose in isolated fish spermatozoa. *J. Cellular Comp. Physiol.* **62**, 243.
- Wallace, J. C., Stone, G. M., and White, I. G. (1964). The influence of some oestrogens and progestogens on the activity of glycerylphosphorylcholine diesterase in the rinsings of the rat uterus. *J. Endocrinol.* **29**, 175.
- Wallace, J. C., and Wales, R. G. (1964). The effect of ions on the metabolism of ejaculated and epididymal ram spermatozoa. *J. Reprod. Fertility* **8**, 187.
- White, I. G., Wallace, J. C., and Stone, G. M. (1963). Studies on the glycerylphosphorylcholine diesterase activity of the female genital tract. *J. Reprod. Fertility* **5**, 298.
- Williams, W. L., and Hamner, C. E. (1963). Stimulation of respiration of rabbit, human and cock spermatozoa by light and certain chemicals. *J. Reprod. Fertility* **6**, 235.
- Yanagisawa, T. (1959). Studies on echinoderm phosphagens. I. Occurrence and nature of phosphagens in sea-urchin eggs and spermatozoa. *J. Fac. Sci. Univ. Tokyo, Sect. IV* **8**, 473.

CHAPTER 4

Mechanisms of Gametic Approach in Plants*

Leonard Machlis and Erika Rawitscher-Kunkel

DEPARTMENT OF BOTANY, UNIVERSITY OF CALIFORNIA, BERKELEY, CALIFORNIA

I.	Introduction	117
II.	Historical Background	118
III.	The Chemotactic Systems	123
	A. Isogametes	123
	B. Physiological and Morphological Anisogametes	125
	C. Free, Nonmotile Female Gametes and Sperm	128
	D. Enclosed, Nonmotile Female Gametes and Sperm	131
	E. Archegonia and Sperm	135
IV.	The Chemotropic Systems	143
	A. Zygomorphs	144
	B. Antheridial Hyphae	149
	C. Trichogynes	152
	D. Pollen Tubes	154
V.	Discussion	155
	References	158

I. Introduction

This chapter is concerned with the answers to the question "How does a sperm find an egg?" Were animals, rather than plants, the organisms involved, the discussion would terminate abruptly. The gametes of animals meet by chance. Although there have been a few observations interpreted as chemotaxis of animal sperm to eggs, reviews of fertilization in animals have been consistent in concluding that sperm chemotaxis has not been demonstrated (Morgan, 1927; Tyler, 1955; Rothschild, 1956;

* This chapter, as requested by the editors, is a reprinting of the article published in 1963 under the same title *In* "International Review of Cytology" (C. H. Bourne and J. F. Danielli, eds.), Vol. 15, pp. 97-138. Academic Press, New York.

The original article has been modified to include subsequent work through July, 1965 and to take into account the fact that Chapter 5, Volume II will include a section on pollen tube chemotropism.

Metz, 1957; Austin, 1961). While some reports discuss sperm chemotaxis in plants, they fail to cite any observations for animals (Runnström *et al.*, 1959). Possible exceptions to the absence of sperm chemotaxis in animals are the reports, so far only in the form of brief abstracts, of Miller and Nelson (1962) and Miller (1963, 1964) that the sperm of the hydroid *Campanularia* react chemotactically to the female gonangium and to seawater from gonangia, and the report by Dickmann (1963) claiming chemotaxis of rabbit spermatozoa.

The question, "How does a sperm find an egg?" when applied to plants immediately becomes a problem in semantics. In contrast to animals, for which the terms sperm and egg can be used without serious difficulty throughout the kingdom, plants have been much more experimental in the evolution of the kinds of cells or structures that contain the gametic nuclei and that are involved in the transport of the nuclei until they are in the same cytoplasmic matrix. Thus, in the flowering plants a pollen tube transports by growth the sperm nuclei to the egg, and in certain fungi an antheridial hypha, on the one hand, or a trichogyne on the other, functions in a similar manner. In certain algae the cells containing the gametic nuclei become connected by the growth of conjugation tubes through which ameboid gametes then move, and in certain fungi vegetative cells function as gametes and meet as the result of hyphal growth. As a consequence the events directly preceding fertilization in plants are more varied than in animals.

The mechanisms by which the various carriers of the gametic nuclei meet each other is the step in sexual reproduction to which this chapter is limited. The subject has been treated previously, but almost always within a larger context and usually for only certain groups of plants (Raper, 1952, 1954, 1957, 1960; "Fortschritte der Botanik," under the heading *Physiologie der Fortpflanzung and Sexualität* [Linskens, 1954–1964], beginning with Vol. 17; Lewin, 1954; Rothschild, 1956; Wiese, 1961; Coleman, 1962; Mascarenhas and Machlis, 1962a; Machlis, 1966). The accumulated evidence indicates that when one or both gametic nuclei are carried by a motile cell, chemotaxis is often involved; when transport involves growth, it is frequently chemotropic in nature. Plants, as will be shown, are not lacking in sexual attraction; they are simply more modest than animals in their display of concupiscence.

II. Historical Background

The general role of chemotaxis and chemotropism in the sexual reproduction of plants was recognized at almost the same time by both of the men dominating plant physiology during the latter half of the 19th century. Although Julius von Sachs did no significant experimental work

in this area of plant physiology, he devoted to it a chapter entitled "The Action of Sexual-Cells upon One Another" in his book "Lectures on the Physiology of Plants" (1882, translated into English by Ward in 1887). The first part of this chapter is a discussion of the role of the male cell and of "nuclein" in sexual reproduction. The second part of the chapter is introduced as follows:

One of the most surprising facts connected with the reproduction processes is the action at a distance, or mutual attraction of the two sexual cells towards one another. I select this expression for the facts to be examined because it is short and at least clearly denotes the matter; though the words "action at a distance" and "attraction" are not to be understood in exactly the same sense as in physics.

In the numerous descriptions given by observers as to the behavior of the antherozoids in the neighborhood of the oosphere, and of swarming gametes and even of antheridia in the neighborhood of oogonia, we meet almost without exception with most definite expressions of the fact that a certain mutual interaction of the sexual cells at a certain distance exists, and this always of such a kind that the union of the two cells is thereby accomplished or promoted.

Sachs considered "Still more remarkable and astounding" than the above the description by de Bary (1881) of the induction of antheridial hyphae by oogonia and the attraction of the antheridial hyphae to the oogonia. Finally, Sachs commented on pollen tubes as follows: "To my mind there can scarcely be a doubt that the pollen-tube of the Phanerogams also is specially influenced by something which induces it to grow with its end containing the fertilizing substance directly towards the opening of the very narrow micropyle, to penetrate eventually to the oosphere. It is remarkable that this matter has as yet scarcely been thought worthy of investigation."

Although Sachs clearly recognized the importance of directed movements in the sexual reproduction of plants, it is Pfeffer who truly initiated this field of study with the publication of his classical paper in 1884, "Locomotorische Richtungsbewegungen durch chemische Reize." Here, in 120 pages, he established that the sperm of liverworts, mosses, club-mosses, horsetails, and ferns react chemotactically to their respective archegonia and to certain chemical substances. The nature and results of these investigations will be discussed in a subsequent section of this review. He also almost completely correlated all previous observations suggesting chemotactic or chemotropic responses of sexual elements (as well as chemotropic responses of vegetative parts of organisms such as hyphae, roots, and root hairs). His review led him to the following conclusions: "Presumably a well-conducted research will uncover among the many stimuli used by plants, also the chemical stimuli. Amongst these must be those which lead the parasites to their hosts; those in-

volved in the general seeking of nourishment—be this via fungi, roots, or root hairs; copulation processes of algae and fungi; and also the conduction of the pollen tube to the egg cell.” (Translated from the German.)

We propose now to consider the evidence leading Pfeffer to the foregoing conclusions and will begin with the phenomenon of group formation by isogametes.

Copulation of isogametes was first observed by Pringsheim (1869) in *Pandorina morum*. The phenomenon was subsequently observed in various other algae. Pfeffer took particular note of the reports of Dodel (1876), de Bary and Strasburger (1877), and Berthold (1880) on the copulation of the isogametes of *Ulothrix zonata*, *Acetabularia mediterranea*, and *Dasycladus clavaeformis*, respectively. Dodel showed that isogametes from neighboring gametangia would fuse, but not those that came from the same gametangium. De Bary and Strasburger observed, apparently for the first time, the sudden group formation (clumping reaction) and subsequent pairing of the isogametes. (Recent publications [Lewin, 1954; Smith, 1955] would appear to erroneously credit Berthold with this discovery.) A few years later Berthold also reported group formation in *Dasycladus clavaeformis*. Pfeffer himself studied the behavior of the isogametes of *Chlamydomonas pulvisculus* and *Ulothrix zonata*; he found no evidence of attraction between the isogametes and did not see how they would profit from an attraction action. Until very recently, as will be discussed later, chemotaxis had not been observed to occur during the copulation of isogametes.

Pfeffer was aware that the reaction of the isogametes of certain brown algae is different from those just discussed. Berthold (1881) had described fertilization in *Ectocarpus siliculosus* (see Fig. 1) and *Scytosiphon lomentarium*. Certain of the isogametes of these plants attach themselves to the substratum by their long flagella, which subsequently shorten, rendering such gametes nonmotile. At this stage, for a few minutes, the motile swimmers gather in great numbers about the now nonmotile, functional female gamete. This attraction of the now functional males as evidenced by their swarming in the hundreds about the functional female gamete occurs only when the latter comes to rest.

The next type of evidence concerns oogamous forms (see Fig. 4). The earliest observation, indeed, the first observation suggesting an attraction between sexual elements in plants, was made by Thuret (1854), who described the clustering of sperm about the free, nonmotile eggs of *Fucus*. He wrote: “It is difficult, when one observes this phenomenon with attention, to disbelieve that a particular impulse does direct the antherozoids towards the body which they are to fertilize.”

(Translated from the French.) The next year Pringsheim (1855) described the great accumulation of sperm about the opening into the oogonium of *Vaucheria* and in 1856 Cohn noted the entrance of numerous sperm into the oogonium of *Sphaeroplea*. All these Pfeffer considered to be evidences of chemotaxis. Indeed, Pfeffer saw a chemotactic response in Pringsheim's (1858) description of fertilization in a nannandrous form of *Oedogonium*. Pringsheim made a point of the fact that the place of attachment of the androspores on or near the oogonial mother cell is definite for each species, and further, that the sperm, after their release from the antheridial cells at the apex of the dwarf males, make their way into the oogonium through a pore.

As noted earlier, the experimental work discussed in Pfeffer's paper was concerned principally with the attraction of the sperm of mosses, liverworts, ferns, and the fern allies to their archegonia and to various chemicals. The background for this work begins with Hanstein's (1865-1866) description of fertilization in the water fern *Marsilea*. He noted that large numbers of sperm gathered in the archegonial opening.

The architecture of the egg-containing apparatus of *Marsilea* is particularly suitable for calling attention to the necessity of distinguishing between an attraction of sperm and a trapping of sperm. The structure in *Marsilea* is illustrated in Fig. 9. The megagametophyte consists of a large basal cell with a small archegonium at its apex. The entire structure is surrounded by a gelatinous layer which has the shape of an old-fashioned milk bottle over the archegonium. The inside is filled with liquid and open at the top. As shown in Fig. 9, sperm become trapped in this gelatinous material. Once in it, they can continue to move, although at a much reduced rate. Some of them manage to get into the interior of the gelatinous bottle, where they then are able to swim back and forth much more freely than when they were moving in the gel. Some sperm may also enter the liquid portion of the bottle directly through the opening at the top. When the archegonium opens, the sperm swimming at random inside the gelatinous bottle now gather at the entrance to the archegonial canal.

Strasburger (1868, 1869-1870a) described a similar situation in the fern *Pteris serrulata*. This fern has archegonia that release mucilage after the neck cells open (Fig. 8). He reported that the sperm move by the archegonial necks as if the archegonia were not present as long as the archegonia are closed. As soon as they open and extrude mucilage, the sperm that touch the mucilage slow down, but then proceed to follow the canal inward. Other sperm do the same until there are as many as a hundred sperm at the base of the canal. Strasburger attributed an attractive action to the mucilage. Pfeffer studied some of these forms

later and, as we will see, it seems more likely that the mucilage is a trap. Once the sperm are trapped, they appear to be attracted down the archegonial canal to the base, where the egg is located. The same year Strasburger (1869–1870b) described the massing of sperm at the entrance to the archegonia of the liverwort *Marchantia polymorpha*.

Pfeffer was quite aware of the necessity of distinguishing between a trapping action and attraction and referred specifically to *Marsilea* as an example, and also to the green alga *Chara*, whose trapping and attracting characteristics were described by de Bary (1871).

Up to now we have considered the evidence on which Pfeffer based his conclusion that chemotaxis is an important element in the sexual reproduction of plants. He did not overlook the possible importance of chemotropism. He was particularly impressed, as Sachs had been, with de Bary's (1881) studies on the reproduction of various water molds such as *Saprolegnia*, *Achyla*, etc. (Fig. 11). De Bary concluded that the oogonium induces the development of antheridial hyphae and speculated (without evidence) that this induction is brought about by chemical substances secreted by the oogonia. He further concluded that for those species growing in water there is a directed growth of the antheridial hyphae toward the oogonia. Again he speculated that this too is brought about by a chemical stimulus originating from the eggs.

Pfeffer then called attention to the report by Fischer (1882) that the hyphae of *Saprolegnia* grow toward insect larvae (Pfeffer was unable to repeat this work) and to Kihlman's (1883) observation that germinated ascospores of *Melanospora parasitica* grow toward the hyphae of its host, *Isaria farinosa*. Also in 1883, but apparently unnoticed by Pfeffer, there appeared a description by Brefeld on the development of tubular connections between pairs of germinating sporidia of rusts. The connection we now know is a somatic sexual fusion, but at the time Brefeld considered it as nonsexual because the sporidia would always develop into mycelia or conidial colonies in the yeast form as long as there were suitable nutrients. Only when the nutrient solutions were exhausted did the hyphal fusions occur.

Hyphal fusions purely vegetative in nature occur in many fungi. There is a substantial literature on chemotropism between fungal hyphae, resulting in such fusions, as well as chemotropism of the hyphae to nutrients, etc. Since sexual reproduction is not involved, this literature will not be considered in this chapter; the reader is referred to the papers and reviews of Fulton (1906), Graves (1916), Stadler (1952), Fischer and Werner (1955), König (1961) and references cited therein.

Pfeffer, like Sachs, suspected that the conduction of the pollen tube to the egg cell is chemotropic in nature, although the evidence at the

time was ambiguous. We will not consider this early literature here, since an extensive review of pollen tube chemotropism prepared in this laboratory has recently been published (Mascarenhas and Machlis, 1962a).

We have in this section described in some detail the nature of the observations suggesting to the various early investigators a chemotactic or chemotropic response of sexual elements.* Similar observations, unsupported by any attempts to actually demonstrate the existence of chemotactic or chemotropic hormones, have been made since the time of Pfeffer. These will be noted in the sections that follow, but without detail unless the response is novel. Rather, attention will be concentrated on the experimental demonstration of the existence of these kinds of sexual hormones and the efforts made toward the determination of their chemical identity.

III. The Chemotactic Systems

Five types of systems having at least one motile gamete will be considered here: first, isogamy in which all gametes are equally and fully motile; second, physiological and morphological anisogamy; third, oogamy in which the egg is released from the oogonium; fourth, oogamy in which the egg is retained within the oogonium; and fifth, those plants in which the egg is encased in a multicellular sex organ, the archegonium, into which the motile sperm must swim to reach the egg.

A. ISOGAMETES

We earlier described group formation by isogametes and noted that chemotaxis appeared not to be involved in this phenomenon. In the many subsequent studies of group formation by isogametes (see reviews by Lewin, 1954; Runnström *et al.*, 1959; Coleman, 1962) the lack of chemotaxis has been continuously recorded. However, Tsubo completed a series of papers (1956, 1957, 1961) on sexual behavior in a new isolate of *Chlamydomonas*, in which he presented evidence that chemotaxis precedes group formation in this species.

The work was initially done with an isolate named *Chlamydomonas moewusii* var. *rotunda*, composed of plus and minus mating types with

* Two ambiguities in relation to this early literature are worth noting. Ward (1888) in a rather difficult sentence on page 331 indicates that Pfeffer made some observations on conjugating *Spirogyra*. We can find no mention of *Spirogyra* by Pfeffer. Fulton (1906) attributed to Pfeffer the observation that the hyphae of *Saprolegnia* turned toward nutrient substances. Pfeffer did observe that the zoospores of *Saprolegnia* are attracted to meat extract and fly legs, but he was unable to verify any directed growth of the hyphae of either *Saprolegnia* or *Penicillium* to similar materials.

the usual clumping and pairing reactions, as described by Lewin (1950, 1954), and the clumping reaction between dead cells of one mating type with living cells of the other, as described by Hutner and Provasoli (1951). When capillary tubes containing cell-free filtrate from sexually active cells of each mating type were placed in sexually active suspensions of each mating type (characterized by their ability to clump), then out of the four combinations, plus cells aggregated about the tip and inside the capillary containing minus cell filtrate; no such reaction occurred with the other combinations (Fig. 3). If capillaries were filled with suspensions of each type, then when the capillary contained minus cells, plus cells gathered at the entrance and also entered the tube and proceeded to clump; the minus cells did not leave the tube. In the converse situation, with plus cells in the tube, the latter actively swam out of the tube into the minus cell suspensions and there made clumps. Comparable responses were obtained when one of the suspensions consisted of dead minus cells. There quite clearly is an attraction of plus cells to the minus cells and, further, the latter release the chemotactic agent into the suspending medium.

Using the same techniques, Tsubo (1961) found no chemotaxis in four other species, including *Chlamydomonas eugametos*, which Moewus (1939) had earlier claimed to display chemotaxis and which Hagen-Seyfferth (1959) claimed responded chemotactically to a number of inorganic salts, sugars, and other substances. Cell-free supernatants of each of the mating types of each of the five species were then tested against sexually active cells (as evidenced by participation in group formation) of each of the mating types of each of the species. Only the supernatant of the minus strain of the *rotunda* variety showed any chemotactic activity. This supernatant not only attracted the plus cells of *rotunda* but also both mating types of *C. moewusii*, *C. eugametos*, and *C. moewusii* var. *reinhardii*; only the mating types of *reinhardii* failed to react. It was shown that the attraction was restricted to sexually active cells, there being no effect on nonsexual cells.

The chemotactic agent was shown to be stable when heated at 130°C for 30 minutes in a sealed tube. On the other hand, the heating of supernatant for a short time in an open dish dissipated the activity; it was also lost from the supernatant when nitrogen gas was bubbled through it for 30 minutes. By using Conway dishes it was shown that the material could diffuse from one solution through the intervening atmosphere into another. Thus, the substance appears to be heat stable and readily volatile. Preliminary testing of various gases dissolved in distilled water under atmospheric pressure at room temperature elicited chemotaxis of various mating types of the several species. The relation of these gases to the chemotactic factor in minus cultures is unknown.

The volatility of the substance is of considerable interest because of a similar characteristic for the chemotactic agent in *Fucus* and the zygotropic agent in *Mucor mucedo* (see later). For the sperm of *Fucus*, *n*-hexane caused chemotaxis. Water saturated with *n*-hexane immobilized the sexually active cells of *Chlamydomonas moewusii* var. *rotunda*.

Although recent workers on group formation by isogametes have failed to find chemotaxis involved in various strains of *Chlamydomonas*, *Ulva*, *Enteromorpha*, *Acetabularia*, *Dasycladus*, etc., displaying this general type of random meeting, it is not surprising that a situation such as that described by Tsubo exists. It is not far removed from the chemotaxis observed in the physiological anisogamy described in the next section.

B. PHYSIOLOGICAL AND MORPHOLOGICAL ANISOGAMETES

We use the term "physiological anisogamy" here to characterize the behavior pattern first described by Berthold (1881) and later confirmed

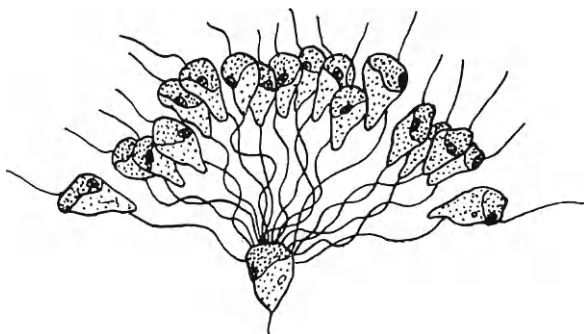


FIG. 1. The attachment by their long flagella of motile isogametes of *Ectocarpus siliculosus* to one that has become nonmotile. (After Berthold, 1881.)

by Hartmann (1934) in which some members of an isogametic suspension from *Ectocarpus siliculosus* become nonmotile and attract to them substantial numbers of the other still motile gametes (Fig. 1). Similar patterns of behavior, with minor differences, have been described for the algae *Chlamydomonas paupera* (Pascher, 1931a) and *Hydrodictyon reticulatum* (Mainx, 1931a) and for the fungi *Synchytrium fulgens* (Kusano, 1931) and *Synchytrium endobioticum* (Köhler, 1930), with the inference that the motile swimmers are attracted to those that become nonmotile. From the illustrations by Schreiber (1931) this would also seem to be true for *Cladostephus spongiosus*, a brown alga. In three morphologically anisogamous organisms it also seems that the female gamete attracts the sperm. In *Cutleria multifida* (another brown alga) the attraction

occurs only after the female gamete has become nonmotile (Hartmann, 1950). Kunieda and Suto (1938) considered the events preceding fertilization in the brown alga *Colpomenia sinuosa* to be similar to Berthold's observations on *Ectocarpus siliculosus*. Lastly, in the water mold *Allomyces* (Machlis, 1958a,b,c) attraction occurs while the female gamete is still motile, although sluggish. Only with *Allomyces*, of all the organisms named above, has any substantial experimental proof of the existence of a chemotactic agent been presented. It should be noted, however, that Hartmann (1950) did one simple experiment. A drop of a suspension of freshly settled female gametes of *Cutleria multifida* was placed at the edge of a coverslip under which there was a suspension of sperm. At the same time a drop of water was placed nearby on the same edge of the coverslip. The sperm soon moved into the first drop, which contained the female gametes, but not into the drop of water.

Judging entirely from the drawings and descriptions on both physiological and morphological anisogamy in the brown algae cited above (*Ectocarpus siliculosus*, *Cladostephus spongiosus*, *Cutleria multifida*, *Colpomenia sinuosa*), it seems that if chemotaxis is here involved there is also an attachment of several gametes directly to the female gamete by their long flagella. All but one of these must subsequently detach, since only one of the sperm fertilizes the egg. If this is true, then the situation would appear to be a combination of chemotaxis and of group formation.

In *Allomyces* the gamete-bearing generation consists of a dichotomously branching mycelium that bears large numbers of small, red, male gametangia and larger, colorless, female gametangia (Fig. 2). In water the gametangia release highly active, motile, orange, male gametes and sluggishly motile, colorless, female gametes. It can be observed that the male gametes cluster in the vicinity of unopened female gametangia.

The first proof that the clustering is a response to a chemotactic agent was obtained by embedding female gametangia in agar and showing that the male gametes are attracted to the gametangia. It was subsequently shown that the male gametes would approach and remain in the vicinity of a dialyzing membrane that had on its other side the supernatant in which female gametangia had released female gametes. The hormone attracting the sperm was named "sirenin."

From the normally hermaphroditic plant, predominantly female and predominantly male isolates were genetically selected on the basis of hybridization studies by Emerson and Wilson (1954). The female plants were then used to produce sirenin and the male plants were used for the production of sperm in adequate concentrations for assaying for sirenin. A quantitative assay based on the number of gametes adhering

to a unit area of membrane in response to diffusion of sirenin through the membrane was developed.

Since the original publications on sirenin a pilot plant for its production has been in operation and a concerted effort has been underway to establish its chemical constitution. Machlis (1963) and Machlis *et al.*

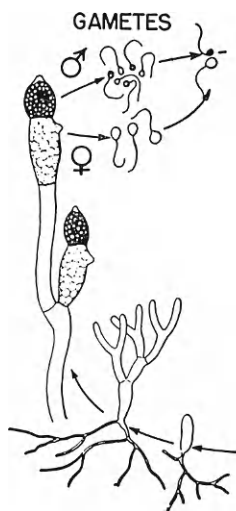


FIG. 2. Diagrammatic representation of the mycelium, gametangia, and gametes of *Allomyces*. The male gametangia and gametes are bright orange; the female counterparts are colorless. (Part of a drawing made by Rafael Rodriguez and reproduced by permission of Arthur T. Brice.)

(1966a) have described the production, isolation, and characterization of sirenin as well as certain esters of sirenin. The hormone was shown to have a molecular weight of 236 and to be an oxygenated sesquiterpene with four degrees of unsaturation and with both of its oxygen functions capable of forming esters.

The availability of pure sirenin made possible the initiation of studies on the mechanism of sperm attraction (Carlile and Machlis, 1965). This first report showed that the male gametes of *Allomyces* are attracted to sirenin concentrations as low as 10^{-10} M. Increasingly strong responses were observed to concentrations up to 10^{-6} M. The response is slightly weaker at 10^{-5} M and very weak at 10^{-4} M. Of particular interest is the observation that the male gametes inactivate sirenin. If a mixture of male gametes at a concentration of 1,200,000/ml and sirenin at 10^{-6} M is made, no sirenin can be detected in the suspending medium at the end of an hour. None of a variety of methods ever resulted in recovery of sirenin from the gametes. The authors suggest that this inactivation enables the

gametes to maintain their sensitivity to concentration gradients of sirenin and that it may be an essential part of the process of chemoreception.

The availability of highly active preparations of hormone A, which induces the formation and chemotropic growth of antheridial initials in *Achlya* (see Section IV,B) and of sirenin, made it possible to test for any common physiological activity of the two hormones (Barksdale *et al.*, 1965). The results showed a complete absence of either parallel or antagonistic effects.

C. FREE, NONMOTILE FEMALE GAMETES AND SPERM

Sexual reproduction in which nonmotile eggs are extruded into the external environment is found principally (but see later) in two orders of the brown algae, the Dictyotales and Fucales, and is characteristic of all the members. Two other orders of the brown algae—the Desmarestiales and Laminariales—also extrude the eggs from the oogonia, but the eggs remain attached to the tips of the oogonia and do not float free; the occurrence of chemotaxis in these forms has not been suggested, although one would expect it to occur. It has been much easier to study the phenomenon with free eggs.

The original observations of Thuret (1854) on the massing of the sperm about the egg of *Fucus* were followed by a series of similar observations and, finally, by the first serious attempt to isolate a sexual chemotactic hormone. The response of sperm to the eggs of *Fucus* is made particularly dramatic because some of the massed, swarming sperm attach themselves by their long flagella to the eggs, spinning them as a result of their continued motion. This was well described by Farmer and Williams (1898) for *Fucus serratus*, *F. vesiculosus*, and *Ascophyllum nodosum*. Discussing the sperm of all three, the authors say: "They at once congregate around them, and attaching themselves to the periphery of the eggs, cause the well known movements by lashing the water with the free cilium."

In the meantime Bordet (1894)* had questioned whether chemotaxis was involved at all. Bordet worked with *Fucus platycarpus*, *F. vesiculosus*, *Himanthalia lorea*, and *Ascophyllum nodosum*, performing a number of experiments. First, he filled capillary tubes with eggs crushed in seawater and found that when the capillary was placed in a dense suspension of sperm none entered the capillary. Similarly, if a drop of

* This report is sometimes erroneously cited. Earlier in the same volume Errera (1894) described briefly the work of Bordet and recommended that it be published in the *Bulletins de l'Académie Royale des Sciences, des Lettres et des Beaux-Arts de Belgique*.

seawater containing crushed eggs was placed adjacent to a drop of sperm suspension and the two connected by a small channel, without mixing, no sperm moved into the egg suspension. In his second experiment, he observed in a drop of sperm under a coverslip that the sperm, after swimming randomly for a while, eventually (3 hours) attached themselves by a flagellum to the surface of the slide or coverslip and then continued with gyrations while attached. Finally, in a drop of egg and sperm he found the sperm to attach indiscriminately to either the glass or the egg surface with no evidence of chemotaxis to the eggs.

Williams (1904), in a report on *Dictyota dichotoma*, described the attachment of the sperm to the egg but noted that the subsequent gyrations of the attached sperm did not cause the egg to revolve as in the Fucales. When eggs were brought into contact with suspensions of sperm some of the eggs became surrounded by several layers of sperm. He considered this to be a clear case of chemotaxis and further noted, ". . . nor can it be said that the crowds of antherozoids are entangled in a mucilaginous envelope, for in the first place their movements are absolutely unrestrained and furthermore, staining with gentian violet fails to demonstrate the presence of such substances."

Robbins (1916) sought a chemotactic agent for *Fucus* sperm by testing a variety of substances such as certain organic acids like lactic, malic, butyric, citric, etc.; various inorganic acids, bases, and salts; and certain organic compounds such as urea, tartrate, sugar, peptone, etc., all with negative results.

Kotte (1923), aware of the doubts raised by Robbins and Bordet as to the chemotaxis of sperm to *Fucus* eggs, repeated the capillary tube tests with centrifuged egg water and obtained negative results. When, however, a drop of this stringy, slimy egg water was placed in the middle of a drop of sperm suspension, the sperm aggregated at the interphase. He claimed this was not a trapping action because a drop of gum arabicum did not elicit aggregation. On the other hand, when the cell-free egg water was solidified in agar, it exerted no attraction, a result explained by Kotte on the basis that the active substance did not diffuse.

Cook *et al.* (1948) resolved a number of the questions raised by the preceding investigations. They showed that: (a) mature eggs do attract sperm that come within about 0.2 mm of the egg and that the attraction is expressed through a cellophane membrane, thus eliminating the idea that the whole phenomenon is the result of chance contact and subsequent sticking by a flagellum of the sperm to the egg or a trapping action; (b) a drop of cell-free filtrate from ripe eggs did attract sperm from an adjacent drop of sperm suspension; (c) the filtrate in capillary tubes did attract sperm in large numbers to the tip and that this response

could be obtained with dilutions up to four times (Fig. 3). The sperm attracted by these methods continued to be actively motile.

They also showed that the filtrate from *F. serratus* attracted the sperm of *F. vesiculosus* and *F. spiralis* as well as its own. In testing certain potential attractants they illustrated a different type of accumulation that can occur as a result of immobilization. Thus, large numbers of sperm collected about a crystal of indoleacetic acid, but they were all nonmotile after arriving at the crystal. They also found that the activity of a filtrate was retained for 10 days at 0°C, but was rapidly lost by boiling and by passage of a stream of hydrogen through the filtrate for 2–3 minutes. It could be recovered in a cold trap inserted in the aspiration train. In a subsequent paper, Cook and Elvidge (1951) attempted unsuccessfully to identify the chemotactic substance.

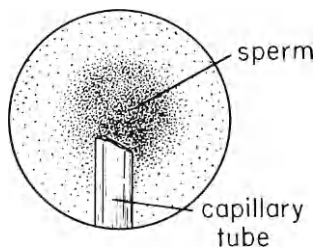


FIG. 3. Typical aggregation of sperm at the tip of a capillary tube containing an attracting substance. This figure is drawn from a photograph in Cook and Elvidge (1951) showing a cloud of sperm of *Fucus serratus* attracted to the egg supernatant of the same species.

When they tried to extract the active principle with light petroleum they found the activity to be greatly enhanced. They subsequently showed that the petroleum had chemotactic activity itself. Upon testing fractions of the petroleum they found *n*-hexane to be a most effective chemotactic agent. Using the mass spectrum as an analytical device and a sample of the natural chemotactic agent of unknown purity obtained by condensation of aspirates of egg water, they concluded that the agent was very probably not *n*-hexane nor any of the other pure substances examined. It is true that the mass spectrum of diethyl ether approached that presumed to be characteristic of the natural substance, but the diethyl ether was active only in excessively high concentrations.

Nothing further has been done to clarify the nature of the attractant in these brown algae. We would note only that more recently Levring (1949) confirmed again the swarming of sperm around the egg in *Homosira banksii* for up to one-half hour, and that as soon as a sperm penetrates the egg the rest become sluggish and soon die. In a subse-

quent paper Levring (1952) postulated that the egg, as a result of penetration by a sperm, releases a substance that causes immotility of the sperm and thereby prevents polyspermy (on this subject see Runnström *et al.*, 1959; Bishop, 1962).

In addition to the brown algae just considered, two other examples somewhat comparable in nature have been observed. *Chlamydomonas coccifera* is a large oogamous chlamydomonad in which the macrogamete is entirely nonmotile. Goroschankin (1905) commented to the effect that "One could observe 2 to 12 microgametes surrounding the macrogamete." (Translated from the German.) According to Pascher (1931b), the egg in the green unicellular alga *Chlorogonium oogamum* escapes from the

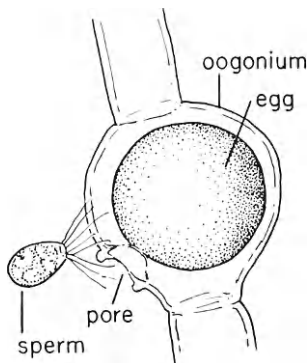


FIG. 4. Oogonium of *Oedogonium cardiacum* with mature egg and nearby sperm. (Drawn from a photograph in Hoffman, 1960.)

oogonial wall and after developing a hyaline receptive spot attracts sperm until they form a dense spherical aggregation.

D. ENCLOSED, NONMOTILE FEMALE GAMETES AND SPERM

It is surprising that so few observations suggesting chemotaxis of sperm have been made on those algae in which the eggs are retained within the oogonia. Chance entrance of a sperm into an oogonium through a pore in the wall of the oogonium would appear to be most unlikely, and the situation seems almost to demand some kind of directional signal for the sperm. In the comparable situation in the fungi, where the hyphal tube bearing the male element must reach the oogonium in order to penetrate it (Fig. 11), evidence for such direction was found early. Since the early investigations described previously, further study has been restricted to *Sphaeroplea* and *Oedogonium*.

The first experiments were done by Pascher (1931a) in connection with *Sphaeroplea*. This curious filamentous green alga develops oogonia

containing large numbers of eggs and antheridia that give rise to large numbers of sperm. If oogonia are placed in a suspension of sperm, the sperm rush into the oogonia. Pascher showed that a cotton fiber soaked in a suspension of ripe oogonia would attract the sperm for 1-2 hours and, by changing the water in which the oogonia were held, that the

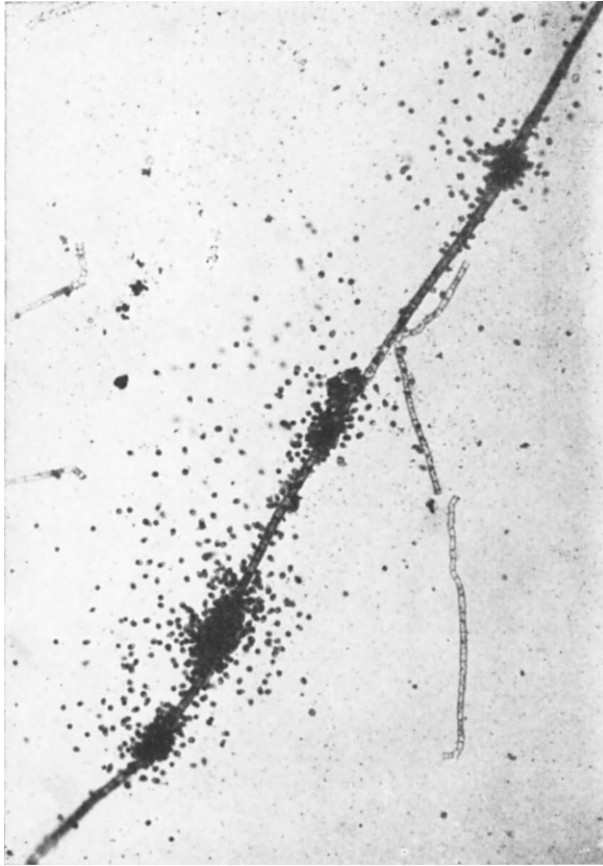


FIG. 5. Filament of *Oedogonium* with oogonial mother cells attracting androspores. (From Rawitscher-Kunkel and Machlis, 1962.)

attractive substance was produced by the oogonia for some 20 hours. Nothing more has been done with *Sphaeroplea*, although it appears to be well designed for studies aimed at the isolation of a chemotactic substance. Both monoecious and dioecious species are known (Fritsch, 1935), and the oogonia are large, each containing a great many eggs, suggesting a substantial production of the chemotactic agent.

There has been a recent spurt of research on *Oedogonium*. This genus of filamentous algae has two basic types of sexual reproduction. The simplest (macrandrous) involves male gametangia that give rise to motile sperm and oogonia each containing an egg (Fig. 4). The sex organs may be borne on the same or on different filaments, depending on the species. In the more complex cycle (nannandrous), the first male element is not a sperm but a motile androspore. Upon reaching the vicinity of the oogonium it attaches itself and develops into a one-celled dwarf male plant which in turn gives rise to motile sperm (Figs. 5, 6,

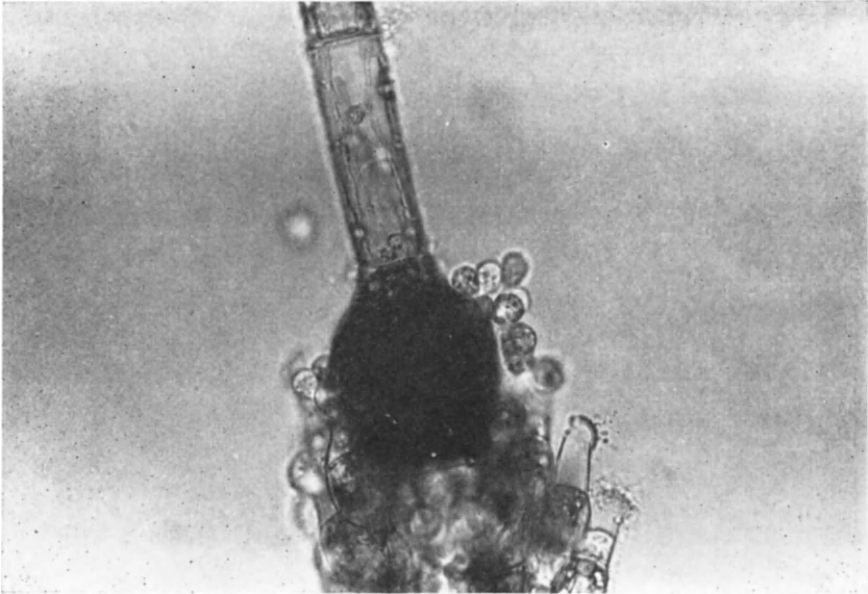


FIG. 6. Sperm of nannandrous *Oedogonium* attracted to a papilla protruded through the oogonial pore. These sperm are actually within a gelatinous matrix (see Fig. 7). (From Rawitscher-Kunkel and Machlis, 1962.)

and 7). In 1960, Hoffman confirmed the previous speculations (Pringsheim, 1858; Pfeffer, 1884; Mainx, 1931b; Tiffany, 1957) on the chemotaxis of the sperm of macrandrous species to oogonia. If either homogenates of fertile female plants or water in which female plants had produced oogonia were placed in capillary tubes and these then placed in a suspension of sperm, the sperm accumulated at the tips and eventually entered the capillaries. If the active material were solidified with 1% agar in a capillary tube and the tip inserted into a sperm suspension, again the sperm accumulated around the tips.

Hoffman (1960) reported without experimental details, although these are described in his thesis (1961), that the chemotactic substance is water soluble on the basis that chloroform and ether, either acidic or basic, failed to extract the activity from water; that it survives boiling for 15 minutes, but is mostly lost when autoclaved for 15 minutes at 15 lb. pressure; that it survives treatment with 4.0 *N* HCl and evaporation to dryness; that there is no loss of activity after freezing for 7 months;

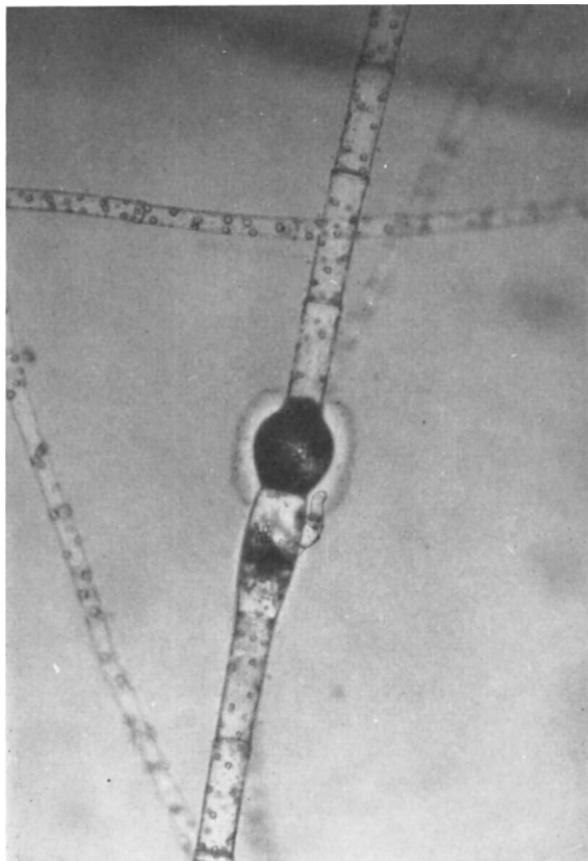


FIG. 7. An oogonium of *Oedogonium* suspended in dilute India ink to make visible the gelatinous matrix into which the sperm are released from the antheridia formed at the apical ends of the dwarf males. (From Rawitscher-Kunkel and Machlis, 1962.)

that it passes through a dialysis membrane; and that attempts at chromatography failed. Of particular interest is the fact that the active fractions from two different species, *O. cardiacum* and an unidentified species, failed to attract each other.

Some of these results have been confirmed (Machlis and Merkel, unpublished). When small pieces of agar encasing ripe oogonia of *O. cardiacum* and *O. geniculatum* are placed in a suspension of their own sperm, they attract the sperm. The active material from *O. cardiacum* is water soluble and not extractable with chloroform, but it is readily destroyed on boiling, and frozen preparations become inactive in a month or so. Working with material from *O. cardiacum* it has been possible to separate the material by paper chromatography using isopropyl water (2:1).

Rawitscher-Kunkel and Machlis (1962) described the hormonal integration of sexual reproduction in a nannandrous species of *Oedogonium*. The androspores released from the male filaments are chemotactically attracted to the oogonial mother cells (Fig. 5). Some evidence for this was presented in the thesis by Hoffman (1961). Water in which oogonial mother cells have developed will attract androspores into capillary tubes, and when such active water is solidified with agar, it remains attractive.

After the androspores become attached to the oogonial mother cells they develop into dwarf males and the oogonial mother cell divides into an oogonium and a suffultory cell. The dwarf males then release sperm (Fig. 6) into the gelatinous sheath surrounding the oogonium (Fig. 7). These sperm can be seen to congregate about a spot on the oogonium where a pore will subsequently appear. The aggregation disappears after fertilization. This is all the evidence presently available that indicates that the sperm in such a nannandrous species are attracted to the point of origin of the pore in the oogonium.

E. ARCHEGONIA AND SPERM

An archegonium (Fig. 8), particularly the type found in ferns, is most readily visualized as a long-necked, round-bottom flask. The neck of the archegonium consists of four tiers of cells that meet at the top and surround a central or axial row of cells consisting of a variable number of neck canal cells in the upper portion, below which is the conspicuous ventral canal cell and, finally, at the base, the large egg cell. A mature archegonium on exposure to water opens by a separation of the apical neck cells followed by the extrusion of the neck canal cells and the ventral cell. The best and perhaps most reliable description of the behavior of an archegonium just prior to fertilization is that by Ward (1954) for the fern *Phlebodium aureum*. Of significance to the chemotactic response of sperm to archegonia is his evidence that a mature archegonium explosively releases the contents of the canal, so that this material ends up at some distance away from the entrance to the

archegonium. Sperm are attracted to the canal and down it to the egg, a path they can traverse without impedance. They are not attracted to the archegonium until it opens. Thus, the attractant comes from within the archegonium. To be sure, the extruded canal material is attractive for a while, but because of its distance from the archegonium, it plays no essential role in fertilization. We are making a particular point of this because, as Ward pointed out, investigators of chemotaxis and of fertilization in the group of plants now under review have for practical reasons tended to use somewhat desiccated archegonia. Such archegonia on exposure to water do not throw the canal contents free of the archegonium. This material is slimy and, capping the archegonium, it then acts as a trap for the sperm and impedes their entrance into the canal.

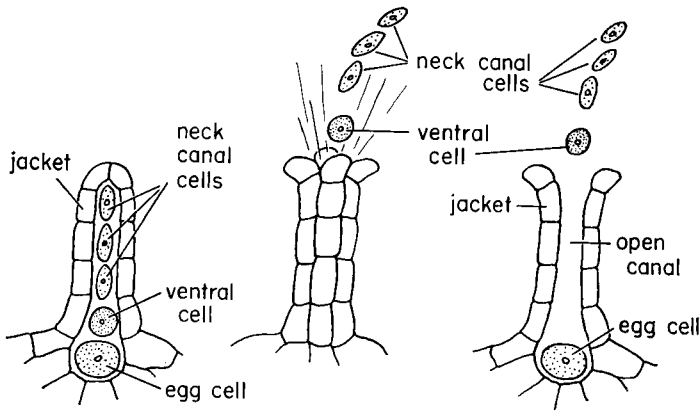


FIG. 8. The structure of an archegonium and the dehiscence of ventral and neck canal cells. Sperm swim into the open canal down to the egg. (After Ward, 1954, in part.)

In contrast to these freely exposed archegonia is the structural arrangement in the water ferns (the Marsileaceae, Fig. 9) and in the Anthocerotales (Proskauer, 1948). These plants (and perhaps others, but we have not explored this possibility exhaustively) have archegonia that are surrounded by gelatinous structures which function as traps for sperm. But here, too, attraction of the sperm appears not to be involved until the archegonia open and empty their canals. After the opening, the sperm trapped in the vicinity respond chemotactically to the stimulus emanating from the canal.

We mentioned earlier that Hanstein (1865–1866) had described the fertilization process in *Marsilea* and that Strasburger (1868, 1869–1870a, b) had done this for the ferns *Pteris serrulata* and *Ceratopteris thalictroides* and the liverwort *Marchantia polymorpha*. Both investigators

noted the trapping action of the gels or extruded slime and the subsequent chemotactic movement of the sperm into the canal. Pfeffer (1884) confirmed their observations by describing chemotactic responses of sperm to the archegonia of the liverworts *Marchantia polymorpha* and *Radula complanata*; the mosses *Funaria hygrometrica*, *Leptobryum pyriforme*, and *Brachythecium rivulare*; the spike moss *Selaginella erythropus*; and the ferns *Marsilea drummondii*, *Pteris serrulata*, *Blechnum*

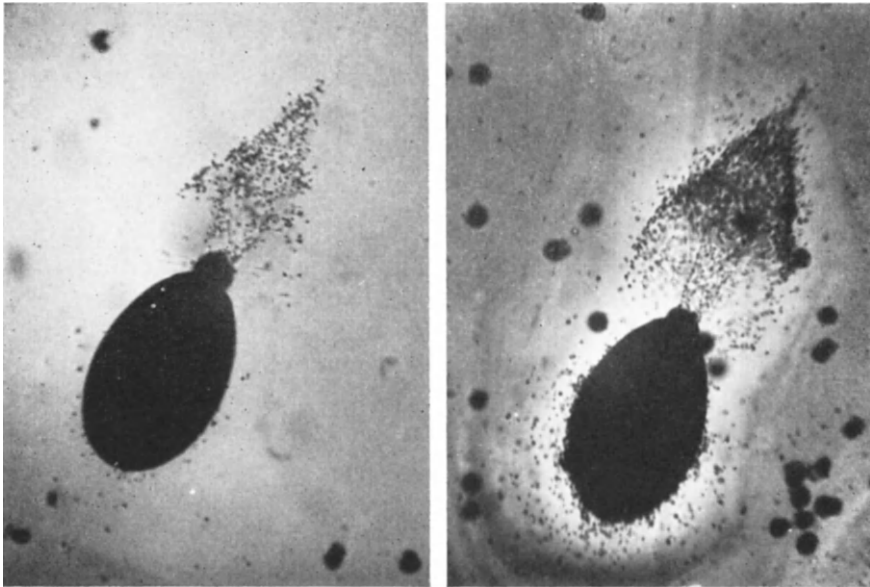


FIG. 9. The trapping mechanism for sperm in *Marsilea*. The megagametophyte is surrounded by a gelatinous envelope which takes the form of a bottle in the region above the archegonium. The nob at the end of the dark, egg-shaped body (basal cell) is the archegonium. The photograph on the left was taken shortly after exposure of the megagametophyte to the sperm, while that on the right is the appearance after extended exposure. The dark, round bodies are microgametophytes which give rise to the sperm.

fraxinum, *Ceratopteris thalictroides*, *Adiantum capillus-veneris*, and *Adiantum cuneatum*.

In this paper, immediately following a rather short introductory section, Pfeffer plunged into a detailed description of numerous experiments on the response of the sperm of *Blechnum fraxinum* and *Adiantum cuneatum* to sodium malate, using a method now referred to as "Pfeffer's capillary method." The method consists in partially filling under a slight vacuum a capillary tube (0.1–0.14 mm in diameter, 7–12 mm

long, and sealed at one end) with a solution and then introducing the open tip into a drop of water containing sperm. The tube thus contains solution in the first 2–4 mm and air in the upper, closed portion. If the tube contains 0.1 % malate, sperm very quickly collect in a cloud about the tip of the tube. The method was later refined by Rothschild (1956), who solidified the solution in the capillary with 1 % agar, thereby preventing spurious results caused by trapping of sperm within the tube, convection currents, hydrodynamic flow, etc. Two of Pfeffer's observations are particularly significant. First, 0.1 % malate (sodium) elicited a strong response, while 0.001 % caused a just detectable (threshold) attraction. Second, by adding malate to the sperm suspension he found that a thirtyfold difference in concentration between the solution in the capillary and the suspension was required for the sperm to react. This indicated that the chemotactic response was related to the gradient of the logarithm of the malate concentration and, as Pfeffer pointed out, followed Weber's Law. It is only later in the paper that Pfeffer explained how he arrived at the conclusion that malate was to be studied in such detail. Once he found sperm to be attracted to archegonia, he then tested them against a variety of materials such as decapitated hairs of higher plants, boiled extracts of various parts of several higher plants, algal filaments, prothallia of ferns, parts of mosses, etc. All of these were very attractive provided the tissues were injured, so that, according to Pfeffer, malate was released.

Since an evaporated grass decoction retained its activity, he concluded that the substance was not volatile and that it was organic because the decoction lost its activity on ashing. The activity was retained in the precipitates obtained with the consecutive addition of barium hydroxide, lead acetate, and calcium nitrate to grass decoction. He then tested the sperm against three mixtures: (a) 0.05–0.15 % each of the K, NH_4 , or Na salts of some 11 organic acids; (b) a mixture of various carbohydrates; and (c) a mixture of amides and amino acids. Only mixture (a) elicited a response. Subsequent testing showed malate to be the effective acid. Subsequently he found maleate to be active, but he did not consider it seriously, since it was not known to occur naturally in plants. Pfeffer did not demonstrate the presence of malic acid in the excretions from archegonia, but he did observe that if sperm and the archegonia with which they should react were contained in a solution of malate (thereby destroying any possible concentration gradients), no reaction took place. By watching the reaction of sperm to the archegonia of *Ceratopteris thalictroides*, whose prothallus is transparent, Pfeffer thought that the receptive spot on the egg was the actual source of the attractant, presumably malate.

Pfeffer also looked into interspecific attraction among ferns. Thus, he observed that the sperm of *Blechnum fraxinum* and *Adiantum cuneatum* entered the archegonia of *Pteris serrulata*, *Adiantum capillus*, and *Ceratopteris thalictroides*. The sperm of *Selaginella erythropus* did not enter the archegonium of *Adiantum capillus-veneris* but, according to Pfeffer, only because they were unable to get through the mucilage at the entrance to the canal; they were attracted to the mucilage.

After establishing these and other facts related to the reaction of the fern sperm to malate, he then tested the sperm of other plants against the mixtures described above using the capillary method. The results were as follows: nothing tested attracted the sperm of two liverworts, *Marchantia polymorpha* and *Radula complanata*, nor those of the water fern *Marsilea drummondii*; the sperm of the mosses *Funaria hygrometrica* and *Leptobryum pyriforme* were attracted by sucrose (0.001–0.1%), but nothing else, and those of *Brachythecium rivulare*, as well as other mosses, also responded to sucrose; finally, the sperm of the spike moss *Selaginella erythropus* were also sensitive to malate.

Buller (1900) found the sperm of another fern, *Gymnogramme martensii*, to be chemotactically attracted to malate, but also to be attracted by relatively high concentrations (0.1 M) of potassium and rubidium salts and phosphates. This response to inorganic ions and many other substances was to be further investigated by Akerman (1910) and Shibata (1911). Before that, however, Lidforss (1904) showed that the sperm of liverworts, for which Pfeffer could find no attractive substance, were attracted by proteins. Using the sperm of *Marchantia polymorpha*, he found them attracted to capillaries containing 0.0005% diastase or albumin or 0.001% hemoglobin. This was confirmed later by Akerman (1910) with sperm from an unnamed species of *Marchantia*, which were attracted to hemoglobin; in addition, he found the sperm to respond to K, Rb, and Cs at a threshold concentration of approximately 0.001 M, to be repelled by NH_4 , Mg, and heavy metals, and to be unaffected by Na and Ca. Lidforss (1905) also found that the sperm of the horsetails *Equisetum arvense* and *E. palustre* were responsive to both malate and maleate at threshold concentrations of 0.0001 M. The same year, the first of a series of papers by Shibata (1905a,b,c) appeared. He reported that the sperm of the quillwort *Isoetes japonica* were attracted by malate and also, at higher concentrations, to succinate, fumarate, and D-tartrate, but not to maleate. For the fern *Salvinia natans*, he observed both malate and maleate to be effective, in conformity with previous reports on the sperm of ferns. For the horsetail *Equisetum arvense*, malate was effective, but neither fumarate nor maleate. The latter contradicts Lidforss' observation, which has since been ignored and probably rightly so, since

Shibata's results have been subsequently confirmed (Rothschild, 1956).

In the first of his short papers Shibata (1905a) pointed out the interesting differential response between the sperm of the ferns and the fern allies—the horsetails and quillworts. The sperm of all of these plants, he noted, are strongly attracted by malate. Those of ferns are also attracted to maleate, but not to fumarate. On the other hand, sperm of *Isoetes* are attracted by fumarate, but not by maleate, while those of the horsetail fail to respond to either fumarate or maleate. Fumarate and maleate are *cis-trans* stereoisomers of each other. The ability of the sperm to differentiate between these two substances led Shibata to suggest that the first step in the chemotactic response involves a reaction of the chemotactic substance with receptor sites (an idea which is to appear again in very recent studies on the sperm of ferns).

Before Shibata's (1911) final paper on this subject (which includes the results of the previous short ones) appeared, Bruchmann (1909) reported on another group of fern allies, the club mosses. Using the sperm of *Lycopodium clavatum*, he obtained positive chemotaxis only to citrate and to boiled prothallial homogenate; malate was inactive. Unfortunately, such sperm have not been tested again.

Shibata's final paper, encompassing an enormous series of tests of the sperm of *Isoetes japonica*, *Equisetum arvense*, and *Salvinia natans* against numerous inorganic salts, acids and bases, a variety of dicarboxylic acids, and a series of alkaloids, confirmed the previous responses to the dicarboxylic acids. In addition, however, he found various cations, including the hydrogen ion and a considerable number of alkaloids, also to be active. To fit these results into a coherent system he postulated the sperm to have three separate and independent types of sensitivities, one for the anions of the dicarboxylic acids, another for cations and alkaloids, and in the case of *Isoetes*, one for the hydroxyl ion.

Since no further work on the chemotaxis of the sperm of archegonium-bearing plants was to be done for forty years, the results so far reviewed are appropriately summarized. The main, significant facts appear to be: (a) the sperm of these plants are attracted to their respective archegonia; (b) the sperm are strongly attracted by certain types of organic substances, all of which are common metabolites or plant substances; and (c) some of the sperm respond to a large assortment of ions and organic substances unrelated to those included in (b). Point (b) refers specifically to the fact that the chemotactically active substances for the sperm of the different groups of plants were shown to be proteins for liverworts; sucrose for mosses; malate and (of the two stereoisomers) maleate, but not fumarate, for the ferns; malate and fumarate, but not maleate, for the quillwort; malate, but neither fumarate nor maleate, for the horsetails; and citrate, but not the other acids, for the club moss.

In 1954, Wilkie published a study on the chemotaxis of the bracken fern (*Pteridium aquilinum*). For the first time, a new method of demonstrating chemotaxis of such sperm was introduced. He constructed on a microscope slide a system of three pools connected by channels, so that sperm could be confronted with a choice of environments. With sperm in one pool and with the flow of liquid tending to run into this pool from the other two pools which contained mature and immature archegonia, respectively, the sperm swam preferentially into the pool with the mature archegonia, as would be expected. They reacted similarly to water in which mature prothallia had been held for 15 minutes. In a second set of experiments, where presumably (the description is not clear) ruptured cells of vegetative prothallia, of the leaves of several plants, of a fruit fly, of an annelid, and of a mollusk were placed in suspensions of sperm, he observed attraction. He suggested, as did Pfeffer, that these sperm are attracted to a substance or substances common to both plants and animals.

The last reports concerned with the kinds of substances that attract the sperm of these archegoniate plants are those of Rothschild (1952, 1956). The main report (Rothschild, 1956) is a chapter in a brief book on fertilization and is sadly lacking in experimental detail. He used Pfeffer's capillary method, but with the solutions solidified with 1% agar, and exposed the sperm of the horsetail *Equisetum arvense*, the quillwort *Isoetes japonica*, and the ferns *Osmunda javanica*, *Pityrogramma sulphurea*, *Pteridium aquilinum*, and *Salvinia natans* (considered by Rothschild not to be a fern) to various numbers of 1% solidified solutions of 23 mostly dicarboxylic acids. The results were consistent with past observations. In addition, it is to be noted that: (a) the sperm of *Equisetum arvense* responded to only one (malate) of the 19 substances to which it was exposed; (b) those of *Isoetes japonica* responded to 17 of the 19 substances with which it was confronted; and (c) the sperm of all 4 of the ferns, to the limited extent that all 4 were tested against each of the various compounds, responded similarly and were attracted to 7 out of the 23 substances to which at least one of them was exposed.

We must at this point attempt to appraise the significance of these studies stimulated by Pfeffer's discovery of the attractive power of malate. Their relation to the naturally occurring chemotactic substance(s) is unknown. No one has attempted to isolate a chemotactic substance from archegonia, nor even to see if there is in archegonia a sufficient concentration of active organic acids to exert a chemotactic response. A detailed study of one plant involving an attempt to isolate the naturally occurring chemotactic substance is needed.

The information amassed does indicate something about the reaction of sperm to various substances. From this point of view, Brokaw (1957,

1958a,b, 1959) has presented new information about the chemotaxis of the sperm of the fern *Pteridium aquilinum* in relation to the physics and chemistry of their reaction to malate. These studies are of interest in relation to the general problem of the control of the direction of movement of flagellated organisms.

In the first set of experiments a drop of 0.1 *N* HCl was placed on a slide in contact with a suspension of sperm containing a pH indicator and various buffers in different experiments (tris, citrate, phosphate, borate, and 10^{-4} *M* sodium L-malate). The sperm aggregated in the boundary where a sharp change in pH occurs and appeared to prefer a pH of 3.5–5.6. Aggregation also occurred between two drops equally buffered at the same pH (6.2), but with a tenfold difference in the concentration of sodium L-malate. Pfeffer (1884) had long ago shown that the gradient of the logarithm of concentration is the important variable in the chemotaxis of these sperm. An analysis of the relative concentration of the malate, bimalate, and malic acid at different pH values shows that bimalate is highest at pH 4.2. Brokaw (1958a) concluded that the bimalate ion is the effective chemotactic agent and pointed out that this agrees with Shibata's (1911) conclusion that the *cis*-configuration is important in chemotaxis of fern sperm. It will be recalled that fumarate (*trans*) was inactive while maleate (*cis*) was active.

The second set of experiments involved the effects of an imposed voltage gradient. If sperm suspended in Tris buffer (pH 8.1) and sodium L-malate are subjected to a voltage gradient (7 v/cm), the sperm swim toward the anode. They do not respond in the absence of malate nor with fumarate or succinate, but maleate can replace malate in agreement with the specificities established by the Pfeffer capillary method. In other experiments where two drops containing different concentrations of malate are brought into contact, the normal response of the sperm to swim into the higher concentration can be reversed by the application of the appropriate voltage gradient. This result is not because the voltage reverses the direction of flow of the bimalate ion, since the voltage gradient that reverses the direction of swimming of the sperm is the opposite of that required to counteract the diffusion gradient of bimalate at constant pH. These results led Brokaw (1958b) to suggest that the sensory elements of the sperm reversibly absorb the bimalate ions. If so, then a voltage gradient would cause them to move to the anode, and a bimalate concentration gradient would cause them to move into the region of higher concentration. He speculated that if the bimalate ions are absorbed on the anterior end of the sperm, then the operating forces (voltage or bimalate concentration gradients) would simply pull

the sperm around until they were swimming in the right direction. It might, perhaps, be possible to confirm this theory by quantitative measurements of the adsorption of malate, succinic, fumaric, and maleic acids by large quantities of sperm. It would, at least, be interesting to try.

The final paper in this series is concerned with the random movement of the fern sperm when not subjected to orienting gradients. Brokaw (1959) found that the movement is random, agreeing with what should be expected if the turning were the result of rotational Brownian movement. This agreement is formal only, since the reciprocal of the rotational diffusion coefficient actually found for the sperm was much too low. He suggested that the cause of the turning might therefore be random differences in the activity of the flagella on different sides of the sperm. No evidence for this conclusion was presented.

IV. The Chemotropic Systems

In contrast to systems with motile gametes, a directional response in those now to be considered always necessitates directed growth. There

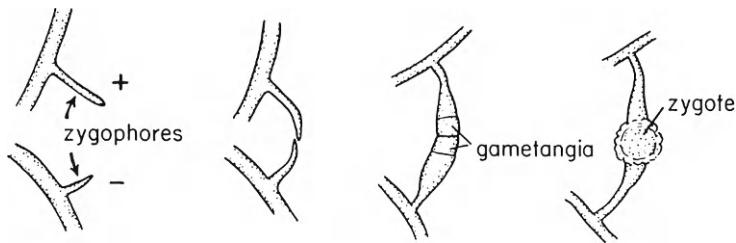


FIG. 10. Stages in the conjugation of *Mucor mucedo*. (After Blakeslee, 1904.)

are four main types. The first consists basically of the growth of a tube from each of the cells on the same or different plants containing one or more gametic nuclei (Fig. 10). These tubes meet and the nuclei then either fuse at the point of juncture or move into one or the other of the originating cells. Systems of this type are found in the conjugating algae, for example *Spirogyra*, in the fungi such as in the Mucorales, in certain yeasts, and in the higher fungi characterized by sexual somatic fusions. Less diffuse in its distribution is the type of reproductive mechanisms found in the aquatic water molds, for example, in the Saprolegniales. Here, antheridial hyphae grow to the egg-containing oogonium (Fig. 11). The opposite situation is found in some of the higher fungi, in which the female gametangium grows a process, the trichogyne, to the male element, which may have various forms (Fig. 12). Basically similar to the directional growth of antheridial hyphae to oogonia is the growth of pollen tubes to the eggs contained within ovules, which are themselves

enclosed in ovaries (Fig. 13). For each of the basic types mentioned above there exists good experimental evidence that the growth of the various sexual elements is directed by chemical agents.

A. ZYGOPHORES

The meeting of gametic nuclei by their migration (of one or both of the sexual partners) through tubes developed by both partners, from the sources of the nuclei, has been studied in depth only in the Mucorales.

One should perhaps mention, however, that Overton (1888) did wonder whether mutual chemical stimuli are involved in the copulation of *Spirogyra*, and we ourselves see a possible chemotropic response in the directional growth of the copulation tubes in certain diatoms as described by Geitler (1928, 1932). Also, it was suggested that the conjugation tubes of certain yeasts may be hormonally directed in their growth toward each other (Winge, 1935; Nickerson and Thimann, 1941, 1943). More recently, Levi (1956) presented experimental evidence for the hormonal control of copulation in *Saccharomyces cerevisiae*. Plus and minus mating types in liquid culture develop hyphal copulatory processes. When these meet, fusion occurs and a zygote is formed. He showed that when a plus cell is placed close to a minus cell on agar, the latter, but not the plus mating type, develops a copulatory tube. In further experiments he showed that an agar surface from which sexually active cells had been removed could now induce the copulatory process in new minus cells, but not in the plus cells. So far, Levi has not been able to prepare an active filtrate, and only once out of several trials was the mating reaction induced across a collodion membrane.

In the Mucorales sexuality is manifested by the development of protuberances on adjacent sexually compatible hyphae. These protuberances are referred to variously as "progametangia," "copulation branches," or "zygophores" (Fig. 10). When they meet, the apex of each is cut off, thus delimiting multinucleate gametangia which subsequently fuse to form the zygote. Blakeslee (1904), in the course of his discovery of heterothallism in the Mucorales, observed that progametangia of *Rhizopus nigricans* were formed after the zygophores were mutually attracted to each other. The chemotropism of the zygophores was named zygotropism by Burgeff (1924), who provided the first experimental evidence for such attraction. He observed that when plus and minus strains of *Mucor mucedo* were allowed to grow toward each other on an agar plate, hyphal swellings considered to be the beginnings of progametangia appeared before actual contact had been made. He confirmed this in an experiment in which a membrane was placed over the mycelium of the

minus strain growing on nutrient agar. On top of the membrane he placed a block of agar containing the plus strain, which then grew out over the membrane. Zygophores were induced in the two strains and grew toward each other, although they were prevented from meeting by the membrane.

Similar experiments with *Rhizopus nigricans*, *Mucor hiemalis*, and *Phycomyces nitens* failed. However, when two strips of agar, one containing the minus strain of *Mucor hiemalis* and the other the plus strain, were placed about 2 mm apart, each produced zygophores which grew toward each other through the air. Burgeff (1924) postulated that each strain produced a volatile and water-soluble substance that could diffuse through agar media or pass through the air and both induce as well as direct the growth of the zygophores. In support of the idea that each mycelium produces an inducing and chemotropic hormone, he did an experiment in which air was passed over a plus culture of *Rhizopus nigricans*, then through a 1-m tube, and then over a minus culture of *Mucor mucedo*. In the latter, young sporangiophores were converted into branched zygophores. Although the evidence for volatile hormones is not here fully convincing, the experiments are described because of the subsequent recurrence of this theme.

It will be noted that Burgeff (1924) did not postulate separate hormones for the induction of zygophores on the one hand and their chemotropic attraction on the other. Experimental proof that these two processes are controlled by separate hormones did not become available until 1960 (Plempel, 1960a). In what follows attention is directed to the zygotropic reaction.

Burgeff's report, as might be expected, initiated a series of investigations on the hormonal basis of reproduction in the Mucorales, which have continued to this day. Verkaik (1930) repeated Burgeff's membrane tests with *Mucor mucedo*, but added nothing significant to what was already known. Ronsdorf (1931) showed that *Phycomyces blakesleeanus* is similar to *Mucor mucedo* in its behavior. Köhler (1935) was concerned only with the induction process in *Mucor mucedo*, as were Hepden and Folkes (1960) and Hepden and Hawker (1961) much later. Köhler's efforts to isolate an active principle failed and led him to suggest that the induction is caused by "some kind of mitogenic ray." When Kehl (1937) found that a continuous current of air passing over a nutrient plate containing plus and minus strains of *Mucor mucedo* appeared to narrow the copulation zone, he concluded that the active principle is transmitted through the atmosphere, and he tested a number of volatile oils for activity with negative results. In a very detailed, extensive investigation of *Pilobolus crystallinus*, Krafczyk (1935) demon-

strated for this species, which grows entirely submerged in the agar in contrast to *Phycomyces*, which has aerial zygothores, that zygothore induction and attraction occur prior to contact. To demonstrate the zygotropic reaction he placed agar blocks, one with a minus mycelium and the other with a plus mycelium, next to each other, with the direction of growth of the mycelia being opposite but parallel. As they approached each other along the common edge, the zygothores clearly turned toward each other.

The first detailed study of zygotropism itself was made by Banbury (1954, 1955). For experimental material he inoculated agar plates with alternate transverse rows of plus and minus spores of *Mucor mucedo*. When the mycelia approached each other zygothores were induced. At this time he cut out rectangular agar blocks that crossed the copulation zone. Each block then contained in the middle a mixture of plus and minus copulating zygothores and at one end minus zygothores and at the other plus zygothores. Thus, a rectangular block was like a magnet with its two poles. If now, under appropriate conditions of humidity, and with the blocks turned on their sides, a plus end was brought close to a minus end, the zygothores grew toward each other, curving if necessary. If two plus ends or two minus ends were brought close to each other, then the zygothores grew away from each other. When such blocks were sealed from each other by glass, mica, or aluminum foil barriers, there was no zygotropism. These last experiments were done to eliminate the possibility of "mitogenic rays" previously suggested by Köhler (1935) as the active agent.

Since the agar blocks in the above tests did not touch each other, Banbury concluded that the active principles were volatile. In keeping with others before him (Ronsdorf, 1931; Krafczyk, 1931, 1935; Buller, 1933), Banbury concluded that there must be two hormones involved. He proposed that one of them is produced by the plus zygothores and that it stimulates elongation in the plus zygothores and inhibits elongation in the minus ones; and that the other is produced by the minus zygothores, stimulating growth in the minus and inhibiting growth in the plus. The inhibitory action explains the mutual attraction (bending toward each other) between plus and minus zygothores. The stimulating action accounts for the growing away from each other of the zygothores of the same mating strain.

Beginning in 1957, Plempel and his associates (Plempel, 1957, 1960a, b, 1963a,b; Plempel and Braunitzer, 1958; Plempel and Dawid, 1961) initiated a vigorous attack on the hormonal regulating system in *Mucor*, working principally with *Mucor mucedo*. The experimental results, which space prohibits recounting in great detail, require the postulation of at

least six hormones. The plus and minus strains when grown separately each release into the nutrient broth during their vegetative growth a progamone. When the cell-free broth is then used to culture the opposite strain, i.e., broth from plus mycelium used as substrate for minus mycelium, the latter then synthesizes the minus gamone; similarly, the plus mycelium produces plus gamone. These gamones when used in the quantitative assay induce the development of the zygophores, the plus gamone being specific for zygophore production by the minus mycelium and the minus gamone for the plus mycelium. Finally, the zygophores produce zygotropic hormones; that from the zygophores on the plus mycelium directs the growth of the zygophores of the minus mycelium toward the former, and vice versa for minus zygotropic hormone.

An extensive series of experiments were carried out on the zygotropic hormones to establish whether or not they are volatile and are transmitted through the gas phase, as suggested by the earlier investigators. When Plempel attacked the problem of zygotropism the experimental conditions were improved by his own isolation of the substances inducing zygophores in the plus and minus strains. It thus became possible to induce zygophores on plus and minus strains grown in separate agar plates.

When this was done by including zygogenic substance in the nutrient agar, the placing of agar blocks containing additional amounts of the active material adjacent to plus or minus zygophores failed to cause bending toward the blocks. From this it was concluded that the purified material containing zygophore-inducing hormones did not include the zygotropic factor. Further, because of the conditions under which the zygophore-inducing material was produced, it seemed likely that the chemotropic factor(s) was formed only after the appearance of the zygophores. Also reported are observations of the reaction when plus or minus *Mucor mucedo* is confronted with the opposite strain of *Phycomyces blakesleeanus*. In the latter the zygophores develop within the agar substrate and rise above it only after contact, whereas in *Mucor mucedo* they develop upward from the substrate prior to contact. The *Mucor* zygophores always developed first. Then, when those of the *Phycomyces* formed, the former bent back toward the substrate where they became encircled by the *Phycomyces* zygophores. Zygophores were not formed. Plempel concluded by considering the possibility of a gaseous zygotropic factor for the *Mucor*, but considered it out of the question for the *Phycomyces*, since the latter grows entirely in the substrate.

These workers then set about establishing the mechanism involved in the transfer of the zygotropic factor in *Mucor mucedo*. First they

unsuccessfully attempted to induce a zygotropic response by diffusion of presumably active material through agar. Plus and minus mycelia alone and mixed were grown on 3% malt extract absorbed into cotton and then induced to form zygophores by the zygogenic substance. Extracts were then obtained by gently pressing the cotton and the mycelium on it. These extracts were tested directly or after various treatments which included: (a) bringing to pH 3 with sulfuric acid or pH 9 with KOH and then extracting with petroleum ether, ether, and acetaldehyde in succession, followed by taking to dryness at 30°C and then dissolving the residue in 0.5 ml water; and (b) concentrating 7.0 ml of the pressed juice by lyophilization to dryness and then dissolving in 0.5 ml of water. The several preparations were tested both by solidifying with agar and placing a small agar cylinder near zygophores or by placing capillaries containing the solutions next to zygophores. In no instance did any curvature toward the test material occur. Were the factor present, it should have diffused through the agar to the zygophores. It was possible that the factor had been destroyed in the preparative procedures. To check this, cheesecloth circles supported by a wire ring about the size of a Petri plate were coated with nutrient agar 2 mm thick. These were seeded with plus spores near one edge on one side and with minus spores on the other side at the other end of a diameter connecting the two seedings. After incubating in a sterile, humid chamber for 3 days, zygophores were induced by the zygogenic substances. If zygotropic substances were produced, they should diffuse through the 2-mm-thick agar disk and cause the zygophores of the two strains to bend 90° toward the surface of the agar disk. This did not happen. Moreover, young zygophores not yet above the agar surface should remain in the medium in response to the attraction. None of this happened. It was concluded that the zygotropic material was not diffusible through agar.

They then tested for transmission through the air. The basic experiments were done with a Petri dish divided in half by a vertical mica sheet through which holes 3.5 mm in diameter (four across the plate) had been drilled. The mica was held in place between glass blocks glued to the bottom of the plate and by the half-covers which met with the mica between them. Rectangular blocks of agar, 12 × 5 mm with plus and minus mycelia were prepared, soaked in zygogenic solution, dried on filter paper, and then placed standing on edge next to the holes in the mica, so that the direction of growth of the zygophores was at right angles to the nutrient agar surface and in the direction of the holes. When minus zygophores faced plus zygophores across the holes, several of the former grew with the necessary curvature through the hole toward a single plus zygophore and established contact. The distance

here was 2.5 mm. With a separation of 4 mm the zygophores of both types nearest to the hole elongated twice as much as those most distant (laterally) from the hole. If for the first 12 hours a minus block faced a minus block, the zygophores stopped growing and began changing into sporangiophores with thickened tips. If then one of the minus blocks was replaced by a plus block, the transformed minus zygophores developed from their tips the long, thin hyphae characteristic of zygophores, which then grew to and through the hole. When two plus blocks or two minus blocks faced each other, no responses in terms of growth toward the holes and accelerated elongation were observed. Most of the zygophores changed into sporangiophores. Additional tests of various kinds further served to confirm the fact that the zygotropic hormones are volatile and transmitted through the atmosphere. They greatly increase the growth in length of the zygophores as much as four to six times compared to those not exposed to the agent and cause them to bend toward each other.

No mention is made of a tendency of like zygophores facing each other to grow back toward their own nutrient agar surfaces, such as described by Banbury. The zygotropic response is explained in terms of two gaseous, organ-specific growth substances. These stimulate elongation of the zygophores of the opposite mating type. If a zygophore is exposed to unequal concentrations on its anterior and posterior flanks, either the elongation on the side receiving the higher concentration is inhibited or the other side receiving the lower concentration is enhanced. Their work suggests that the zygotropic substances are unstable and readily oxidized in air. This destruction is postulated to be the basis for the maintenance of concentration gradients.

The other major line of attack by Plempel and his associates on the hormonal system in *Mucor mucedo* has been on the chemistry of the gamones or zygogenic hormones. This work is included in the earlier literature citations, but a discussion of it is not germane to the subject of this chapter.

B. ANTHERIDIAL HYPHAE

One might expect two separate chemotropic responses to occur in this type of reproduction. Fig. 11 shows the commonly reported growth of antheridial hyphae to a developing oogonium. Upon reaching the oogonium, the antheridial hyphae become applied to the oogonial wall. Usually a transverse wall is then formed cutting off an antheridium. From the latter there develop fertilization tubes that penetrate the oogonial wall and grow to the eggs, a growth process which might also be expected to be directed. It is only for the growth of the antheridial

hyphae that evidence for a hormonal control of the direction of growth has been adduced.

The idea that antheridial hyphae grow to developing oogonia in response to chemical signals from the oogonia began, as discussed earlier, with de Bary (1881) during his studies of sexual reproduction in *Saprolegnia* and related forms. Subsequently Couch (1926) attempted without success to demonstrate a chemotropic agent in *Dictyuchus monosporus*. No reaction was obtained with a brei or when male and female plants were separated by water or by membranes. In 1940, Bishop showed beyond doubt that secretions from the female plants of *Sapromyces reinschii* induce the development of antheridial hyphae on the male plants. He presumed (but did no experiments to prove) that the directional growth of the antheridial hyphae is caused by diffusible substances. The proof was finally made by Raper (1939, 1940) as part of

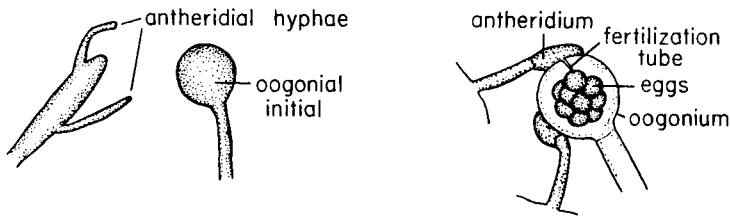


FIG. 11. Stages in the sexual reproduction of *Achlya*.

his now classical experiments demonstrating the involvement of at least seven hormones in the integration of sexual reproduction in *Achlya bisexualis* and *A. ambisexualis*. The full story has been reviewed (Raper, 1952, 1957), and attention will be concentrated here on the evidence for the chemotropic hormone (hormone C of Raper).

After the antheridial hyphae have been induced by secretions from the female plant, the antheridial hyphae in turn induce the development of oogonia. The oogonial initials then secrete hormone C that attracts the antheridial hyphae to the developing oogonium. The existence of hormone C was shown by two different methods. In one the male and female plants were separated from each other by a cellophane membrane. It was observed that the antheridial hyphae became appressed to the membrane opposite oogonial initials. When antheridial hyphae were induced by solutions of the inducing hormones, they grew in random directions. The second method consists essentially of hormone C passing into a microaquarium containing male plants with antheridial hyphae. The solution entered through a fine capillary tube. There was clear directional growth of the hyphae to the capillary. Unfortunately, most

of the subsequent work by Raper and his group was directed toward the assay and chemistry of the antheridium-inducing hormone A. Subsequent reports (Raper, 1950; Barksdale, 1960) show that the hormonal system worked out by Raper and his associates is operative in several other species of *Achlya* and *Thraustotheca*, including homothallic as well as heterothallic species. According to Raper (1960), Barksdale is attempting to isolate hormone A, but nothing has yet been done toward isolating the chemotropic factor. With the wealth of information now available on the control of sexuality in these organisms, an attempt to isolate and chemically characterize the chemotropic factor should be blessed with success.

Barksdale (1963a,b) undertook a study of the production of the antheridial-inducing hormone (hormone A of Raper) by various strains of *Achlya*. In the course of these investigations she found that hormone A not only induced the antheridial hyphae but also attracted them, a function attributed by Raper to his hormone C. Barksdale found that particles of either polystyrene or polyvinyl plastic would absorb hormone A. These particles were then quite clearly able to attract the antheridial hyphae. When these hormone-treated particles were placed on top of a cellophane membrane overlaying the male strain, they induced the formation of antheridial branches beneath the membrane. They also caused certain other effects considered to be a function of hormone C, according to Raper. Thus it appears that hormone A is responsible for the activities attributed to hormone C, and therefore that it has chemotropic activity as well as other hormonal effects. In discussing the work of Fischer and Werner (1955) below, reference will continue to be made to hormone C, even though its existence is now uncertain. Also now in doubt is the need of antheridial hyphae to touch a membrane or an oogonium before cutting off antheridia (Barksdale, 1963a).

Fischer and Werner (1955) reported on chemotropism of the vegetative hyphae and antheridial hyphae of *Saprolegnia mixta* and *S. ferax*. Their method consisted in growing mycelia on nutrient-deficient medium (0.2% malt extract solidified with 2% agar). The solutions to be tested were solidified with agar and small cylinders of these solidified solutions were then placed just in front or just behind the growing front. The radius of the influence of the solutes as evidenced by hyphae growing to the agar cylinder was used as a measure of the chemotropic effect. Of a great many substances tested, only protein hydrolyzate, casein hydrolyzate, or a mixture of at least five to seven amino acids induced chemotropism. Although no one amino acid was found indispensable, neither would any one by itself exert a chemotropic effect. When the block of active material was placed just behind the front, the hyphal tips made a

full 180° curvature back to the block. In similar experiments, antheridial hyphae were attracted by agar blocks containing 20 mg casein hydrolyzate per milliliter of solidified agar. Such blocks also attracted antheridial hyphae away from developing oogonia. The antheridial hyphae attracted away from oogonia dedifferentiate into vegetative hyphae in contrast to the effect of Raper's hormone C, which, after the hyphae touch either a membrane or the oogonial wall, cut off antheridia. The authors suggest that the developing oogonial initials release a mixture of amino acids or protein fragments which serve as the chemotropic factor. One wonders whether the antheridial hyphae attracted by the casein hydrolyzate would have formed antheridia had there been a membrane for them to touch. It seems unlikely that the actual chemotropic agent attracting antheridial hyphae is comparable to a protein hydrolyzate. In Raper's experiments the medium included 0.1% peptone in contrast to the deficient media of Fischer and Werner. Thus, amino acids were available. Raper showed that antheridial hyphae were not attracted to the vegetative hyphae, but specifically to the developing oogonia. For hormone C to be an amino acid mixture would require the developing oogonia to be specialized organs for the secretion of amino acids or protein fragments in higher concentrations than that supplied in the medium unless one assumes the medium supply to have been depleted by growth of the mycelium. This could presumably be checked by providing substantially higher concentrations of hydrolyzate in the nutrient medium.

Although it seems to us that the chemotropic factor from developing oogonia is unlikely to be as unspecific as a mixture of amino acids, the matter should be explored, and methods of so doing seem to be at hand. There is nothing that says that an antheridial hypha cannot display a general chemotropic response to nutrients, also exhibited by vegetative hyphae, as well as a chemotropic response to a specific factor.

Of some interest is the suggestion of Fischer and Werner (1955) as to the mechanism of the chemotropic response. They postulated on the basis of various kinds of evidence that the nutrient uptake causes the development of a thicker, less elastic wall on the side of the tip toward the nutrient source, thus resulting in growth toward the source. It is pointed out that on media containing protein hydrolyzates the hyphae tend to be short and thick, while on media deficient in protein but rich in carbohydrate the hyphae are long and thin.

C. TRICHOCYNES

Work involving chemotropism in this system has been done only with certain ascomycetous fungi. The basic pattern of reproduction involves

the development on hyphae of an ascogonium, usually a coiled, septate, structure representing the female (Fig. 12). From this, a tube, which may in some species branch, develops. The tube—a trichogyne—eventually contacts the nonmotile male element, whose contents then enter the trichogyne and migrate into the ascogonium. The ascogonium may become ensheathed by hyphae originating from basal cells of the ascogonium or nearby vegetative hyphae. The male elements may be almost any kind of cell from the proper mating type, but specifically, in the work to be reviewed here, are nonmotile, unicellular bodies cut off from the parent plant and called variously, depending on matters not pertinent to the present review, “oidia,” “spermatia,” or “conidia.” If chemotropism were involved, it would be expected at two times in the process, one during the approach of the trichogyne to the male element and the second at the time of the ensheathment of the ascogonium by the enveloping hyphae.

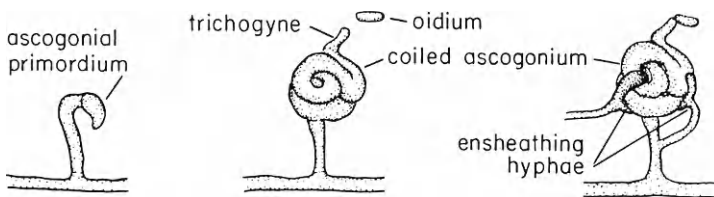


FIG. 12. Stages in the asexual reproduction of *Ascobolus stercorarius*. (After Bistis, 1956, 1957.)

The possible involvement of hormones in the induction of sexual activity in these fungi was a matter of considerable controversy recounted in detail by Raper (1952). Among these early reports the first suggestion of a chemotropic growth of a trichogyne was made by Dodge (1912) when describing reproduction in *Ascobolus carbonarius*: “There can be no question, however, as to the specific attraction between the trichogyne and the conidium, and I shall call it the antheridial conidium.” A similar observation was made by Backus (1939). He, too, performed no experiments to substantiate his idea that in *Neurospora sitophila* the trichogyne sought out the conidium. Backus also observed that conidia, which by themselves can germinate and grow into a mycelium, would not germinate in the presence of a mycelium of the opposite compatibility strain.

Definite evidence for the chemotropic growth of a trichogyne was provided by Zickler (1937, 1952) in *Bombardia lunata*. In this organism the male element is a spermatium. In contrast to the conidia above, they will not germinate and grow vegetatively on nutrient agar, nor do they

require sexual activation as do the oidia to be described later in relation to *Ascobolus stercorearius*. Each of the compatibility strains produces both ascogonia and spermatia. The trichogyne arising from an ascogonium grows initially in a random fashion, and then if spermatia from the opposite mating type are present, the trichogyne grows directly to the spermatia, making in some cases a full right angle bend. Zickler suspended spermatia in water and then filtered them off. The filtrate, either in capillary tubes or in agarized blocks, attracted the trichogynes and did so even after autoclaving. Vegetative hyphae did not respond chemotropically, indicating that the chemotropic factor was specific for the trichogyne. On the other hand, mycelial extracts of the correct mating type were as effective as spermatial extracts in attracting trichogynes. This is not unexpected, since almost any part of the appropriate compatibility-type partner can serve as a male element in these organisms.

Finally, Bistis (1956, 1957) provided evidence that in *Ascobolus stercorearius* there is a multihormonal mechanism controlling and regulating sexual reproducing. In this work the male element consisted of oidia which are multinucleate individual cells obtainable free of the mycelium on which they are produced (Fig. 12). These are capable of growing vegetatively when placed on nutrient agar. In the presence of mycelium of their own or the opposite compatibility class, their germination and vegetative growth is inhibited. An oidium induces ascogonial development only after it itself has been sexually activated by exposure for about 4 hours to a mycelium of the opposite compatibility type. After activation it then induces the development of ascogonial initials, which develop into multicellular organs, the terminal cell of which grows out as a trichogyne. The trichogyne grows to the oidium and fuses with it. Evidence for this chemotropic growth of the trichogyne was direct. After a trichogyne started growing toward an oidium, the latter was moved. The trichogyne then turned until directed toward the new position of the oidium. This could be repeated until the trichogyne failed to grow anymore. An unactivated oidium is unable to attract a trichogyne.

After fusion, lateral hyphae from the base of an ascogonium and nearby vegetative hyphae grow to the ascogonium and ensheath it. This is considered to be a chemotropic response, but no evidence other than the observation of the approach of these investing hyphae is presented.

D. POLLEN TUBES

As noted earlier, Sachs and Pfeffer were inclined to the view that the growth of a pollen tube down through the stigma and style and then into an ovule either through the micropyle or through the chalaza is chemically directed, that is, a chemotropic response (Fig. 13). A chemo-

tropic response of pollen tubes was first actually demonstrated by Molisch (1890, 1893) by a method used by almost all subsequent investigators. Pollen grains and test material, for example, ovules, are placed about 1 mm from each other on the surface of nutrient agar. The response, when it occurs, is dramatic, as shown in Fig. 14, which also shows the response to ovules and to calcium in a different type of system used by Mascarenhas and Machlis (1962b, 1964).

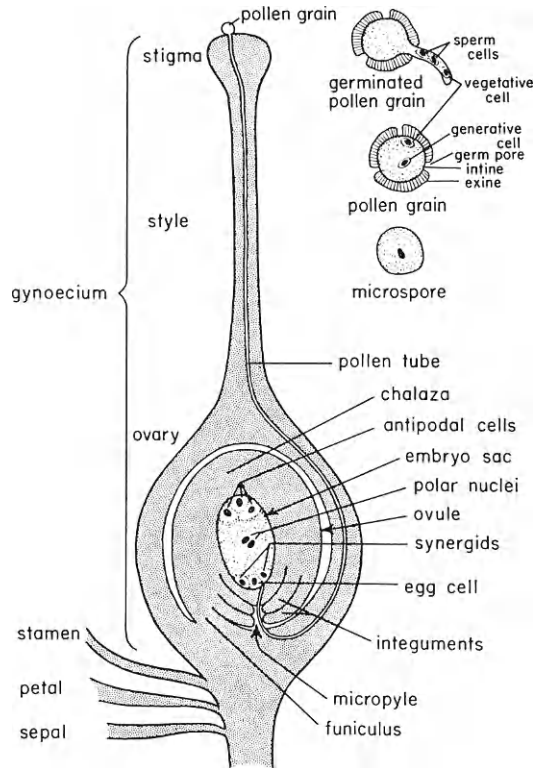


FIG. 13. The path of a pollen tube to the egg cell.

Mascarenhas and Machlis (1962a) reviewed in some detail the subject of the chemotropic growth of pollen tubes. (The subject will be discussed in Chap. 5 of Vol. II and therefore will not be further considered here.)

V. Discussion

This chapter makes quite clear that chemotactic and chemotropic reactions participate in the sexual reproduction of a widespread variety of plants. The evidence varies from the simple observation of aggregation

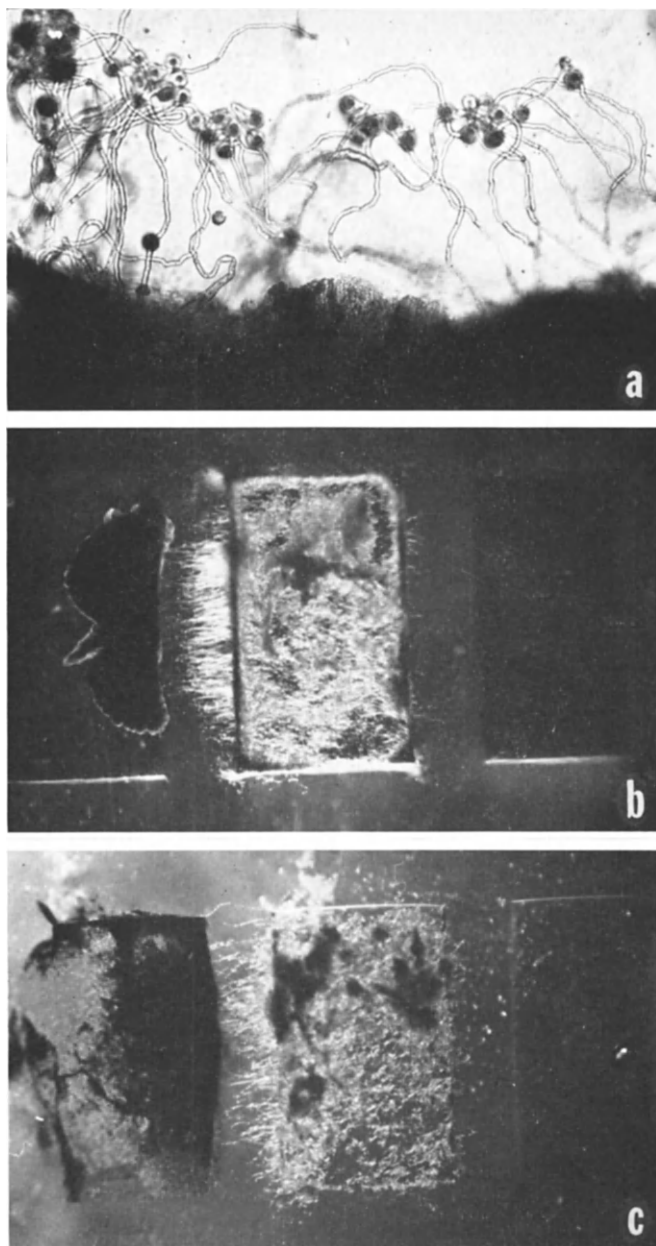


FIG. 14. The directed growth of pollen tubes to ovules and to calcium. In (a) pollen grains were placed approximately 1 mm from a clump of ovules (at the bottom of the picture) on the surface of nutrient agar. In (b) and (c) excess pollen was placed in the middle one of three adjacent depressions formed in nutrient agar.

of sperm and the apparent directed growth of various sexual structures to conclusive evidence obtained with cell-free extracts, partially purified preparations and, in the case of pollen, presumably the factor itself. It would appear to be only a matter of time and effort to demonstrate directed movements in the sexual reproduction of many additional species. While this would be interesting, the more fundamental need is to identify chemically the substances effecting the responses. Unfortunately, producing these substances in sufficient amounts to permit the determination of chemical structure is more engineering than research and the chemistry is better done in well-equipped chemical laboratories than in the usual biological laboratory. Nevertheless, such efforts are underway, as pointed out by Raper (1960). And, as he aptly says, at least the financing of such ventures is more promising now than in the past. For those who would seriously consider the effort it is worth remembering that in addition to the intrinsic value of such knowledge there is always the chance that new types of biologically active compounds may be revealed. The fungi, particularly, have an esteemed reputation for providing man with compounds that have come to have widespread scientific and practical significance.

Although alluded to only incidentally, there is an increasing amount of documentation for the fact that sexual reproduction in plants is a complex process whose steps are correlated and controlled by hormones. This is obvious for the flowering plants, although not even the flower-inducing hormone florigen is characterized chemically. In the ferns, in addition to the chemotactic seduction of the sperm into the archegonium, a factor has been isolated and partially purified that induces the formation of the antheridia (Näf, 1962). And in the algae and fungi, where so long Raper's demonstration of multihormonally controlled sexual reproduction in *Achlya* stood in isolated splendor, there now is good company furnished by complex systems in *Mucor*, in *Oedogonium*, and in *Ascobolus*. For these systems, just as for the more limited area of directed movements, the need is for chemical identification of the hormones, so that one may proceed to a study of the biochemical and biophysical mechanisms through which they act.

We should also comment on the specificity of the chemotactic and chemotropic substances. Because sexual reproduction is so highly specific, particularly with respect to gametic nuclei that fuse, one on first thought

Ovules were placed against the wall (1 mm thick) separating the left depression from the pollen containing depression in (b) while solid CaCO_3 was similarly placed in (c). Nothing was placed in the right compartments. (From Mascarenhas and Machlis, 1962a,b,c, and 1964.)

might expect the agents of directed movement of the sexual elements to also be very specific. This may be more the exception than the rule. If, as is indicated, the chemotropic factor for the directed growth of pollen tubes is calcium, then it cannot even claim the name of "hormone." Long ago, but not retested since, Pfeffer showed that the sperm of various ferns responded chemotactically to the same archegonium. In the same vein, Plempel found the zygophores of *Mucor mucedo* and *Phycomyces blakesleeanus* to respond chemotropically to each other; Tsubo showed that the agent from the minus strain of one species of *Chlamydomonas* attracted both mating types of three other species; and the egg water of one species of *Fucus* was found to attract the sperm of two other species. On the other hand, there is a high degree of specificity indicated for the attractants of the sperm of *Oedogonium*. Although the meager evidence available does not particularly support a high degree of specificity, neither have any very critical tests been made in this area.

ACKNOWLEDGMENTS

We are pleased to acknowledge the support of the National Science Foundation through research grant GB1107. We are also indebted to Joseph Mascarenhas for preparing the photographs of the megagametophyte of *Marsilea*, to Emily Reid for the diagrammatic drawings, and to Professors R. Emerson and J. Proskauer for reading and criticizing portions of the manuscript.

REFERENCES

- Akerman, A. (1910). *Z. Botan.* **2**, 94.
 Austin, C. R. (1961). *Intern. Rev. Cytol.* **12**, 337.
 Backus, M. P. (1939). *Bull. Torrey Botan. Club* **66**, 63.
 Banbury, G. H. (1954). *Nature* **173**, 499.
 Banbury, G. H. (1955). *J. Exptl. Botany* **6**, 235.
 Barksdale, A. W. (1960). *Am. J. Botany* **47**, 14.
 Barksdale, A. W. (1963a). *Mycologia* **40**, 164.
 Barksdale, A. W. (1963b). *Mycologia* **40**, 627.
 Barksdale, A. W., Carlile, M. J., and Machlis, L. (1965). *Mycologia* **57**, 138.
 Berthold, G. (1880). *Botan. Ztg.* **38**, 648.
 Berthold, G. (1881). *Mitt. Zool. Stat. Neapel* **2**, 401.
 Bishop, D. (1962). *Physiol. Rev.* **42**, 1.
 Bishop, H. (1940). *Mycologia* **32**, 505.
 Bistis, G. (1956). *Am. J. Botany* **43**, 389.
 Bistis, G. (1957). *Am. J. Botany* **44**, 436.
 Blakeslee, A. F. (1904). *Proc. Am. Acad. Arts Sci.* **40**, 206.
 Bordet, J. (1894). *Bull. Acad. Roy. Sci. Lettres et Beaux-arts Belg.* [3] **27**, 288.
 Brefeld, O. (1883). "Untersuchungen aus dem Gesamtgebiet der Mykologie," Vol. 5. A. Felix, Leipzig.
 Brokaw, C. J. (1957). *Nature* **179**, 525.
 Brokaw, C. J. (1958a). *J. Exptl. Biol.* **35**, 192.
 Brokaw, C. J. (1958b). *J. Exptl. Biol.* **35**, 197.
 Brokaw, C. J. (1959). *J. Cellular Comp. Physiol.* **54**, 95.

- Bruchmann, H. (1909). *Flora (Jena)* **99**, 193.
- Buller, A. H. R. (1933). "Researches on Fungi," Vol. 5, Chap. 1. Longmans, Green, New York.
- Buller, R. (1900). *Ann. Botany (London)* **14**, 543.
- Burgeff, H. (1924). *Botan. Abhandl.* **4**, 5.
- Carlile, M. J., and Machlis, L. (1965). *Am. J. Bot.* **52**, 478.
- Cohn, F. (1856). *Ann. Sci. Nat. Botan. Biol. Vegetale* [4] **5**, 187.
- Coleman, A. W. (1962). In "Physiology and Biochemistry of Algae" (R. A. Lewin, ed.), p. 711. Academic Press, New York.
- Cook, A. H., and Elvidge, J. A. (1951). *Proc. Roy. Soc. (London), Ser. B* **138**, 97.
- Cook, A. H., Elvidge, J. A., and Heilbron, Sir I. (1948). *Proc. Roy. Soc. (London), Ser. B* **135**, 293.
- Couch, J. N. (1926). *Ann. Botany (London)* **40**, 848.
- de Bary, A. (1871). *Monatsber. Deut. Akad. Wiss. Berlin* p. 233.
- de Bary, A. (1881). *Abhandl. Senckenberg. Naturforsch. Ges.* **12**, 225.
- de Bary, A., and Strasburger, E. (1877). *Botan. Ztg.* **35**, 713.
- Dickmann, Z. (1963). *J. Exptl. Biol.* **40**, 1.
- Dodel, A. (1876). *Jahrb. Wiss. Botan.* **10**, 417.
- Dodge, B. O. (1912). *Bull. Torrey Botan. Club* **39**, 139.
- Emerson, R., and Wilson, C. M. (1954). *Mycologia* **46**, 393.
- Errera, M. (1894). *Bull. Acad. Roy. Sci. Lettres et Beaux-arts Belg.* [3] **27**, 863.
- Farmer, J. B., and Williams, J. L. (1898). *Phil. Trans. Roy. Soc. (London), Ser. B* **190**, 623.
- Fischer, A. (1882). *Jahrb. Wiss. Botan.* **13**, 286.
- Fischer, F. G., and Werner, G. (1955). *Z. Physiol. Chem.* **300**, 211.
- Fritsch, F. E. (1935). "The Structure and Reproduction of the Algae," Vol. I. Cambridge Univ. Press, London and New York.
- Fulton, H. R. (1906). *Botan. Gaz.* **41**, 81.
- Geitler, L. (1928). *Arch. Protistenk.* **61**, 419.
- Geitler, L. (1932). *Arch. Protistenk.* **78**, 1.
- Goroschankin, J. (1905). *Flora (Jena)* **94**, 420.
- Graves, A. H. (1916). *Botan. Gaz.* **62**, 337.
- Hagen-Seyfferth, M. (1959). *Planta* **53**, 376.
- Hanstein, J. (1865-1866). *Jahrb. Wiss. Botan.* **4**, 197.
- Hartmann, M. (1934). *Arch. Protistenk.* **83**, 110.
- Hartmann, M. (1950). *Pubbl. Staz. Zool. Napoli* **22**, 120.
- Hepden, P. M., and Folkes, B. P. (1960). *Nature* **185**, 254.
- Hepden, P. M., and Hawker, L. E. (1961). *J. Gen. Microbiol.* **24**, 155.
- Hoffman, L. R. (1960). *Southwestern Naturalist* **5**, 111.
- Hoffman, L. R. (1961). Studies of the morphology, cytology, and reproduction of *Oedogonium* and *Oedocladium*. Ph.D. Dissertation, Univ. Texas, Austin, Texas.
- Hutner, S. H., and Provasoli, L. (1951). In "Biochemistry and Physiology of Protozoa" (A. Lwoff, ed.), p. 27. Academic Press, New York.
- Kehl, H. (1937). *Arch. Mikrobiol.* **8**, 379.
- Kihlman (1883). *Acta Soc. Sci. Fennica* **13**, 12; cited by Pfeffer, W. (1884) p. 469.
- Köhler, E. (1930). *Zentr. Bakteriell. Parasitenk. Abt. II* **82**, 1.
- Köhler, F. (1935). *Planta* **23**, 358.
- König, E. (1961). *Arch. Mikrobiol.* **40**, 395.
- Kotte, W. (1923). *Ber. Deut. Botan. Ges.* **41**, 24.
- Krafczyk, H. (1931). *Ber. Deut. Botan. Ges.* **49**, 141.
- Krafczyk, H. (1935). *Beitr. Biol. Pflanz.* **23**, 349.

- Kunieda, H., and Suto, S. H. (1938). *Botan. Mag. (Tokyo)* **52**, 539.
- Kusano, S. (1931). *Japan. J. Botany* **5**, 35.
- Levi, J. D. (1956). *Nature* **177**, 753.
- Levring, T. (1949). *Physiol. Plantarum* **2**, 45.
- Levring, T. (1952). *Physiol. Plantarum* **5**, 528.
- Lewin, R. (1950). *Nature* **166**, 76.
- Lewin, R. A. (1954). In "Sex in Microorganisms" (D. H. Weinrich, I. F. Lewis, and J. R. Raper, eds.), p. 100. Am. Assoc. Advance. Sci., Washington, D.C.
- Lidforss, B. (1904). *Jahrb. Wiss. Botan.* **41**, 65.
- Lidforss, B. (1905). *Ber. Deut. Botan. Ges.* **23**, 314.
- Linskens, H. (1954-1964). In "Fortschritte der Botanik" (E. Bünning and E. Gäumann, eds.), Vols. 17-27. Springer, Berlin.
- Machlis, L. (1958a). *Physiol. Plantarum* **11**, 181.
- Machlis, L. (1958b). *Nature* **181**, 1790.
- Machlis, L. (1958c). *Physiol. Plantarum* **11**, 845.
- Machlis, L. (1963). In "Physiology of Reproduction," *Proc. 1961 Biol. Colloquium* (F. L. Hisaw, Jr., ed.), p. 79. Oregon State Univ., Corvallis, Oregon.
- Machlis, L., Nutting, W. H., Williams, M. W., and Rapoport, H. (1966a). *Biochemistry* **5**, 2147.
- Machlis, L. (1966b). In "The Fungi" (G. C. Ainsworth and A. S. Sussman, eds.), Academic Press, New York.
- Mainx, F. (1931a). *Arch. Protistenk.* **75**, 502.
- Mainx, F. (1931b). *Z. Botan.* **24**, 481.
- Mascarenhas, J. P., and Machlis, L. (1962a). *Vitamins and Hormones* **20**, 347.
- Mascarenhas, J. P., and Machlis, L. (1962b). *Am. J. Botany* **49**, 482.
- Mascarenhas, J. P., and Machlis, L. (1962c). *Nature* **196**, 292.
- Mascarenhas, J. P., and Machlis, L. (1964). *Plant Physiol.* **39**, 70.
- Metz, C. (1957). In "The Beginnings of Embryonic Development" (A. Tyler, R. C. von Borstel, and C. Metz, eds.), p. 23. Am. Assoc. Advance. Sci., Washington, D.C.
- Miller, R. L., and Nelson, L. (1962). *Biol. Bull.* **123**, 477.
- Miller, R. L. (1963). *Biol. Bull.* **125**, 386.
- Miller, R. L. (1964). *Biol. Bull.* **127**, 381.
- Moewus, F. (1939). *Arch. Protistenk.* **92**, 485.
- Molisch, H. (1890). *Anz. Oesterr. Akad. Wiss. Math.-Naturw. Kl.* **26**, 11.
- Molisch, H. (1893). *Sitzber. Heidelberg Akad. Wiss. Math.-Naturw. Kl. Abhandl.* **102**, 423.
- Morgan, T. H. (1927). "Experimental Embryology," Columbia Univ. Press, New York.
- Näf, U. (1962). *Ann. Rev. Plant Physiol.* **13**, 507.
- Nickerson, W. J., and Thimann, K. V. (1941). *Am. J. Botany* **28**, 617.
- Nickerson, W. J., and Thimann, K. V. (1943). *Am. J. Botany* **30**, 94.
- Overton, C. E. (1888). *Ber. Deut. Botan. Ges.* **6**, 68.
- Pascher, A. (1931a). *Jahrb. Wiss. Botan.* **75**, 551.
- Pascher, A. (1931b). *Beih. Botan. Zentr. Abt. I* **48**, 466.
- Pfeffer, W. (1884). *Untersuch. Botan. Inst. Tübingen* **1**, 363.
- Plömpel, M. (1957). *Arch. Mikrobiol.* **26**, 151.
- Plömpel, M. (1960a). *Planta* **55**, 254.
- Plömpel, M. (1960b). *Naturwissenschaften* **47**, 472.
- Plömpel, M. (1963a). *Naturwissenschaften* **50**, 226.
- Plömpel, M. (1963b). *Planta* **59**, 492.

- Plempel, M., and Braunitzer, G. (1958). *Z. Naturforsch.* **13b**, 302.
- Plempel, M., and Dawid, W. (1961). *Planta* **56**, 438.
- Pringsheim, N. (1855). *Monatsber. Deut. Akad. Wiss. Berlin* 133.
- Pringsheim, N. (1858). *Jahrb. Wiss. Botan.* **1**, 1.
- Pringsheim, N. (1869). *Monatsber. Deut. Akad. Wiss. Berlin* 721.
- Proskauer, J. (1948). *Ann. Botany (London)* [N.S.] **12**, 237.
- Raper, J. R. (1939). *Am. J. Botany* **26**, 639.
- Raper, J. R. (1940). *Am. J. Botany* **27**, 162.
- Raper, J. R. (1950). *Botan. Gaz.* **112**, 1.
- Raper, J. R. (1952). *Botan. Rev.* **18**, 447.
- Raper, J. R. (1954). In "Sex in Microorganisms" (D. H. Weinrich, I. F. Lewis, and J. R. Raper, eds.), p. 42. Am. Assoc. Advance. Sci., Washington, D.C.
- Raper, J. R. (1957). *Symposia Soc. Exptl. Biol.* **11**, 143.
- Raper, J. R. (1960). *Am. J. Botany* **47**, 749.
- Rawitscher-Kunkel, E., and Machlis, L. (1962). *Am. J. Botany* **49**, 177.
- Robbins, W. J. (1916). *Biol. Bull.* **30**, 125.
- Ronsdorf, L. (1931). *Planta* **14**, 482.
- Rothschild, Lord (1952). *Intern. Rev. Cytol.* **1**, 257.
- Rothschild, Lord (1956). "Fertilization." Methuen, London.
- Runnström, J., Hagström, B. E., and Perlmann, P. (1959). In "The Cell" (J. Bracket and A. E. Mersky, eds.), Vol. 1, p. 327. Academic Press, New York.
- Sachs, J. von (1882). "Vorlesungen über Pflanzenphysiologie." Engelmann, Leipzig.
- Sachs, J. von (1887). "Lectures on the Physiology of Plants" (English translation by H. M. Ward). Oxford Univ. Press, London and New York.
- Schreiber, E. (1931). *Ber. Deut. Botan. Ges.* **49**, 235.
- Shibata, K. (1905a). *Jahrb. Wiss. Botan.* **41**, 516.
- Shibata, K. (1905b). *Botan. Mag. (Tokyo)* **19**, 39.
- Shibata, K. (1905c). *Botan. Mag. (Tokyo)* **19**, 79.
- Shibata, K. (1911). *Jahrb. Wiss. Botan.* **49**, 1.
- Smith, G. (1955). "Cryptogamic Botany," 2nd ed., Vol. 1. McGraw-Hill, New York.
- Stadler, D. R. (1952). *J. Cellular Comp. Physiol.* **39**, 449.
- Strasburger, E. (1868). *Botan. Ztg.* **26**, 822.
- Strasburger, E. (1869–1870a). *Jahrb. Wiss. Botan.* **7**, 390.
- Strasburger, E. (1869–1870b). *Jahrb. Wiss. Botan.* **7**, 409.
- Thuret, G. (1854). *Ann. Sci. Nat. Botan.* [4] **11**, 197.
- Tiffany, L. H. (1957). *Botan. Rev.* **23**, 47.
- Tsubo, Y. (1956). *Botan. Mag. (Tokyo)* **69**, 1.
- Tsubo, Y. (1957). *Botan. Mag. (Tokyo)* **70**, 327.
- Tsubo, Y. (1961). *J. Protozool.* **8**, 114.
- Tyler, A. (1955). In "Analysis of Development" (B. H. Willier, P. Weiss, and V. Hamburger, eds.), p. 170. Saunders, Philadelphia, Pennsylvania.
- Verkaik, C. (1930). *Proc. Koninkl. Ned. Akad. Wetenschap.* **33**, 656.
- Ward, H. M. (1888). *Ann. Botany (London)* **2**, 319.
- Ward, M. (1954). *Phytomorphology* **4**, 1.
- Wiese, L. (1961). In "Fortschritte der Zoologie" (H. Bauer, ed.), Vol. 13, p. 119. Fischer, Stuttgart.
- Wilkie, D. (1954). *Exptl. Cell Res.* **6**, 384.
- Williams, L. (1904). *Ann. Botany (London)* **18**, 183.
- Winge, Ö. (1935). *Compt. Rend. Trav. Lab. Carlsberg, Ser. Physiol.* **21**, 77.
- Zickler, H. (1937). *Z. Induktive Abstammungs-Vererbungslehre* **73**, 403.
- Zickler, H. (1952). *Arch. Protistenk.* **98**, 1.

CHAPTER 5

Gamete Surface Components and Their Role in Fertilization*

Charles B. Metz

INSTITUTE OF MOLECULAR EVOLUTION, UNIVERSITY OF MIAMI,
CORAL GABLES, FLORIDA

I. Introduction	163
II. Specificity of Fertilization	164
A. Specificity and Membrane Fusion	165
B. Self-Sterility	165
III. The Spermatozoon	166
A. Antigenic Structure of the Mammalian Spermatozoon	168
B. The Aspermatogenic Antigen	171
C. Blood Group Antigens	173
IV. The Egg	177
V. Sea Urchin Gamete Surfaces and Fertilization	179
A. Fertilizin and Antifertilizin	181
B. Cytofertilizin	193
C. Antigens of Sea Urchin Gametes	195
VI. Amphibian Fertilization	211
A. Role of the Egg Jelly	211
B. Role of Egg Cortex Antigens	220
VII. Summary and Conclusions	222
References	224

I. Introduction

The passage of the spermatozoon to the egg surface involves important and in some cases essential preliminaries in fertilization. At the morphological level, these preliminaries include the sperm acrosomal reaction, penetration of any extracellular egg membranes, and, finally, the attachment of the sperm to the egg plasma membrane. Physiological

* Contribution No. 063 from the Institute of Molecular Evolution. During preparation of this manuscript, support was received from the National Aeronautics and Space Administration (NsG 689), the National Science Foundation (GB 3899), and the National Institutes of Health (5T1 HD 26-04).

preliminaries include effects on sperm motility and respiration (see Metz, 1957b), contributions to the specificity of fertilization and, in the case of amphibians, some essential but as yet undefined function. These morphological and physiological events, and indeed the initiation of egg activation itself, must result from interaction of sperm and egg substances. Such substances evidently constitute a part of or are released from the extracellular layers and from the surfaces of the gametes themselves.

It is the purpose here to review certain of these early events of fertilization and to examine the gametes, and especially their surfaces, in terms of molecular components that may function in fertilization. Sperm motility and respiration, the acrosomal reaction, and egg membrane lysins are considered elsewhere in this volume (see Chaps. 2, 3, and 6, respectively). Accordingly, the discussion is confined to general treatments of fertilization specificity, the sperm surface with emphasis on mammalian studies, and the egg surface. Finally, sea urchin and amphibian gamete surfaces and their relation to fertilization are treated in detail.

II. Specificity of Fertilization

In a number of cases fertilization specificity appears to result from at least two highly specific sperm-egg interacting systems. The first of these is the fertilizin-antifertilizin system (see Section V,A), which probably aids in binding the sperm to the egg (Tyler, 1959) and may initiate the acrosomal reaction (Dan, 1956; Haino and Dan, 1961; Collier, 1959; Metz, 1957a; Piatigorsky and Austin, 1962). Unfortunately, only a limited specificity study of the initiation of the acrosomal reaction has been made (Dan, 1956).

The second specificity system concerns lysins from the sperm acrosome which dissolve a passage for the sperm through extracellular egg membranes. Well-established examples of the latter are found in the mollusks *Megathura* (Tyler, 1939; Krauss, 1950), *Haliotus* (Tyler, 1939), and *Mytilis* (Berg, 1949, 1950; Hauschka, 1963, 1964) and the echinoderms *Mellita quinquesperforata* (Brookbank, 1958) and *Arbacia punctulata* (Hathaway *et al.*, 1960). Sperm penetration of follicle cell layers about the mammalian egg results from a comparable lytic action of hyaluronidase from the sperm acrosome (see Austin, 1961; Mann, 1964), and a lysin may well permit penetration of the zona pellucida by the sperm (e.g., Austin, 1961). In reference to hyaluronidase, Katsh (1960b) found, as might be expected, that hyaluronidases from different species are immunologically different. It would be of interest to test these for specificity of enzymic action on hyaluronic acids from different

sources. Any such differences would suggest a specificity contribution for mammalian fertilization. The acrosomal reaction and egg membrane lysins from sperm are reviewed in detail in Chapter 6.

A. SPECIFICITY AND MEMBRANE FUSION

One point of particular interest is whether sperm and egg plasma membrane fusion is the ultimate site of fertilization specificity. Presumably acrosomal membrane and egg plasma membrane interaction and fusion occur in successful hybrid crosses, as indicated in the electron micrograph of Franklin (1965) in the cross *Mellita quinquiesperforata* male $\times$ *Arbacia punctulata* oocytes. But to test critically for a role of plasma membrane interaction and fusion in fertilization specificity, other sources of specificity must be eliminated. Hultin (1948a,b) and others (see Tyler and Metz, 1955, for references) attempted this in sea urchins by removing the membranes, probably including the vitelline membrane, with trypsin. Such treated eggs give higher percentages of cross-fertilization than normal eggs, but in the combinations tested by Tyler and Metz (1955) none succeeded with trypsin-treated eggs that failed completely with normal eggs. This failure of trypsin to extend cross-fertilization to new combinations at least is in accord with the view that the sperm acrosomal membrane-egg plasma membrane interaction and fusion involve a relatively high order of specificity in sea urchins. It should be noted, however, that in alkaline medium, echinoderm cross-fertilizability can be extended to new combinations (Loeb, 1915). Finally, in some hybrid combinations the spermatozoon activates but fails to enter the egg and may even detach (see Wiese, 1966, for review).

B. SELF-STERILITY

The specificity role of gamete membranes is strikingly seen in the self-sterility of some hermaphroditic ascidians. As shown in the classical studies of Castle and of Morgan (see Tyler, 1948), sperm of *Ciona* fail to fertilize eggs from the same individual but do fertilize eggs from most others. This self-sterility apparently involves the egg chorion, because removal of this membrane renders the eggs fertilizable by sperm of the same animal (Morgan, 1923). The nature of this barrier to self-fertilization has not as yet been worked out at the molecular level, but the information so far available implies molecular differences not only between the egg membranes from different individuals but an exquisite complimentary specificity of the sperm as well. Apparently specificity of sperms of different individuals has not been compared by methods independent of the fertilization reaction itself. Localization of the barrier to self-fertilization in the egg chorion implies a mechanical block to

fertilization that is overcome by lysins from the sperm in successful combinations. However, Morgan (see Tyler, 1948) was unable to demonstrate an immediate action of sperm extracts on eggs from the same or other individuals. Penetration of the external egg membranes in tunicates appears to be a complicated process. The mitochondrion is lateral to the nucleus in *Ciona* (Ezell, 1963) and *Ascidia* (Schabtach and Ursprung, 1965) and is lost prior to penetration of the chorion in the latter species (Ursprung and Schabtach, 1965).

In ascidians an important barrier to cross-fertilization also resides in the extracellular egg membranes. Minganti (1959) obtained activation of eggs in 15 different hybrid combinations of sperms and eggs. These crosses were successful only with demembranated eggs. Six of the crosses involved *Ciona*, the subject of most of the self-sterility studies.

III. The Spermatozoon

The classical comparative studies demonstrated an almost limitless variation in spermatozoon gross morphology (see Wilson, 1925; Austin, 1965), and the recent ultrastructural investigations revealed striking variations in the subcellular organization as well. In fact, of the complex of organelles found in conventional spermatozoa, only the nucleus and a small amount of attendant cytoplasm is of constant occurrence in functional spermatozoa. For example, the acrosome is apparently absent in many osteichthian fishes (Ginsburg, 1963; Chap. 7, Vol. II, this publication; Tyler, 1966), and several groups lack mitochondria, flagella, and in some cases apparently even centrioles. Notable among these are certain crustaceans (Brown, 1966; Moses, 1961; Yasuzumi, 1960; Reger, 1964). The shrimp sperm (Fig. 1) is a rather extreme example.

In spite of the variation in form and internal structure, the outer boundary of the spermatozoon is a triple-layered, unit membrane in electron micrographs and corresponds to the plasma membrane of other cells. Unlike the egg, the mature spermatozoon is never surrounded by extracellular layers resolvable with light or electron optics (see Chaps. 6 and 7). At most, some macromolecular material is adsorbed to the sperm surface (Weil, 1961, 1962). Even some mammalian sperm which are remarkably resistant to extraction and disintegration evidently have the usual unit membrane surface structure (Bedford, 1964; Fawcett, 1958, 1965). The evidence for the albuminoid-like sheath postulated by Green (1940) is explained as an unusually insoluble class of basic proteins associated with nuclear DNA (Bril-Petersen *et al.*, 1963).

Regardless of the ultrastructural constancy of the sperm cell membrane, differences in the sperm surface are expected at the molecular level. Such differences should occur among species to account for fer-

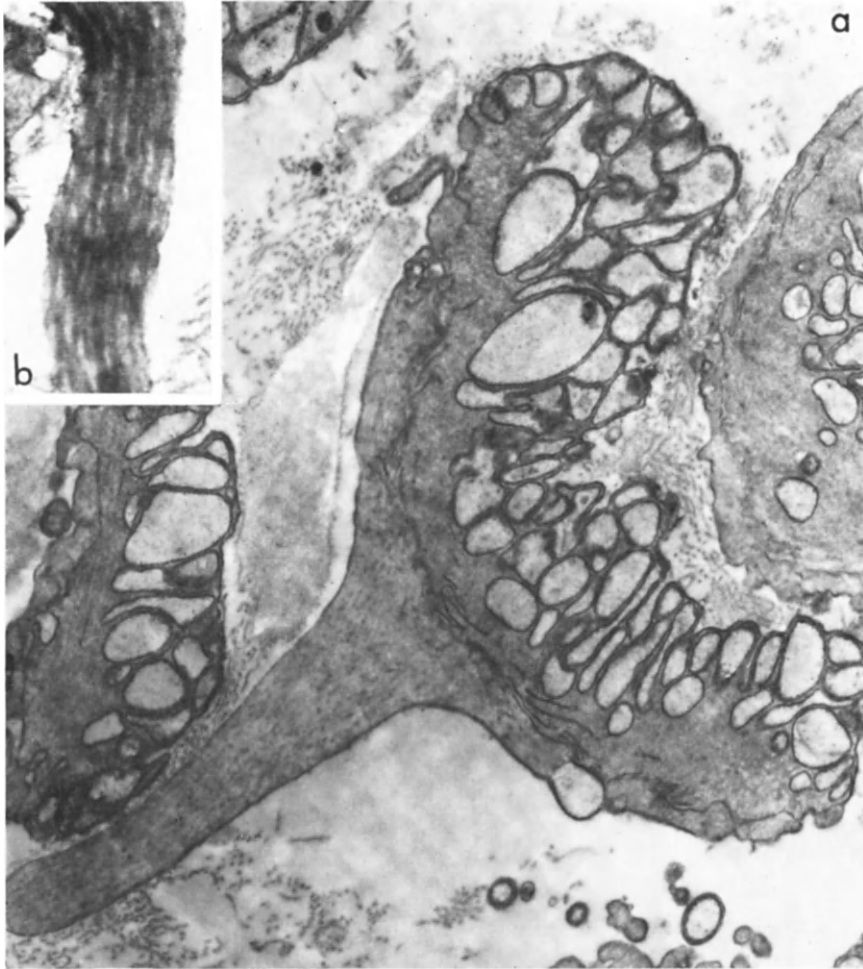


FIG. 1. Spermatozoon of the shrimp *Palaemonetes paludosus*. The cell consists of a cup-shaped "head" and a "spike." A layer of large vesicles lines the concave side of the "head." The vesicles are apparently embedded in the nuclear (Feulgen-positive) material that forms the interior of the head. The spike consists of longitudinally oriented fibers that are cross-banded with a periodicity of 260 Å. The sperm head appears to attach to the egg in *in vitro* mixtures of gametes. (a) Longitudinal section of the spermatozoon O₂O₂, Araldite. (b) Longitudinal section of spike to show fibers with cross-banding. O₂O₂, Araldite. Magnification: (a) $\times 25,000$; (b) $\times 44,000$. (From G. G. Brown, unpublished.)

tilization specificity. Differences might occur among different genotypes within a species. This should be the case in ascidians. Differences might be found among individual sperms from one heterozygous individual, and differences among regions of the same sperm cell might be expected.

Finally, such differences could result from "built-in" macromolecular components or from adsorption of specific substances from the male or even female reproductive fluids. Differences of most of these types have been demonstrated. The most striking examples have been found in mammals by means of immunological methods.

A. ANTIGENIC STRUCTURE OF THE MAMMALIAN SPERMATOZOON

A large literature dates from Landsteiner (1899) concerning the immunological properties of spermatozoa, and much of this is assembled in bibliographies by Tyler (1961) and Tyler and Bishop (1963). Spermatozoa readily incite production of antibodies when injected into an appropriate foreign species, and, by virtue of their specificity, such antibodies can be employed to identify, localize, and isolate specific sperm antigens and determine whether they perform an essential role in fertilization. For current concepts concerning antigens, antibodies, and their interaction, see standard works and reviews such as Cushing and Campbell (1957), Kabat and Mayer (1961), Cohen and Porter (1964), Nisonoff and Inman (1965), and Singer (1965).

For purposes of the immunological discussion, sperm-surface antigens (combining sites) are those antigens so situated that their combination with antibody under appropriate conditions results in sperm agglutination (Köhler and Metz, 1960).

Henle *et al.* (1938) made the first real progress in the study of sperm-surface antigens. Anti-bull sperm serum prepared by injecting rabbits gave agglutination and complement-fixation reactions with sperm from the bull and certain other species (Henle, 1938). Using antisera absorbed with isolated bull sperm heads and tails, these investigators demonstrated heat-labile tail specific and head specific surface antigens. A third antigen common to both heads and tails was demonstrated by complement-fixation tests using heated (100°C) material. It is uncertain whether this heat-stable antigen is in a surface or subsurface location.

Finally, Henle *et al.* (1938) demonstrated exposure of a subsurface antigen by treatment of bull sperm with sonic vibrations. Evidently the antigen is protected and unavailable for combination with antibody in the intact cell. This suggests, in addition, that antibodies do not penetrate into the intact bull sperm. A similar conclusion was reached for sea urchin sperm (Flake and Metz, 1962) from other evidence.

Cross-agglutination tests using appropriate absorbed sera have demonstrated three sperm-surface antigens on rabbit, guinea pig, and mouse sperms (Smith, 1949). Data for rabbit sperm are given in Table I. Similar results have been reported with sea urchin sperm (Köhler and Metz, 1960; Section V,C).

TABLE I
SPERM-AGGLUTINATING ACTION OF GOAT ANTIRABBIT SPERM SERUM BEFORE
AND AFTER ABSORPTION^{a,b}

Serum	Test sperm						
	Rabbit	Guinea pig	Mouse	Ferret	Dog	Bull	Fowl
R	+	+	+	+	+	—	—
R-GP	+	—	+				
R-M	+	+	—				
R-R	—	+	+				

^a (R) Goat antirabbit sperm serum; (R-GP) serum absorbed with washed guinea pig sperm; (R-M) serum absorbed with washed mouse sperm; (R-R) serum absorbed with washed rabbit sperm. The positive reactions with R-R are unexpected. If the serum is absorbed with rabbit sperm homogenate, no cross-reactions are obtained with R-R. This result suggests that subsurface rabbit sperm antigens are present on the surfaces of the other species.

^b From data of Smith, 1949.

With the advent of gel-diffusion precipitin techniques (Ouchterlony, 1948; Grabar and Williams, 1953), studies on the antigenic composition of soluble seminal antigens advanced rapidly. Pernot (1956) found six soluble sperm tail antigens and one antigen common to heads and tails in alkaline extracts prepared from isolated guinea pig sperm fragments. The seminal plasma contained 11 antigens, 6 of which were present in sperm tail extract. The single antigen common to heads and tails was not present in seminal plasma or blood serum. Only three of the seminal plasma antigens showed identity with blood serum antigens. Katsh and Katsh (1961) reported separation of four soluble guinea pig sperm antigens by chromatography. Further analysis indicates that one is hyaluronidase, another contains nucleic acid, and the remaining two are protein and polysaccharide, respectively.

A degree of tissue specificity comparable to that of the guinea pig has been reported in the bull (Larson *et al.*, 1954; Pernot and Szumowski, 1958). More recently Hunter and Hafs (1964) and Matousek (1964b) reported up to seven antigens in bull seminal plasma and saline extracts of seminal sperm. Absorption of anti-bull seminal spermatozoan rabbit serum with blood serum, seminal plasma, or selected secretions from the accessory organs removed most, but not all, of the precipitins for sperm extract antigens and sperm agglutinins. Using immunoelectrophoretic techniques, Mittal *et al.* (1965) reported 20 precipitin bands with frozen-thawed whole bull semen. Weil *et al.* (1956) obtained up to 12 precipitin bands in gel-diffusion experiments with human seminal

plasma and 5 bands with rabbit seminal plasma when diffused against the homologous antiserinal plasma serum. Segal (see Tyler and Bishop, 1963) found increasing numbers of precipitin bands with increasing age in rat testis extracts. Rao and Sadri (1960) reported up to 17 antigens in buffalo semen. Rao and Sadri (1959) also obtained 15 precipitin bands with human seminal plasma and 7 with sperm. Four of the latter were shared with seminal plasma. Finally, it is of considerable theoretical as well as practical interest that isoantibodies and even autoantibodies can be demonstrated by agglutination and precipitin reactions in man. These cases are rare and are usually associated with infertility. The reactions are not related to blood group (see Rümke and Hellenga, 1959; Tyler and Kakabayashi, 1962; Katsh, 1963; Katsh and Katsh, 1965b; Tyler and Bishop, 1963).

The studies of Weil and collaborators (1956, 1958) are of particular interest. These investigators were unable to demonstrate antigenic differences between extracts of washed sperm and the seminal plasma in both man and rabbit. Antiserinal plasma serum failed to give precipitin bands with rabbit seminal plasma following absorption with rabbit sperm. Likewise, antiserinal sperm serum failed to give precipitates with rabbit sperm extracts after absorption with seminal plasma. They reasoned from this apparent identity of soluble antigens in seminal sperm extracts and seminal plasma that the sperm extract antigens are really seminal plasma antigens adsorbed to the sperm. This view was supported by experiments showing that the accessory glands, notably the seminal vesicles (rabbit), yielded four of the five antigens found in seminal plasma (Weil *et al.*, 1956) and the observation that epididymal sperm failed to fix complement in the presence of either antiserinal sperm or seminal plasma. In the experiments seminal sperm and seminal plasma both fixed complement, although not in high titer. Human testicular (spermatocele) spermatozoa also showed very much less complement-fixing action and, in addition, failed to absorb the precipitating antibody from antiserinal plasma serum (Weil and Rodenburg, 1960). Subsequent study has centered on a dominant antigen(s), the "spermatozoan-coating antigen" (SCA). This antigen has been found by immunofluorescence to be absent from testicular (man) and epididymal sperm (rabbit), to be present on seminal sperm, and to be a product of the seminal vesicle (rabbit) (Weil and Rodenburg, 1962). Epididymal sperm can adsorb the antigen from seminal plasma (Weil, 1962). Once adsorbed, the antigen(s) is not readily removed from the sperm (Weil, 1961) and becomes a sperm-surface antigen as the sperms pass into the secretion of the seminal vesicle. In a sense this phenomenon is analogous to coatings applied to eggs as they pass through the female reproductive

tract. As has been pointed out (Owen, in Weil, 1962), certain erythrocyte antigens (J of cattle; Lewis in man) are also attached to the cells secondarily by adsorption from the blood plasma.

The seminal vesicle origin of the (SCA) is of considerable interest. The question of equal interest is what, if any, role this antigen may have in fertilization. Tyler (see discussion of Weil, 1962) has suggested that the antigen may be involved in the capacitation and decapacitation phenomenon (Austin, 1952; Chang, 1957; Williams *et al.*, 1964) in the rabbit. In this connection it would be of some interest to determine if spermatozoa are still coated with the SCA after passage through the female reproductive tract (e.g., capacitated), and if so, whether capacitated sperm adsorb the antigen from seminal plasma (e.g., during decapacitation). In any event, the SCA cannot be essential for fertilization, since epididymal sperm, which lack the antigen, are capable of fertilizing eggs (Austin and Bishop, 1957; Bedford, 1965).

The SCA has been demonstrated as yet only in man and rabbit, but a comparable antigen(s) may be present in the bull (Matousek, 1964b), ram, and guinea pig. In the latter, Hathaway and Hartree (1963), in addition to isolating acrosomes from the sperm, obtained six precipitin lines with seminal plasma and four with alkaline sperm extract in immunodiffusion experiments using antisperm extract serum. The sperm-extract lines were confluent with four of the seminal plasma lines, so it is possible that these are seminal plasma antigens that adsorb to spermatozoa. The same reasoning can be applied to the six soluble antigens of the guinea pig that are present in both the seminal plasma and the sperm (Pernot, 1956).

Weil (1961) and others have demonstrated mammalian sperm antigens of other than seminal plasma origin, which are presumably an integral part of the sperm cell membrane. In many mammals these antigens are refractory to extraction. An exception is the Chinese hamster, from which Pikó and Tyler (1962), using antiepididymal sperm serum, obtained 10 gel-diffusion precipitin lines with frozen-thawed extract of epididymal sperm, 6 with alkaline extract (acrosomal extract), and 5 with seminal plasma.

B. THE ASPERMATOGENIC ANTIGEN

Another antigen(s) of unusual interest is that responsible for aspermatogenesis. As first clearly demonstrated by Freund *et al.* (1953), injection of a mixture of guinea pig testicular tissue and "adjuvant" (see Katsh, 1964, for role of the adjuvant) into male guinea pigs results in isoimmunization. The most striking effect is degeneration of the germinal epithelium of the injected male guinea pigs. Only the active germinal

tissue is affected. Sertoli and Leydig cells are normal, and spermatogenesis can recur (see Katsh and Katsh, 1965b). The effect can be obtained, but with difficulty, without the use of the adjuvant (Bishop, 1961). It can be obtained with washed guinea pig sperm, testicular tissue from roosters and monkeys, human semen (Katsh and Bishop, 1958), and brain extracts (Katsh and Katsh, 1965a,b). The effect has also been reported in rats (McCartney, 1923) and Japanese quail (Wentworth and Mellen, 1964).

The aspermatogenic phenomenon has many facets of interest, including fertility control, autoimmunization, and histocompatibility, which are beyond the scope of this review (see Bishop, 1961; Tyler and Bishop, 1963; Katsh and Katsh, 1965b). However, the aspermatogenic antigen first appears in secondary spermatocytes and is a component of the periodic acid-Schiff (PAS) positive acrosomal granule (Katsh, 1960a; Willson and Katsh, 1965). Since "capacitation" of sperm may involve an alteration of the acrosome and release of acrosomal material (see Austin, 1961, 1965), the aspermatogenic antigen becomes of interest in terms of a possible role in fertilization.

The chemistry of the antigen has received considerable attention considering the laborious assay procedure required for its study. In view of the PAS positive character of the acrosome, a polysaccharide or glycoprotein is a possibility, and this has been substantiated in part at least. The active material has been extensively but incompletely purified (Freund *et al.*, 1955; Katsh, 1960a; Brown *et al.*, 1965). It is not inactivated by heating, fails to diffuse through dialysis membranes, and is not digested by proteolytic enzymes, lipase, lysozyme, or β -glucuronidase (Katsh and Katsh, 1961). The purified preparations contain 10–15% reducing sugars and 4–7% nitrogen (Freund *et al.*, 1955; Brown *et al.*, 1965). Chromatography of hydrolysates yields 13–18 amino acids (Brown *et al.*, 1965; Kirkpatrick and Katsh, 1964) and galactose, glucose, and mannose (Brown *et al.*, 1965). Unexpectedly, the material was not antigenic after digestion with DNase and nearly inactive following RNase treatment (Katsh and Katsh, 1961). This result is at least consistent with the 4.6% of 257.5 $m\mu$ absorbing material in the purified preparation of Brown *et al.* (1965). It appears, then, that the aspermatogenic antigen is a relatively high molecular weight, heat-stable carbohydrate-amino acid complex. Resistance to proteolytic enzymes indicates that antigenicity does not depend upon the integrity of a substantial protein fraction. Sensitivity to RNase and DNase has yet to be explained.

No role in fertilization can as yet be assigned to the aspermatogenic antigen. It seems clear from the work of Katsh and Katsh (1961) that this antigen is distinct from hyaluronidase, which is also largely, if not

entirely, confined to the acrosome (Mancini *et al.*, 1964). Ram sperm acrosomal glycoproteins have recently been extracted (alkali) and characterized by Hartree and Srivastava (1965). As yet there is no information with respect to any relation between these and the aspermatogenic antigen(s) in the guinea pig.

C. BLOOD GROUP ANTIGENS

In addition to the species sperm antigens so far considered, the possibility of individual antigenic differences among sperms of different individuals within the species is of interest. Such individual differences were found between inbred strains of mice (Snell, 1944, 1962) by isoimmunization and cross-agglutination tests. Additional individual differences in mammalian sperm have been sought in relation to known antigenic differences in other cell types, notably blood group antigens.

Blood group substances were first demonstrated in human semen by Yamakami (1926). Landsteiner and Levine (1926) clearly showed that the A and B antigens were present on spermatozoa from men of the corresponding blood group. Recent interest concerns presence of the antigens with respect to the secretor phenotype and the possibility of demonstrating two classes of sperms (e.g., A and B) from individual men heterozygous for blood group (e.g., AB). In spite of the conflicting report (see Edwards *et al.*, 1964, for clarification) of Levine and Celano (1961), it seems clear that the AB blood group antigens are found on seminal sperm of secretors but not on seminal sperm of nonsecretors of the appropriate blood group (Table IV). Other cell types present in semen give positive blood group tests in both secretors and nonsecretors (Edwards *et al.*, 1964; Boettcher, 1965). The origin of the blood group antigens on sperms has not been established. The relation to the secretor phenotype suggests that the antigens are derived secondarily by adsorption from the seminal plasma, since seminal plasmas of nonsecretors lack blood group antigens (e.g., Wiener, 1945). This view is supported to the extent that Holborow *et al.* (1960) failed to detect A antigen on sperm of four group A adults and testis of two infants by immunofluorescence. The sperm from the four adults is stated by Edwards *et al.* (1964) to be testicular sperm. The secretor status of the individuals is not given. Edwards *et al.* (1964) obtained a positive mixed agglutination of spermatocele sperm from a group A secretor with anti-A serum. Presumably these were epididymal sperms. Finally, Boettcher (1965) reported that washed group O cells from a nonsecretor can adsorb A and H substances from seminal plasma of a type A-secretor individual.

If the blood group antigens found on sperm are indeed adsorbed from reproductive secretions, it would seem unlikely that the haploid geno-

type of individual sperm would be recognizable in men heterozygous for blood group. Nevertheless, Gullbring (1957), using the mixed-agglutination test, reported two classes of sperm from AB individuals. One gave a positive test with anti-A serum only, the other with anti-B serum only (Table II). Popivanov and Vulchanov (1962) reported separation of sperms from AB individuals into two classes by agglutination with anti-A serum, and Shahani and Southam (1962) distinguished separate type A and B sperms from individual AB men by immunofluorescent staining of dried sperm smears. The latter study (Table III) is convincing except for the fact that no differences were found between secretors and non-secretors. In view of both the theoretical and applied significance of

TABLE II
DEMONSTRATION OF BLOOD GROUP ANTIGENS A AND B ON SPERMATOZOA OF GROUP AB PERSON BY "MIXED AGGLUTINATION"^{a,b}

Spermatozoa and antiserum	Red cell suspension			
	A	B	AB	O
S + anti-A	+	—	+	—
S + anti-B	—	+	+	—
S + anti-A and anti-B	+	+	+	—

^a Spermatozoa (S) from blood group AB man was treated with antiserum and then washed until the washings contained no trace of antiserum. Red blood cells of designated blood group were then added to the sperm suspension. (+) red cells agglutinated to spermatozoa.

^b Gullbring, 1957.

these reports, it is surprising that they have not been followed up with more exhaustive studies. If true, these results constitute a long-sought example of specific phenotypic differentiation according to the haploid genotype of the individual spermatozoon, and at least in secretors provides a really practical key to the problem of physically separating sperms into two classes, each carrying a different and identifiable chromosome (e.g., linkage group) of an homologous pair from an heterozygous individual.

In view of the above interest, the probability of antigenic differentiation of sperms according to haploid genotypes in heterozygotes requires consideration in terms of the biochemical events of meiosis and spermiogenesis. In the mouse no detectable RNA synthesis occurs in association with the X or Y chromosome during meiosis or spermiogenesis (Monesi, 1965). This would seem to preclude the possibility of antigenic differences between X- and Y-bearing sperms specified by mRNA from the respective chromosomes following the reduction division. Autosomal

RNA synthesis stops at early spermatid stages, but protein synthesis continues into late stages. Thus, it is likely that some mRNA is synthesized by the haploid set of autosomes following the reduction division, and this could specify antigens of the haploid autosomal genotype. The terminal protein is arginine-rich histone (see Monesi, 1965, for references). The mRNA in the spermatid is long-lived (at least a week), thus it is quite possible that appreciable amounts of long-lived mRNA are carried over from prereductional (diploid) stages. Any such mRNA and its specification products (antigens) would have to be inactivated

TABLE III
DEMONSTRATION OF TWO CLASSES OF SPERM, GROUP A AND GROUP B, FROM BLOOD
GROUP AB INDIVIDUALS^{a, b}

Experiment No.	Antisera applied	Results	
		Fluorescein stain anti-A	Rhodamine stain anti-B
1	Anti-A-F		
	Anti-B-R	Some positive	Some positive
2	Anti-A-F	Some positive	
3	Anti-B-R		Some positive
4	Anti-A untagged, followed by anti-A-F	Negative	—
5	Anti-A untagged, followed by anti-B-R	—	Some positive
6	Anti-B untagged, followed by anti-B-R	—	Negative
7	Anti-B untagged, followed by anti-A-F	Some positive	—
8	Anti-A-anti-B untagged, followed by anti-A-F	Negative	—
9	Anti-A-anti-B untagged, followed by anti-B-R	—	Negative

^a (Anti-A-F) Fluorescein-conjugated group A serum; (Anti-B-R) rhodamine-conjugated anti-blood group B serum.

^b Shahani and Southam, 1962.

or destroyed during later stages to permit phenotypic expression of the haploid genotype exclusively.

These considerations do not seem to favor the possibility of differentiation of individual sperms according to their haploid genotype in the mouse. It remains to be determined if the mouse is typical of mammals in general.

In addition to the ABO system, other blood type antigens have been studied to a limited extent. Edwards *et al.* (1964) demonstrated M and N antigens by mixed agglutination on sperms from men of corresponding M-N blood type (Table IV). Apparently, no attempt other than that of

Edwards *et al.* (1964) in Table IV has been made to detect two classes of sperms in semen of type MN individuals.

Several workers have examined for Rh antigens on sperm. Gullbring (1957) reported positive and negative mixed-agglutination tests using anti-D serum and washed spermatozoa from group O, Rh⁺ and Rh⁻ individuals, respectively. Majsky and Hraba (1960) also distinguished between spermatozoa from Rh⁺ and Rh⁻ individuals with anti-D serum in a more extensive series of tests. Levine and Celano (1961) and Quin-

TABLE IV
BLOOD GROUP ANTIGENS A, B, M, N, AND TJA ON HUMAN SPERMATOZOA^{a,b}

Specimen	Blood type	Antigens shown by mixed agglutination				
		A	B	M	N	TJA
4900	A ₁ M secretor	+	—	+	—	w
Spermatocele	A ₁ M secretor	+	—	+	—	
Cam BS	A ₁ M secretor	+		+		w
Lon Y	A ₁ secretor	+				
989	A ₁ B MN secretor	+	w	+	w	w
9972	B N secretor	—	+	w	w	
806	A ₁ MN nonsecretor	—	—	w	w	w
14564	A ₂ MN nonsecretor	—	—	+	w	
Lon G	A ₂ nonsecretor	—	—			
Lon 2/8	B nonsecretor	—	—			
Lon W	O	—	—			
705	O M	—	—	+	—	w
3502	O MN	—	—	+	w	w
3363	O N	—	—	—	w	w

^a (+) Definite-mixed agglutination; (w) weak-mixed agglutination; (—) no-mixed agglutination.

^b Edwards *et al.*, 1964.

livan and Masouredis (1962) were unable to demonstrate Rh antigen with anti-D serum on spermatozoa from Rh⁺ individuals. Edwards *et al.* (1964) found the Tja antigen on all sperms tested (Table IV), but failed to obtain positive reactions for the sex-linked Xga antigen with spermatozoa from men with Xga positive blood.

Attempts have been made to demonstrate the blood group antigens of cattle on bull spermatozoa. Docton *et al.* (1952) tested the spermatozoa of three bulls for a series of erythrocyte antigens using complement-fixation and absorption tests. They reported that in all three bulls the spermatozoa possessed most (including the J antigen), but not all, of

the antigens that were present on the erythrocytes of the same bull. However, Matousek (1964a) was unable to confirm this. He reported finding the J antigen in seminal plasma, but not on sperm. Menge *et al.* (1962) also found the J antigen in seminal plasma, but were unable to find evidence for erythrocyte antigens on sperm of bulls or rabbits.

The possibility of histocompatibility antigens on sperm is of particular interest (Eichwald, 1962; Snell, 1962). Barth and Russell (1964) were unable to demonstrate the H-2 antigen or the Y-chromosome histocompatibility antigen(s) in mouse sperm (strain C57BL/6) by immunofluorescence. The test for the latter antigen was not critical, since antibody against Y antigen could not be demonstrated in the test serum. However, Katsh *et al.* (1964) found that female mice (strain C57B1/6J) showed decreased or increased tolerance of skin grafts from isogenic males following injection with sperm from the same isogenic males. The effect obtained depended upon the time relations of the treatment and are consistent with a sensitization or desensitization, respectively, of the treated females. Comparison of the effectiveness of sperm and male spleen cells indicate that the sperm cells contain a larger amount of Y-chromosome histocompatibility antigen.

IV. The Egg

External to the plasma membrane, a structure called the "vitelline membrane" surrounds the egg. Additional layers composed of jellies, shells, chorions, or follicle cells may be present. These may be of egg, ovarian, or oviducal origin. It should be understood, however, that these names are primarily descriptive and do not necessarily reflect homologies of function or origin. For example, the *Nereis* egg jelly results from rupture of jelly-precursor granules within the egg (Costello, 1949) at fertilization, the sea urchin egg jelly is produced by the egg or follicle cells while in the ovary, whereas the amphibian egg jelly is a product of the oviduct.

It is not the objective here to undertake an exhaustive comparative study of these structures, but only to consider certain cases insofar as they are known to function in fertilization. Considering the prominence of these structures, relatively little is known about them with respect to a role in fertilization, except in the sea urchin and amphibian. In most other cases the membranes external to the vitelline membrane are usually regarded as barriers to fertilization and to contribute positive factors only insofar as the mechanism for overcoming them provides increased specificity to fertilization (see Section II). The follicle cell layer (corona radiata) and zona pellucida of mammalian eggs are examples (Austin, 1965).

A suggestion of particular interest (Tyler, 1962,) is that the egg surface (plasma membrane) extends beyond the vitelline membrane and into the egg jelly (fertilizin) in the form of microvilli, and that sperm-egg interaction involves a direct interaction between the antifertilizin of the sperm surface and the fertilizin at the surface of these microvilli. A number of cases have been studied sufficiently to provide evidence regarding the microvilli.

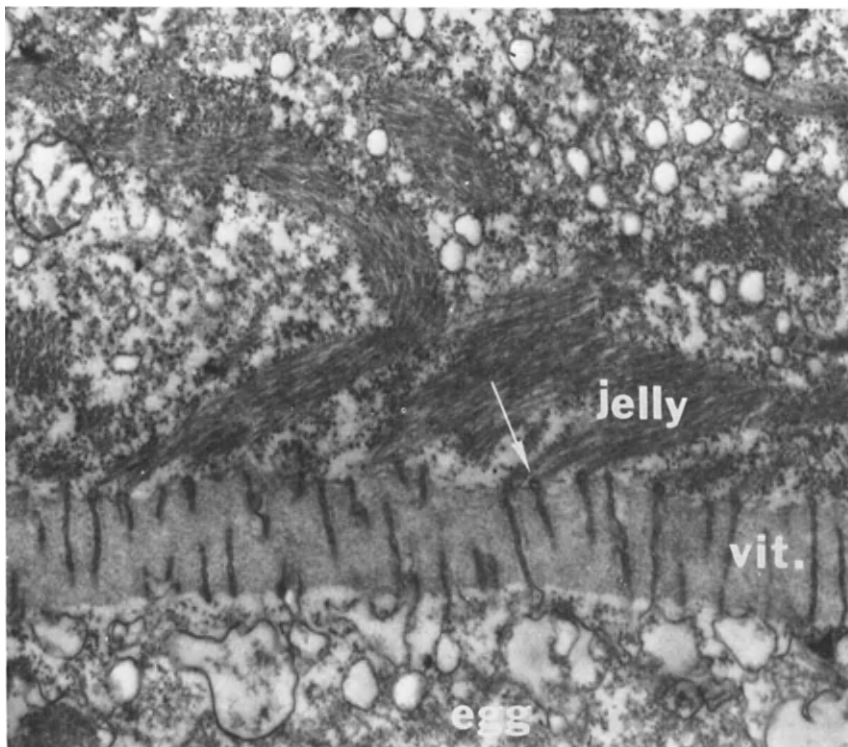


FIG. 2. Ovarian egg of *Mytilus edulis*. Numerous microvilli penetrate the vitelline membrane (vit.). Fibrous material believed to be the future egg jelly layer has "flamelike" relation to the tips of the microvilli (arrow). Magnification: $\times 25,000$. (From Franklin, unpublished.)

In *Mytilus* (Dan, 1962; Humphreys, 1962, 1964; Reverberi and Mancuso, 1961), microvilli appear to extend through the vitelline membrane, and in the immature ovarian egg close association between the microvilli and the jelly is evident, as seen in Fig. 2 (Franklin, unpublished). The jelly is either secreted by the tips of the microvilli or is preferentially deposited in this position. Microvilli also appear to penetrate the thick vitelline membrane and extend into the thin jelly layer in *Spisula* (Reb-

hun, 1962) and *Urechis* (Tyler, 1962). However, some caution should be exercised in concluding complete penetration of the vitelline membrane by microvilli, for in the mollusk *Barnea*, the tips of the microvilli are actually covered by a thin layer of vitelline membrane material (Pasteels and de Harven, 1962).

The *Sabellaria* egg is surrounded by several layers (Pasteels, 1965). Proceeding from the egg surface, these are a thick compound vitelline membrane, a layer of vesicles, and the egg jelly. From examination of the mature egg, it would appear that the microvilli do not penetrate the vitelline membrane. However, as seen in Fig. 3 (Franklin, unpublished), a wealth of branching microvilli do penetrate the vitelline membrane of the immature ovarian egg. Later, the exposed tips of these microvilli pinch off, forming isolated "vesicles," and the microvilli retract to some extent (Fig. 3). Thus these vesicles are fragments of the cell surface outside the vitelline membrane, a rather unusual form of microvillar extension. The mature *Hydroides* egg has a similar arrangement of membranes, including the layer of vesicles at the surface of the vitelline membrane. This vesicular layer in *Hydroides* (outer-border layer of Colwin and Colwin, 1960, 1961) may also arise by pinching off of microvilli.

In the amphibian, *Triturus*, Hope *et al.* (1963) found that the microvilli extend through the vitelline membrane in developing oocyte stages, but that as the egg matures these microvilli retract. The final, ripe egg is covered with a continuous vitelline membrane without microvilli.

The ascidian egg is of interest because the chorion constitutes the barrier to self- and cross-fertilization (see Section II). This egg (e.g., *Ciona*, *Styella*) is partially or completely surrounded by an inner layer of "test" cells and an outer layer of follicle cells. The two cell layers are separated by the chorion. Conklin (1905) thought that the test cells actually enter the egg at early stages of development and are later extruded. However, it appears (Reverberi and Mancuso, 1960; Kessel and Kemp, 1962; Kessel, 1965) that these cells are simply embedded in pockets of the egg surface and subsequently assume their final position after the egg rounds up. The chorion is probably a product of the follicle cells, although this is not fully established.

V. Sea Urchin Gamete Surfaces and Fertilization

Fertilization mechanisms have been studied more extensively in the sea urchin than in any other metazoan. The egg is surrounded by a jelly layer which extends many microns from the egg surface. The vitelline membrane is so thin and closely applied to the plasma membrane that it is not readily resolved in electron micrographs. Many microvilli project

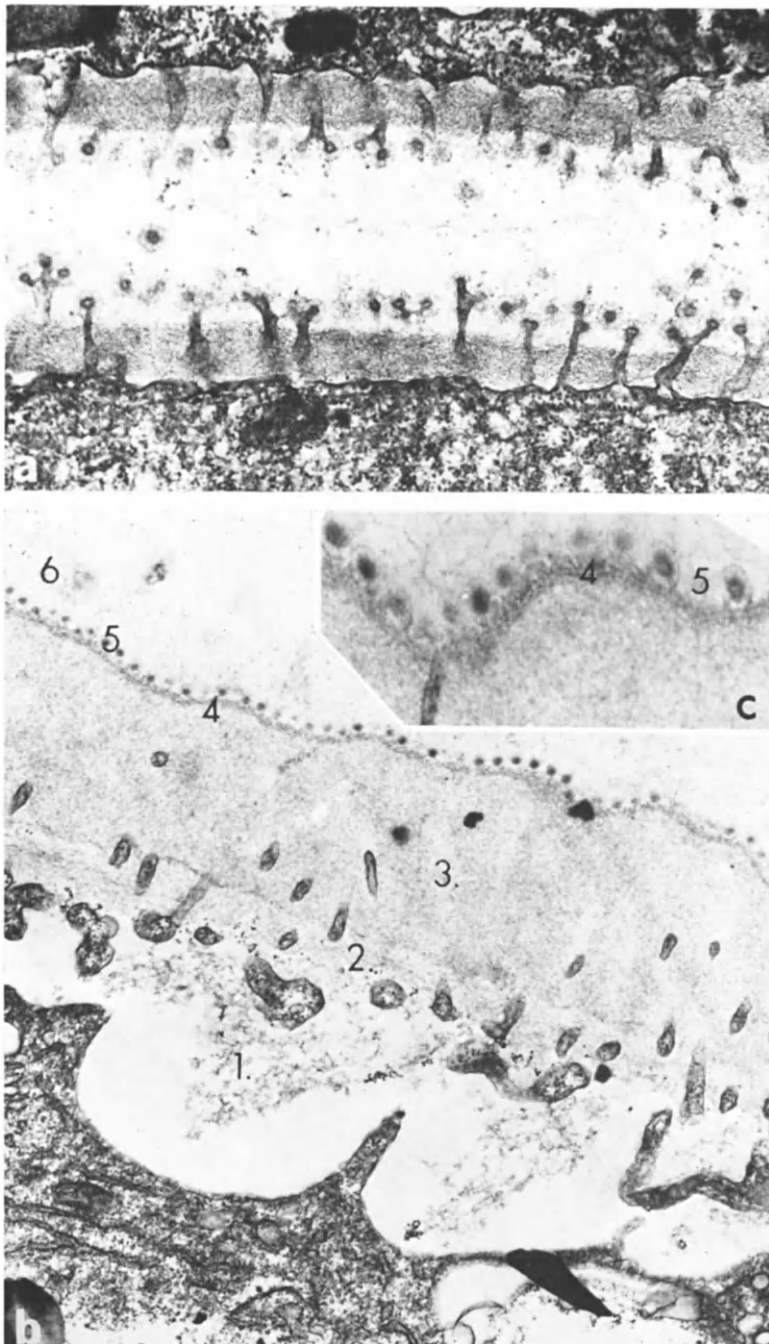


FIG. 3. *Sabellaria vulgaris*. (a) Section of ovary. The surfaces of two adjacent eggs are shown. Numerous branching microvilli penetrate the vitelline membranes. The tips of these microvilli pinch off to form the row of spheres (outer-border

from the surface of oocytes, but the mature egg surface is smooth (Franklin, 1965). Cortical granules are located just beneath the plasma membrane or in pockets of the plasma membrane (see Monroy, 1965, for discussion).

The sea urchin egg jelly has received much attention because it constitutes the fertilizin of the fertilizin-antifertilizin system. This system has dominated thinking about the sperm and egg surfaces in relation to fertilization for many years. It will now be considered in some detail.

A. FERTILIZIN AND ANTIFERTILIZIN

Sea urchin sperms rapidly agglutinate in the supernatant seawater from egg suspensions. This striking phenomenon was first investigated seriously by F. R. Lillie (1913, 1919), who attributed it to the action of an egg substance, "fertilizin." The earlier studies have been reviewed repeatedly (Metz, 1957a,b, 1961a,b; Tyler, 1948, 1959; Rothschild, 1956; Runnström, 1949; Runnström *et al.*, 1959). These will be summarized here and emphasis given to recent investigations.

The sperm isoagglutination results from an "antigen-antibodylike" combination of the fertilizin with a complementary sperm-surface substance, "antifertilizin." Evidence is that treatment with an excess of sperm lowers the sperm-agglutinating capacity of the fertilizin solution, absorbs out carbohydrate (Monroy, 1965), a major component of fertilizin, and also absorbs ^{35}S when the fertilizin carries this label (Hathaway and Metz, 1961). In addition, fertilizin agglutination in the sea urchin, but not in other forms, reverses spontaneously on standing. Such "reversed" sperm does not reagglutinate on addition of more fertilizin, if

layer of *Hydroides*, described by Colwin and Colwin, 1960, 1961) seen at the surface of the vitelline membrane in the mature egg (b and c). The space between the vitelline membranes of the two eggs is filled with jelly. Specimen collected at Woods Hole, Massachusetts. Similar figures were obtained with material from Miami, Florida. (b) Mature unfertilized egg after 2 minutes in seawater. The cortical granules have ruptured, microvilli have separated from the egg surface, and the vitelline membrane has begun to elevate. The innermost layer of the vitelline zone (1) is being laid down, apparently from cortical granule material. The vitelline membrane of the mature egg consists of a proximal layer of tangentially oriented fibrous material (2), a thick amorphous layer (3), a narrow layer of tangentially oriented fibers (4), and the layer of cytoplasmic spheres (5) derived from the tips of the microvilli. The egg jelly (6) extends peripherally from the vitelline membrane. (c) Mature unfertilized egg after 20 minutes in seawater to show details of outer surface of vitelline membrane, including the row of cytoplasmic spheres. One microvillus projects to the edge of the membrane. (4) Outer tangential fibrous layer. (5) Layer of cytoplasmic spheres (outer-border layer of Colwin and Colwin, 1960, 1961). Specimen collected at Miami, Florida. Magnification: (a) $\times 30,000$; (b) $\times 19,000$; (c) $\times 50,000$. (From Franklin, unpublished.)

an excess of fertilizin is used in the initial treatment. This reversal is attributed to conversion of fertilizin to a "univalent" form by action of the sperm. Failure of the sperm to reagglutinate is believed to result from combination of the univalent fertilizin with the sperm-surface antifertilizin. Such blocked antifertilizin cannot combine with multivalent, agglutinating fertilizin (Tyler, 1941; Metz, 1942). Finally, material with some of the properties attributable to antifertilizin can be obtained in solution by extracting sperm in various ways. Such "antifertilizin" extracts neutralize the sperm-agglutinating action of fertilizin and precipitate the egg jelly, the location of most of the fertilizin in the sea urchin egg.

The fertilizin agglutination reaction is largely species specific. In cross tests comparing the specificity of sperm agglutination and cross-fertilization, Tyler (1949) found a close parallel. The specificity of the sperm agglutination implies that the sperm-surface antifertilizin, as well as fertilizin, is highly specific. However, sperm extracts that neutralize fertilizin and precipitate egg jellies are less specific in action (Metz, 1961a). Whether this is due to a reduction in specificity of antifertilizin by extraction or to the extraction of some other agent or agents has yet to be resolved. Until this problem is resolved, some question must remain concerning the identity of sperm-surface antifertilizin and the "antifertilizin" in sperm extracts.

Fertilizin agglutination of sperm has been demonstrated in a number of organisms, with representatives in several of the major phyla. However, the actual number of cases is small, and the routine agglutination tests have failed to give positive results in a number of species. In certain of these, special methods have subsequently given convincing evidence for the existence of fertilizin. Notable examples are starfish, in which fertilizin agglutination ordinarily occurs only in the presence of a metal-binding agent (Metz, 1945, 1957b), and in the echiuroid worm *Urechis*, where fertilizin can be detected by its effect on the fertilizing capacity of the spermatozoa (Tyler, 1941).

Calcium is evidently necessary for agglutination in some (Loeb, 1915; Vasseur, 1949), but not all, species (Dan, 1954) and can enhance fertilizin agglutination of sperm. Systems comparable to, if not homologous with, the fertilizin-antifertilizin systems have also been described in plants, notably in the unicellular alga *Chlamydomonas* (Wiese, 1961; Volume II, this publication). Finally, in the absence of a soluble, diffusible isoagglutinin, insoluble or fixed substances comparable to fertilizin and antifertilizin may exist rather widely. The mating-type substances in *Paramecium* (Metz, 1954; Hiwatashi, Chap. 6, Vol. II, this publication) and yeast (Brock, 1959) are examples. It appears, then, that a fertilizin-antifertilizin system is widely, if not universally, distributed among animals, and probably microorganisms and plants as well.

1. Role in Fertilization

In view of the specificity of fertilizin and antifertilizin in the sperm agglutination reaction and its wide occurrence, it is little wonder that many workers, beginning with F. R. Lillie (1913), have attributed a role in fertilization to fertilizin and antifertilizin. Studies attempting to demonstrate an essential role concern the source of fertilizin and antifertilizin, the effect of removal and addition of these materials on fertilization, and, finally, the effect of certain fertilizin and antifertilizin blocking or inhibiting agents on the fertilizability of gametes. These studies are reviewed below.

a. Source of Fertilizin. In most species that have been studied critically, fertilizin is the jelly coat surrounding the egg, or at least a major component of it. This jelly slowly dissolves, charging the surrounding medium with the agglutinin. An interesting variation is seen in the annelid *Nereis*. No substantial amount of jelly surrounds the unfertilized *Nereis* egg, yet sperm agglutinin appears in the supernatant in small amounts (Lillie, 1912). Upon fertilization, jelly-precursor granules are released from the egg cortex. These granules convert to an extensive layer of jelly about the fertilized egg (Costello, 1949). This release of jelly coincides with the appearance of a large amount of agglutinin in the medium (Lillie, 1912).

It is still uncertain whether the sea urchin egg jelly originates from the egg or from the follicle cells (see Metz, 1957b). It seems immaterial as far as fertilization is concerned which of these alternatives is correct. In the amphibian (Section VI,A), jelly layers that are clearly produced by the oviducts can perform an essential function in fertilization. Whatever its source, fertilizin seems to be rather firmly bound to, or to be a constituent of, the egg surface, in addition to composing the jelly layer. This fertilizin-coated surface is believed by Tyler (1963) to include extensions of the egg plasma membrane (microvilli) that project through the vitelline membrane (see Section IV). The evidence for close association of fertilizin with the plasma membrane is found in the fact that eggs from which the jelly and probably the vitelline membrane have been removed by trypsin still agglutinate with antifertilizin from sperm (Table V). In view of questions concerning the specificity of sperm-extract antifertilizin (Metz, 1961a), repetition of the experiment using an antiserum specific for fertilizin would be desirable. Finally, the sea urchin egg proper clearly can yield a sperm-agglutinating material (see Section V,B).

b. Removal of Fertilizin from Eggs. It is clear that sea urchin eggs from which the jelly and all traces of soluble sperm agglutinin have been removed by acid or trypsin treatment can still be fertilized (Tyler

and Metz, 1955). As noted above, such eggs still agglutinate with antifertilizin. The gradual loss of agglutinability parallels loss of fertilizability in trypsin-treated eggs (Table V). To the extent, then, that agglutination of eggs with sperm extract is a test for the presence of fertilizin, the experiments support the view that fertilizin at the egg surface is essential for fertilization.

In other studies, Hagström (1956) also tested the effect of jelly removal on fertilizability of sea urchin eggs. He used the "fertilization-rate" method for assaying the fertilizability of the treated eggs. This

TABLE V
AGGLUTINABILITY BY ANTIFERTILIZIN AND FERTILIZABILITY OF EGGS OF
Strongylocentrotus AFTER SHORT AND LONG EXPOSURES TO TRYPSIN^a

Time of treatment in 0.05 % trypsin	Degree of agglutination after 1 hour in solution of antifertilizin	Percentage fertilization ^b						
		1	1/3	1/9	1/27	1/81	1/243	1/729
0	All eggs agglutinated and show precipitation membranes	100	100	100	95	80	15	5
10 minutes	95 % of eggs agglutinated in clumps of 5-20 eggs	98	80	50	20	3	0	0
6 hours	About 99 % of eggs are free: 1 % are stuck together in pairs	25	5	1	0	0	0	0

^a Tyler and Metz, 1955.

^b Ca. 500 eggs inseminated in 4 ml of the following concentrations of semen. Measured in %.

method involves treating egg suspensions with an excess of sperm, killing the sperm at intervals by exposure to detergent, and subsequently determining the percentage of fertilized eggs. Within the brief period of exposure of sperm to eggs, this method is, at least in part, a measure of how rapidly the sperm reach the egg surface (Tyler, 1959). Hagström (1956) found that the fertilization rate is increased by removal of the egg jelly; the dejellied eggs fertilized more rapidly than normal eggs, but given sufficient time, both treated and control eggs yielded the same percentages of fertilized eggs with the relatively high sperm concentrations employed. Hagström concluded that the jelly constitutes a barrier to fertilization and a polyspermy-preventing mechanism rather than an essential intermediary in fertilization. Hagström's result is not surprising, since the sperms might be expected to be slowed in their motion by the

viscous egg jelly. In addition, as will be seen below, fertilizin does inhibit the fertilizing capacity of sperm under certain conditions, and this inhibition can be interpreted as evidence for an essential role of fertilizin in fertilization.

c. Removal of Antifertilizin from Sperm. The counterpart to jelly removal from the egg is removal of antifertilizin from the sperm. Tyler and O'Melveny (1941), again using acid treatment, removed some antifertilizin from sea urchin sperm and found a reduced fertilizing capacity of the sperm. The respiratory rate, used as a measure of injury to the sperm, was not reduced proportionally. Tyler and O'Melveny (1941) concluded that removal of antifertilizin lowers the fertilizing capacity of sperm and that, therefore, the antifertilizin is important, if not essential, in fertilization.

d. Treatment with Fertilization Inhibitors. This subject has been reviewed extensively (Metz, 1961a,b). The value of inhibitors as tools for the elucidation of biological mechanisms at the molecular level is a function of their specificity of action. Since specificity of action, except in very few cases, is difficult to prove in living systems, the effect of inhibitors must be interpreted with caution. Frequently the failure of an inhibitor to have an effect can be more meaningful than a positive result.

Among the more specific agents that have been studied are fertilizin and antifertilizin themselves. As outlined above, fertilizin agglutination in sea urchins reverses spontaneously. Such free, "reversed" sperm fail to reagglutinate on further addition of fertilizin. This fact is interpreted to mean that the sperm-surface antifertilizin is blocked by combination with fertilizin. Such blocked, reversed sperm show a reduction in fertilizing capacity. The effect is very striking in some forms. In *Arbacia*, a species in which the effect is not pronounced, it can be demonstrated by the use of trypsin-treated eggs, as seen in Table VI (Tyler and Metz, 1955). The reduction in fertilizing capacity following fertilizin treatment is interpreted to mean that the sperm-surface antifertilizin must be exposed for interaction with the egg and, therefore, performs a significant, if not essential, role in fertilization.

In the reciprocal experiment, treatment of eggs with sperm extract ("antifertilizin"), the fertilizability of the eggs is reduced. Interpretation of this effect is complicated by the fact that the egg jelly is precipitated in the form of a membrane that can act as a mechanical barrier to the sperm. This difficulty is overcome, in part at least, by the use of jellyless (trypsin-treated) eggs, which also show reduced fertilizing capacity following sperm-extract treatment (Tyler and Metz, 1955).

Other specific inhibitors include antisera. Rabbit antisperm serum is rendered univalent by digestion with papain followed by reduction

(Porter, 1959). Following treatment with some, but not all, such non-agglutinating anti-whole sperm sera, sea urchin sperm fail to agglutinate on subsequent addition of fertilizin. Evidently the univalent antibody blocks the sperm-surface antifertilizin in these cases. Univalent antiserum treatment also markedly lowers the fertilizing capacity of the sperm. This is true both for antisera that inhibit fertilizin agglutination and those that do not (Metz *et al.*, 1964; Section V). Nonagglutinating antisera prepared against sperm extracts also inhibit the fertilizing capacity of

TABLE VI
FERTILIZING CAPACITY OF FERTILIZIN-AGGLUTINATED AND REVERSED SPERM ON TRYPSIN-TREATED EGGS OF *Arbacia*^{a,b}

Dilution of 1/3 % semen	Fertilization (%) of:			
	Control eggs inseminated with		Trypsin-treated eggs inseminated with	
	Control sperm	Fertilizin- treated sperm	Control sperm	Fertilizin- treated sperm
undiluted	100	95	100	4
3-fold	100	98	98	3-4
9-fold	100	99	99	2.5
27-fold	99.5	50	35	2
81-fold	90	80	7	1.5
243-fold	95	20	20	0.2
729-fold	80	3	5	0.2

^a Trypsin treatment of eggs was for 3 hours in 0.05 % solution in seawater. For fertilizin treatment 4 ml of 1 % sperm was added to 8 ml of a solution of titer 256. At the same time 4 ml of 1 % sperm was added to 8 ml of seawater. After 20 minutes serial, threefold dilutions of 4 ml of the sperm suspensions were prepared as indicated (column 1) and 2 drops of washed trypsin-treated or control eggs (ca. 500) were added immediately after each dilution.

^b Tyler and Metz, 1955.

sperm (Tyler, 1946). However, sperm extracts such as those used for immunization ordinarily contain several antigens (Metz and Köhler, 1960), any one or a combination of which could be involved in the fertilization-inhibiting action. These tests, then, do not give unequivocal evidence for a role of sperm antifertilizin in fertilization.

Antisera to sea urchin egg jelly have been tested for effects on fertilization and early development. As expected, antijelly sera precipitate the egg jelly in the form of a membrane about the egg. In spite of this, Perlmann (1957) reported that the antijelly serum had little effect on fertilization rate. On the other hand, antiegg homogenate serum markedly

lowered the fertilization rate, and absorption with jelly had little effect on this action. It appears from Perlmann's studies that antiegg jelly serum does not affect the fertilizability of eggs. Nevertheless, this is not conclusive evidence that fertilizin has no role in fertilization, for the antigenic sites on the fertilizin molecule could be different from those that interact with the sperm. Antijelly sera do have other effects on eggs. Notable among these are an increase in the surface tension of jellyless eggs (Tyler and Brookbank, 1956b), precipitation of the hyaline layer of jellyless fertilized eggs (Tyler and Brookbank, 1956b), and an inhibition of cleavage (Tyler and Brookbank, 1956a,b).

A number of agents unrelated to the gametes have been found to inhibit fertilization, and in two of these, namely, *Arbacia* dermal secretion and polyphenols from *Fucus*, the mechanism of action has been studied. Both of these seem to act at the egg surface and are not toxic to sperm or fertilized eggs.

"Dermal secretion" is a fluid released upon injury to the integument of adult *Arbacia*. This material has a powerful fertilization-inhibiting action (Oshima, 1921; Pequegnat, 1948; Metz, 1960) and is probably the inhibiting agent that Lillie (1914) attributed to *Arbacia* blood.

The dermal secretion also inhibits fertilizin agglutination of sperm by inactivating fertilizin (Metz, 1959). The activity for both effects is destroyed by heating (100°C), does not diffuse through cellophane, and can be recovered from $(\text{NH}_4)_2\text{SO}_4$ -precipitates (Metz, 1961a). The crude preparations contain at least three antigens in common with *Arbacia* sperm (Metz, 1961a). However, dermal secretion does contain a second heat-stable inhibitor which inactivates or precipitates the egg-agglutinating activity of sperm extracts (Metz, 1959).

Sperm washed from dermal secretion possess full fertilizing capacity, whereas normal or acid dejellied eggs do not. Apparently, then, the fertilization inhibitor acts on the egg, not the sperm. In spite of the inactivating action on fertilizin in solution described above, dermal secretion does not inactivate fertilizin when in the form of natural jelly about the eggs. This would suggest that fertilization inhibition is not mediated through an inactivation of fertilizin. This observation is confirmed to the extent that fertilizin does not neutralize the fertilization-inhibiting action of the dermal secretion, as seen in Table VII.

It appears, then, that dermal secretion can inactivate the reactive sites of fertilizin, but probably inhibits fertilizability of eggs through some other means, possibly by an action on the vitelline membrane. This follows from the fact that trypsin-treated eggs, which probably lack the vitelline membrane, are not irreversibly inhibited (Metz, 1960) by exposure to dermal secretion.

TABLE VII
EFFECT OF FERTILIZIN ON FERTILIZATION-INHIBITING ACTION OF DERMAL SECRETION (DS)^{a,b}

DS dilution	DS diluted in fertilizin			DS diluted in seawater			Seawater + fertilizin			Seawater alone		
	Cleavage %	Sperm agglutination		Cleavage %	Sperm agglutination		Cleavage %	Sperm agglutination		Cleavage %	Sperm agglutination	
1/2	0 (200+)	-		0 (200+)	-		99 (136)	+++		100 (200+)	+	
1/10	0 (200+)	(+++ to +++)		0 (200+)	±							
1/50	13 (159)	++++		6 (140)	+							
1/250	97 (119)	++++		86 (131)	+							

^a Aliquots of a dermal secretion (DS) preparation were diluted serially in seawater and in fertilizin, as indicated; 2 drops of unfertilized *Arbacia* eggs were then added to 0.8-ml samples of each DS dilution, and finally 5 ml 0.0003 % *Arbacia* sperm were added to each sample. Values in parentheses are the number of eggs counted. To test for fertilizin excess in the mixtures, 2-drop samples of the supernatant from each mixture were withdrawn after insemination of the eggs. These were mixed with 2 drops 1-2 % sperm and the agglutinating action recorded on a - to +++++ scale in the table. The sperm-agglutination titer of the original fertilizin solution was > 729.

^b Metz, 1960.

"Polyphenols" in plant extracts also inhibit fertilization. Harding (1951) found that sea urchin eggs failed to fertilize in extracts of the alga, *Fucus*. This inhibition has since been studied by several investigators (see Metz, 1961a,b; Branham and Metz, 1960). The inhibiting activity of *Fucus* extracts is heat stable and nondialyzable. The active material is probably a polyphenol (Esping, 1957a,b) similar to tannins.

Contrary to earlier reports (Runnström and Hagström, 1955), Branham and Metz (1960) found that *Fucus* extracts inhibited fertilizin agglutination of sperm as well as fertilization. In contrast to *Arbacia* dermal secretion, the *Fucus* extracts inhibited agglutination by combination with the antifertilizin of the sperm surface, not by combination with fertilizin.

Arbacia sperm had full fertilizing capacity after washing from *Fucus* extracts in most experiments. Normal eggs failed to fertilize even after repeated washings following exposure to *Fucus* extract. Trypsin-pre-treated eggs fertilized readily after the extract treatment. Commercial tannic acid solutions (6×10^{-5} to 3×10^{-3} gm/ml) had effects similar to those of *Fucus* extracts on fertilizin agglutination of sperm and fertilization (Branham and Metz, 1960).

Evidently, then, these preparations contain material which inactivates the antifertilizin of the sperm surface and fails to inactivate fertilizin. Fertilization inhibition, however, results from action on the egg, again possibly through action on the vitelline membrane.

In summary, then, fertilizin from eggs and antifertilizin from sperm constitute a specific egg-sperm interacting system of wide, if not universal, occurrence. Removal of these surface substances reduces the fertilizing capacity of the gametes. Blocking of fertilizin and antifertilizin by some, but not all, inhibiting agents also reduces fertilizing capacity. The experiments for the most part, then, support the view that fertilizin and antifertilizin are essential for fertilization. The conflicting results with certain inhibitors, especially dermal secretion and *Fucus* extract, may yet be resolved. It is likely that a fertilizin-antifertilizin interaction initiates the acrosomal reaction, contributes substantially to the specificity of fertilization, and operates in attachment of the gametes.

2. Chemistry of Fertilizin and Antifertilizin

The chemistry of sea urchin fertilizin has been reviewed rather extensively (Metz, 1957b; Monroy, 1965; Vasseur, 1952a; Runnström *et al.*, 1959; Tyler, 1956). Fertilizin is an acid polysaccharide-amino acid complex. The acidic properties (isoelectric point below 2.0) are attributed to the sulfate content of approximately 20–25% found in most species. Fertilizin contains about 20% amino acids, including most, if not all, of the commonly occurring forms. Finally, hydrolysates yield one or a

few monosaccharides. Of these, fucose is the main sugar in two-thirds of the species examined (see Monroy, 1965). The intramolecular arrangement of the components is not completely understood. The sulfate is probably esterified to carbohydrate. Since only half of the carbohydrate is readily susceptible to periodate oxidation, Vasseur (1952b) suggested a branching carbohydrate chain. The arrangement of the amino acids is least clear. No distinct protein moiety has been demonstrated. The amino acids, probably as peptides, may be interspersed with sugars, as suggested by Tyler (1949). The *Arbacia* fertilizin molecule has a molecular weight of about 300,000 and an axial ratio of 20:1 (Tyler, 1956).

The reactive sites of the fertilizin molecule that combine specifically with sperm-surface antifertilizin apparently do not involve SH, S-S, phenolic, or amino groups (see Metz, 1957b). Oxidation with H_2O_2 converts fertilizin to the univalent form without destroying the reactive sites. Periodate oxidation destroys the capacity of fertilizin to combine with sperm. Therefore, the carbohydrate moiety may be involved. It is also possible that the acidic sulfate groups are components of the reactive sites, since fertilizin combines with many basic materials. In any event, the specificity of the agglutination reaction requires a fairly high order of configurational organization.

The most recent interest in fertilizin chemistry concerns the conversion of fertilizin to the univalent, nonagglutinating form. Such conversion can be achieved by treatment with heat, proteolytic enzymes, X-rays, ultraviolet light, or H_2O_2 (Tyler, 1941, 1948; Metz, 1942, 1957b). The action of the enzymes implies cleavage of peptide bands. In addition, sea urchin fertilizin probably becomes univalent on spontaneous reversal of agglutination. In this case, at least, the cleavage is not a simple splitting of the molecule. As shown by Hathaway and Metz (1961), reversal of agglutination is associated with release of a large fertilizin fragment from the sperm. This fragment will not combine with sperm. It is tempting to compare this to the inert "Fraction III" (Porter, 1959) that is split from 7 S antibody by proteolytic enzymes.

Additional information on univalent fertilizin has been obtained in this laboratory by Stern (1966). Data comparing univalent (H_2O_2 -treated) and multivalent *Lytechinus variegatus* fertilizin is given in Table VIII. The conversion does not result in release of sulfate or carbohydrate that will diffuse through cellophane. Therefore, it appears that the action of H_2O_2 involves some kind of major cleavage or dissociation of the molecule into fragments or subunits. This view was supported by the results of cellulose acetate strip electrophoresis. As seen in Fig. 4, multivalent *Lytechinus* fertilizin gives one strong metachromatic band at the origin. Comparable treatment of univalent fertilizin yields no band

at the origin and four strong bands migrating toward the anode. Evidently, then, multivalent fertilizin does not move on cellulose acetate under the conditions of the experiment. Treatment with H_2O_2 converts the metachromatic, nonmotile, multivalent material to a migrating form(s). The appearance of four bands with different electrophoretic mobilities indicates that the multivalent fertilizin is fragmented in a nonrandom fashion and implies formation of at least four subunits. It then becomes of interest to determine how many of these bands possess combining sites for sperm-surface antifertilizin. Absorption with sperm results in a preparation that at most gives only two of the four bands. The experiment is complicated by the fact that sperm supernatant controls, in the absence of fertilizin, produced a strong metachromatic band in the position of two of the univalent fertilizin bands. Thus, at least two of the fragments appear to have combining sites for sperm. The possi-

TABLE VIII
COMPARISON OF MULTIVALENT WITH UNIVALENT *Lytechinus variegatus* FERTILIZIN^a

	Multivalent	Univalent
Fucose %	26.3 $\pm$ 4 %	24.7 $\pm$ 4 % ^b
$s_{20,w}^\circ \times 10^{13}$	8.60	3.05
Viscosity, relative to H_2O , 30°C	1.42	1.03 ^c

^a Stern, 1966

^b 4 % error based on fucose determinations of commercial fucose.

^c Value for sample containing 0.1 mg/ml. Similar results are obtainable with other concentrations.

bility is not excluded that, in addition, one or two fragments without sperm-combining sites are also present and represent the inert fragment of Hathaway and Metz (1961).

Antifertilizin chemistry has received little attention in recent years. Earlier studies (see Runnström *et al.*, 1942; Metz, 1957b; Tyler, 1948) indicated a relatively heat-stable acidic protein of molecular weight under 10,000. More recent investigations show that "antifertilizin" extracts prepared by freeze-thawing sperm are usually mixtures of four or more antigens (see Section V,C). The antifertilizin (egg jelly-precipitating) activity is readily sedimented at 30,000 *g* (Metz and Köhler, 1960) and appears to be associated with particulate material. Evidently it is not the same as the egg jelly-precipitating agent in extracts prepared by heating sperm suspensions, although it is possible that the heat-stable agent is the active component of the particulate material prepared by freeze-thawing. In any event, neither appears to be a basic protein (Metz, 1949, 1957b), as suggested by Hultin (1947, 1949).



FIG. 4. Cellulose acetate electrophoresis of *Lytechinus variegatus* egg jelly solution. The egg jelly solution was prepared by acid (4.9 pH) treatment of a washed egg suspension. (a) The native, multivalent jelly material has not migrated from the origin. (b) An aliquot of (a) after oxidation (2% H_2O_2 , 30 minutes). No jelly remains at the origin, and the migrating material has separated into three to four components. (From Stern, 1966.)

Material with egg jelly-precipitating activity can also be extracted from sea urchin eggs (Tyler, 1940). This activity is also associated with particulate material (Monroy and Runnström, 1952). The relation of this egg antifertilizin to sperm antifertilizin is uncertain, and no role in fertilization has been assigned to it (see Metz, 1957b).

B. CYTOFERTILIZIN

Lillie (1914) believed that fertilizin is secreted by the egg and that this production ceases upon fertilization. Loeb (1915), Tyler and Fox (1940), and others, however, showed that unfertilized sea urchin eggs yield no additional sperm agglutinin following removal of the egg jelly. Accordingly, Motomura's (1950) discovery of the release of sperm agglutinin from jellyless eggs was unexpected. He called this agglutinin "cytofertilizin." Motomura (1950) obtained it from unfertilized sea urchin eggs by boiling or aging in seawater, from jellyless fertilized eggs by shaking to break the fertilization membrane, from NaSCN-demembrated eggs upon fertilization, and, finally, from hatching blastulae (Motomura, 1953a,b). Byers (1951) failed to demonstrate cytofertilizin in *Arbacia punctulata*, but recently Gregg (1966) and Gregg and Metz (1966) found sperm-agglutinating activity in supernatants from acid de-jellied and trypsin-demembrated eggs following fertilization. The last washings from the eggs before fertilization and supernatants from unfertilized controls were free of sperm agglutinin. Therefore, the agglutinating action of the fertilized egg supernatant could not be the result of incomplete removal of the egg jelly. Hagström (1956) and Runnström *et al.* (1959) obtained sperm agglutinin from *Arbacia lixula*, *Paracentrotus lividus*, *Psammechinus miliaris*, and *P. microtuberculatus* by aging jellyless, unfertilized eggs (12–30 hours) in seawater or by heating. It seems clear, then, that a sperm agglutinin can be obtained from the sea urchin egg after removal of the egg jelly, and that this agglutinin is released at fertilization. Since the supernatants from jellyless, fertilized eggs contain a number of substances (see below), including the sperm agglutinin, these supernatants will be called "fertilization product" in preference to "cytofertilizin" (Ishihara, 1964).

The biological properties and relation to egg jelly fertilizin have been examined to a limited extent. The sperm-agglutinating action of *Paracentrotus lividus* fertilization product is specific to the extent that it failed to agglutinate *Arbacia lixula* and *Psammechinus microtuberculatus* sperm (Hagström, 1956). Fertilization product agglutination of *A. punctulata* sperm, like egg jelly agglutination, reverses spontaneously. Following reversal, fertilization product-agglutinated sperm failed to re-agglutinate with either fertilization product or egg jelly fertilizin (Table

IX, Gregg, 1966; Gregg and Metz, 1966). This fact indicates that both agents agglutinate sperm by combination with the same sperm-surface receptor site, antifertilizin. The complementary reciprocal experiment has not been performed. Likewise, tests for initiation of the acrosomal reaction and inhibition of fertilizing capacity of sperm remain to be done. The fertilization product preparations appear to increase sperm motility (Gregg, 1966; Gregg and Metz, 1966). The release of fertilization product from the egg correlates well with cortical granule breakdown as emphasized by Hagström (1956), and it is highly probable that fertilization product is a mixture of cortical granule components. Brookbank (1959b) demonstrated fertilizin haptens associated with certain ATPase-bearing granules from unfertilized *Strongylocentrotus purpuratus* and *Lytechinus variegatus* eggs. The relation of this material to cytofertilizin or fertilization product is as yet uncertain.

TABLE IX
INHIBITION OF *Arbacia punctulata* FERTILIZIN AGGLUTINATION OF SPERM BY
PRETREATMENT WITH FERTILIZATION PRODUCT (FP)^{a,b}

	Sperm + (FP)	Sperm + Seawater (SW)
+ Fertilizin	—	+++
+ (FP)	—	+
+ (SW)	—	—

^a One drop of approximately 0.5% *Arbacia* sperm was mixed with 2 drops fertilization product (FP) (supernatant from trypsin-pretreated, fertilized *Arbacia* eggs). After reversal of agglutination, sperm was tested for reagglutinating capacity by addition of 1 drop of fertilizin, fertilization product, or seawater.

^b Gregg, 1966; Gregg and Metz, 1966.

Chemical studies on fertilization product and comparisons with jelly solutions have been undertaken. Motomura (1950) distinguished between jelly and fertilization product on the basis of different pH optima for extraction from unfertilized eggs. Hagström (1956) was unable to confirm Motomura's acid extraction experiments. The cortical granules are rich in polysaccharide (Monné and Slautterback, 1950; Monné and Harde, 1951; Osanai, 1964). Therefore, it is not surprising that such material is found in the medium following cortical granule breakdown of demembrated eggs (Oketa, 1963; Ishihara, 1964). Comparison of fertilization product with egg jelly indicates some differences in carbohydrate composition (Ishihara, 1964). Experiments of Gregg (1966) and Gregg and Metz (1966) show that *Arbacia punctulata* fertilization product contains several components. Cellulose acetate strip electrophoresis and toluidine blue metachromatic staining yielded three components, two of which correspond in position to two components in *Arbacia* egg jelly

solution. Immunodiffusion tests show that fertilization product preparations can contain four antigens, at least one of which is indistinguishable from one of the two precipitin bands of egg jelly solutions (Fig. 5). Finally, fertilization product gives a precipitin band with antisperm serum. These materials were prepared with great care to avoid contamination with products of sperm or egg cytolysis. Obviously, chemical comparisons between egg jelly fertilizin and fertilization product will have to be made on the basis of the individual components in the two kinds of preparations. Whether the sperm agglutinin in fertilization product performs a role in fertilization by virtue of its capacity to combine with antifertilizin is uncertain.

C. ANTIGENS OF SEA URCHIN GAMETES

The antigenic constitution of sea urchin gametes has been investigated rather extensively (see Metz, 1961a; Perlmann, 1959; Runnström *et al.*, 1959; Tyler, 1949; for review of earlier work). These studies show that both the sperm and the egg possess a number of antigens, and that certain of these are involved in fertilization.

1. *Antigens of the Sperm and Their Role in Fertilization*

a. Number and Distribution of Antigens. Tyler and O'Melveny (1941) demonstrated that rabbits are readily immunized against sea urchin sperm. Using cross-agglutination-absorption tests, Tyler (1949) demonstrated two sperm-surface antigens in *Strongylocentrotus purpuratus*. Subsequently, Köhler and Metz (1960) distinguished three different sperm-surface antigens in *Arbacia punctulata* and *Lytechinus variegatus* sperm by the same method (Table X). Probably additional sperm-surface antigens could be resolved by extension of this procedure to other cross-agglutinating species.

Arbacia sperm, like bull (Henle *et al.*, 1938) and probably human (Rümke and Hellinga, 1959) sperm, possess "regional" surface antigens. Thus Köhler and Metz (1960) gave evidence for tail specific, head specific, and common head-tail-surface antigens in *Arbacia* sperm.

Apart from the number and distribution of sperm-surface antigens, the question of their solubility and extractability is of interest. Köhler and Metz (1960) examined this question by absorbing anti-whole sperm serum with sperm extracts and testing for sperm-agglutinating action. As seen in Fig. 6, sperm extracts absorb some, but not all, of the agglutinins from anti-whole sperm serum.

Sperm "ghosts" remaining after extraction also absorbed some, but not all, of the sperm agglutinins. Evidently, then, some sperm-surface antigens are insoluble or are built into the sperm surface to the extent

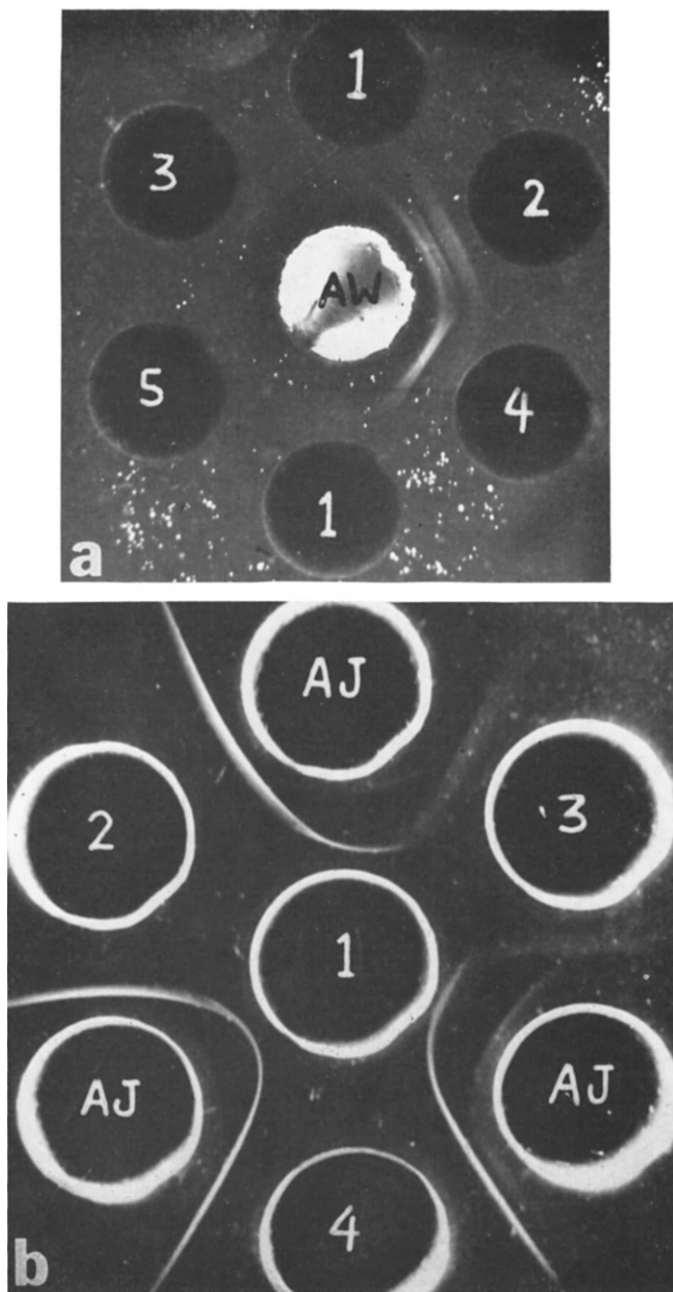


FIG. 5. Agar-gel immunodiffusion tests comparing egg jelly solution and "fertilization product." (a) At least four antigens can be present in fertilization product.

TABLE X
SPERM-AGGLUTINATING ACTION BEFORE AND AFTER ABSORPTION WITH SPERM OF
VARIOUS SPECIES^{a,b}

Sera		Immune sera tested with:					
Prepared against sperm	Absorbed with sperm	A	L	E	M	P	<i>Asterias</i>
A	—	+	+	+	0	+	0
A	A	0	0				
A	L	+	0	0			
A	E	+	+	0			
A	E + L	+	0				
L	—	+	+	+	+	+	+
L	L	0	0				
L	A	0	+	0			
L	E	+	+	0			
L	E + A	0	+	0			
E	—	+	+	+	+		+
E	L	0	0	+			
E	A	0	+				
E	A + L	0	0	?			
<i>Asterias</i>	—	0	0		0		+

^a (A) *Arbacia*. (L) *Lytechinus*. (E) *Echinarachnius*. (M) *Mellita*. (P) *Plagiobrissus*. Control (preinjection) sera failed to agglutinate sperm in tests parallel to those in the table.

^b Köhler and Metz, 1960.

that they cannot be extracted by the methods employed. All of the sperm tail-surface antigens are soluble, whereas the sperm head antigens are largely insoluble. It is of interest also that the insoluble sperm-surface antigens of *Arbacia* are shared with *Lytechinus* sperm, whereas the soluble antigens are not.

(b) Fertilization product and egg jelly have at least one common antigen. The jelly solution has an additional, weak antigen (precipitin band close to antiserum well). Trace amounts of the main jelly antigen are present in this sample of supernatant from trypsin-pretreated unfertilized eggs (well 3). (From Gregg, 1966; Gregg and Metz, 1966.)

(AS) anti-*Arbacia* sperm serum. (AJ) anti-*Arbacia* egg jelly serum. (AW) anti-whole *Arbacia* egg serum. (1) Egg jelly solution (0.66 mg/ml). (2) Supernatant of trypsin-pretreated fertilized eggs (2.18 mg/ml). (3) Supernatant of trypsin-pretreated unfertilized eggs (1.14 mg/ml). (4) Supernatant acid seawater-pretreated fertilized eggs (0.96 mg/ml). (5) Supernatant acid seawater-pretreated unfertilized eggs (1.28 mg/ml).

Finally, fertilizin neutralizes some of the soluble sperm antigens (Köhler and Metz, 1960). Gel-diffusion precipitin analysis reveals that the sperm extracts contain a number of antigens. Four are regularly found (Metz and Köhler, 1960), and additional antigens can be demonstrated in concentrated antigen preparations (Metz, unpublished). The four antigens readily demonstrated in sperm extracts are designated "a," "b," "c," and "d," beginning at the antigen well in agar-diffusion plates

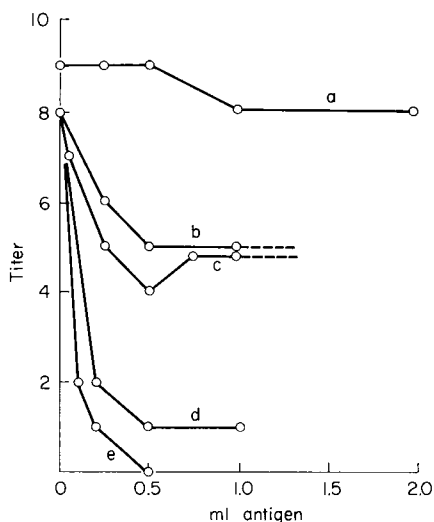


FIG. 6. Effect of absorption with increasing amounts of antigen on sperm-agglutinating action of anti-*Arbacia punctulata* sperm serum. Curve (a) Absorbing antigen prepared by heating (100°C, 1 minute) sperm. Curve (b) Absorbing antigen prepared by extracting sperm in pH 3.0 seawater. Curve (c) Absorbing antigen prepared by Mickle disintegration of sperm. Curve (d) Absorbing antigen: isolated sperm heads. Curve (e) Absorbing antigen: whole intact sperm. Absorbing antigen for curves (a), (b), and (c) centrifuged 3000 g. Each curve represents a different experiment. Ordinate: titer = $-\log_2$ of highest antiserum dilution giving sperm agglutination. Abscissa: ml antigen added to 0.5 ml antiserum. (From Köhler and Metz, 1960.)

(Fig. 7). Attempts at localization and some properties of these antigens have been presented in a series of reports (Metz and Köhler, 1960; Bischoff and Metz, 1962a,b; Flake and Metz, 1962; Shivers and Metz, 1962; Kloetzel and Metz, 1963). In some experiments the relative positions of the "a" and "b" bands appear to be reversed. At present, this inconsistency can only be attributed to minor differences in preparation of sperm extracts and to the use of different antisera. With this reservation, the following relations apply.

The "a"-band antigen is a subsurface sperm head antigen, the "b" band represents a head-surface antigen, the "c" band is confined to the sperm tail interior, and the "d" band antigen is distributed over both the sperm head and tail. These relations are diagrammed in Fig. 7. The "b" antigen is of particular interest because it is associated with the egg jelly-precipitating activity of sperm and the material that neutralizes the fertilization-inhibiting action of univalent antisperm serum. This material is readily sedimented at 30,000 g. As yet no finer localization of sea urchin sperm antigens has been made. It will be of particular interest to explore the antigenic structure of the acrosome surface both before and

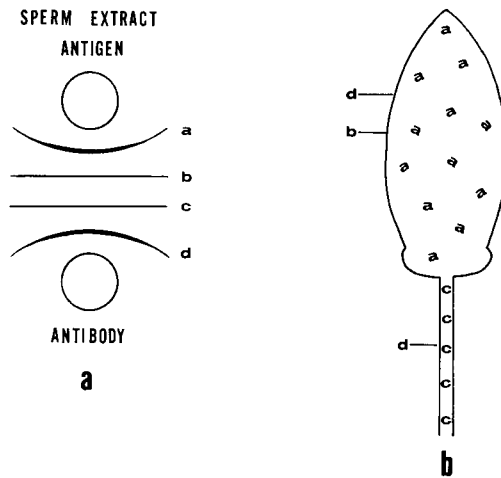


FIG. 7. Distribution of soluble antigens of *Arbacia punctulata* sperm. (a) Diagram of precipitin-band pattern obtained by diffusing *Arbacia* sperm extract, prepared by freeze-thawing, against anti-*Arbacia* sperm rabbit serum. (b) Location of the precipitating antigens in or on the sperm.

after the acrosomal reaction. This work will probably require the high resolution obtainable with ferritin-conjugated antibody in conjunction with electron microscopy. Baxandall (1964) and Metz (unpublished) give evidence that this is practicable.

b. Role of Sperm Antigens in Fertilization. Although the number and distribution of the sperm antigens is of interest, the main concern is the possible role of the antigens in fertilization. Since antibodies combine with and presumably block the antigens to which they are specific, combination with the antibody should prevent the antigen from engaging in interactions essential for fertilization. Tyler and O'Melveny (1941) found that antiserum treatment lowered the fertilizing capacity of sea urchin sperm. Unfortunately, this experiment is complicated by the fact

that normal, multivalent antibody strongly agglutinates sperm. This mechanical trapping is sufficient explanation for the reduction in fertilizing capacity. This difficulty was overcome by the use of antiserum rendered nonagglutinating by photooxidation (Tyler, 1945). As seen in Table XI, such sera markedly and specifically lowered the fertilizing capacity of *Lytechinus pictus* and *Urechis* sperm (Tyler, 1946). The antisera used in these experiments were prepared against sperm extracts. As seen above, sea urchin sperm extracts can contain at least two sperm-surface antigens. In addition, the sperm possess insoluble surface antigens. More than one of these could have a role in fertilization. Accord-

TABLE XI
PERCENT FERTILIZATION OF *Lytechinus* AND *Urechis* EGGS BY HOMOLOGOUS SPERM
TREATED WITH "UNIVALENT" ANTIBODIES^{a,b}

Ml of serum-treated sperm (0.1 %) used for insemination	Sperm and eggs of:				
	<i>Lytechinus pictus</i>			<i>Urechis caupo</i>	
	L1	N1	U1	U1	L1
0.013	—	—	—	$\frac{1}{2}$	20
0.025	0	10	15	2	55
0.05	0	30	35	5	95
0.1	0	85	75	12	100
0.2	0	95	99	15	99
0.4	0	100	100	30	100
0.8	1	100	99	—	—

^a Percentage fertilization of ca. 400 eggs in 5 ml of seawater by sperm treated 15 minutes with photooxidized rabbit antisera vs. antifertilizin of *Lytechinus* (L1) and of *Urechis* (U1) and normal rabbit serum (N1), using 1 vol of 1 % sperm to 9 vol of half-strength serum adjusted to seawater salinity. Photo-oxidation: 352 cu. mm O₂ uptake per ml serum for L1, U1, and N1. Original agglutinative titers: 512 for L1, 1024 for U1. Titters for inhibition of agglutination: 6 for L1, 8 for U1.

^b Tyler, 1946.

ingly, Metz *et al.* (1964) undertook an analysis of the effect of antisperm serum on fertilization in *Arbacia punctulata*. Sperm were treated with antibody made univalent by the papain digestion-reduction procedure (Porter, 1959). Univalent anti-whole sperm serum strongly reduced the fertilizing capacity of *Arbacia* sperm. Preliminary absorption with *Arbacia* or *Lytechinus* (Fig. 8), but not *Echinarachnius* (Fig. 9), sperm removed the fertilization-inhibiting action. It is concluded from these observations that the "fertilization antigen(s)" of the *Arbacia* sperm is also present on *Lytechinus* sperm. It is likely that this is a sperm-surface antigen, for Flake and Metz (1962) found that multivalent antibodies do not diffuse

into *Arbacia* sperm. Evidently, the *Arbacia* sperm-specific antigen is not essential in fertilization. Likewise, the antigen shared with *Echinarachnius* is not essential. This last conclusion was confirmed by Williams and Metz (1963), employing specific antibody dissociated from *Echinarachnius* sperm following absorption to *Echinarachnius* sperm. Unfortunately, the antigens defined by cross-absorption agglutination tests have not yet been related to the regional or soluble and insoluble antigens.

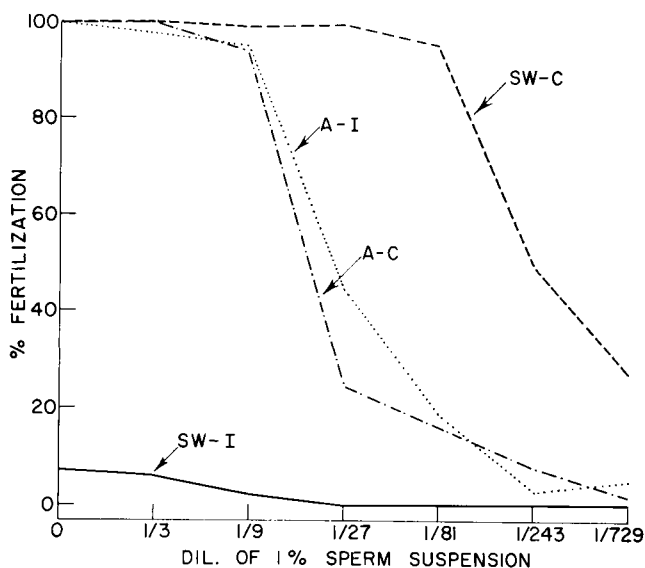


FIG. 8. Fertilizing capacity of *Arbacia* sperm following treatment with *Lytechinus* sperm-absorbed, papain-digested, anti-*Arbacia* sperm antibody. Equal samples of papain-digested immune and control γ -globulin were absorbed with living *Lytechinus* sperm and seawater. To test for effect on fertilization, 2 drops of each of the γ -globulin preparations were mixed with 1 drop of each sperm dilution. After 5 minutes 1 drop of concentrated eggs was mixed with the sperm by flooding with 5 ml of seawater. (SW-I) Seawater-treated immune γ -globulin. (SW-C) Seawater-treated control γ -globulin. (A-I) *Lytechinus* sperm-absorbed immune γ -globulin. (A-C) *Lytechinus* sperm-absorbed control γ -globulin. (From Metz *et al.*, 1964.)

The fertilization antigen(s) of *Arbacia* sperm may be soluble. Absorption of antisperm serum with *Arbacia* sperm extracts can neutralize the fertilization-inhibiting action of the antiserum (Fig. 10). However, the effect can be removed by centrifugation of the sperm extract at 30,000 g, and the sediment is identified as the soluble "b" antigen, the same material that reacts with fertilizin. The relation here is not fully resolved (Metz *et al.*, 1964), for preliminary examination of the 30,000 g sperm-

extract pellet revealed sperm fragments and probably considerable nucleoprotein. The nature of this material remains to be investigated, but it does appear that the "fertilization antigen(s)" is not present in the supernatant following centrifugation at gravity forces usually employed to sediment all particulates.

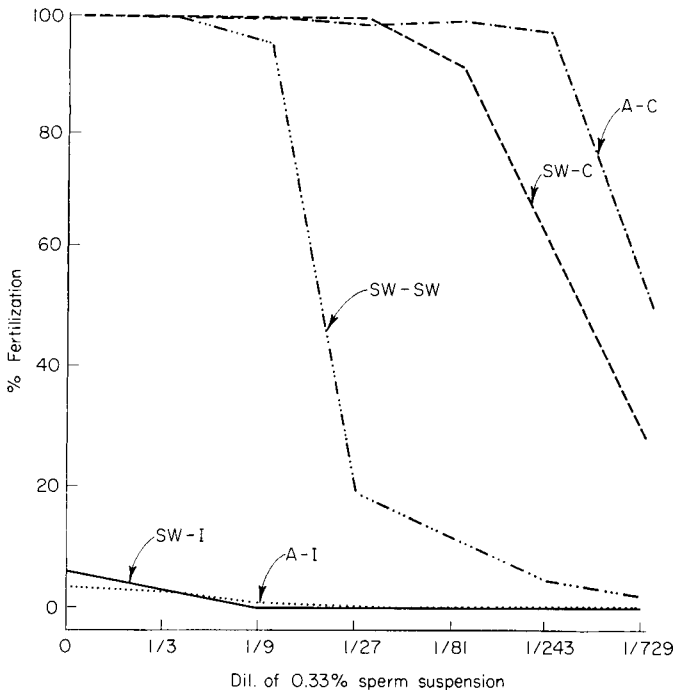


FIG. 9. Fertilizing capacity of *Arbacia* sperm following pretreatment with *Echinarachnius* sperm-absorbed, papain-digested anti-*Arbacia* sperm antibody. Equal samples of papain-digested immune and control γ -globulin were absorbed with living *Echinarachnius* sperm and seawater. To test for effect on fertilization, 2 drops of each of the γ -globulin preparations were mixed with 1 drop of each sperm dilution. Five minutes later 1 drop of concentrated eggs was mixed with the sperm by flooding with 5 ml of seawater. (SW-I) Seawater-treated immune γ -globulin. (SW-C) Seawater-treated control γ -globulin. (A-I) *Echinarachnius* sperm-absorbed immune γ -globulin. (A-C) *Echinarachnius* sperm-absorbed control γ -globulin. (SW-SW) Sperm treated with seawater only. (From Metz *et al.*, 1964.)

It is clear from the above evidence that one or more sperm antigens are closely involved with an essential step or steps in fertilization. As yet no information is available concerning what this step may be. It does not necessarily involve blocking sperm-surface antifertilizin, since some sera fail to inhibit fertilizin agglutination but do inhibit the fertilizing capacity

of sperm. It is possible that antibody treatment inhibits or prematurely initiates the acrosomal reaction, prevents the release of the egg jelly lysin (Brookbank, 1958; Hathaway *et al.*, 1960), prevents membrane fusion, or blocks some essential component of the egg-activating mechanism.

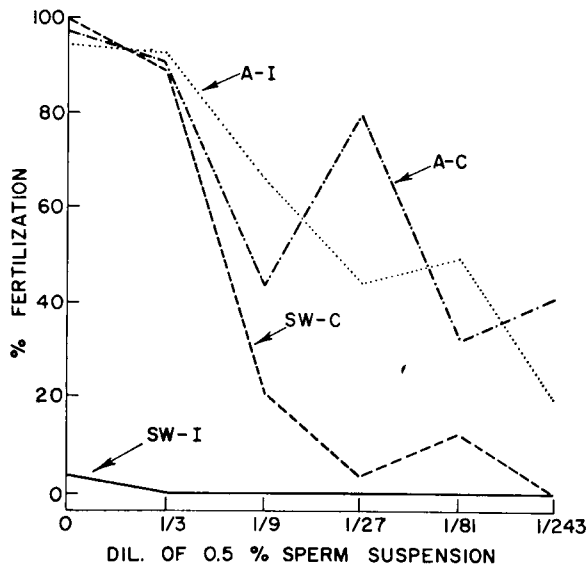


FIG. 10. Fertilizing capacity of *Arbacia* sperm following treatment with *Arbacia* sperm-extract treated, papain-digested antibody. Equal samples of papain-digested immune and control γ -globulin were absorbed with sperm extract (prepared by freeze-thawing a 25% sperm suspension, followed by 10,000 g centrifugation) and seawater. To test for effect on fertilization, 2 drops of each of the γ -globulin preparations were mixed with 1 drop of each sperm dilution. After 5 minutes, 2 drops of concentrated eggs were mixed with the sperm by flooding with 3 ml of seawater. (SW-I) Seawater-treated immune γ -globulin. (SW-C) Seawater-treated control γ -globulin. (A-I) Sperm extract-absorbed immune γ -globulin. (A-C) Sperm extract-absorbed control γ -globulin. (From Metz *et al.*, 1964.)

2. Antigens of the Sea Urchin Egg

Sea urchin eggs contain many antigens. Those related to fertilization have been studied most extensively by Perlmann (e.g. 1959) and co-workers. Others have examined special features such as the role of antigens in cleavage and development (Went and Mazia, 1959; Brookbank, 1959a; Tyler and Brookbank, 1956a,b). Much of this work has been reviewed recently (Metz, 1961a,b; Tyler, 1957, 1959).

Using the Ouchterlony agar-diffusion technique, Perlmann (1953) resolved at least 10 precipitin bands in *Paracentrotus lividus* egg ho-

mogenates and Metz and Köhler (1960) recognized 9 distinct bands in immunoelectrophoresis of *Arbacia* egg material. Acrylamide-gel electrophoresis resolves a larger number of protein-staining bands (Metz, unpublished), so that additional antigens can be expected. Rather than attempt to characterize, localize, and ascribe a physiological role to the many antigens, the approach for the most part has been to examine the effects of antisera on eggs, fertilization, and development, and to attempt to interpret such effects in terms of specific antigens. Treatment with anti-egg serum is reported to result in a number of morphological and physiological effects on sea urchin eggs. These include precipitation of

TABLE XII
CHEMICAL PROPERTIES OF A ANTIGEN FROM *Paracentrotus lividus* EGGS^{a,b}

Some qualitative data on partially purified A antigen:		
Glucose +	Hexosamine —	Ribonucleic acid —
Mannose +	Sialic acids —	Sulfate +
Galactose —	Amino acids +	Phosphate —
Fucose —		

^a Easily soluble in water, insoluble in ethanol. Not susceptible to the action of trypsin or pepsin. Heat stable at pH 3.5–8. Slowly hydrolyzed at pH 1–2 at 100°C. Partially susceptible to IO_4^- (pH 3.5, 50°C). Electrophoretic mobility $-1.6 \times 10^{-5} \text{ cm}^2 \times \text{volt}^{-1} \times \text{sec}^{-1}$ (pH 7.1, phosphate, $\mu = 0.1$).

^b Perlmann, 1959.

the egg jelly and agglutination of eggs (Perlmann, 1954; Baxandall *et al.*, 1964a), precipitation of the ectoplasmic (hyaline) layer of fertilized, demembrated eggs (Tyler and Brookbank, 1956b), and, finally, wrinkling, blistering, and cytolysis (Perlmann, 1954; Tyler and Brookbank, 1956b). The physiological effects reported include increase in respiration (Tyler and Brookbank, 1956b; Brookbank, 1959a), inhibition of fertilization, parthenogenetic activation (Perlmann, 1954; Baxandall *et al.*, 1964a), and inhibition of cleavage (Tyler and Brookbank, 1956a,b). The action of antiserum on egg jelly has been reviewed above (see Section V,A). It clearly involves a precipitation of the jelly material, called the "J" antigen by Perlmann (1954). The effects of antiserum that are of particular interest to fertilization are parthenogenetic activation of the egg and inhibition of fertilization.

a. Parthenogenesis. Perlmann (1954) reported parthenogenetic activation of *Paracentrotus lividus* and *Arbacia lixula* eggs with anti-whole egg homogenate serum. In his first studies, activation was usually partial (e.g., cortical granules failed to break down), and few eggs actually cleaved. Removal of the jelly (Perlmann, 1956) and exposure to anti-serum at low temperature (4°C) resulted in an increase in percentage of

cleavage (Perlmann, 1957). More recently Baxandall *et al.* (1964a,b) reported close to 100% activation of eggs from certain females using a combination of cold and antiserum treatment. The egg-activating action is attributed to an interaction between antibody and A, or activating, antigen. Evidence, including inactivation by periodate (Perlmann and Perlmann, 1957a), is presented (Table XII) to show that this A antigen is a heat-stable carbohydrate different from the J (jelly) antigen (Perlmann and Perlmann, 1957a; Baxandall *et al.*, 1964a). The parthenogenetic action is specific to the extent that absorption with *P. lividus*, but not *A. lixula* or *Sphaerechinus granularis*, egg homogenate neutralized the egg-activating action of anti-*P. lividus* egg serum (Baxandall *et al.*, 1964a). Recently Baxandall *et al.* (1964a,b) examined antiserum-treated eggs with the electron microscope and undertook localization of antigens with ferritin-conjugated antibody. They reported an increase in size and number of surface papillae and the appearance of a wide ($0.1\ \mu$), diffuse layer of material outside the plasma membrane (Fig. 11). No trace of an orthodox vitelline membrane was found. The first evidence of activation was cortical granular breakdown inside the egg. In fully activated eggs most, but not all, of the cortical granules ruptured peripherally, elevating a membrane. This is clearly not a normal sea urchin vitelline membrane, as seen, for example, by Franklin (1965), but is probably a product of the egg itself or perhaps a highly modified vitelline membrane. It is identical in structure to a layer that appears beneath it and which is identified as the hyaline layer (Fig. 12). It differs also from the membrane formed in response to the activating action of butyric acid. The latter has the same dimensions and substructure as the normal fertilization membrane (Baxandall, personal communication). Later, well-defined astral tubules are found in the interior of the egg cytoplasm. Electron micrographs of eggs treated with appropriate ferritin-conjugated antibody indicate that the A antigen is located in the $0.1\ \mu$ -thick layer of material outside the plasma membrane of the antiserum-treated egg (Baxandall *et al.*, 1964b). Unfortunately, attempts to confirm the parthenogenetic action of antiserum on sea urchin eggs have not succeeded to the full satisfaction of others. In particular, Tyler (1959) reported only formation of an "hyaline" (ectoplasmic) layer and nuclear dissolution following antiserum treatment in a number of experiments. Tyler and Brookbank (1956b) and Brookbank (1959a) obtained an increase in respiration following treatment of unfertilized eggs with antijelly and antiegg sera. In a few experiments the increase was four- to fivefold, and, therefore, comparable to the increase normally obtained at fertilization. However, the increased respiration was not accompanied by the increase in phosphate uptake (Brookbank, 1964) that follows conven-

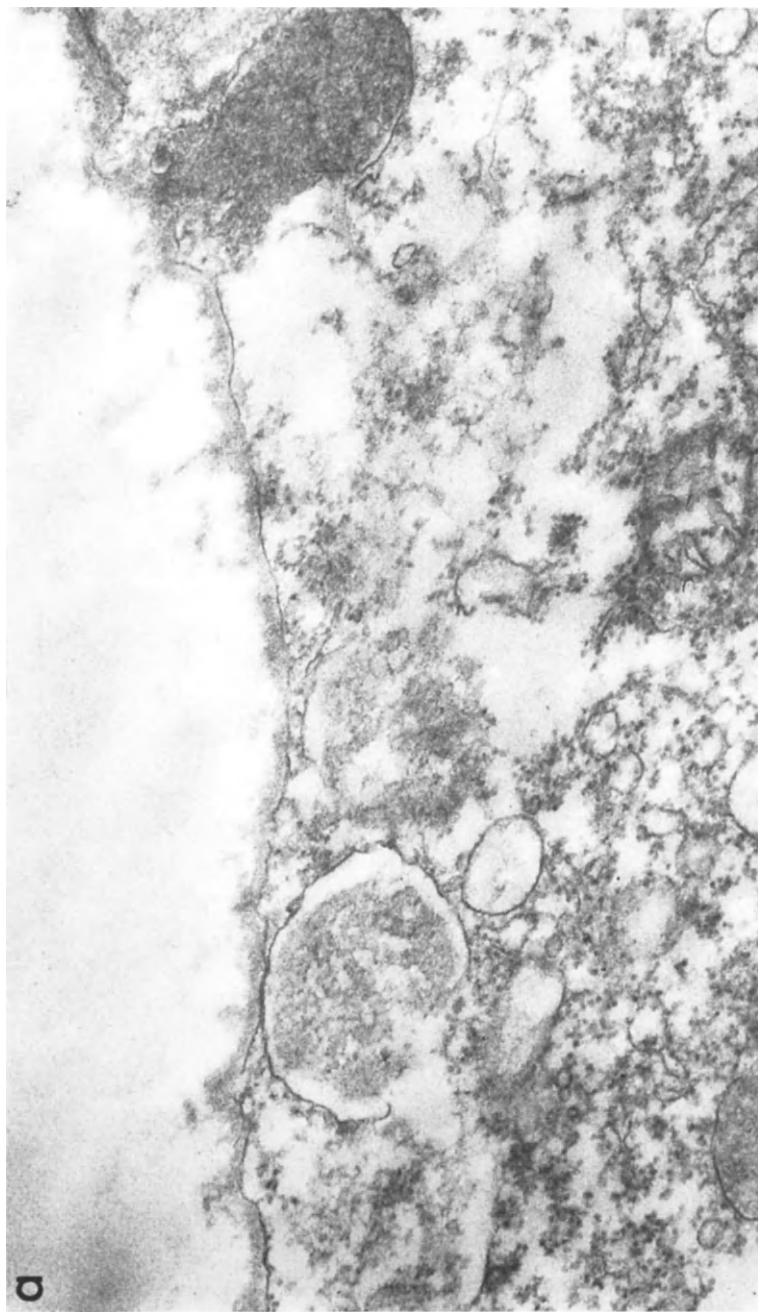


FIG. 11. Morphological effects of antibody on unfertilized *Paracentrotus lividus* eggs. (a) Control egg treated with pre-injection γ -globulin. Magnification: $\times 50,000$. (From Baxandall, unpublished.) (b) Unactivated egg which has been treated with γ -globulin from an antiserum against homogenate of heated (100°C , 2 hours) jellyless eggs. Outside the plasma membrane (PM) is a diffuse layer at least $0.1\ \mu$ wide, and more of this material appears to have diffused into the jelly layer

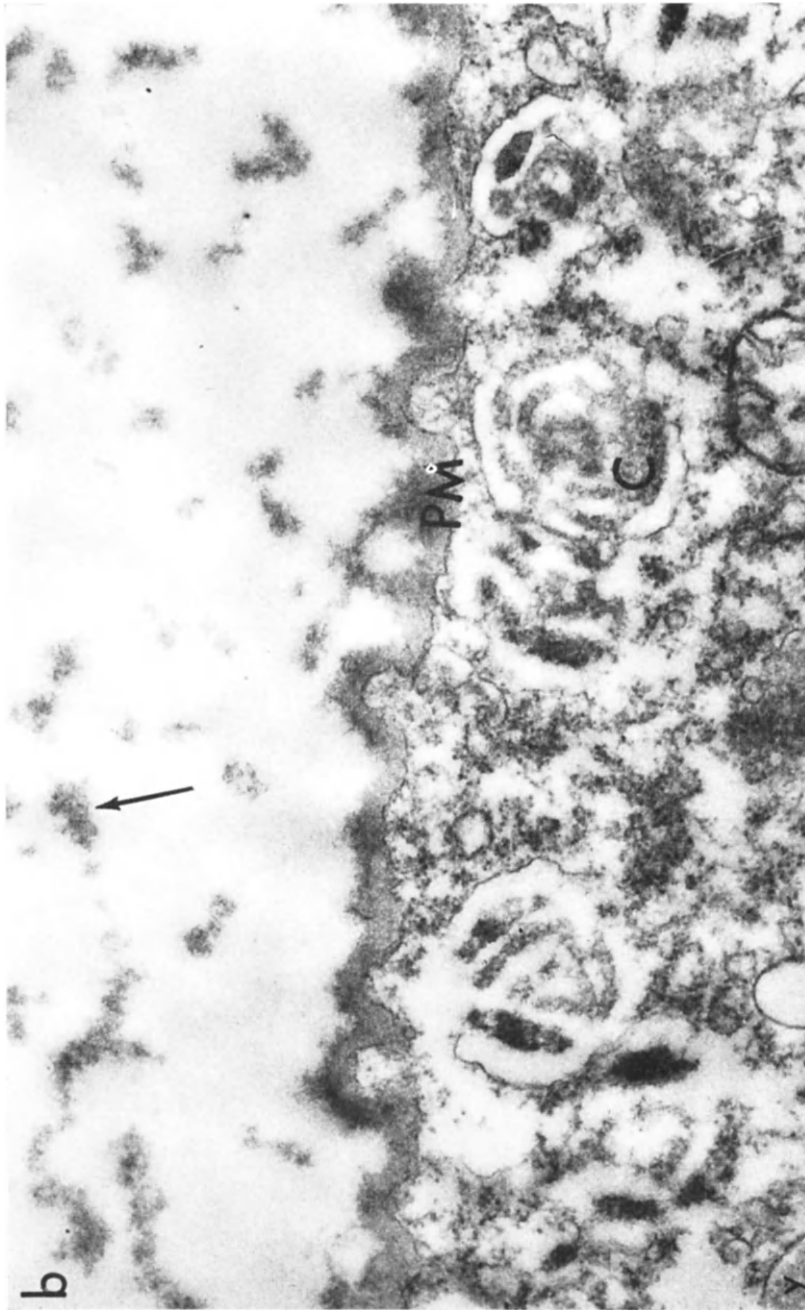


FIG. 11 (Continued).
(arrow). The egg surface has more papillae than in the untreated eggs. Cortical granule (C), yolk granule (Y). Magnification: $\times 50,000$. (From Baxandall *et al.*, 1964a.)

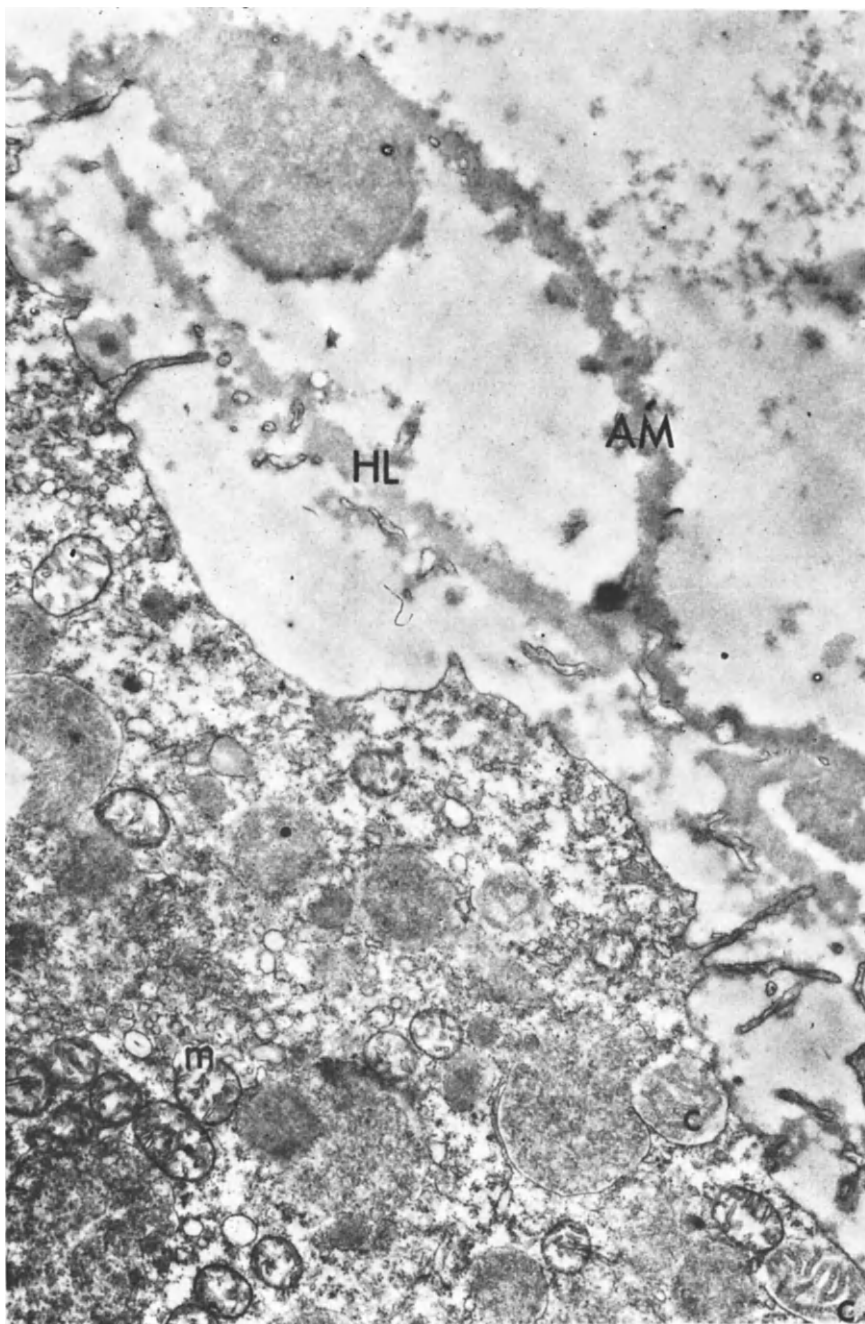


FIG. 12. An unfertilized egg parthenogenetically activated by the γ -globulin from antiserum against a homogenate of total jellyless eggs. A wide activation membrane (AM) has been formed and at places appears to be diffusing into the

tional parthenogenetic activation and fertilization (Whiteley and Chambers, 1960). In fact, antiserum treatment inhibited phosphate uptake by fertilized eggs. The reasons for the lack of agreement are not completely clear. They could involve differences in the biological material, differences in approach, and differences in definition. Such differences should be easily resolved by a joint effort. In any event, Perlmann's claims are of fundamental importance and, as he is aware (Perlmann, 1959), could be the key to the initiation of development that has been sought for over 50 years.

b. Inhibition of Fertilization. Another effect of antiserum, namely, inhibition of fertilization, is of particular interest. Perlmann (1954) reported that antiserum-treated eggs failed to fertilize. Perlmann and co-workers (Perlmann and Hagström, 1955; Perlmann, 1957, 1959; Perlmann and Perlmann, 1957a,b) concluded on the basis of absorption experiments that the effect is not due to the J (egg jelly) or A (activating) antigen, but that fertilization inhibition involves interaction of specific antibody with an additional antigen, the "F," or fertilization, antigen. The presumption is that the F antigen(s) is a link in a chain of early fertilization changes in the egg. Perlmann and Perlmann (1957a,b), presented some evidence, including partial inactivation by heating, that the A and F antigens are different. However, as indicated elsewhere (Metz, 1961a), more conclusive evidence would be desirable. Trypsin does not inactivate the fertilization-inhibiting antigen (Perlmann, 1959). As Perlmann and Perlmann (1957b) are aware, the inhibition of fertilization by antiserum is probably a complex reaction and could involve several specific antigen-antibody interactions. In any event, one of the next steps in the analysis should be a confirmation of the fertilization-inhibiting action using univalent antibody. This would simplify the interpretation to the extent that it would eliminate precipitation and cross-linked lattice formation as contributing factors to fertilization inhibition (see Metz, 1961a; Shivers and Metz, 1962). See under *d* below.

c. Cortical Damage. A final effect of antiserum studied by Perlmann and collaborators is damage to the egg cortex. This was particularly evident in eggs that were fertilized after antiserum treatment, and it involved several abnormalities, including poor fertilization membrane formation and failure of cortical granule breakdown. Perlmann and Perlmann (1957b) ascribed the effects to action of antibody on one or

jelly coat. The layer (HL) structurally similar to the activation membrane is homologous with the hyaline layer. Most of the cortical granules have opened up and released their contents, but a few remain more or less intact (c). (m) Mitochondrion. Section stained with lead acetate. Magnification: $\times 25,000$. (From Baxandall *et al.*, 1964a.)

more cortical, or "C," antigens. Significant effects were not obtained with antijelly serum. Unheated, but not heated, egg homogenate neutralized the cortical-damaging action of antisera. This heat lability distinguished the C antigen from the A, and probably the F, antigens. The antigen was not affected by trypsin (Perlmann, 1959). Again, it would be interesting to compare the effects of normal 7 S antibody and univalent antibody for cortical-damaging action.

The A, C, and F antigens are all distinct from egg jelly and, therefore, must be located in the egg surface, egg cortex, or deeper layers of the egg cytoplasm. Immunoelectron microscopy, using ferritin-conjugated anti-egg homogenate serum, showed ferritin particles primarily in the 0.1 μ -layer of diffuse material found just external to the plasma membrane (Baxandall *et al.*, 1964b). Sera absorbed with heated egg homogenate (A antigen) produced a little ferritin attachment in the same region. Remarkably, the plasma membrane showed very little ferritin labeling, even in trypsin-treated (vitelline membraneless) eggs (unpublished result cited by Baxandall *et al.*, 1964b).

From the material presented in this section it is clear that sea urchin sperms and eggs both have a number of surface as well as subsurface antigens. It is evident that some of these are not essential in fertilization and that others are closely related to essential steps in the initial events of fertilization. Some progress has been made in attempts to localize and characterize these antigens. With further work, a more precise localization and more specific characterization should result. Finally, a well-defined role in fertilization should eventually be revealed.

d. Effects of Univalent Antibody. Metz and Thompson (1967) have investigated the effect of univalent (papain digested) antibody on the morphology, fertilizing capacity, and cleavage of *Lytechinus variegatus* eggs. Univalent anti-jellyless egg homogenate rabbit antibody failed to produce the morphological effects or fertilization inhibition characteristically obtained following treatment of eggs with the parent multivalent antibody. However, when univalent antibody pretreated eggs were subsequently treated with anti-rabbit γ -globulin sheep serum (antiglobulin or Coombs test), morphological effects and fertilization inhibition resulted. These results have been confirmed by Graziano and Metz (1967) using *Arbacia punctulata*. From this it appears that the morphological effects and fertilization inhibiting action of anti-jellyless egg homogenate antibody depend upon cross-linking of egg antigens, not simply to combination with and blocking of specific antigenic sites. This implies that there is no "fertilization antigen" at the egg surface, at least in the sense of a specific molecular configuration (combining site) that is antigenic in the rabbit and has an essential role in fertilization.

The cleavage inhibiting action of anti-egg jelly antibody was reduced,

but not completely eliminated, by papain digestion in both *L. variegatus* (Metz and Thompson, 1967) and *Arbacia punctulata* (Cocanour and Metz, unpublished). Papain digested (univalent) anti-egg jelly antibody, like papain digested anti-jellyless egg homogenate antibody, failed to inhibit fertilization of *L. variegatus* eggs (Metz and Thompson, unpublished).

VI. Amphibian Fertilization

The early literature records some experimental studies on amphibian fertilization and particularly parthenogenesis, but the brevity of the natural breeding seasons of most species and the difficulty of obtaining ripe, ovulated eggs seriously handicapped the earlier research. With the development of the hormonal method of inducing ovulation (see Rugh, 1934) amphibian material became available for nearly continuous study of fertilization mechanisms, as well as other developmental problems.

In most amphibians eggs are ovulated in the germinal vesicle stage, and maturation proceeds to the second meiotic metaphase, where development ordinarily stops (*Triturus viridescens*, Humphries, 1956; *Rana pipiens*, Subtelny and Bradt, 1961). Maturation appears to be precocious in *Bufo vulgaris* (Barthélémey, 1922), but only second meiotic metaphase eggs are physiologically ripe in most species. Earlier meiotic stages cannot be activated by sperm or parthenogenetic treatment (Subtelny and Bradt, 1961). Maturation up to the second meiotic metaphase is normally completed during passage through the upper one-third of the oviduct (*Rana pipiens*, Rugh, 1952; *Triturus viridescens*, Humphries, 1956). Ovulated eggs will mature in the coelom if the oviducts are ligated (Subtelny and Bradt, 1961), and coelomic and even ovarian eggs will undergo maturation *in vitro* under special conditions (Barthélémey, 1922; Tchou-Su, 1950; Tchou-Su and Wang, 1956). Clearly, then, fertilization and activation experiments are meaningful only if the eggs have been allowed to pass through the upper oviduct or one of the special techniques have been employed to insure maturation of the eggs to ripeness.

A. ROLE OF THE EGG JELLY

With such precautions it is found that coelomic eggs are not fertilizable, although they can be activated parthenogenetically (see Subtelny and Bradt, 1961, for references), whereas uterine eggs, which have passed through the oviduct, fertilize readily. Since the eggs can be fertilized only after exposure to the oviducal environment, attention has focused on the oviducal contribution. The extraneous jelly layers of the egg arise from the oviducal gland cells, and since these layers are de-

posited around the egg during its passage through the oviduct, interest has centered on the role of the jelly material in fertilization.

An apparently essential role of egg jelly was confirmed by Good and Daniel (1943) and Subtelny and Bradt (1961), who found in *Triturus torosus* and *Rana pipiens*, respectively, that enrobement of coelomic eggs with egg jelly rendered the eggs fertilizable. Finally, Tchou-Su and Wang (1956) and Kambara (1953) showed that oviducal toad (*Bufo bufo asiatica*) eggs could not be fertilized after removal of the jelly layers, but that reenrobement with jelly restored fertilizability. In Kambara's (1953) experiments the action of jelly was not highly specific,

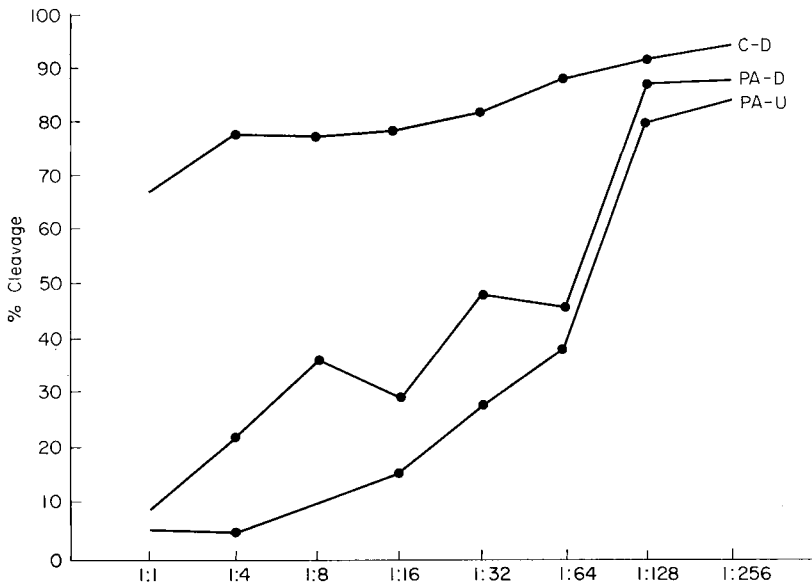


FIG. 13. Cleavage of *Rana pipiens* eggs following treatment with papain-digested (univalent) anti-egg jelly serum. Eggs were treated with univalent antibody for 5 minutes, washed in Holtfreter's solution, and then inseminated. (C-D) Papain-digested control serum. (PA-D) Papain-digested anti-jelly serum. (PA-U) Undigested anti-jelly serum. Each point represents the average of four experiments. (From Shivers and Metz, 1962.)

since it could be duplicated with gelatin or agar but not with gum arabic.

Additional evidence for a role of the egg jelly in fertilization comes from experiments with antisera prepared by immunizing rabbits with jelly from uterine eggs. Fertilizability of *Rana pipiens* eggs pretreated with such antisera was markedly reduced (Shaver and Barch, 1960). This inhibiting action evidently results from blocking of jelly-receptor sites and not by mechanical interference with sperm penetration (e.g.,

by precipitating the egg jelly), because antiserum rendered nonprecipitating (univalent) by papain digestion also inhibited fertilization, as seen in Fig. 13 (Shivers and Metz, 1962). Likewise the antigen(s) involved are specific to the egg jelly, since absorption of the antijelly serum with sperm, or extracts of a variety of tissues, including ovary, failed to affect the inhibition of fertilization induced by antijelly serum (Shaver *et al.*, 1962).

Clearly, then, the egg jelly has some essential role in fertilization. It becomes of interest to determine what jelly component or components are essential and what role they perform. The amphibian egg jelly is not an homogeneous capsule. For example, examination of living material reveals three distinct jelly layers in *Rana pipiens* (e.g., Nace *et al.*, 1961) and four in *Hyla arborea japonica* (Katagiri, 1963). Histochemical study shows that there are five different types of oviducal glands that contribute

TABLE XIII
HISTOCHEMICALLY DISTINGUISHABLE LAYERS OF *Triturus viridescens* EGG JELLY^a

Reaction to	Inner	Jelly layer					Outer
		J ₁	J ₂	J ₃	J ₄	J ₅	
PAS		++	++	++	++	++	
Alcion blue		++	++	±	++	—	
Toluidine blue		++	++	—	++	—	
metachromasia							
Toluidine blue		+	—	—	+	—	
metachromasia after							
alcohol dehydration							

^a Constructed from Humphries, 1964.

the jelly in *Triturus viridescens*. These glands are arranged in sequential regions of the oviduct (Humphries and Hughes, 1959). They evidently deposit the five histochemically distinguishable layers of egg jelly recently described by Humphries (1964) in this species (Table XIII).

Likewise, at least five antigenic components have been found in each of seven other amphibian egg jellies by agar-gel precipitin tests (Shivers, 1965). Again in *R. pipiens* some components are not uniformly distributed throughout the jelly capsule, but are confined to certain jelly layers (Shivers, 1962), as seen in Fig. 16.

Attempts to assess the role of the several jelly components in fertilization have proceeded in two ways. The first involves correlating fertilizability with the jelly layers present in eggs removed from various levels of the oviduct or with eggs from which jelly layers have been removed by mechanical or chemical means. The second concerns the use of anti-jelly sera specific for particular jelly antigens.

Kambara (1953) employed the first approach, using eggs removed from various levels of the oviduct of the toad, *Bufo bufo asiatica*. Four jelly layers are visible in spawned eggs of this species. Eggs lacking the two outer layers (A and B of Kambara, 1953) fertilized readily. However, eggs with only the innermost layer (D layer; lacking layers A, B, and C) did not fertilize when inseminated. It appears, then, that the C layer (second from the egg) or a combination of C plus D are essential for fertilization in this species. *Rana temporaria* eggs are also enveloped by four visible layers. Removal of the outermost and part of the next layer by mechanical dissection or trypsin did not affect fertilizability (Katagiri, 1962). *Hyla arborea japonica* eggs can be fertilized after

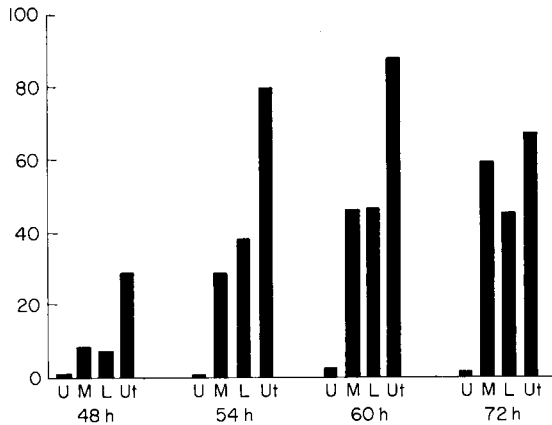


FIG. 14. Fertilizability of eggs of *Rana pipiens* (in percent cleaving eggs) taken from various levels of reproductive tract at different intervals after initial stimulation to ovulation by pituitary gland. (U) Upper oviducal segment. (M) Middle oviducal segment. (L) Lower oviducal segments. (Ut) Ovisac (uterus). (From Glick and Shaver, 1963.)

mechanical removal of the outer three of the four visible jelly layers (Katagiri, 1963).

Glick and Shaver (1963) employed this approach in even greater detail. They removed *Rana pipiens* eggs from the upper, middle, and lower thirds of the oviduct and from the uterus at four different time intervals after pituitary-induced ovulation (see Fig. 14). Eggs from the upper third rarely fertilized, regardless of postinjection age, whereas eggs from the middle and lower levels showed intermediate fertilizability, and eggs from the uterus consistently yielded the highest percentage. The low fertilizability of eggs from lower levels of the reproductive system at 48 hours (Fig. 14) can be attributed to maturation underripeness. However, from 54–72 hours no significant increase in

fertilizability resulted. Therefore, it is assumed that eggs from all regions are ripe and have recovered from any physiological inhibition (e.g., CO₂ intoxication [Dalcq *et al.*, 1936]) by 54 hours. Failure of the eggs from the upper oviduct to fertilize must then be attributed to absence of some essential oviducal factor(s). Contribution(s) from the middle and possibly lower levels of the oviduct supply this factor(s). These progressive changes in fertilizability correlate with antigenic differences in the jelly from the several regions. In *R. pipiens*, Barch and Shaver (1963) found that the upper and lower oviducal segments each had two unique antigens absent from the other regions. Unfortunately, antisera to test for unique middle-segment antigens were unavailable. However, the middle segment contained some antigens in common only with the upper oviduct and others shared only with the lower region. Both the unique and common antigens in these oviducal extracts evidently represent antigens (receptor sites) present in the egg jelly, because anti-egg jelly serum produced the same precipitin-band pattern in agar-gel diffusion tests.

Treatment of eggs with antisera prepared against any of the three oviducal levels resulted in reduction of fertilizing capacity (Barch and Shaver, 1963). Antiserum against the middle oviduct was least inhibitory. It is not clear whether the lower activity of this antiserum is due to differences in the fertilization role of the middle oviducal antigen(s) or simply to a lower antibody titer of the serum employed. Cross-absorption experiments did not produce consistent results (Barch and Shaver, 1963). However, these experiments do confirm that the oviduct is the source of an egg capsular component(s) essential for fertilization. This conclusion would be more rigorous if nonprecipitating, univalent antisera (e.g., Shivers and Metz, 1962) were employed, since the antioviducal sera can precipitate the egg jelly and cause disaggregation of the egg mass (Barch and Shaver, 1963).

Shivers (1962) attempted further localization of the essential *R. pipiens* jelly component or components using fluorescein-conjugated antibody. As indicated above, *R. pipiens*, as well as other amphibian egg jellies, contain at least five antigens. Three of these are species specific (Shivers, 1965), at least within the system of cross tests employed. The two nonspecific antigens are shared with two or three additional species (see Fig. 15). The heterologous (*R. clamitans*, *R. sylvatica*) antisera produced appreciable fluorescence only in the innermost of the three jelly layers (Fig. 16). As expected, anti-*R. pipiens* sera gave strong fluorescence of all three jelly layers (Fig. 16). Following absorption with heterologous (*R. clamitans*, *R. sylvatica*) jelly, the anti-*R. pipiens* jelly sera produced fluorescence in the outer and middle layers, but not the innermost layer (Shivers, 1962). Thus, the innermost of the three

readily visible jelly layers contains only nonspecific antigens, whereas the middle and outer layers contain the species-specific antigens. These antijelly sera caused no fluorescence of the egg proper, confirming that the egg jelly antigens are confined to the jelly itself.

To evaluate the role of the specific and nonspecific jelly antigens, and therefore the innermost as opposed to the two outer jelly layers, Shivers (1961, 1962) tested heterologous (*R. clamitans*, *R. sylvatica*, and *R. catesbiana*) antijelly sera, and jelly-absorbed anti-*R. pipiens* jelly sera for fertilization-inhibiting action. All of the heterologous antisera (anti-*R. clamitans*, anti-*R. sylvatica*, and anti-*R. catesbiana* jelly), inhibited fertilization of *R. pipiens* eggs. Likewise, anti-*R. pipiens* jelly serum,

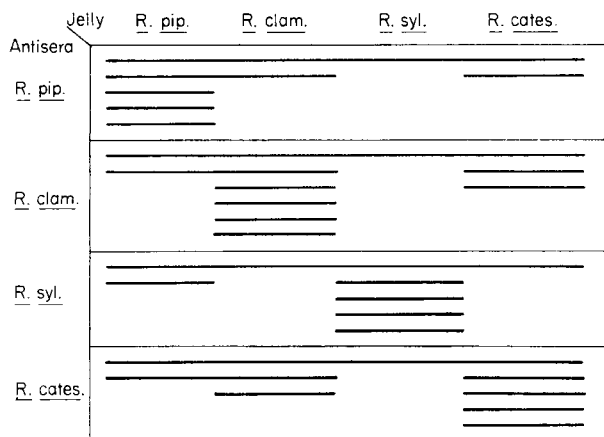


FIG. 15. Interrelationships of antigens in egg jellies of species of *Rana* as determined by agar-gel diffusion analysis. Antigens were determined by reacting the antijelly serum of each species with the various jellies. Common antigens are represented by continuous lines. Lines for each species represent a minimum number of antigenic components. (From Shivers, 1965.)

even following absorption with heterologous jelly, strongly inhibited fertilization of *R. pipiens* eggs. As expected, this antiserum failed to inhibit fertilization following absorption with homologous antigen (*R. pipiens* jelly). As Shivers (1965) noted, the immunologically cross-reacting species considered here will cross-fertilize to some extent. Therefore, the "nonspecific reactions" discussed are still within the fertilization specificity of the system investigated.

These results indicate that at least two jelly antigens are involved in fertilization. One of these is a nonspecific antigen confined to the innermost jelly layer. The other is a species-specific antigen in one or both of the outer jelly layers. Combination of either of these antigens with antibody results in inhibition of fertilization. Since these experiments

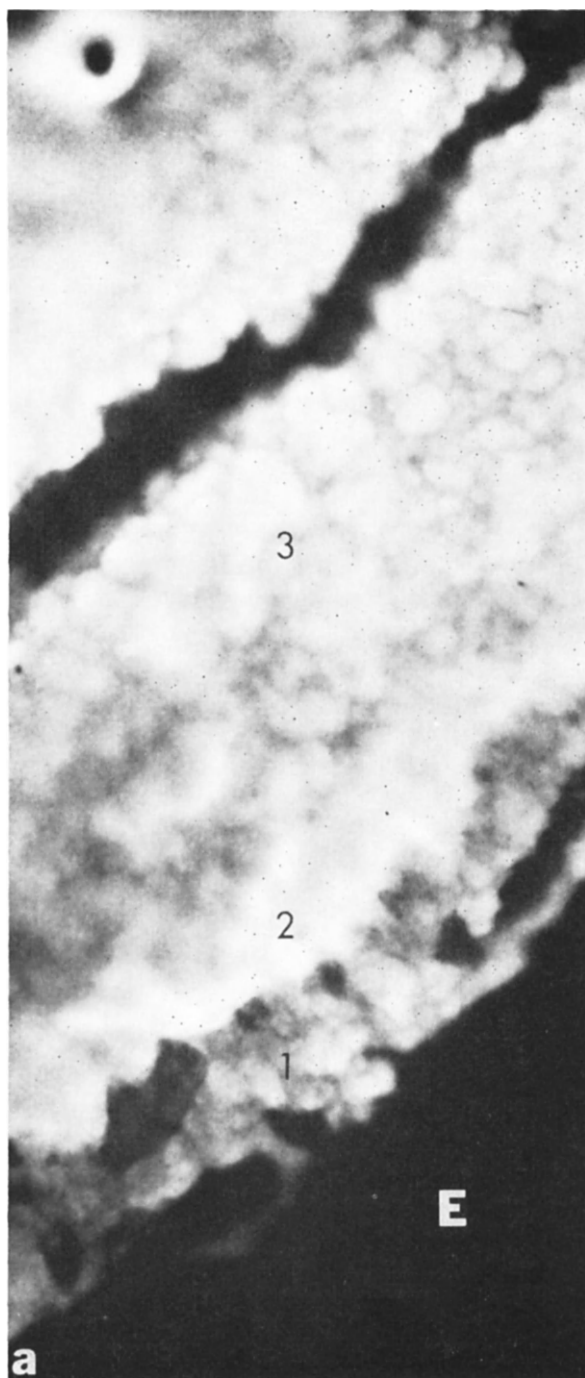


FIG. 16. Localization of antigens in jelly layers of *Rana pipiens* eggs. (a) Egg treated with fluorescein-conjugated anti-*R. pipiens* jelly serum. All jelly layers fluoresce. (E) Egg cytoplasm. (1), (2), (3) Inner, middle, and outer jelly layers,



respectively. Jelly of a second egg appears in the upper right. (b) Egg treated with fluorescein-conjugated anti-*R. sylvatica* jelly serum. Only the innermost layer (1) fluoresces. (From Shivers, 1962, and unpublished.) Approximately $\times 2000$.

were performed with multivalent antibody, fertilization could result from blocking antigenic sites essential for fertilization or from alteration of physical properties of the jelly, e.g., precipitation. At least some antigens have sites essential for fertilization, since univalent, nonprecipitating anti-*R. pipiens* jelly serum inhibits fertilization (Shivers and Metz, 1962). The species-specific and nonspecific antigens of Shivers should now be tested separately for fertilization-inhibiting action using appropriate univalent antibody.

Comparison of Shivers' results with those of Glick and Shaver (1963) are of interest. Eggs removed from the upper oviduct should be coated with at least a part of the innermost jelly layer. Since such eggs do not fertilize, they evidently lack an essential jelly component, at least in effective quantity. Yet, combination of this jelly material with antibody results in fertilization inhibition, according to Shivers (1961), indicating presence of an essential jelly component. This inconsistency may be resolved by repetition of the experiments with univalent antibody. However, the data admit the interesting possibility that two separate egg jelly components are essential in fertilization and that they must act in sequence. The two essential antigens found by Shivers (1961) are obvious candidates for this scheme.

In view of the essential role of the amphibian egg jelly in fertilization, the chemistry of this material should be of unusual interest. However, as seen above, visual, histochemical, and immunological examination shows the amphibian egg jelly to be inhomogeneous and to consist of several macromolecular components. Therefore, analysis of total jelly samples can give guidelines only to the composition of major individual constituents. These constituents must be isolated and analyzed separately to provide information of value for understanding fertilization mechanisms. Analyses of total jelly material have been made in several species and indicate a fairly complete spectrum of amino acids and a number of monosaccharide components (e.g., Folkes *et al.*, 1950; Minganti, 1954; Minganti and D'Anna, 1958).

The role of the essential jelly components in fertilization is not yet evident. However, it appears that the jelly does not have to be organized or oriented in a highly specific pattern at the egg surface, because mechanical enrobement of coelomic eggs or reenrobement of denuded uterine eggs results in fertilizability. Furthermore, at least one essential jelly factor required for fertilization in *Bufo bufo asiatica* (Kambara, 1953) and in *Rana pipiens* (Glick and Shaver, 1963) is located and functions at a distance from the egg surface. This suggests that the action of the jelly is primarily upon the sperm. As suggested by Shivers (see Shaver, 1966), this action may be comparable to the acrosomal

reaction of invertebrate sperms (see Chap. 6) or capacitation changes in mammalian sperms (Austin, 1951, 1963; Bedford, 1963; Chang, 1951). This view is supported by the observation that coelomic eggs give evidence of activation when inseminated in the presence of uterine eggs (Kambara, 1953) or egg jelly solution (Shivers, unpublished data; Shaver, 1966). Although additional experimentation will be necessary for complete understanding of the role of the frog egg jelly in fertilization, the information already at hand establishes this as a rare example of an agent(s) clearly not of gametic origin which performs a direct, essential, and possibly species-specific role in fertilization.

B. ROLE OF EGG CORTEX ANTIGENS

As seen above, the amphibian egg jelly performs some essential function in fertilization. The egg surface and cortex would also be expected to contain substances essential for fertilization, and Nace and Lavin (1963) recently showed this to be the case. The amphibian egg is uniquely favorable for study of the separate roles of jelly and egg cell proper in fertilization because coelomic eggs, which lack the jelly, can be removed from one female and subjected to experimental treatment. Subsequently, they can be placed in the body cavity of a second female and finally recovered from the uterus properly enrobed with jelly, including any jelly factors essential for fertilization (see Lavin, 1964, for reference). The technique, as recently improved by Lavin (1964), is simple, and control eggs give a high percentage of fertilization even if held in Holtfreter's solution for over 12 hours before transfer to the second female.

If coelomic eggs are treated with certain antisera before passage to the second female, subsequent fertilization and development are markedly affected. It should be noted that no antisera have been reported to have parthenogenetic action in the amphibian (Nace and Lavin, 1963). As seen in Table XIV, antisera to embryo and oocyte extracts are particularly inhibitory to fertilization. Agar-diffusion precipitin analysis of these various sera and the immunizing antigens showed one major antigenic component in common, "antigen F." Considerable purification of this antigen was achieved by ammonium sulfate precipitation and DEAE-cellulose column fractionation of ovary extracts. Antisera to such purified antigen F produce one main precipitin band when diffused against ovary extract and strongly inhibit fertilization and cleavage (Table XIV). This inhibition clearly results from action on the egg, because treatment of the sperm with the antiserum had no inhibitory effect on fertilization. Nace and Lavin (1963) concluded that the antibody either prevents sperm-egg interaction or blocks some essential step

TABLE XIV
ACTIVATION AND CLEAVAGE OF INSEMINATED UTERINE EGGS EXPOSED TO VARIOUS TREATMENTS PRIOR TO PASSAGE
THROUGH THE OVIDUCT^{a,b}

Treatment	No. of tests	No. of eggs	No. activated	% activated	Normal cleavage	Cleavage, as % activated	Abnormal cleavage	Abnormal as % total cleavage
Standard Holtfreter's solution	8	457	350	77	263	75	21	7
Normal rabbit serum	12	758	531	70	366	69	15	4
Rabbit antiserum to:								
Adult frog serum	7	362	174	48	71	41	34	32
Oviduct supernatant	3	174	91	52	51	56	8	14
Large oocyte supernatant	4	258	62	24	10	16	18	64
Neurula supernatant	2	119	23	19	0	0	12	100
Tailbud supernatant	2	147	25	17	3	12	6	67
Oocyte lysozyme	2	171	119	69	102	87	12	10
50 % (NH ₄) ₂ SO ₄ -precipitate from large oocyte supernatant (did not contain antifertilizin)	2	118	4	3	0	0	0	0
Fraction obtained from large oocyte supernatant (antifertilizin)	9	453	78	17	2	3	6	75

^a Rotation was taken to be indicative of activation.

^b Nace and Lavin, 1963.

in the response mechanism of the egg. They found in one experiment that the antibody treatment resulted in inhibition of eggs to parthenogenetic activation. This suggests blocking at some step in the activation-response mechanism.

Immunofluorescence studies show localization of antigen F in the cytoplasm and especially the cortex of mature oocytes. Tests for antigen F in follicle cells and oviduct were negative. Therefore, antigen F is distinct from another antigen, the "large oocyte supernatant" (LOS) antigen (Nace *et al.*, 1961) found in eggs, follicle cells, and oviduct, according to Nace and Lavin (1963). The LOS antigen extract (Nace *et al.*, 1961) is the same as the source material for antigen F, namely 10,000 g supernatant of ovary homogenate. Presumably the LOS antigen is separated from antigen F during precipitation of the latter.

Other antigens of the egg proper may also be involved in fertilization, for antisera lacking antibodies to antigen F also inhibit fertilization. In fact, the most inhibitory antiserum recorded by Nace and Lavin (1963) lacked antibody to antigen F. It is likely, then, that further analysis will reveal a constellation of egg antigens that function in fertilization. Identification and localization of these antigens and understanding of their interrelations and functions should contribute much to knowledge of molecular mechanisms in fertilization.

VIII. Summary and Conclusions

During its final approach to the egg the sperm is exposed to gradients of substances diffusing from the egg, and at least to a limited extent, the reverse is also true. Probably, in response to one or more such egg substances, the spermatozoon undergoes the acrosomal reaction. This results in release of egg membrane lysins and exposure of a new and possibly qualitatively different acrosomal surface for interaction with the egg. Finally, this acrosomal surface contacts and fuses with the egg plasma membrane to unite the two gametes.

These events are characterized by a high order of species and in some cases even individual specificity (e.g., self-sterility in hemophroditic ascidians). Such specificity implies interaction of sperm and egg macromolecules in enzyme-substrate or antigen-antibodylike fashion.

Appropriate study reveals that both the sperm and egg surfaces are complex. The sperm-limiting membrane is the plasma membrane of the cell and presents a typical trilaminar appearance on ultrastructural examination. No visible accessory coats or membranes are found. Nevertheless, immunological study shows that the surface of the plasma membrane is a composite of substances, or at least molecular patterns

(e.g., antigens), some of which have limited regional distribution. Some of these antigens, at least in certain mammals, are derived secondarily by absorption from the fluids of the male accessory glands and may be considered analogous to extracellular coats of secondary origin that surround eggs of many species. The sperm acrosomal region is of special interest. A study of its surface structure at the molecular level before and after the acrosomal reaction should be most rewarding.

More recent work on mammalian and especially human sperm has concentrated on examination for known antigens specified by the genotype of the individual. Prominent among these are erythrocyte antigens. The antigens of the ABO system are certainly present on the sperms of secretor individuals of the appropriate genotype. Absence of these antigens from sperm of nonsecretors suggests that these antigens are adsorbed to sperm secondarily from the seminal plasma.

On the basis of cytochemical study of spermiogenesis, it seems unlikely that sperms in heterozygous individuals would show antigenic differences specified by the haploid genotype. It is not surprising, then, that attempts to demonstrate antigenic differences between X- and Y-bearing sperms have so far failed, and efforts to show two classes of sperms attributable to the haploid genotype in heterozygous individuals leave something to be desired. The most encouraging results are from studies of sperms from blood group AB men. The studies of mammalian sperm-surface antigens are most interesting, but, unfortunately, it will be extremely difficult to relate these antigens to direct involvement in fertilization until techniques for *in vitro* fertilization of the mammalian egg have been improved.

Unlike the sperm, the egg usually has one or more extracellular layers. The vitelline membrane is immediately outside the plasma membrane. Additional layers may include egg jelly coats, follicle cell layers, a chorion, or a combination of these. The terms are descriptive and do not necessarily indicate homologies of function or origin in diverse species.

Interaction of sperm and egg surfaces and the role of surface substances in fertilization have been investigated most thoroughly in sea urchins and amphibians among metazoans. The thinking on this subject has been dominated by the fertilizin-antifertilizin concept for over 50 years. Fertilizin is the major component of the sea urchin egg jelly. In solution it specifically agglutinates the sperm of the species by combination with a sperm-surface substance, antifertilizin. This specific isoagglutination has been demonstrated in some, but not all, species tested in several phyla. Special methods have revealed the same or comparable systems in other groups. Accordingly, it appears that a fertilizin-anti-

fertilizin system is of wide, if not universal, occurrence. Evidence from a variety of experimental approaches indicates that a fertilizin-antifertilizin interaction is an important and probably essential step in fertilization. The fertilizin-antifertilizin interaction probably initiates the acrosomal reaction, functions in attachment and fusion of the gametes, and contributes a primary specificity factor in fertilization. In addition, surface antigens of the sperm, and probably the egg as well, have some essential, but as yet unknown, role in sea urchin fertilization.

The amphibian egg jelly is produced by the oviduct. One or more of the jelly components is essential in fertilization. The exact function of the jelly is unknown, but it appears to affect the sperm, possibly by a mechanism comparable to initiation of the acrosomal reaction. Immunological studies show that some essential jelly components are antigenic. In addition, egg surface or cortical antigens also appear to have some essential role in fertilization.

It appears, then, that the morphological events leading up to the initiation of development are known with a high precision, but the molecular events are only imperfectly understood. Details of the acrosomal reaction and sperm-egg fusion are especially in need of additional study at the molecular level. Since several laboratories are active in this field, all the essential molecular events leading up to and including sperm-egg fusion should soon be defined in detail. Likewise, knowledge of the biochemical consequences of egg activation are under intensive study. These, too, should be thoroughly understood in the near future. But the chain of molecular events leading from the primary activation-initiating reaction through the propagated-response mechanisms of the egg to the metabolic and morphogenetic consequences of fertilization still remain obscure.

REFERENCES

- Austin, C. R. (1951). Observations on the penetration of the sperm into the mammalian egg. *Australian J. Sci. Res. (B)* 4, 581-586.
- Austin, C. R. (1952). The "capacitation" of the mammalian sperm. *Nature* 170, 326-327.
- Austin, C. R. (1961). "The Mammalian Egg." Blackwell, Oxford.
- Austin, C. R. (1963). Acrosome loss from the rabbit spermatozoon in relation to entry into the egg. *J. Reprod. Fertility* 6, 313-314.
- Austin, C. R. (1965). "Fertilization." Prentice-Hall, Englewood Cliffs, New Jersey.
- Austin, C. R., and Bishop, M. W. H. (1957). Preliminaries to fertilization in mammals. In "The Beginnings of Embryonic Development" (A. Tyler, R. C. Von Borstel, and C. B. Metz, eds.), pp. 71-107. Am. Assoc. Advance. Sci., Washington, D.C.
- Barch, S. H., and Shaver, J. R. (1963). Regional antigenic differences in frog oviduct in relation to fertilization. *Am. Zoologist* 3, 157-165.

- Barth, R. F., and Russell, P. S. (1964). The antigenic specificity of spermatozoa. I. An immunofluorescent study of the histocompatibility antigens of mouse sperm. *J. Immunol.* **93**, 13-19.
- Barthélémy, M. H. (1922). Sur la maturation *in vitro* et l'activation par piqûre des oeufs ovariens de *Rana fusca* à l'époque de la ponte. *Compt. Rend.* **175**, 1248-1249.
- Baxandall, J. (1964). Sperm antigens in sperm and fertilized eggs. *Proc. 3rd European Regional Conf. Electron Microscopy B*, 463-464. Publishing House Czech. Acad. Sci., Prague.
- Baxandall, J., Perlmann, P., and Afzelius, B. A. (1964a). Immuno-electron microscope analysis of the surface layers of the unfertilized sea urchin egg. I. Effects of the antisera on the cell ultrastructure. *J. Cell Biol.* **23**, 609-628.
- Baxandall, J., Perlmann, P., and Afzelius, B. A. (1964b). Immuno-electron microscope analysis of the surface layers of the unfertilized sea urchin egg. II. Localization of surface antigens. *J. Cell Biol.* **23**, 629-650.
- Bedford, J. M. (1963). Morphological reaction of spermatozoa in the female reproductive tract of the rabbit. *J. Reprod. Fertility* **6**, 245-255.
- Bedford, J. M. (1964). Fine structure of the sperm head in ejaculate and uterine spermatozoa of the rabbit. *J. Reprod. Fertility* **7**, 221-228.
- Bedford, J. M. (1965). Development of the fertilizing ability of spermatozoa in the rabbit epididymis. *J. Reprod. Fertility* **10**, 286-287.
- Berg, W. E. (1949). Some effects of sperm extracts on the eggs of *Mytilus*. *Am. Naturalist* **83**, 221-226.
- Berg, W. E. (1950). Lytic effects of sperm extracts on the eggs of *Mytilus edulis*. *Biol. Bull.* **98**, 128-138.
- Bischoff, E. R., and Metz, C. B. (1962a). Neutralization of the fertilization inhibitors in anti-*Arbacia* sperm serum by sperm extracts. *Biol. Bull.* **123**, 470-471.
- Bischoff, E. R., and Metz, C. B. (1962b). Immunological identification of an egg agglutinin in *Arbacia* sperm extracts. *Biol. Bull.* **123**, 470-471.
- Bishop, D. W. (1961). Aspermatogenesis induced by testicular antigen uncombined with adjuvant. *Proc. Soc. Exptl. Biol. Med.* **107**, 116-120.
- Boettcher, B. (1965). Human ABO blood group antigens on spermatozoa from secretors and non-secretors. *J. Reprod. Fertility* **9**, 267-268.
- Branham, J. M., and Metz, C. B. (1960). Inhibition of fertilizin agglutination and fertilization in *Arbacia* by *Fucus* extracts. *Biol. Bull.* **122**, 194-207.
- Bril-Petersen, E., and Westenbrink, H. G. H. (1963). A structural basic protein as a counterpart of deoxyribonucleic acid in mammalian spermatozoa. *Biochim. Biophys. Acta* **76**, 152-154.
- Brock, T. D. (1959). Biochemical basis of mating in yeast. *Science* **129**, 960-961.
- Brookbank, J. W. (1958). Dispersal of the gelatinous coat material of *Mellita quinquesperforata* eggs by homologous sperm and sperm extracts. *Biol. Bull.* **115**, 74-80.
- Brookbank, J. W. (1959a). The respiration of unfertilized sea urchin eggs in the presence of antisera against fertilizin. *Biol. Bull.* **116**, 217-225.
- Brookbank, J. W. (1959b). The presence of fertilizin haptens within the unfertilized sea urchin egg. *Biol. Bull.* **117**, 63-67.
- Brookbank, J. W. (1964). The effect of antisera against fertilizin on the uptake of orthophosphate by sea urchin eggs. *Biol. Bull.* **126**, 1-7.
- Brown, G. G. (1966). Ultrastructural studies of sperm morphology and sperm-egg interaction in the decapod *Callinectes sapidus*. *J. Ultrastruct. Res.* **14**, 425-440.

- Brown, P. C., Holborow, E. J., and Glynn, L. E. (1965). The aspermatogenic antigen in experimental allergic orchitis in guinea pigs. *Immunology* **9**, 255-260.
- Byers, H. L. (1951). Failure to obtain "cytofertilizin" from *Arbacia* eggs. *Biol. Bull.* **101**, 218.
- Chang, M. C. (1951). Fertilizing capacity of spermatozoa deposited into the Fallopian tubes. *Nature* **168**, 697-698.
- Chang, M. C. (1957). A detrimental effect of seminal plasma on the fertilizing capacity of sperm. *Nature* **179**, 258-259.
- Cohen, S., and Porter, R. R. (1964). Structure and biological activity of immunoglobulins. *Advan. Immunol.* **4**, 287-342.
- Collier, J. R. (1959). The effect of homologous fertilizin on the sperm of *Strongylocentrotus purpuratus*. *Acta Embryol. Morphol. Exptl.* **2**, 163-170.
- Colwin, A. L., and Colwin, L. H. (1960). Egg membrane lytic activity of sperm extract and its significance in relation to sperm entry in *Hydroides hexagonus* (Annelida). *J. Biophys. Biochem. Cytol.* **7**, 321-328.
- Colwin, A. L., and Colwin, L. H. (1961). Changes in the spermatozoon during fertilization in *Hydroides hexagonus* (Annelida). II. Incorporation with the egg. *J. Biophys. Biochem. Cytol.* **10**, 255-274.
- Conklin, E. G. (1905). The organization and cell-lineage of the ascidian egg. *J. Acad. Nat. Sci.* **13** (Pt. 1), 5-119.
- Costello, D. P. (1949). The relations of the plasma membrane, vitelline membrane, and jelly in the egg of *Nereis limbata*. *J. Gen. Physiol.* **32**, 351-366.
- Cushing, J. E., and Campbell, D. H. (1957). "Principles of Immunology." McGraw-Hill, New York.
- Dalcq, A., Pasteels, J., and Brachet, J. (1936). Données nouvelles *Asterias glacialis*, *Phascolion strombi*, *Rana fusca* et considérations théoriques sur l'inertie de l'oeuf vierge. *Mémoires du Musée Royal d'Histoire Naturelle de Belgique* (2), fasc. 3, 881-912.
- Dan, J. C. (1954). Studies on the acrosome. III. Effect of calcium deficiency. *Biol. Bull.* **107**, 335-349.
- Dan, J. C. (1956). The acrosome reaction. In "International Review of Cytology" (G. H. Bourne and J. F. Danielli, eds.), Vol. 5, pp. 365-393. Academic Press, New York.
- Dan, J. C. (1962). The vitelline coat of the *Mytilus* egg. I. Normal structure and effect of acrosomal lysin. *Biol. Bull.* **123**, 531-541.
- Docton, F. L., Ferguson, L. C., Lazear, E. J., and Ely, F. (1952). The antigenicity of bovine spermatozoa. *J. Dairy Sci.* **35**, 706-709.
- Edwards, R. G., Ferguson, L. C., and Coombs, R. R. A. (1964). Blood group antigens on human spermatozoa. *J. Reprod. Fertility* **7**, 153-161.
- Eichwald, E. J. (1962). Sex-specific graft rejection and selective fertility control. In "Proceeding of the Conference on Immuno-Reproduction" (A. Tyler and K. A. Lawrence, eds.), pp. 175-179. Population Council, New York.
- Esping, U. (1957a). A factor inhibiting fertilization of sea urchin eggs from extracts of the alga *Fucus vesiculosus*. I. The preparation of the factor inhibiting fertilization. *Arkiv Kemi* **11**, 107-115.
- Esping, U. (1957b). A factor inhibiting fertilization of sea urchin eggs from extracts of the alga *Fucus vesiculosus*. II. The effect of the factor inhibiting fertilization on some enzymes. *Arkiv Kemi* **11**, 117-127.
- Ezell, S. D. (1963). The lateral body of *Ciona intestinalis* spermatozoa. *Exptl. Cell Res.* **30**, 615-617.

- Fawcett, D. W. (1958). The structure of mammalian spermatozoon. In "International Review of Cytology" (G. H. Bourne and J. F. Danielli, eds.), Vol. 7, pp. 195-232. Academic Press, New York.
- Fawcett, D. W. (1965). The anatomy of the mammalian spermatozoon with particular reference to the guinea pig. *Z. Zellforsch. Mikroskop. Anat. Abt. Histochem.* **67**, 279-296.
- Flake, G. P., and Metz, C. B. (1962). Soluble surface and subsurface antigens of the *Arbacia* sperm. *Biol. Bull.* **123**, 472-473.
- Folkes, B. F., Grant, R. A., and Jones, J. K. N. (1950). Frog spawn mucin. *J. Chem. Soc. Pt. III*, pp. 2136-2140.
- Franklin, L. E. (1965). Morphology of gamete membrane fusion and of sperm entry into oocytes of the sea urchin. *J. Cell Biol.* **25**, 81-100.
- Freund, J., Lipton, M. M., and Thompson, G. E. (1953). Aspermatogenesis in the guinea pig induced by testicular tissue and adjuvants. *J. Exptl. Med.* **97**, 711-726.
- Freund, J., Thompson, G. E., and Lipton, M. M. (1955). Aspermatogenesis, anaphylaxis, and cutaneous sensitization induced in the guinea pig by homologous testicular extract. *J. Exptl. Med.* **101**, 591-603.
- Ginsburg, A. S. (1963). Sperm-egg association and its relationship to the activation of the egg in salmonid fishes. *J. Embryol. Exptl. Morphol.* **11**, 13-33.
- Glick, R. N., and Shaver, J. R. (1963). The fertilizability of frog eggs from different levels of the female reproductive tract. *Exptl. Cell Res.* **32**, 615-618.
- Good, G. M., and Daniel, J. F. (1943). Fertilization of coelomic eggs of *Triturus torosus*. *Univ. Calif. Publ. Zool.* **51**, 149-158.
- Grabar, P., and Williams, C. A., Jr. (1953). Méthode permettant l'étude conjuguée des propriétés électrophorétiques d'un mélange de protéines. Application au sérum sanguin. *Biochim. Biophys. Acta* **10**, 193-194.
- Graziano, K. D., and Metz, C. B. (1967). Failure of papain digested antibody to inhibit fertilization of *Arbacia punctulata* eggs, *Exptl. Cell Res.* (in press).
- Green, W. W. (1940). The chemistry and cytology of the sperm membrane of sheep. *Anat. Record* **76**, 455-473.
- Gregg, K. W. (1966). A comparison of fertilizin and cytofertilizin from eggs of *Arbacia punctulata*. Masters thesis, Emory Univ., Atlanta, Georgia.
- Gregg, K. W., and Metz, C. B. (1966). A comparison of fertilizin and cytofertilizin from eggs of *Arbacia punctulata*. *Assoc. Southeast. Biol. Bull.* **13**, 34.
- Gullbring, B. (1957). Investigation on the occurrence of blood group antigens in spermatozoa from man, and serological demonstration of the segregation of characters. *Acta Med. Scand.* **159**, 169-172.
- Hagström, B. E. (1956). On "cytofertilizin" from sea urchins. *Exptl. Cell Res.* **11**, 160-168.
- Haino, K., and Dan, J. C. (1961). Some quantitative aspects of the acrosomal reaction to jelly substance in the sea urchin. *Embryologia (Nagoya)* **5**, 376-383.
- Harding, C. B. (1951). The action of polysaccharides on fertilization in sea urchins. *Exptl. Cell Res.* **2**, 403-415.
- Hartree, E. F., and Srivastava, P. N. (1965). Chemical composition of the acrosome of ram spermatozoa. *J. Reprod. Fertility* **9**, 47-60.
- Hathaway, R. R., and Hartree, E. F. (1963). Observations on the mammalian acrosome: experimental removal of acrosomes from ram and bull spermatozoa. *J. Reprod. Fertility* **5**, 225-232.
- Hathaway, R. R., and Metz, C. B. (1961). Interactions between *Arbacia* sperm and S³⁵-labelled fertilizin. *Biol. Bull.* **120**, 360-369.

- Hathaway, R. R., Warren, L., and Flaks, J. G. (1960). Egg jelly dispersal by *Arbacia* sperm extracts. *Biol. Bull.* **119**, 319.
- Hauschka, S. D. (1963). Purification and characterization of *Mytilus* egg membrane lysin from sperm. *Biol. Bull.* **125**, 363.
- Hauschka, S. D. (1964). Hyaline membrane lysis in *Mytilus edulis* eggs. *Biol. Bull.* **127**, 373.
- Henle, W. (1938). The specificity of some mammalian spermatozoa. *J. Immunol.* **34**, 325-336.
- Henle, W., Henle, G., and Chambers, L. S. (1938). Studies on the antigenic structure of some mammalian spermatozoa. *J. Exptl. Med.* **68**, 335-352.
- Holborow, E. J., Brown, P. C., Glynn, L. E., Hawes, M. D., Gresham, G. A., O'Brien, T. F., and Coombs, R. R. A. (1960). The distribution of the blood group A antigen in human tissues. *Brit. J. Exptl. Pathol.* **41**, 430-437.
- Hope, J., Humphries, A. A., and Bourne, G. H. (1963). Ultrastructural studies on developing oocytes of the salamander *Triturus viridescens*. I. The relationship between follicle cells and developing oocytes. *J. Ultrastruct. Res.* **9**, 302-324.
- Hultin, T. (1947). On the question of sperm antifertilizin. *Pubbl. Staz. Zool. Napoli* **21**, 153-163.
- Hultin, T. (1948a). Species specificity in fertilization reaction. I. The role of the vitelline membrane of sea-urchin eggs in species specificity. *Arkiv Zool.* **40A**, 1-9.
- Hultin, T. (1948b). Species specificity in fertilization reaction. II. Influence of certain factors on the cross-fertilization capacity of *Arbacia lixula* (L.). *Arkiv Zool.* **40A**, 1-8.
- Hultin, T. (1949). Agglutination of sea urchin eggs by nucleoproteins. *Arkiv Kemi* **1**, 419-423.
- Humphreys, W. J. (1962). Electron microscope studies on eggs of *Mytilus edulis*. *J. Ultrastruct. Res.* **7**, 467-487.
- Humphreys, W. J. (1964). Electron microscope studies on the fertilized egg and the two-cell stage of *Mytilus edulis*. *J. Ultrastruct. Res.* **10**, 244-262.
- Humphries, A. A., Jr. (1956). A study of meiosis in coelomic and oviducal oocytes of *Triturus viridescens*, with particular emphasis on the origin of spontaneous polyploidy and the effects of heat shock on the first meiotic division. *J. Morphol.* **99**, 97-136.
- Humphries, A. A., Jr. (1964). A study of the jelly envelopes of the oocyte of the newt, *Triturus viridescens*, with particular reference to their reactions to several procedures of polysaccharide cytochemistry. *Am. Zoologist* **4**, 403.
- Humphries, A. A., Jr., and Hughes, W. N. (1959). A study of the polysaccharide histochemistry of the oviduct of the newt, *Triturus viridescens*. *Biol. Bull.* **116**, 446-451.
- Hunter, A. G., and Hafs, H. D. (1964). Antigenicity and cross-reactions of bovine spermatozoa. *J. Reprod. Fertility* **7**, 357-365.
- Ishihara, K. (1964). Release of acid polysaccharides following fertilization of sea urchin eggs. *Exptl. Cell Res.* **36**, 354-367.
- Kabat, E. C., and Mayer, M. M. (1961). "Experimental Immunochemistry." Thomas, Springfield, Illinois.
- Kambara, S. (1953). Role of jelly envelopes of toad eggs in fertilization. *Annotationes Zool. Japan.* **26**, 78-84.
- Katagiri, C. (1962). On the fertilizability of the frog egg. II. Changes of the jelly envelopes in water. *Japan. J. Zool.* **13**, 365-373.
- Katagiri, C. (1963). Fertilizability of the egg of *Hyla arborea japonica*, with special reference to the change of jelly envelopes. *Zool. Mag. (Tokyo)* **72**, 23-28.

- Katsh, S. (1960a). Localization and identification of antispermatogenic factor in guinea pig testicles. *Intern. Arch. Allergy Appl. Immunol.* **16**, 241-275.
- Katsh, S. (1960b). The anaphylactogenicity of testicular hyaluronidase and a species difference in testicular hyaluronidase demonstrated by isolated organ anaphylaxis. *Intern. Arch. Allergy Appl. Immunol.* **17**, 70-79.
- Katsh, S. (1963). Studies on immunological aspects of reproduction in laboratory animals and man. *Proc. 7th Intern. Conf. on Planned Parenthood, Singapore*, pp. 513-526.
- Katsh, S. (1964). Adjuvants and aspermatogenesis in the guinea pig. *Intern. Arch. Allergy Appl. Immunol.* **24**, 319-331.
- Katsh, S., and Bishop, D. W. (1958). The effects of homologous testicular and brain and heterologous testicular homogenates combined with adjuvant upon the testes of guinea pigs. *J. Embryol. and Exptl. Morphol.* **6**, 94-104.
- Katsh, S., and Katsh, G. F. (1961). Antigenicity of spermatozoa. *Fertility and Sterility* **12**, 522-537.
- Katsh, S., and Katsh, G. F. (1965a). Aspermatogenic antigen from brain. *Experientia*, **XXI** 8, 442-443.
- Katsh, S., and Katsh, G. F. (1965b). Perspectives in immunological control of reproduction, past, present and future. *Pacific Medicine and Surgery (Ortho-symposium)*, **73**, 28-43.
- Katsh, G. F., Talmage, D. W., and Katsh, S. (1964). Acceptance or rejection of male skin by isologous female mice: effect of injection of sperm. *Science* **143**, 41-42.
- Kessel, R. G. (1965). Intranuclear and cytoplasmic annulate lamellae in tunicate oocytes. *J. Cell Biol.* **24**, 471-487.
- Kessel, R. G., and Kemp, N. E. (1962). An electron microscope study on the oocyte, test cells and follicular envelope of the tunicate, *Molgula manhattensis*. *J. Ultrastruct. Res.* **6**, 57-76.
- Kirkpatrick, C. H., and Katsh, S. (1964). Amino-acid content of antispermatogenic antigen. *Nature* **201**, 197-198.
- Kloetzel, J. A., and Metz, C. B. (1963). Studies on the soluble antigens of the sperm of *Arbacia punctulata*. *Biol. Bull.* **125**, 363-364.
- Köhler, K., and Metz, C. B. (1960). Antigens of the sea urchin sperm surface. *Biol. Bull.* **118**, 96-110.
- Krauss, M. (1950). Lytic agents of the sperm of some marine animals. I. The egg membrane lysin from sperm of the giant keyhole limpet, *Megathura crenulata*. *J. Exptl. Zool.* **114**, 239-278.
- Landsteiner, K. (1899). Zur Kenntnis der spezifisch auf Blutkörperchen wirkenden Sera. *Centralblatt* **25**, 546-549.
- Landsteiner, K., and Levine, P. (1926). On group specific substances in human spermatozoa. *J. Immunol.* **12**, 415-418.
- Larson, B. L., Gray, R. S., and Salisbury, G. W. (1954). The proteins of bovine seminal plasma. II. Ultracentrifugal and immunological studies and comparison with blood and milk serum. *J. Biol. Chem.* **211**, 43-52.
- Lavin, L. H. (1964). The transfer of coelomic eggs between frogs. *J. Embryol. Exptl. Morphol.* **12**, 457-463.
- Levine, P., and Celano, M. J. (1961). The question of D (Rho) antigenic sites on human spermatozoa. *Vox Sanguinis* **6**, 720-723.
- Lillie, F. R. (1912). The production of sperm iso-agglutinins by ova. *Science* **36**, 527-530.
- Lillie, F. R. (1913). The mechanism of fertilization. *Science* **38**, 524-528.
- Lillie, F. R. (1914). Studies of fertilization. VI. The mechanism of fertilization in *Arbacia*. *J. Exptl. Zool.* **16**, 523-590.

- Lillie, F. R. (1919). "Problems of Fertilization." Univ. of Chicago Press, Chicago, Illinois.
- Loeb, J. (1915). On the nature of the conditions which determine or prevent the entrance of the spermatozoon into the egg. *Amer. Naturalist* **49**, 257-285.
- Majsky, A., and Hraba, T. (1960). Demonstration of D (Rho) agglutinogens in human spermatozoa. *Folia Biol. (Prague)* **6**, 342-347.
- McCartney, J. L. (1923). Studies on the mechanism of sterilization of the female by spermotoxin. *Am. J. Physiol.* **63**, 207-217.
- Mancini, R. E., Alonso, A., Barquet, J., Alvareg, B., and Nemirovsky, M. (1964). Histo-immunological localization of hyaluronidase in the bull testes. *J. Reprod. Fertility* **8**, 325-330.
- Mann, T. (1964). "Biochemistry of Semen and the Male Reproductive Tract." Methuen, London.
- Matousek, J. (1964a). Antigenic characteristics of spermatozoa from bulls, rams and boars. I. Erythrocytic antigens in bull spermatozoa. *J. Reprod. Fertility* **8**, 1-3.
- Matousek, J. (1964b). Antigenic characteristics of spermatozoa from bulls, rams and boars. III. Absorption analysis, precipitins and fructolysis in relation to the antigenicity of bull spermatozoa. *J. Reprod. Fertility* **8**, 13-21.
- Menge, A. C., Stone, W. H., Tyler, W. J., and Casida, L. E. (1962). Immunological studies on fertility and sterility. V. Effect of antibodies against semen and erythrocytes on rabbit embryos. *J. Reprod. Fertility* **3**, 331-341.
- Metz, C. B. (1942). The inactivation of fertilizin and its conversion to the "univalent" form by X-rays and ultraviolet light. *Biol. Bull.* **82**, 446-454.
- Metz, C. B. (1945). The agglutination of starfish sperm by fertilizin. *Biol. Bull.* **89**, 84-94.
- Metz, C. B. (1949). Agglutination of sea urchin eggs and sperm by basic proteins. *Proc. Soc. Exptl. Biol. Med.* **70**, 422-424.
- Metz, C. B. (1954). Mating substances and the physiology of fertilization in ciliates. In "Sex in Microorganisms," pp. 284-334. Am. Assoc. Advance. Sci., Washington, D.C.
- Metz, C. B. (1957a). Mechanisms in fertilization. In "Physiological Triggers" (T. H. Bullock, ed.), pp. 17-45. Am. Physiol. Soc., Washington, D.C.
- Metz, C. B. (1957b). Specific egg and sperm substances and activation of the egg. In "Beginnings of Embryonic Development" (A. Tyler, R. C. Von Borstel, and C. B. Metz, eds.), pp. 23-69. Am. Assoc. Advance. Sci., Washington, D.C.
- Metz, C. B. (1959). Inhibition of fertilizin agglutination of sperm by the dermal secretion from *Arbacia*. *Biol. Bull.* **116**, 472-483.
- Metz, C. B. (1960). Investigation of the fertilization inhibiting action of *Arbacia* dermal secretion. *Biol. Bull.* **118**, 439-450.
- Metz, C. B. (1961a). Use of inhibiting agents in studies on fertilization mechanisms. *Intern. Rev. Cytol.* **11**, 219-253.
- Metz, C. B. (1961b). Fertilization studies using inhibitors. In "Germ Cells and Earliest Stages of Development" (S. Ranzi, ed.), pp. 175-196. Symp. Intern. Inst. Embryol., Pallanza, Italy, 1960. Fondazione A. Baselli, Milan.
- Metz, C. B., and Köhler, K. (1960). Antigens of *Arbacia* sperm extracts. *Biol. Bull.* **119**, 202-209.
- Metz, C. B., Schuel, H., and Bischoff, E. R. (1964). Inhibition of the fertilizing capacity of sea urchin sperm by papain digested, non-agglutinating antibody. *J. Exptl. Zool.* **155**, 261-272.
- Metz, C. B., and Thompson, P. H. (1967). Effect of papain digested, univalent anti-

- body on the morphology, cleavage, and fertilizing capacity of sea urchin eggs. *Exptl. Cell Res.* (in press).
- Minganti, A. (1954). Sui glicide della gelatina ovulare degli Anfibi. *Ric. Sci.* **24**, 1658–1661.
- Minganti, A. (1959). Lo sviluppo embrionale e il comportamento dei cromosomi in ibridi tra 5 Specie di Ascidie. *Acta Embryol. Morphol. Exptl.* **2**, 269–301.
- Minganti, A., and D'Anna, T. (1958). Sulla composizione ovulare de *Discoglossus pictus*. *Ric. Sci.* **10**, 2090–2094.
- Mittal, K. K., Salisbury, G. W., Graves, C. N., and Rasmusen, B. A. (1965). Antigens of bovine semen and the influence of specific rabbit anti-bull-semen serum on metabolic activity of bull spermatozoa. *J. Reprod. Fertility* **10**, 29–34.
- Monesi, V. (1965). Synthetic activities during spermatogenesis in the mouse: RNA and protein. *Exptl. Cell Res.* **39**, 197–244.
- Monné, L., and Harde, S. (1951). On the cortical granules of the sea urchin egg. *Arkiv Zool., Ser. 2* **1**, 487–498.
- Monné, L., and Slautterback, D. B. (1950). Differential staining of various polysaccharides in sea urchin eggs. *Exptl. Cell Res.* **1**, 477–491.
- Monroy, A. (1965). "Chemistry and Physiology of Fertilization." Holt, New York.
- Monroy, A., and Runnström, J. (1952). A cytoplasmic fraction acting on the surface layers of the *Arbacia punctulata* eggs. *Exptl. Cell Res.* **3**, 10–18.
- Morgan, T. H. (1923). Removal of the block to self-fertilization in the ascidian *Ciona*. *Proc. Natl. Acad. Sci.* **9**, 170–174.
- Moses, M. J. (1961). Spermiogenesis in the crayfish *Procambarus clarkii*. *Biophys. Biochem. Cytol.* **9**, 222–228.
- Motomura, I. (1950). On the secretion of fertilizin in the eggs of a sea urchin, *Strongylocentrotus pulcherrimus*. *Sci. Rep. Tôhoku Univ., Ser. 4* **18**, 554–560.
- Motomura, I. (1953a). Secretion of sperm agglutinin in the fertilized eggs of sea urchins. *Exptl. Cell Res.* **5**, 187–190.
- Motomura, I. (1953b). Secretion of the sperm agglutinin in the fertilized and denuded eggs of the sea urchin, *Strongylocentrotus nudis*. *Sci. Rep. Tôhoku Univ., Ser. 4* **20**, 93–97.
- Nace, G. W., and Lavin, L. H. (1963). Heterosynthesis and autosynthesis in the early stages of Anuran development. *Am. Zoologist* **3**, 193–207.
- Nace, G. W., Suyama, T., and Smith, N. (1961). Early development of special proteins. In "Germ Cells and Earliest Stages of Development" (S. Ranzi, ed.), pp. 564–603. Symp. Intern. Inst. Embryol., Pallanza, Italy, 1960. Fondazione A. Baselli, Milan.
- Nisonoff, A., and Inman, F. P. (1965). Structural basis of the specificity of antibodies. In "Reproduction: Molecular, Subcellular, and Cellular" (M. Locke, ed.), pp. 39–64. Academic Press, New York.
- Oketa, K. (1963). Studies on the acid production at fertilization of sea urchin eggs. *Exptl. Cell Res.* **30**, 93–97.
- Osanaï, K. (1964). Cortical granule as a body containing cyto-fertilizin in the sea urchin egg. *Sci. Rep. Tôhoku Univ., 4th Ser., Biol.* **30**, 119–131.
- Oshima, H. (1921). Inhibitory effect of dermal secretion of the sea urchin upon the fertilizability of the egg. *Science* **54**, 578–580.
- Ouchterlony, O. (1948). Antigen-antibody reactions in gels. *Arkiv Kemi* **26B**, 1–9.
- Pasteels, J. J. (1965). Etude au microscope électronique de la réaction corticale. I. La réaction corticale de fécondation chez *Paracentrotus* et sa chronologie. II. La réaction corticale de l'oeuf vierge de *Sabellaria alveolata*. *J. Embryol. Exptl. Morphol.* **13**, 327–340.

- Pasteels, J. J., and de Harven, E. (1962). Etude au microscope électronique du cortex de l'oeuf de *Barnea candida* (mollusque bivalve), et son évolution au moment de la fécondation, de la maturation, et de la segmentation. *Extrait Arch. Biol.* 73 (3), 446-490.
- Pequegnat, W. (1948). Inhibition of fertilization in *Arbacia* by blood extract. *Biol. Bull.* 95, 69-82.
- Perlmann, P. (1954). Study on the effect of antisera on unfertilized sea urchin eggs. *Exptl. Cell Res.* 6, 485-490.
- Perlmann, P. (1956). Response of unfertilized sea urchin eggs to antiserum. *Exptl. Cell Res.* 10, 324-353.
- Perlmann, P. (1957). Analysis of the surface structures of the sea urchin egg by means of antibodies. *Exptl. Cell Res.* 13, 365-390.
- Perlmann, P. (1959). Immunochemical analysis of the surface of the sea urchin egg—an approach to the study of fertilization. *Experientia* 15, 41-52.
- Perlmann, P., and Hagström, B. E. (1955). Effect of antiserum on fertilization of the sea urchin egg. *Exptl. Cell Res., Suppl.* 3, 274-280.
- Perlmann, P., and Perlmann, H. (1957a). Analysis of the surface structures of the sea urchin egg by means of antibodies. II. The J and A antigens. *Exptl. Cell Res.* 13, 454-474.
- Perlmann, P., and Perlmann, H. (1957b). Analysis of the surface structures of the sea urchin egg by means of antibodies. III. The C and F antigens. *Exptl. Cell Res.* 13, 475-487.
- Pernot, E. (1956). Recherches sur les constituants antigéniques des spermatozoïdes de Cobayes. *Bull. Soc. Chim. Biol.* 38, 1041-1054.
- Pernot, E., and Szumowski, P. (1958). Etude électrophorétique et immuno-électrophorétique des protéines du plasma séminal de taureau. *Bull. Soc. Chim. Biol.* 40, 1423-1434.
- Piatigorsky, J., and Austin, C. R. (1962). Relationship of fertilizin to the acrosome reaction in *Arbacia*. *Biol. Bull.* 123, 473.
- Pikó, L., and Tyler, A. (1962). Antigenic analysis of Chinese hamster sperm. *Am. Zoologist* 2, 548.
- Popivanov, R., and Vulchanov, V. H. (1962). Segregation of man's AB-group spermatozoa in A- and B-spermatozoa through agglutination with immune anti-A rabbit serum. *Z. Immunitäts Forschung* 124, 206-210.
- Porter, R. R. (1959). The hydrolysis of rabbit gamma-globulin and antibodies with crystalline papain. *Biochem. J.* 73, 119-126.
- Quinlivan, W. L. G., and Masouredis, S. P. (1962). Rho (D) antigen content of human spermatozoa. *Immunology* 5, 267-277.
- Rao, S. S., and Sadri, K. K. (1959). Immunological studies with human semen and cervical mucus. *Proc. Sixth Intern. Conf. on Planned Parenthood, New Delhi*, pp. 313-318.
- Rao, S. S., and Sadri, K. K. (1960). The antigenic composition of buffalo semen. *J. Comp. Pathol. Therap.* 70, 1-9.
- Rebhun, L. I. (1962). Electron microscope studies on the vitelline membrane of the surf clam, *Spisula solidissima*. *J. Ultrastruct. Res.* 6, 107-122.
- Reger, J. F. (1964). The fine structure of spermatozoa from the isopod *Asellus militaris* (Hay). *J. Ultrastruct. Res.* 11, 181-192.
- Reverberi, G., and Mancuso, V. (1960). The constituents of the egg of *Ciona intestinalis* (Ascidians) as seen at the electron microscope. *Acta Embryol. Morphol. Exptl.* 3, 221-235.

- Reverberi, G., Mancuso, V. (1961). The constituents of the egg of *Mytilus* as seen at the electron microscope. *Acta Embryol. Morphol. Exptl.* **4**, 102-121.
- Rothschild, Lord (1956). "Fertilization." Wiley, New York, and Methuen, London.
- Rugh, R. (1934). Induced ovulation and artificial fertilization in the frog. *Biol. Bull.* **66**, 22-29.
- Rugh, R. (1952). "Experimental Embryology. A Manual of Techniques and Procedures." Burgess, Minneapolis, Minnesota.
- Rümke, P. H., and Hellinga, G. (1959). Autoantibodies against spermatozoa in sterile men. *Am. J. Clin. Pathol.* **32**, 357-363.
- Runnström, J. (1949). The mechanism of fertilization in metazoa. In "Advances in Enzymology," Vol. 9, pp. 241-327. Wiley (Interscience), New York.
- Runnström, J., and Hagström, B. (1955). Studies on the action of the "Fucus fertilization inhibitor" on sea urchin eggs and sperm. *Exptl. Cell Res.* **8**, 1-14.
- Runnström, J., Hagström, B. E., and Perlmann, P. (1959). Fertilization. In "The Cell" (J. Brachet and A. E. Mirsky, eds.), pp. 327-397. Academic Press, New York.
- Runnström, T., Tiselius, A., and Vasseur, E. (1942). Zur Kenntnis der Gamonwirkungen bei *Psammechinus miliaris* und *Echinocardium cordatum*. *Arkiv Kemi* **15A**, 1-18.
- Schabtach, E., and Ursprung, H. (1965). The fine structure of the sperm of a tunicate *Ascidia nigra*. *J. Exptl. Zool.* **159**, 357-366.
- Shahani, S., and Southam, A. L. (1962). Immunofluorescent study of the ABO blood group antigens in human spermatozoa. *Am. J. Obstet. Gynecol.* **84**, 660-666.
- Shaver, J. R. (1966). Immunobiological studies of the jelly-coats of anuran eggs. *Am. Zool.* **65**, 75-87.
- Shaver, J. R., and Barch, S. H. (1960). Experimental studies on the role of jelly coat material in fertilization in the frog. *Acta Embryol. Morphol. Exptl.* **3**, 180-189.
- Shaver, J. R., Barch, S. H., and Shivers, C. A. (1962). Tissue-specificity of frog egg-jelly antigens. *J. Exptl. Zool.* **151**, 95-103.
- Shivers, C. A. (1961). Immunological studies of the species-specificity of egg jellies of the frog. Ph.D. dissertation, Mich. State Univ.
- Shivers, C. A. (1962). Localization of the inhibitory effect of anti-jelly serum on fertilization in frog eggs by fluorescein-tagged antibody. *Am. Zoologist* **2**, 448.
- Shivers, C. A. (1965). The relationship of antigenic components in egg jellies of various amphibian species. *Biol. Bull.* **128**, 328-336.
- Shivers, C. A., and Metz, C. B. (1962). Inhibition of fertilization in frog eggs by univalent fragments of rabbit antibody. *Proc. Soc. Exptl. Biol. Med.* **110**, 385-387.
- Singer, S. J. (1965). Structure and function of antigen and antibody proteins. In "The Proteins" (H. Neurath, ed.), Vol. III, pp. 269-357. Academic Press, New York.
- Smith, A. V. (1949). The antigenic relationship of some mammalian spermatozoa. *Proc. Roy. Soc. (London), Ser. B* **136**, 472-479.
- Snell, G. D. (1944). Antigenic differences between the sperm of different inbred strains of mice. *Science* **100**, 272-273.
- Snell, G. D. (1962). Histocompatibility and cellular immunity. In *Proc. Conf. on Immuno-reprod.* (A. Tyler and K. A. Lawrence, eds.), pp. 161-166. Population Council, New York.

- Stern, S. (1966). Physical and chemical comparison of univalent and multivalent sea urchin fertilizins. Ph.D. dissertation, U. of Miami, Coral Gables, Florida.
- Subtelny, S., and Bradt, O. (1961). Transplantation of blastula nuclei into activated eggs from the body cavity and from the uterus of *Rana pipiens*. *Develop. Biol.* 3, 96-114.
- Tchou-Su (1950). Etude cytologique de la parthénogénèse expérimentale osmotique sur l'oeuf intra-ovarien mûré in vitro chez le crapaud (*Bufo bufo asiaticus*). *Chinese J. Exptl. Biol.* 3, 1-39.
- Tchou-Su, and Wang, Yu-lan (1956). Etudes expérimentales sur le rôle du mucus des oviduces dans la fécondation chez le crapaud, et la considération générale sur le mécanisme de la pénétration spermatique. *Acta Exptl. Biol. Sinica* 5, 75-122.
- Tyler, A. (1939). Extraction of an egg membrane lysin from sperm of the giant keyhole limpet *Megathura crenulata*. *Proc. Natl. Acad. Sci.* 25, 317-323.
- Tyler, A. (1940). Agglutination of sea urchin eggs by means of a substance extracted from the eggs. *Proc. Natl. Acad. Sci.* 26, 249-256.
- Tyler, A. (1941). The role of fertilizin in the fertilization of eggs of the sea urchin and other animals. *Biol. Bull.* 81, 190-204.
- Tyler, A. (1945). Conversion of agglutinins and precipitins into "univalent" (non-agglutinating or non-precipitating) antibodies by photodynamic irradiation of rabbit antisera vs. pneumococci, sheep red cells and sea urchin sperm. *J. Immunol.* 51, 157-172.
- Tyler, A. (1946). Loss of fertilizing power of sea urchin and *Urechis* sperm treated with "univalent" antibodies vs. antifertilizin. *Proc. Soc. Exptl. Biol. Med.* 62, 197-199.
- Tyler, A. (1948). Fertilization and immunity. *Physiol. Rev.* 28, 180-219.
- Tyler, A. (1949). Properties of fertilizin and related substances of eggs and sperm of marine animals. *Am. Naturalist* 83, 195-219.
- Tyler, A. (1956). Physico-chemical properties of the fertilizins of the sea urchin *Arbacia punctulata* and the sand dollar *Echinarachnius parma*. *Exptl. Cell Res.* 10, 377-386.
- Tyler, A. (1957). Immunological studies of early development. In "The Beginning of Embryonic Development" (A. Tyler, R. C. von Borstel, and C. B. Metz, eds.), pp. 341-382. Am. Assoc. Advance. Sci., Washington, D.C.
- Tyler, A. (1959). Some immunobiological experiments on fertilization and early development in sea urchins. *Exptl. Cell Res. Suppl.* 7, 183-199.
- Tyler, A. (1961). Approaches to the control of fertility based on immunological phenomena. *J. Reprod. Fertility* 2, 473-506.
- Tyler, A. (1962). Immunologic aspects of fertilization and early development of animal eggs. Commemorative Volume for the 25th Anniversary of the Institut Sperimentale Italiano "L. Spallanzani" per la Fecondazione Artificiale. pp. 1-16.
- Tyler, A. (1963). The manipulation of macromolecular substances during fertilization and early development of animal eggs. *Am. Zoologist* 3, 109-126.
- Tyler, A., and Bishop, D. W. (1963). Immunological phenomena. In "Mechanisms Concerned with Conception" (C. Hartman, ed.), pp. 397-482. Macmillan, New York.
- Tyler, A., and Brookbank, J. W. (1956a). Antisera that block cell division in developing eggs of sea urchins. *Proc. Natl. Acad. Sci.* 42, 304-308.
- Tyler, A., and Brookbank, J. W. (1956b). Inhibition of division and development of sea urchin eggs by antisera against fertilizin. *Proc. Natl. Acad. Sci.* 42, 308-313.

- Tyler, A., and Fox, S. W. (1940). Evidence for the protein nature of the sperm agglutinins of the keyhole limpet and the sea urchin. *Biol. Bull.* **79**, 153-165.
- Tyler, A., and Kakabayashi, N. (1962). Naturally occurring and immune antibodies in relation to infertility in humans. In *Proc. Conf. on Immuno-reprod.* (A. Tyler and K. A. Lawrence, eds.), pp. 155-160. Population Council, New York.
- Tyler, A., and Metz, C. B. (1955). Effects of fertilizin-treatment of sperm and trypsin-treatment of eggs on homologous and cross fertilization in sea-urchins. *Pubbl. Staz. Zool. Napoli* **27**, 128-145.
- Tyler, A., and O'Melveny, K. (1941). The role of antifertilizin in the fertilization of sea urchin eggs. *Biol. Bull.* **81**, 364-374.
- Tyler, P. (1966). Ultrastructural studies concerning the occurrence of acrosomes on the spermatozoa of some holostean and teleost fishes. Masters thesis. Univ. Miami, Coral Gables, Florida.
- Ursprung, H., and Schabtach, E. (1965). Fertilization in tunicates: loss of the paternal mitochondrion prior to sperm entry. *J. Exptl. Zool.* **159**, 379-384.
- Vasseur, E. (1949). Effect of calcium ions on the agglutination of *Strongylocentrotus droebachiensis*. *Mull. Arkiv Kemi* **1**, 105-116.
- Vasseur, E. (1952a). "The Chemistry and Physiology of the Jelly Coat of the Sea Urchin." Independent publication. Kihlstromstryck, A. B., Stockholm.
- Vasseur, E. (1952b). Periodate oxidation of the jelly coat substance of *Echinocardium cordatum*. *Acta Chem. Scand.* **6**, 376-384.
- Weil, A. J. (1960). Immunological differentiation of epididymal and seminal spermatozoa of the rabbit. *Science* **131**, 1040-1041.
- Weil, A. J. (1961). Antigens of the adnexal glands of the male genital tract. *Fertility Sterility* **12**, 538-543.
- Weil, A. J. (1962). Spermatozoa-coating antigens. In *Proc. Conf. Immuno-reprod.* (A. Tyler and K. A. Lawrence, eds.), pp. 43-50. Population Council, New York.
- Weil, A. J., and Finkler, A. E. (1958). Antigens of rabbit semen. *Proc. Soc. Exptl. Biol. Med.* **98**, 794-797.
- Weil, A. J., Kotsevalov, O., and Wilson, L. (1956). Antigens of human seminal plasma. *Proc. Soc. Exptl. Biol. Med.* **92**, 606-610.
- Weil, A. J., and Rodenburg, J. M. (1960). Immunological differentiation of human testicular (spermatocele) and seminal spermatozoa. *Proc. Soc. Exptl. Biol. Med.* **105**, 43-45.
- Weil, A. J., and Rodenburg, J. M. (1962). The seminal vesicle as the source of the spermatozoa-coating antigen of seminal plasma. *Proc. Soc. Exptl. Biol. Med.* **109**, 567-570.
- Weiner, A. S. (1945). "Blood groups and Transfusion." Thomas, Springfield, Illinois.
- Went, H. A., and Mazia, D. (1959). Immuno-chemical study of the origin of the mitotic apparatus. *Exptl. Cell Res. Suppl.* **7**, 200-218.
- Wentworth, B. C., and Mellen, W. J. (1964). Active immunity induced and spermatogenesis suppressed by testicular antigen in the male Japanese quail *Coturnix coturnix japonica*. *J. Reprod. Fertility* **8**, 215-223.
- Whiteley, A. H., and Chambers, E. L. (1961). The differentiation of a phosphate transport mechanism in the fertilized egg of the sea urchin. *Symp. Germ Cells and Development, 1961* (S. Ranzi, ed.), pp. 387-401. Inst. Intern. d'Embryol. and Fondazione, F. A. Baselli, Milan.
- Wiese, L. (1961). Gamone. *Fortschr. Zool.* **13**, 119-145.
- Wiese, L. (1966). Geschlechtsbestimmung. *Fortschr. Zool.* **18**, 139-206.

- Williams, D. E., and Metz, C. B. (1963). Inhibition of fertilization by specific antibodies dissociated from sperm. *Biol. Bull.* **125**, 365.
- Williams, W. L., Weinman, D. E., and Hamner, C. E. (1964). Effects of the female reproductive tract on spermatozoa. *Proc. Vth Intern. Congr. Animal Reproduction and Artificial Insemination* **3**, 367-370. Casciago (Varese).
- Willson, J. T., and Katsh, S. (1965). Cyto-immunological studies of the guinea pig sperm antigens. *Z. Zellforsch.* **65**, 16-26.
- Wilson, E. B. (1925). "The Cell in Development and Heredity." Macmillan, New York.
- Yamakami, K. (1926). The individuality of semen, with reference to its property of inhibiting specifically iso-hemo agglutination. *J. Immunol.* **12**, 185-189.
- Yasuzumi, G. (1960). Spermatogenesis in animals as revealed by electron microscopy. VII. Spermatid differentiation in the crab, *Eriocheir japonicus*. *J. Biophys. Biochem. Cytol.* **7**, 73-78.

CHAPTER 6

Acrosome Reaction and Lysins

Jean Clark Dan

DEPARTMENT OF BIOLOGY, OCHANOMIZU UNIVERSITY,
OTSUKA, BUNKYO-KU, TOKYO, JAPAN

I. Introduction	237
II. General Aspects of the Acrosome Reaction	239
A. Components of the Reaction	239
B. Occurrence of Acrosome Reactions	240
C. Conditions Conducive to Reaction	241
III. Acrosome Formation during Spermiogenesis	244
A. Golgi Origin of Acrosomes	244
B. Fine-Structure Studies of Acrosome Formation	249
C. Discussion	254
IV. Fine Structure of Acrosomes	257
A. Echinoderms	257
B. Bivalve Mollusks	260
C. Polychaet Annelids	264
D. Arthropods	264
E. Vertebrates	267
V. Fine-Structure Studies of Acrosomal Changes	270
A. Invertebrates	270
B. Vertebrates	280
VI. Lysins	281
A. Isolation and Characterization of Vitelline-Coat Lysins	281
B. Localization in Acrosome	282
C. Effect on Egg Surface	283
D. Hyaluronidase	284
E. "Zona Lysin"	284
VII. General Considerations	284
A. Trigger Mechanism	284
B. Process Membrane	285
C. Perforatorium	287
References	288

I. Introduction

The earliest illustrations of sperm cells show an apical structure which Waldeyer (1870) called the "perforatorium." By 1887, Benda had determined that this organelle is derived from a granule located in a

vacuole within the Golgi complex. In 1898, Lenhossek suggested "acrosome" as a more appropriate name for this structure.

The work of Loeb (1901) on artificial parthenogenesis, performed during the first decade of the present century, convinced him that a lytic substance (enzyme?) carried by the spermatozoon causes a superficial cytolysis of the egg cortex. This proposal, with its implication of a chemical function for the acrosome, brought into question the earlier idea of a mechanical perforation of the egg surface. The widespread interest that was generated by the Fertilizin Theory of F. R. Lillie (1912, 1919) further tended to discredit the older term, the criticism being that it represented a primitive and outmoded way of describing the role of the spermatozoon. Its eclipse was never quite total, however, perhaps because the sharply pointed, spiral, and even barbed tips of many sperm cells persistently suggested a mechanical function.

During the following decade Bowen (e.g., 1924) intensively investigated the role of the Golgi complex in the synthesis of enzymes and particularly in the formation of the acrosome. The close connection evident in this common genesis led him to suggest that the significant acrosomal component might well be an enzyme for activating the cytoplasm of the unfertilized egg. Subsequent attempts to discover such an enzyme (Hibbard, 1928; Wintrebert, 1929), the expansion and modification of Lillie's (1912, 1919) original concept in the immunological studies of Tyler and his co-workers (1948), and the further variations on this theme that gave rise to the gamone concept (Hartmann *et al.*, 1940) all combined in the next 20 years to submerge the identity of the individual spermatozoon in the effort to isolate the chemical components of the fertilization reaction.

The appearance of the phase-contrast microscope not only restored to the spermatozoon its anatomy but made possible for the first time close observation of its behavior. Such study revealed that in many species well-defined changes take place in the structure of the acrosome when the spermatozoon makes contact with the surface of an egg. The current use of thin sectioning and electron microscopy to investigate this aspect of the sperm cell is bringing to light a variety of patterns, representing substances of different textures arranged in characteristic formations. The evidence so far obtained indicates that these substances interact in specific ways when the spermatozoon is appropriately stimulated, enabling it to make its way through the protective coats surrounding the egg and deliver its contribution of nuclear material to the zygote cytoplasm.

These high-magnification studies of sperm morphology have brought the term "perforatorium" back into use, but with a restricted meaning,

to describe sharply pointed, edged, or hooked elements formed of fibrous material which have been found in the acrosomal region of certain vertebrate spermatozoa (Burgos and Fawcett, 1956). The fact that acrosomes which include such structures are likely to lack other commonly observed acrosomal components suggests that some spermatozoa may, after all, use these tools to "bore into the egg," although probably not in the purely mechanical sense that this word implies (see Section VII).

It remains to be determined whether acrosomes include an activating enzyme, as proposed by Loeb (1901, 1915) and Bowen (1924), which would specifically catalyze the "sluggish" processes of the unfertilized egg and thereby initiate development. The evidence from studies of artificial parthenogenesis suggests that this is not the case. Moreover, attempts to activate unfertilized eggs by exposing them to sperm extracts have been consistently unsuccessful. This failure cannot be considered conclusive, in view of the delicate nature of enzymes, the preciseness of conditions governing their activity, and the fact that an activating enzyme would be expected to act within the egg cytoplasm rather than on the outer surface of its plasma membrane. Until at least the existence of a substance with such properties can be demonstrated, however, it seems appropriate to assume that the main function of the acrosome is to introduce the sperm nucleus into the egg. The following account will attempt to summarize the information accumulated in recent years concerning the structure of acrosomes and the changes which they undergo in performing this function.

II. General Aspects of the Acrosome Reaction

A. COMPONENTS OF THE REACTION

As in all studies of fertilization, the problem of what is going on in the acrosome is most open to attack when fertilization takes place externally and when adequate amounts of readily reacting gametes are consistently available. Particularly among the vertebrates, where such knowledge might have practical application, the special conditions under which fertilization takes place make it extremely difficult to follow the sequence of changes undergone by the acrosome as the spermatozoon approaches the egg surface (see Vol. II, Chap. 8). For this reason, most of the extant information concerns the changes that occur in the acrosomes of marine mollusks, annelids, echinoderms, and an enteropneust.

The concept of the acrosome reaction had its origin in phase-contrast microscopic observations of living spermatozoa fixed with formaldehyde under various experimental conditions. The results of investigations at

this level will be described first, in order to provide a basis for discussing the findings of thin-section studies.

Among invertebrates it is generally found that the acrosome undergoes a change when the spermatozoon swims into contact with the protective covering surrounding the egg. At low magnifications this change is recognized as a loss of the often-prominent structure at the apex of the sperm head and its replacement by a projection of some sort. This projection may be very short, as in the echinoids, extremely long and slender, as in the sea cucumbers and the lamprey, or of intermediate length, depending apparently on the characteristics of the jelly layer or vitelline coat around the egg.

In addition to forming such a projection, the reaction of the acrosome is believed to release a lytic substance, probably an enzyme, which specifically attacks the material of the protective coat. The information relating to this branch of fertilization studies is summarized in Section VI.

A third aspect of acrosomal activity concerns the role of the perforatorium. Evidence from fertilization studies of mammalian material indicates that this structure must be exposed, by the removal of the acrosomal components which originally surround it, before the spermatozoon can pass through the innermost egg envelope. The precise nature of its function, however, has not yet been determined. This question is discussed in Section VII.

B. OCCURRENCE OF ACROSOME REACTIONS

In many species the acrosome is so conspicuous a feature of the sperm head that its presence or absence at the time of sperm entry into the egg can be determined directly, by light microscopy (Fig. 1). When a long process is formed, it visibly links the sperm head with the egg surface. In the case of the echinoids, it is not possible to see the condition of the very small acrosome as a fertilizing spermatozoon enters an egg, but experiments show that contact with the egg jelly is highly effective in inducing the acrosome to react. Such direct and indirect observations, including the results of thin-section studies, fail to include any well-authenticated case of a spermatozoon entering or found within an egg with its original acrosomal structure intact. On the basis of the existing evidence, it can therefore be concluded that a change in the acrosomal complex, when one is present, regularly precedes sperm entry in normal fertilization.

Evidence is accumulating, however, that the spermatozoon of teleost fishes lacks an acrosome. Porte and Follenius (1960) found that none is formed during spermiogenesis in the guppy, *Levistes reticulatus*. More-

over, a recent study by Ginsburg (1963) on fertilization in salmonids affords physiological evidence that no acrosome is present in these fishes. The data of her experiments, supplemented by electron microscopy of whole spermatozoa, led her to the conclusion that the spermatozoon of the higher teleosts has lost its acrosome as the result of the evolutionary development of the egg micropyle. The work of Sakai (1961), on dechorionated eggs of the teleost *Oryzias latipes*, showed

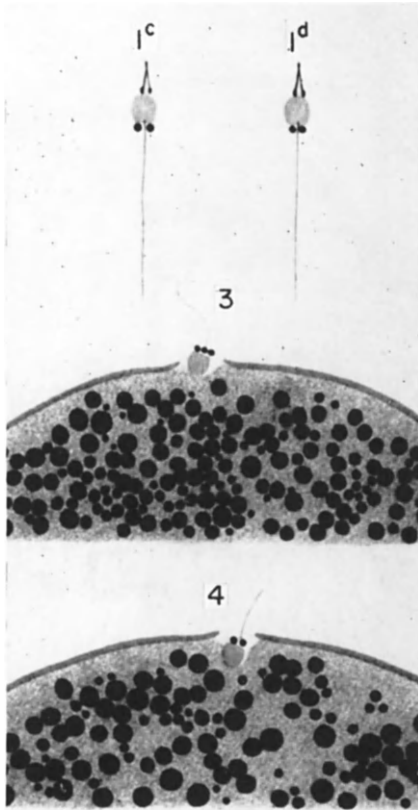


FIG. 1. Early stages in fertilization of the *Mytilus edulis* egg, as drawn by Meves (1915). Note the absence of the acrosome in entering spermatozoa; Meves comments, "sein Substanz muss sehr rasch auflösen." ("its substance must dissolve very rapidly.") Eggs were fixed 2.5 minutes after insemination.

that any part of the egg surface is capable of reacting with spermatozoa, refuting the possibility that there is an area below the micropyle which is specially adapted to such a purpose. This situation in the teleosts poses an interesting problem in connection with the role of the acrosomal process in sperm entry (see Chap. 7).

C. CONDITIONS CONDUCTIVE TO REACTION

The primary requisite for acrosomal reactivity is good physiological condition of the spermatozoon. In echinoderms, percentages of experi-

mentally induced reaction fall off gradually with aging of "dry sperm," and rapidly with the lapse of time in dilute suspensions. Bivalve sperm suspensions retain their reactivity much longer; in both groups the period of reactivity is greatly extended at low temperatures. Starfish spermatozoa are particularly liable to be adversely affected if the adult animals have been kept in tanks with inadequate flow of seawater, but this damage is reversed to some extent by adding $5 \times 10^{-3} M$ EDTA to the seawater with which the dry sperm is diluted. In general, spermatozoa obtained by natural or induced spawning are more reactive than those secured by extirpating the testes.

The available evidence indicates that lowering the pH of the medium inhibits the acrosome reaction of sea urchin spermatozoa (Nakano, personal communication). It is not yet certain, however, whether this effect is specific for the acrosomal function or whether it merely reflects a more general cellular injury. Increasing the alkalinity of the seawater to pH 8.6–9.2 has been found to induce a normal-appearing acrosomal reaction in the spermatozoa of the following marine invertebrates: sea urchins (Dan, 1952), sea cucumber and polychaet *Sabellaria* (Colwin and Colwin, 1955a,c), pelecypods (Dan and Wada, 1955), and gastropods (Dan and Wada, unpublished). When the spermatozoa of the sea urchin *Pseudocentrotus* are suspended in seawater of pH 9.5, they stick to each other at random and irreversibly and die after a short time. This evidence that excessive alkalinity damages the cell surface suggests that the acrosome-stimulating effect of the mildly alkaline medium may have a direct connection with the state of the sperm plasma membrane (see Section VII,A).

It seems not out of place at this point to recall that the early experiments, particularly those of Loeb (1915), on the physiology of the fertilization reaction showed that fertilization in the sea urchin is reversibly blocked by insufficient alkalinity and the absence of calcium. Conversely, Loeb (1915) showed that the sea urchin egg can be activated by the spermatozoa of starfish, brittle stars, and holothurians "if the alkalinity is a trifle higher than that of ordinary sea water" ("50 cc sea water + 0.6 cc N/10 NaOH" = pH 8.9). It can hardly be without significance that the one general condition which is widely effective in inducing the acrosome reaction affords a means of bringing about cross-fertilization between otherwise noninteracting gametes.

In view of Loeb's insistence on the importance of calcium in fertilization, it is not surprising to find that the absence of calcium inhibits the acrosome reaction in all marine species which have been tested*

* With the sole exception of the irregular echinoid, *Chlypeaster*.

(echinoids, asteroids, pelecypods, gastropods). This inhibition is specific for acrosomal reactivity: the swimming activity of sea urchin spermatozoa is unimpaired or even enhanced by the removal of calcium from the medium, and their life span is longer in calcium-free than in normal seawater (Tyler, 1953). In the (freshwater) sturgeon (Dettlaff and Ginsburg, 1963), however, the acrosome reaction will take place without calcium if magnesium is present.

Since unfertilized eggs cannot be artificially activated in a calcium-free medium (Moser, 1939), the failure of the acrosome to react cannot be considered the sole cause of the universally observed failure of fertilization in the absence of calcium, but it is certainly a fundamental one.

Suspended in their normal medium, the spermatozoa of most marine invertebrates will swim about until their energy resources are exhausted, without any particular change occurring in their acrosome. The natural condition which induces the characteristic reaction in each species is contact with whatever constitutes the outermost covering of the egg. If this is a layer of jelly, as in echinoderm eggs, a purified solution of the jelly substance will elicit the reaction (Piatigorsky and Austin, 1962). When a spermatozoon of the polychaet *Hydroides* (Colwin and Colwin, 1961c) approaches the jellyless surface of the egg, its acrosome reacts at the outer layer of the vitelline coat.

In the lamprey (Kille, 1960), what appears to be an acrosome reaction occurs spontaneously when the spermatozoa come to the end of their swimming life span, about 60 seconds after they are suspended in (fresh) water (or sooner if they collide with an egg or other solid object). The readiness with which this reaction occurs in the absence of eggs or egg-derived substances is in marked contrast to the stability that generally characterizes the acrosomes of marine spermatozoa and suggests that the hypotonicity of the freshwater medium may exert a direct effect on the acrosome in these spermatozoa. In marine animals, also, the acrosome may react on contact with indifferent objects, but only when the cells are freshly suspended and swimming actively.

Attempts to induce the acrosome reaction experimentally in *Mytilus edulis* have shown (Wada *et al.*, 1956) that while the supernatant seawater from dense egg suspensions had little effect, nearly all the acrosomes reacted when 10% by volume of isotonic CaCl_2 was added to a sperm suspension. In some cases nearly all the acrosomes reacted when dense sperm suspensions were kept at about 4°C for 2 hours. Both of these treatments exert a marked effect only on the acrosome: the spermatozoa continue to swim actively, and no recognizable structural change occurs in other parts of the cell.

Exposure to 0.05% merthiolate, on the other hand, instantly immobilizes *Mytilus* spermatozoa (Dan, 1956), but does not kill them immediately. After 1–3 minutes, their acrosomes undergo a reduced “reaction,” which is closely followed by the death of the cell. When *Mytilus* spermatozoa are treated with a low concentration of formaldehyde (e.g., 1–2% in seawater), the acrosome either undergoes an incomplete reaction (Dan and Wada, 1955; Rothschild and Tyler, 1955) or a peculiar change in which the acrosomal contents appear to swell *in situ*, causing a distorted elongation of the original structure.

The acrosome reaction, then, occurs when spermatozoa in the best physiological condition, swimming actively in a calcium-containing medium, come into contact with an egg of their own species. A normal-appearing reaction can be induced experimentally in many species by increasing the alkalinity of the medium, and particularly among the echinoderms by treatment with dissolved egg jelly. A low percentage of reaction on contact with an indifferent object is widely observed, and the acrosomes of *Mytilus* spermatozoa react to chilling and an excess of calcium.

Table I lists the species in which a change in the acrosome has been observed; in all except the mammals this change results in the formation of some sort of acrosomal process. All the invertebrates are marine animals. The acrosome reaction was induced by mixing spermatozoa with eggs of the same species, or by treating them with alkaline (pH 9.2) seawater, excess calcium, the supernatant seawater from dense egg suspensions, or plain river water (lamprey).

III. Acrosome Formation during Spermiogenesis

A. GOLGI ORIGIN OF ACROSOMES

Cytological investigations based on appropriate staining methods (e.g., Pollister, 1930; Moriber, 1956) have shown that the small and relatively numerous Golgi elements of spermatocytes, the “dictyosomes,” unite to form a single “acroblast” in each spermatid. Electron microscopy (Burgos and Fawcett, 1955; Kaye, 1962) demonstrates that these consist of cup-shaped arrays of the agranular cytomembranes characteristic of the Golgi complex together with associated vesicles of various sizes. Typically, one of these vesicles lying within the cup becomes filled with finely particulate material of moderate density and increases in diameter, or several such units may unite to form the “proacrosomal granule.” In some cases the vesicle may appear only partly filled by the granule, and in others it may contain very little electron-scattering material.

TABLE I
SPECIES IN WHICH REACTION OF THE ACROSOME HAS BEEN OBSERVED

Annelida	
Polychaeta	
<i>Nereis limbata</i>	Metz and Morrill, 1955a
<i>N. mirabilis</i>	Dan, 1956
<i>N. japonica</i>	Takashima and Takashima, 1963
<i>Platynereis dumerilii</i>	Dan, 1956
<i>Perinereis cultrifera</i>	Dan, 1956
<i>Sabellaria vulgaris</i>	Colwin and Colwin, 1955a
<i>Hydroides hexagonus</i>	Colwin <i>et al.</i> , 1957
<i>Pomatoleios crosslandi</i>	Dan (unpublished)
Mollusca	
Gastropoda	
<i>Turbo stenogyryus</i>	Wada, personal communication
<i>T. cornutus</i>	Wada, personal communication
<i>Lunella coronata coreensis</i>	Wada, personal communication
<i>Calcar haematrugum</i>	Wada, personal communication
<i>Monodonta labio</i>	Wada, personal communication
<i>Tegula pfeifferi</i>	Wada, personal communication
<i>Umbonium</i> sp.	Wada, personal communication
<i>Scutus scapha</i>	Dan, 1956
<i>Megathura crenulata</i>	Dan, unpublished
<i>Clypidina</i> sp.	Wada, personal communication
Pelecypoda	
<i>Mytilus edulis</i>	Dan and Wada, 1955
<i>Lithophaga curta</i>	Dan and Wada, 1955
<i>Spondylus cruentus</i>	Dan and Wada, 1955
<i>Crassostrea echinata</i>	Dan and Wada, 1955
<i>C. nippona</i>	Dan and Wada, 1955
<i>C. gigas</i>	Dan and Wada, 1955
<i>Trapezium sublaevigatum</i>	Dan and Wada, 1955
<i>Chama retroversa</i>	Dan and Wada, 1955
<i>Petricola japonica</i>	Dan and Wada, 1955
<i>Mactra veniformis</i>	Dan and Wada, 1955
<i>M. sulcataria</i>	Dan and Wada, 1955
<i>Zurfaea subconstricta</i>	Dan and Wada, 1955
Cthropoda	
Arrustacea	
<i>Menippe mercenaria</i>	Brown, 1966
<i>Callinectes sapidus</i>	Brown, 1966
Xiphosura	
<i>Xiphosura polyphemus</i>	André, 1963
Echinodermata	
Crinoidea	
<i>Comanthus japonica</i>	Dan, unpublished

TABLE I (Continued)

Asteroidea	
<i>Asterias amurensis</i>	Dan, 1954
<i>A. forbesi</i>	Colwin and Colwin, 1955c
	Metz and Morrill, 1955
<i>A. vulgaris</i>	Colwin and Colwin, 1955c
<i>Asterina pectinifera</i>	Dan, 1954
<i>Astropecten scoparius</i>	Dan, 1954
<i>Pycnopodia helioanthoides</i>	Dan, unpublished data
Holothuroidea	
<i>Holothuria atra</i>	Colwin and Colwin, 1955a
<i>Thyone briareus</i>	Colwin and Colwin, 1955d
<i>Parastychopus californicus</i>	Dan, unpublished
Ophiuroidea	
<i>Ophioplocus japonicus</i>	Dan, 1956
<i>Ophiarachnella gorgonia</i>	Dan, 1956
Echinoidea	
<i>Clypeaster japonicus</i>	Dan, 1956
<i>Astriclypeus manni</i>	Dan, 1956
<i>Echinocardium cordatum</i>	Afzelius and Murray, 1957
<i>Arbacia punctulata</i>	Bernstein, 1962
<i>Anthodiaris crassispina</i>	Dan, 1954
<i>Hemicentrotus pulcherrimus</i>	Dan, 1952
<i>Pseudocentrotus depressus</i>	Dan, 1952
<i>Strongylocentrotus purpuratus</i>	Collier, 1959
<i>S. droebachiensis</i>	Afzelius and Murray, 1957
<i>S. franciscanus</i>	Dan, 1958
<i>Mespilia globulus</i>	Dan, 1956
<i>Temnopleurus toreumaticus</i>	Nakano, personal communication
<i>Diadema setosum</i>	Dan, 1956
<i>Lytechinus variegatus</i>	Dan, unpublished data
Enteropneusta	
<i>Saccoglossus kowalevskii</i>	Colwin and Colwin, 1954
Vertebrata	
Cyclostomata	
<i>Lampetra planeri</i>	Kille, 1960
<i>L. fluviatilis</i>	Kille, 1960
Pisces	
<i>Acipenser stellatus</i>	Dettlaff and Ginsburg, 1963
<i>A. guldenstädti</i>	Dettlaff and Ginsburg, 1963
Mammalia	
Rabbit	Moricard, 1960
Rat	Pikó and Tyler, 1964
Golden hamster	Yanagimachi, 1964
Pig	Dickmann, 1964

When the proacrosomal granule or vesicle attains a certain size (often ca. $1\ \mu$ in diameter), it moves away from the Golgi membranes and becomes attached to the nuclear envelope, where it continues to undergo changes which finally give rise, at the end of spermiogenesis, to the acrosome characteristic of the mature spermatozoon.

The early cytochemical studies of spermiogenesis showed that the proacrosomal granule is the only component of the spermatid which gives a positive reaction with the periodic acid-Schiff (PAS) test (Leuchtenberger and Schrader, 1950; Clermont and Leblond, 1955). Subsequent electron microscopic investigations of acrosome formation confirm at high magnifications that the PAS-positive material seen with the light microscope is associated with this granule. In the particularly favorable case of the large crayfish spermatozoon, to be described below, it has been possible to follow the staining specificity of the acrosomal regions differentiated during the later stages of spermiogenesis.

An extensive study of the staining properties of mammalian spermatids and spermatozoa has recently been made by Onuma and Nishikawa (1963). Combining several enzyme-digestion and specific staining techniques (Table II), they arrived at the conclusion that the PAS-

TABLE II
TESTS FOR PRESENCE OF GLYCOGEN AND ACID MUCOPOLYSACCHARIDES IN THE
DEVELOPING ACROSOME OF BOAR SPERMATIDS

Fixative	Treatment	Result
Orth Maximov Zenker	Digestion by saliva (1 hour) followed by PAS staining (glycogen)	No change in positive PAS reaction
Orth Maximov Zenker	Bauer's chromic acid-Schiff staining (glycogen)	No staining
Orth	Hyaluronidase digestion (37°C) for 2-24 hours, PAS staining (acid mucopolysac- charide)	No change in positive PAS reaction
Buffered formalin	Staining with toluidine blue (acid mucopoly- saccharide)	No metachromasia

positive material of the acrosome during spermiogenesis and in ejaculated spermatozoa is neither glycogen nor acid polysaccharide. On comparing the effectiveness of 12 fixatives in preserving this material (Table III), they found that it is strongly extracted with fat solvents, suggesting that it may be a glycolipid.

Table III

Effect of Components of Fixatives on Stability of PAS-Positive Material in Acrosomes of Boar Spermatis

Fixative	Components of fixing fluids										PAS staining		
	Distilled water	Potassium bichromate	Sodium sulfate	Formalin	Mercuric chloride	Picric acid	Glacial acetic	Acetone	Alcohol	Chloroform	Acrosomal vesicle	Acrosomal granule	Golgi
Orth	o	o	o	o							+	+++	+
Regaud's first fluid	o	o		o							+	+++	+
Maximov	o	o	o	o	o						+	+++	+
Tellyesnitsky's first	o	o					o				-	+++	+
Hamazaki's chrome-KEG	o	o	o				o				-	+++	+
Regaud's second fluid	o	o		o			o				-	+++	+
Zenker	o	o	o		o		o				-	+++	+
Bouin	o			o		o	o				-	+++	+
Acetone								o			-	++	+
Alcohol									o		-	++	+
Carnoy's first fluid							o			o	-	-	+
Carnoy's second fluid							o		o	o	-	-	+

Hartree and Srivastava (1965) removed the acrosomes from washed ejaculated ram spermatozoa by treating them with the anionic detergent Hyamine 2389 (0.05%). The isolated acrosomal material was separated into lipid and glycoprotein fractions by extraction with methanol and chloroform. The lipid fraction was found to have a high content of phospholipid, consisting mainly of plasmalogen and lecithin in a ratio of 1.1:1.2, which is close to that of whole ram sperm. Analysis of the glycoprotein fraction gave values of 777 mg/gm for amino acids, 73 mg/gm for sugars, 33 mg/gm for phospholipid, and 18 mg/gm for nucleic acid. The amount of sialic acid found in the isolated acrosomes closely approaches that characterizing whole spermatozoa, indicating that all the sialic acid of the sperm cell is located in the acrosome.

These values agree rather closely with those obtained by a similar analysis of material extracted from ram spermatozoa by treatment with dilute (0.0125 *N*) NaOH, which is believed to dissolve the acrosome without significantly affecting other parts of the cell (Hathaway and Hartree, 1963). This agreement gives reason to conclude that the material isolated by both methods fairly well represents the composition of the acrosome. It would appear inevitable, however, that any method which removed the acrosome would involve dissolution of the plasma membrane. The similarity of the phospholipid found in the acrosomal fraction to that of intact ram spermatozoa, as well as the presence of "all the sialic acid," suggests the possibility that the dissolution products of the whole plasma membrane are included in the acrosomal fraction.

B. FINE-STRUCTURE STUDIES OF ACROSOME FORMATION

Recent use of the electron microscope has disclosed a number of variations in the structural details of acrosome differentiation as it takes place in particular animals. Burgos and Fawcett (1955) found that in the late stages of cat spermiogenesis, the material of the proacrosomal granule spreads over the whole anterior part of the nucleus as a continuous thin cap. This result settled a long controversy as to whether the "cap," "capitea galea," "head cap," etc., is a separate structure from the acrosome in mammalian spermatozoa, and led to the suggestion that the name "acrosomal cap" would prevent this confusion.

Horstmann (1961), studying the formation of the human spermatozoon, reported that, as in the cat, the head cap represents thinly extended material of the acrosomal granule, which is synthesized by the Golgi complex of the early spermatid. In view of the close juxtaposition of the spermatid acrosome with the cytoplasm of the surrounding Sertoli cell, this author suggested that there may be an exchange of materials in this region during spermatid differentiation.

An investigation of spermiogenesis in the domestic chicken (Nagano, 1962) showed that a membrane-bounded acrosomal granule migrates from the acroblast and becomes affixed to the nuclear surface. In mid-spermiogenesis, before the nucleus has begun to elongate, a spherical depression is formed in the nuclear envelope beneath the center of the acrosomal vesicle (Fig. 2A). A small mass of very dense material appears in the depression, in contact with the inwardly bulging acrosomal vesicle. As spermiogenesis proceeds, the acrosomal vesicle first spreads laterally and then becomes conical (Fig. 2B), while the nuclear depression narrows and deepens and the dense granule begins to elongate within it. An extensive invagination forms in the posterior side of the acrosomal vesicle, in line with the depression which now extends well into the nucleus (Fig. 2C). Its common lumen is filled by a rod of dense material; the author suggests that this may represent a perforatorium.

In a paper describing some points in toad spermiogenesis, Burgos and Fawcett (1956) reported that the acrosomal vesicle of the spermatid contains only faintly apparent granules in a clear matrix and that no PAS-positive material can be detected in the testis at the stage in which the mammalian testis gives a fairly strong positive PAS reaction. The acrosomal vesicle differentiates to form a conical cap over the anterior end of the elongating spermatid; this eventually becomes completely filled with moderately dense particulate material. Later in spermatid differentiation, a variable number of bundles of finely fibrous elements appear in the space left between this cap and the nucleus by the con-

densation of the nuclear contents. The bases of these bundles surround the nucleus, and their tips converge within the apex of the acrosomal cap to form a sharply pointed perforatorium.

The differentiation of the acrosome in the house cricket has been investigated in detail by Kaye (1962). He found that the acroblast of the early spermatid gives rise to a single proacrosomal granule, which attains a diameter of about 1μ in close association with the Golgi membranes and then moves toward the nucleus (Fig. 3A,B). During this migration the side of the granule facing the nucleus flattens and is joined by a single, circular "interstitial membrane" of unknown origin. The center of the membrane is closely applied to the granule, while its periphery extends free in the cytoplasm. When the granule attaches it-

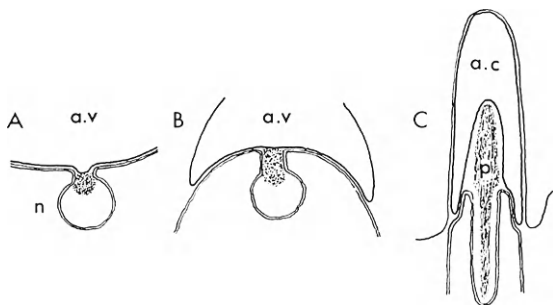


FIG. 2. Spermiogenesis in the domestic chicken; stages in differentiation of the structure believed to be the perforatorium. (Sketched from electron micrographs of Nagano, 1962.) (A) Midspermiogenesis: formation of the depression in the nucleus under the center of the acrosomal vesicle; appearance of the dense granule. (B) Later stage: the nuclear depression is enlarged; the granule is beginning to elongate. (C) Late spermatid stage: the acrosomal cap of the nearly mature spermatozoon covers the rod. (a.c) Acrosomal cap. (a.v) Acrosomal vesicle. (n) Nucleus. (p) Rod believed to represent the perforatorium. (Courtesy of Academic Press, New York.)

self to the nuclear envelope, this membrane lies between the two and covers a considerable area of the nuclear surface around the granule (Fig. 3C).

During this phase of granule attachment, the spermatid elongates and the Golgi membranes of the acroblast migrate to the posterior end of the cell.

After it is attached to the nucleus, the spheroidal granule becomes a much-flattened cone (Fig. 3D), and its substance segregates into a denser basal periphery and a less dense central mass. An invagination begins to appear in the basal surface of the cone; the interstitial membrane is missing in this region. At midspermiogenesis the height of the acrosomal cone has increased, as well as the size of the invagination

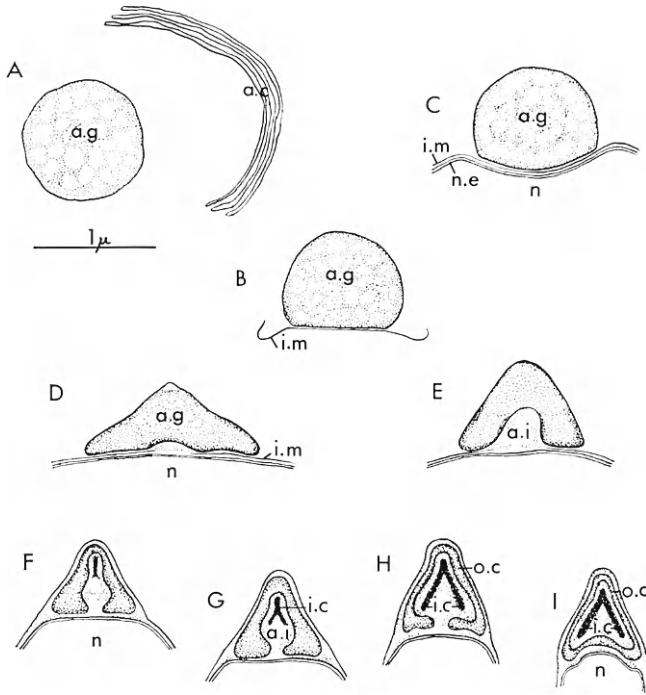


FIG. 3. Spermiogenesis in the cricket. (Sketched from electron micrographs of Kaye, 1962.) (A) Full-sized proacrosomal granule, still close to the Golgi membranes of the acroblast in the early spermatid. (B) Proacrosomal granule migrating toward the nucleus; appearance of the interstitial membrane. (C) The proacrosomal granule is applied to the nucleus; the interstitial membrane lies between the two bodies. (D) The granule flattens into the form of a cone; the center of the base begins to invaginate. (E) Midspermiogenesis: the height of the cone and the size of the invagination are increased; amorphous material appears in the invagination. (F, G, H) Further increase in the size of the invagination and decrease in the volume of the contents of the acrosomal vesicle. The amorphous material within the invagination is organized into a strongly staining inner cone. (I) Mature spermatozoon: the basal part of the acrosomal vesicle extends inward, across the mouth of the invagination, enclosing the inner cone within the outer cone (acrosomal vesicle). (a.c) Golgi membranes of the acroblast. (a.g) Proacrosomal granule. (a.i) Invagination of the acrosomal vesicle. (i.c) Inner cone. (i.m) Interstitial membrane. (n) Nucleus. (o.c) Outer cone. (n.e) Nuclear envelope. (Courtesy of Academic Press, New York.)

(Fig. 3E). The subsequent changes (Fig. 3F,G,H) involve further expansion of the invagination to about nine-tenths of the height of the acrosome, so that the original vesicle forms a thin-walled outer cone, and the organization into a hollow inner cone of amorphous material which appears in the invagination. In the mature spermatozoon, the basal part

of the outer cone extends across the opening of the invagination, completely isolating the inner cone within this closed-off pocket in the acrosomal vesicle (Fig. 3I).

Reger (1963) followed the course of spermiogenesis in the tick, *Amblyomma dissimili*. In the early spermatid, the nucleus first migrates to one side of the cell, and a flat acrosomal vesicle then forms between the nuclear envelope and the plasma membrane by the fusion of numerous dictyosomal vesicles. This vesicle at first contains sparsely distributed particulate material; as spermiogenesis proceeds, it expands along the inner surface of the plasma membrane, and becomes even more flattened and completely filled with dense material. By the end of spermiogenesis it is greatly expanded, and the inner surfaces of its membrane are in intimate contact, giving the impression that all the Golgi-derived material has been used up in forming this vesicular membrane. The membrane is thinner and more clearly defined than the adjacent plasma membrane. An interesting aspect of acrosome formation in this animal is that the early acrosomal vesicle is at first in contact with both the nuclear and plasma membranes; the nucleus then moves away and the acrosomal vesicle continues to develop as an inner lining of the plasma membrane. This is, in a way, a reversal of the usual procedure.

The peculiar organization of decapod spermatozoa was responsible for a good deal of confusion in recognizing their component parts, until the recent appearance of studies combining cytochemistry with electron microscopy. These studies established the identity of the acrosome and described its fine structure in a crab (Yasuzumi, 1960) and two crayfishes (Moses, 1961; Yasuzumi *et al.*, 1961). The following account will be based on the observations of Moses.

Although the structure of decapod spermatozoa includes some unique aspects, the formation of the acrosome follows the general course seen in the other examples which have been studied. One exceptional feature, however, is that no typical Golgi membranes can be found in the *Procambarus* spermatid. There are, instead, aggregations of agranular vesicles (described as "agranular vesicular endoplasmic reticulum" by Yasuzumi *et al.*, 1961) which fuse into clustered tubelike structures and are believed to carry out the Golgi function in this case. The acrosome is formed in the vicinity of these elements. It is first recognized as a clear area near the cell periphery, in which appear scattered clusters of dense 200-Å particles. (In *Cambaroides*, these are apparently derived from larger granules found near the mitotic apparatus after the second meiotic division.) Fairly early in spermiogenesis the plasma membrane doubles inward from the cell surface to enclose this mass of clear material (Fig. 4A). A thin layer of dense material lines the membrane along the outer

surface of this developing acrosomal vesicle; its interior often includes a round mass of the 200-Å particles. This granule is cytochemically identified as the proacrosomal granule by its positive PAS-reaction; it also stains intensely with fast green.

As spermiogenesis proceeds, the aggregations of agranular vesicles are transferred, with the rest of the cytoplasm, to the sustentacular cell (Fig. 4B). The acrosomal granule is replaced by a dense layer which lines the inner surface of the large lens-shaped vesicle, except for a

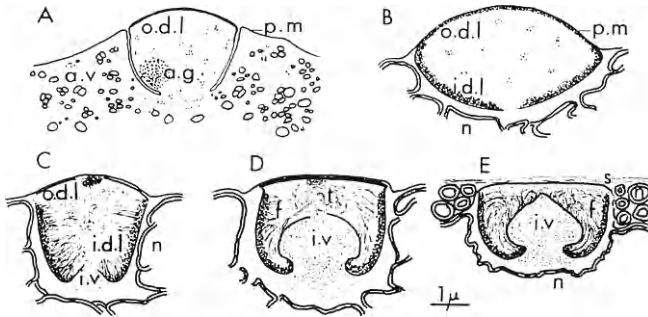


FIG. 4. Spermiogenesis in the crayfish, *Procambarus* (Moses, 1961). (A) Early spermatid: infolding of the plasma membrane encloses the clear material at the cell periphery to form the acrosomal vesicle. The outer membrane is lined by a thin layer of osmiophilic material; the proacrosomal granule is attached to the inner membrane. (B) The acrosomal vesicle is lens shaped; the material of the proacrosomal granule is distributed as a dense layer along the inner side of the vesicle. (C) Latter part of spermiogenesis: the inner dense layer is in the form of a bowl, with the invagination forming at the center. (D) Nearly mature spermatid: the invagination is filled with finely particulate material; the vesicle contains fibrillar and fine tubular elements. (E) End of spermiogenesis: the spermatozoon is surrounded by a shell of polysaccharide; the acrosomal vesicle contains only fibrillar material. (a.g) Proacrosomal granule. (a.v) Agranular vesicles. (f), (t) Fibrillar and tubular material in vesicle. (i.d.l) Inner dense layer. (i.v) Invagination in the acrosomal vesicle. (n) Nucleus. (o.d.l) Outer dense layer. (p.m) Plasma membrane. (s) Polysaccharide shell. (Courtesy of Academic Press, New York.)

small gap at its center. This layer has the same staining properties as did the granule, whereas the somewhat less conspicuous dense layer lining the outer side of the acrosomal vesicle, although it is continuous with the inner layer, fails to stain with either fast green or the PAS method.

During the latter half of spermiogenesis, the side of the acrosome toward the nucleus assumes the shape of a deep bowl, with an incompletely closed place at its center which becomes the site of an invagination (Fig. 4C). The concavity thus formed outside the vesicle gradually

enlarges and becomes filled with very finely and homogeneously particulate material. Within the vesicle filamentous strands extending from the dense peripheral layer mingle with cloudy masses of the fine particles. At this time the contents of the acrosomal vesicle stain faintly PAS-positive and fast green-negative; the dense layer lining its inner side is strongly positive for both, and the outer lining layer is negative for both.

In the nearly mature spermatid, continued invagination of the acrosomal vesicle gives rise to a double-walled structure (Fig. 4D). The finely particulate material is now concentrated in the cavity of the invagination and the adjacent space between acrosome and nucleus. It is found to be faintly acidophilic (in *Cambaroides*, lightly fast green staining). Among the filaments within the vesicle are slender tubules (in *Cambaroides*, the layer of dense particles forming the inner wall of the vesicle has changed into a mat of tubules at this stage). The appearance of shrinkage spaces between the layer lining the outer wall of the vesicle and the filamentous and tubular contents suggests that the latter are not integrally connected with this outer layer.

The mature spermatozoon is surrounded by an extracellular coating of amorphous, PAS-positive material of sustentacular-cell origin, which ruptures or dissolves in freshwater or saline solution. Immediately beneath this shell lies the acrosomal vesicle (Fig. 4E), its contents enclosed by the single membrane which is here doing double duty as both plasma and acrosomal vesicle membrane. The staining properties of the various acrosomal components remain unchanged.

C. DISCUSSION

Faced with the structural variety which characterizes acrosomes in general, one is relieved to discover some common aspects in their modes of formation. The most widely significant of these is the consistently observed synthesis of the proacrosomal granule by the Golgi apparatus of the spermatid. Even the exception to this rule seen in the nonconforming crayfish spermatid is only superficially exceptional, in that the proacrosomal granule is derived from a subcellular system which substitutes in performing the Golgi function in these cells, in the absence of a typical Golgi complex.

Another constant feature of spermiogenesis is that the acrosome-forming role of the Golgi complex appears to end when an adequate amount of its secretion product has been synthesized. In most cases the proacrosomal granule is reported to move toward the nucleus, while the Golgi complex migrates in the opposite direction and is eventually sloughed off from the spermatid. The crayfish achieves a similar result by rearranging the boundary between spermatid and supporting cells.

There is no direct evidence at present to indicate that the nucleus plays a primary role in the later differentiation of the acrosome, in spite of its propinquity, and the fact that its contents during this period are in the process of being condensed and, so to speak, packaged for transportation, suggests that their physiological activity may well be at a low level. The course of acrosome formation in the chicken spermatid appears to show that the nucleus undergoes deformation to accommodate a part of the acrosome, suggesting that its role is rather passive than active in this case.

As consistently observed as the Golgi origin of the proacrosomal granule is its PAS-positive staining. The strong reaction with fast green of the crayfish granule indicates that it also includes basic protein, and the fat-solvent extraction of the PAS-positive material observed by Onuma and Nishikawa (1963) indicates that lipid plays an important part in the chemical constitution of the acrosomal granule as well as the completed acrosome. This evidence that all three major classes of organic compounds are represented in the acrosome precursor makes it apparent that it is futile to hope for a simple chemical explanation of acrosomal function.

A striking feature of the later stages of acrosomal differentiation, as observed in invertebrates, is the segregation of the material within the acrosomal vesicle into various parts, each with a constant configuration and, as will be shown in Section V, a definite role when the acrosome reacts. In many spermatozoa, the acrosomal vesicle in the latter half of spermiogenesis measures less than $1\ \mu$ in diameter, so that the chemical characteristics of its parts cannot be investigated by ordinary cytochemical techniques. At present the recognition of differences in the nature of the various regions has to depend on differences in their appearance in electron micrographs, usually after osmium fixation.

The crayfish spermatid is an exception, however, in that its acrosomal complex is large enough to permit observation of its parts with the light microscope. Toward the end of spermiogenesis, as described above, it is possible to identify a dense layer lining the outer surface of the acrosome which is negative to both fast green and PAS staining, an inner dense layer which is positive to both, an internal mass of filaments and tubules which is PAS-positive and fast green-negative, and an external mass of "faintly acidophilic" particulate material (Moses, 1961). This proof that the products of crayfish acrosomal differentiation, which appear in electron micrographs as regions with different textures and densities, actually represent substances having different chemical characteristics is a particularly important contribution to the effort to understand the acrosome.

How the homogeneously distributed material of the proacrosomal granule gives rise to these arrays is not yet known. One observable fact which may point in a general way toward an explanation is that, together with the nucleus, the acrosome undergoes a considerable decrease in volume during spermiogenesis. A severalfold reduction in the water content of the proacrosomal granule might bring about a segregation of its components, and enzyme action or interactions among labile constituents may well occur. At present even speculation about this process of acrosomal differentiation is hampered by our ignorance of the rate at which it takes place.

As baffling as the problem of how the contents of the acrosomal vesicles sort themselves out is the question of where the substances originate which appear in the spaces between the nuclear and acrosomal membranes. Assuming that nothing is contributed by the nucleus, there are two possible sources of this material: the surrounding cytoplasm, including that of supporting cells when such are present, and the interior of the acrosomal vesicle.

In connection with these possibilities, it may be pointed out that the acrosomal vesicle in most cases is very closely applied to the nuclear envelope, but the apparent absence of a structural connection between the two is confirmed by the extensive movements of the acrosomal complex over the nuclear surface (Kaye, 1962; Onuma, 1963). There are no cytoplasmic inclusions of a Golgi or mitochondrial nature in the neighborhood of the acrosomal vesicle during the period when material is accumulating in the invagination cavities of either acrosome (cricket) or nucleus (chicken), or in the space between them (toad). Toward the end of spermiogenesis the cytoplasm of the supporting cells is separated from the acrosomal vesicle by two plasma membranes; there is no evidence that material is transferred across these membranes. The increase in the size of the acrosomal invagination occurs *pari passu* with a steady decrease in the overall size of the acrosomal complex (Fig. 3). This implies that the increase in volume of the invagination is exactly balanced by a decrease in volume of the vesicular contents and argues against an outside source of the material that appears in the invagination.

In the cricket, the texture and staining properties of the material in the invagination cavity are similar to those of the vesicle contents, and of a layer lying between the outer side of the acrosomal vesicle and the plasma membrane. The membrane of the crayfish acrosomal vesicle appears to be incompletely closed in the region where invagination takes place; the material which finally fills the invagination cavity has the same finely particulate appearance as one component of the vesicular contents in the preceding stage.

Although there are still too many imponderables involved to permit making a decision, the weight of evidence seems to support the idea that the material within the acrosomal invagination originates inside the acrosomal vesicle. The major objection to this conclusion is that the vesicular membrane would presumably prevent the outward movement of large-molecular units to make up the various electron-scattering materials found in this location. More precise information about the nature of this membrane will help to solve this problem, as well as others discussed in Section VII.

IV. Fine Structure of Acrosomes

This section will summarize the information which has been accumulated concerning the structure of the fully formed acrosome in mature spermatozoa. All the observations reported here have been made with the electron microscope on thin-sectioned material (Fig. 5).

A. ECHINODERMS

Among the five classes of echinoderms, the spermatozoa of all but the echinoids have spheroidal heads, with the acrosome set into the nucleus, so that one side of it is exposed at the center of the distal surface (Fig. 5F,G,H,I). In the crinoids, it extends into an inconspicuous cone (Fig. 5J). The sperm heads among the echinoids (Afzelius, 1955; Bernstein, 1962) are uniformly conical, with the acrosome forming the apex of the cone (Fig. 5A-E). In all cases the plasma membrane of the cell lies in direct contact with the surface of the acrosomal complex wherever the latter is not enclosed in nuclear envelope.

The details of acrosomal structure to be reported will be those observed in the sea urchin *Pseudocentrotus depressus* and the starfish *Asterias amurensis*, since these are the forms in which the acrosome reaction has been closely followed, making it possible to assign, at least provisionally, a function to the various parts of the intact acrosome.

1. *Pseudocentrotus depressus*

As was first described by Afzelius (1955), the sea urchin acrosome consists of a granule about $0.25\ \mu$ in diameter made up of homogeneously particulate material and capping a pouch-shaped depression in the apex of the nucleus which contains randomly oriented fibrous material in a nonelectron-scattering matrix (Fig. 6a,b,c). Subsequent observations (Dan *et al.*, 1964) have shown that several substances located in this region take part in the acrosome reaction. Over the sperm apex (Fig. 6d) a thin layer of osmiophilic material is interposed between the plasma membrane and the surface of the granule as far as a ring

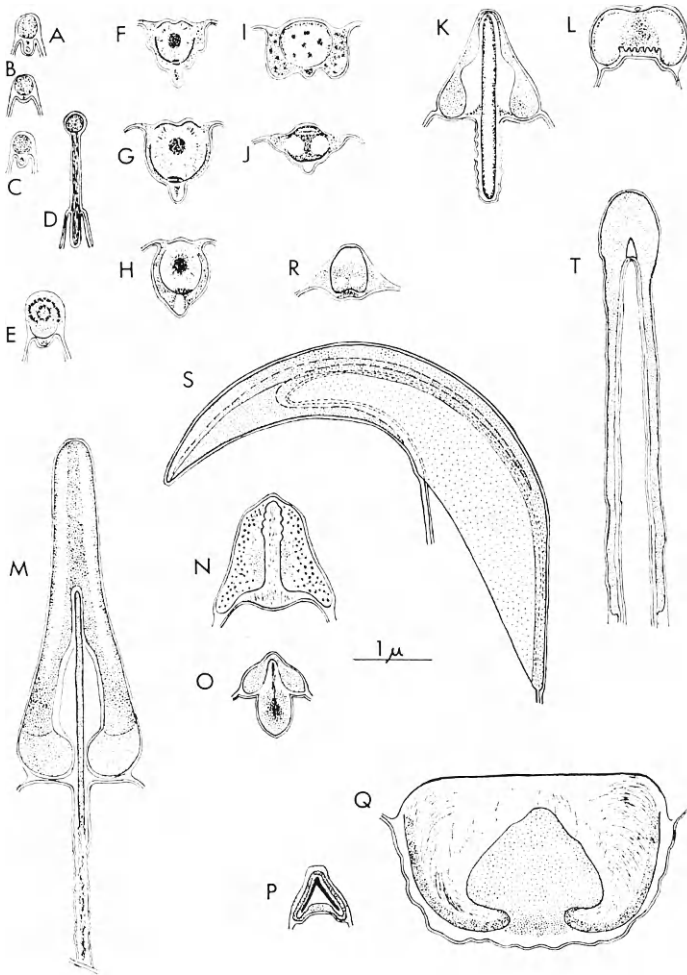


FIG. 5. Fine structure of representative acrosomes. (Drawn to single scale from electron micrographs.) (A) *Strongylocentrotus droebachiensis* (Afzelius, 1955). (B) *Arbacia punctulata* (Bernstein, 1962). (C) *Pseudocentrotus depressus* (Dan *et al.*, 1964). (D) *Echinocardium cordatum* (Afzelius, 1955). (E) *Diadema setosum* (Hagiwara, in prep.). (F) *Asterias amurensis*. G. *Asterina pectinifera* (Hagiwara *et al.*, in press). (H) *Parastychopus californicus*. (I) *Gorgonocephalis eucnemis*. (J) *Comanthus japonicus* (Hagiwara, in prep.). (K) *Nereis japonicus* (Takashima and Takashima, 1963). (L) *Hydroides hexagonus* (Colwin and Colwin, 1961a). (M) *Mytilus edulis* (Niiijima and Dan, 1965a). (N) *Barnea candida* (Pasteels and de Harven, 1962). (O) *Crassostrea virginica* (Galtsoff and Philpott, 1960). (P) *Acheta domestica* (Kaye, 1962). (Q) *Procambarus clarkii* (Moses, 1961). (R) *Saccoglossus kowalevskii* (Colwin and Colwin, 1963a). (S) *Rattus norvegicus* (Pikó and Tyler, 1964). (T) Rabbit (Hadek, 1963b).

encircling the equator of the granule. Here it gives place to a bowl-shaped system of layers surrounding the inner side of the granule (Fig. 6e,f,g). The layer (e), composing the sides of the bowl, is more resistant to disruptive influences than is (f), which forms its flat base. Lining the inside of the bowl is an osmiophobic layer (g) in immediate contact with the surface of the granule. The material of the granule appears to be homogeneous except where it is coextensive with (f)

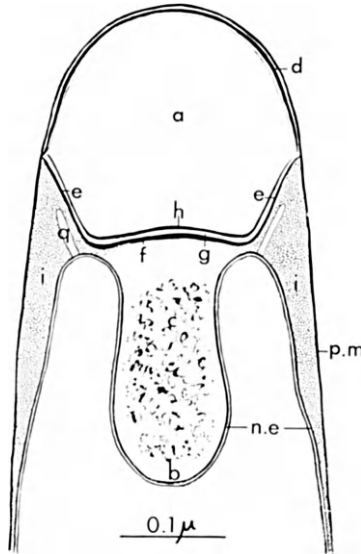


FIG. 6. Fine structure of sea urchin acrosome (*P. depressus*). (a) Acrosomal granule. (b) Nonelectron-scattering matrix in nuclear depression. (c) Unoriented fibrous material. (d-i) Components of acrosomal process (see text). (n.e) Nuclear envelope. (q) Peglike elements. (p.m) Plasma membrane.

across (g). Here, at low magnifications, there is a differentiated surface layer (h) at least as conspicuous as (f), and high resolution may reveal a periodic differentiation consisting of several faint layers parallel to the surface.

At the rim of the bowl, (e) is in contact with the plasma membrane around the granule. Filling the narrow space bounded outwardly by the plasma membrane and inwardly by the tip of the nucleus and the layer (e) is a substance (i) of medium density and homogeneity. Embedded in this material is a ring of peglike elements, (q), which extend toward the apex from the rim of the nuclear depression. The profiles of these elements were first interpreted by Afzelius (1955) as sections through a collarlike structure; he later determined from appropriate cross-sections that there are about 18 of these elements in the spermat-

zoon of *Strongylocentrotus droebachiensis* (Afzelius, personal communication).

2. *Asterias amurensis* (Hagiwara *et al.*, in press)

The most conspicuous component of the *A. amurensis* acrosome is a mass (diameter ca. $0.6\ \mu$) of material which is hyaline and highly refringent in the living cell and which, on fixation, condenses into irregular strands arranged radially around a moderately electron-opaque granule (Figs. 7 and 8a,b). Between the (a) material and the plasma membrane, over the anterior surface of the acrosome, is a thin, osmiophilic layer (c). The posterior sides of the mass are bounded by laminate material (d) having a definite outer surface, but continuous inside with the (a) material. These layers form a bowl, the base of which consists of two thicknesses of the layer complex (d'), with their rough sides apposed, so that this central disc has a smooth inner surface not continuous with the (a) material.

Encircling the mass of (a) material, so that one of its edges is directly under the plasma membrane (p.m) at the anterior face of the sperm head, is a thick (ca. $0.1\ \mu$) band of rather densely particulate material (e), which appears very dark with phase contrast in the living cell. This is in contact peripherally with the nuclear envelope (n.e), which curves inward around the posterior edge of the (e) band and follows the contour of the bowl-shaped layers (d) toward the center of the nucleus, where it forms a small pouched-shaped "subdepression" similar to that seen in the sea urchin, and similarly containing randomly oriented, finely fibrous material (f) in a nonelectron-scattering matrix. A somewhat heterogeneous substance of medium density (g) lies between the outer side of the bowl and the nuclear envelope.

The suggestion has been made (Colwin and Colwin, 1961a, 1963a) that the use of the term "acrosome" be limited to refer to the acrosomal vesicle and its contents. This usage does not seem to be practicable, however, in the case of the echinoderms, since no vesicle can be recognized in the acrosomal complex.

B. BIVALVE MOLLUSKS

The three species, representing different orders of the Pelecypoda, which have been investigated by electron microscopy (*Crassostrea virginica*: Galtsoff and Philpott, 1960; *Barnea candida*: Pasteels and de Harven, 1962; *Mytilus edulis*: Nijima and Dan, 1965a) are alike in the general design of their acrosomes. A prominent acrosomal vesicle is given the form of a hollow cone by an invagination of its posterior side. The lumen of this invagination is continuous with a depression in

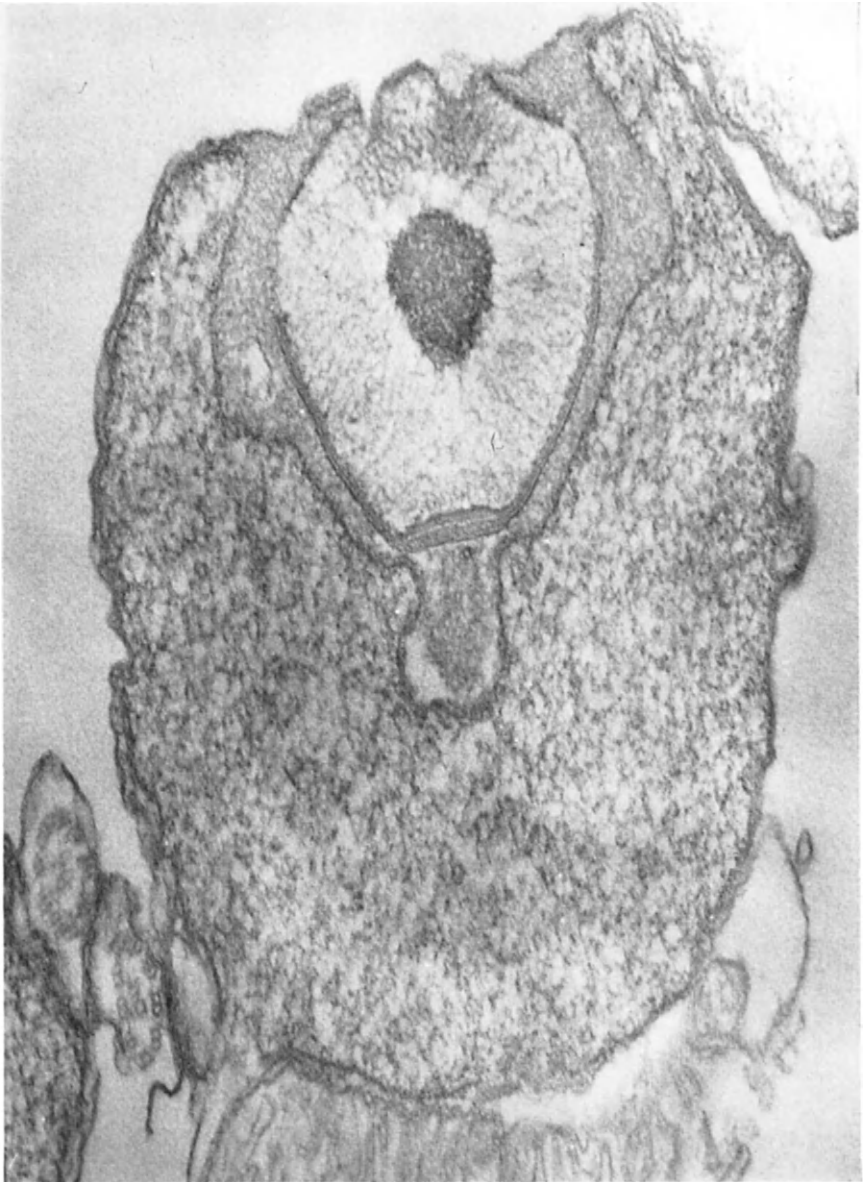


FIG. 7. Sagittally sectioned spermatozoon of *Asterias amurensis*. See Fig. 8 and text for description of components. Magnification: $\times 100,000$. (Courtesy of Y. Hagiwara.)

the anterior side of the nucleus which contains fibrous material in a matrix. Extending through the axis of this common lumen is a bundle of aligned fibrils organized anteriorly into a compact rod. Within the vesicle several substances are distinguishable by their different textures. All of the vesicular surface not in contact with the nucleus is closely invested by the plasma membrane.

1. *Mytilus edulis*

The acrosomal vesicle of the *Mytilus* spermatozoon has the shape of an extended hollow cone (Fig. 9a), its base forming a ring which rests against the anterior face of the nucleus. The characteristically shaped

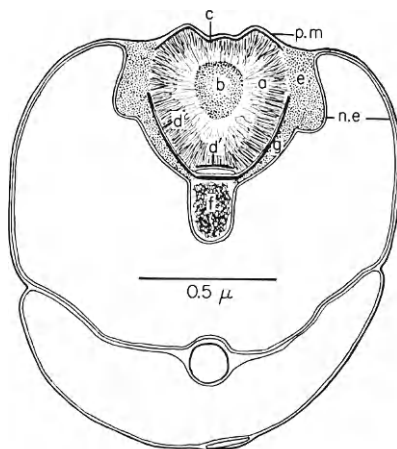


FIG. 8. Fine structure of starfish acrosome (*A. amurensis*). (a-g) Components of acrosomal process (see text). (n.e) Nuclear envelope. (p.m) Plasma membrane.

invagination of this vesicle (a') passes as a narrow pore through the center of the ring, expands anteriorly, and then narrows again to form a blind tube which fits closely over the apical end of the rod (b). The latter maintains a uniform diameter of about $50 \text{ m}\mu$ throughout its length within the invagination and extends into a slender tubule formed by the nuclear envelope. This tubule passes through the center of the nucleus and connects with the posterior pocket containing the centrioles and the base of the flagellum; most of its extent is loosely filled with unoriented material (c) of a density similar to that of the organized rod and continuous with its basal end.

The acrosomal vesicle itself contains a minimum of two substances distinguishable by their different appearance (after osmium fixation), or possibly three, on the basis of their location in the vesicle. One of these (d), which makes up an irregular layer lining the outer wall of the vesicle from the boundary of the basal ring (e) to the apex, consists

of loosely aggregated particles interspersed with very fine tubules extending more or less perpendicularly from the inner surface of the vesicle membrane. Around the expanded part of the invagination lumen, this layer (f) is about $200\text{ m}\mu$ thick and completely fills the space between the outer and inner (invaginated) sides of the acrosomal vesicle. In this region the tubules are more conspicuous than they are in the thinner part of the layer.

A second substance (g), visually indistinguishable from material (h) located in the ring-shaped base of the vesicle, forms a slightly tapered

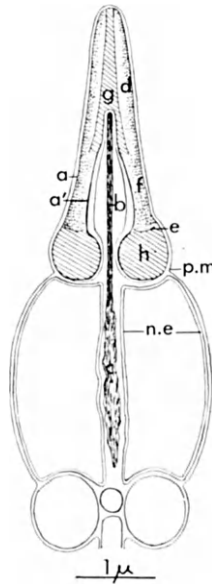


FIG. 9. Fine structure of *Mytilus* acrosome. (a) Acrosomal vesicle. (a') Invaginated part of acrosomal vesicle. (b) Rod. (c) Unorganized fibrous material. (d) Layer of particulate material and fine tubules lining the outer part of the acrosomal membrane. (e) Partition between the basal ring and the lining layer. (f) Thick part of the lining layer. (g, h) Particulate material in the apex and base of the vesicular lumen. (n.e) Nuclear envelope. (p.m) Plasma membrane.

cylindrical strand that reaches from the apex of the invagination to the tip of the acrosome. In view of their similar texture and identical behavior when the acrosome reacts (see Section V), it seems probable that (g) and (h) represent, respectively, the apical and basal locations of a single substance.

2. *Barnea candida* (Pasteels and de Harven, 1962)

The *Barnea* acrosomal vesicle (Fig. 5N) takes the form of a broad cone with a simple tubular invagination that extends through the vesicu-

lar axis to end in contact with the inner surface of the apex. The vesicle itself contains a finely particulate component concentrated around the wall of the tubular invagination, and a large amount of spongy material made up of coarse granules connected by fine fibrils.

The shallow nuclear depression is sparsely filled with fibrillar material which extends into the invaginated tubule and is organized apically into a rather compact rod about $60\text{ m}\mu$ in diameter. The tip of this rod is capped by the two layers of acrosomal membrane and the plasma membrane.

C. POLYCHAET ANNELIDS

The spermatozoa of the three species which have been investigated, *Hydroides hexagonus* (Colwin and Colwin, 1961a), *Nereis japonica* (Takashima and Takashima, 1963), and *N. limbata* (Fallon and Austin, 1963), are alike in having a prominent acrosomal vesicle occupying the anterior part of the sperm head. In *Hydroides* (Fig. 5L), the vesicle contains a central granule, and its membrane is lined with finely particulate material. In its adnuclear wall is a cluster of short tubular invaginations filled with particulate periacrosomal material (see Chap. 7 for details).

The acrosomal vesicle of the *Nereis* spermatozoon (Fig. 5K) is very deeply invaginated, so that it closely resembles a bivalve acrosome, particularly that of *Barnea*. Its most striking feature, however, is a closed-tubular rod, about $100\text{ m}\mu$ in diameter and $2.5\text{ }\mu$ long, which extends through the center of the invagination and into a deep pocket in the nucleus, where its basal end is attached to the nuclear envelope. The acrosomal vesicle is completely filled with particulate material; the orifice of the invagination is blocked by an annular mass of particles which fits closely around the shaft of the rod and appears to lack a bounding membrane.

D. ARTHROPODS

Of the tremendous number of species included in the class Insecta, only the cricket (Fig. 5P) has been selected for electron microscopic study of its acrosome (Kaye, 1962). Its structure will remain difficult to interpret, however, until information is obtained concerning the changes which it undergoes as it approaches the egg.

Bawa (1964) found that the spermatozoon of the thysanuran *Thermobia* (Insecta) is a much-elongated cell ($600\text{--}650\text{ }\mu$); a centriole at its forward end gives rise to the locomotor apparatus ($9 + 2$ filaments), which extends the whole length of the cell. The nucleus parallels it throughout, except posteriorly, where it gives place to an elongated

acrosome. This location of the acrosome at the hind end, with respect to the direction of locomotion of the sperm cell, led Bawa to question its role in fertilization. An explanation of this apparent anomaly may lie in the conditions under which fertilization takes place. According to Wigglesworth (1953), insect spermatozoa generally proceed under their own power to the spermatheca, from which they are expelled by muscular action directly into contact with the micropyle of the egg as it passes down the oviduct. In this situation, it may well be an advantage to have the acrosome at the posterior end of these extremely long cells.

The cylindrical spermatozoon of the tick (*Amblyomma*), as described by Reger (1963), is about $300\ \mu$ long, with a deep invagination (ca. $50\ \mu$) at its posterior end. The acrosome, a completely flattened vesicle, is closely applied to the plasma membrane in the invagination and around its opening. This acrosome is the simplest of these organelles which has so far been described in any animal.

The fine structure of the spermatozoon has recently been described in three crabs: *Eriocheir japonicus* (Yasuzumi, 1960), *Callinectes sapidus*, and *Menippe mercenaria* (Brown, 1966). The acrosomes of the three genera show the same general pattern, while varying considerably with respect to the shapes and textures of the component parts. In *Menippe* (Fig. 10), a posterior invagination of the acrosomal vesicle forms a blind tubule, which ends anteriorly in contact with a mushroom-shaped apical "cap." Surrounding the mouth of the invagination, immediately inside the vesicle, a thin, collarlike element is applied to the tubule membrane.

The surface of the apical cap is the only part of the acrosome not covered by nuclear membrane. The cap consists chiefly of a thick plate of dense material, perforated at its center by the end of the axial tubule. A thin, highly osmiophilic layer covers the surface of the cap, immediately beneath the outermost membrane. In both of the other genera also, this region shows strong osmiophilia; Yasuzumi (1960) has suggested that it may include a lipoprotein.

The tubule is distinguished from the rest of the acrosome by its failure to give a positive PAS reaction. Its lumen contains fibrous elements; its membrane is surrounded by several materials which are clearly different from the homogeneous major component of the nearly spherical acrosomal vesicle. This complicated acrosome presents a startling contrast to the simple one just observed in the tick.

André (1963) reported that the acrosomal vesicle of the *Xiphosura polyphemus* (horseshoe crab) spermatozoon is a somewhat flattened hemisphere, with a conical invagination in the side toward the nucleus (Figs. 11A and 12A,C). It contains a centrally located hyaline com-



FIG. 10. Electron micrograph of sagittally sectioned crab acrosome (*Menippe mercenaria*). (Courtesy of G. G. Brown.)

ponent and a denser peripheral substance. The nucleus is traversed by an elongate organelle, the tip of which lies in the apex of the vesicular invagination. Emerging from the posterior side of the nucleus alongside the centriole, it describes an ascending spiral, winding usually six times around the nucleus within a space interposed between the two membranes of the nuclear envelope (Figs. 11B and 12B). The diameter of its basal end is 150–170 $m\mu$; this tapers to 50–60 $m\mu$ at its tip. In cross

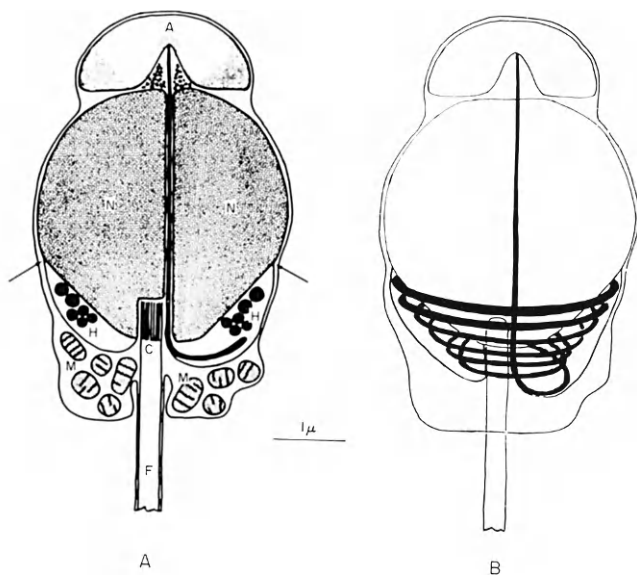


FIG. 11. Fine structure of horseshoe crab spermatozoon (*Xiphosura polyphemus*, André, 1963). (A) Sagittal section of sperm head. (B) Three-dimensional diagram to show coiling of the rod about the posterior part of the nucleus. (A) Acrosome. (C) Centriole. (F) Flagellum. (H) Cross sections of six helices of the rod in the space between the two membranes of the nuclear envelope. (M) Mitochondria. (N) Nucleus.

section it is seen to consist of a bundle of regularly arranged fibrils ca. 80 Å in diameter, surrounded by a single membrane.

Reger (1964) studied the fine structure of the spermatozoon of an isopod, *Asellus militaris*. He found that this very aberrant, nonmotile sperm cell has no acrosome at all.

E. VERTEBRATES

The only information concerning the fine structure of amphibian acrosomes is derived from the study of spermiogenesis in *Bufo* (Burgos

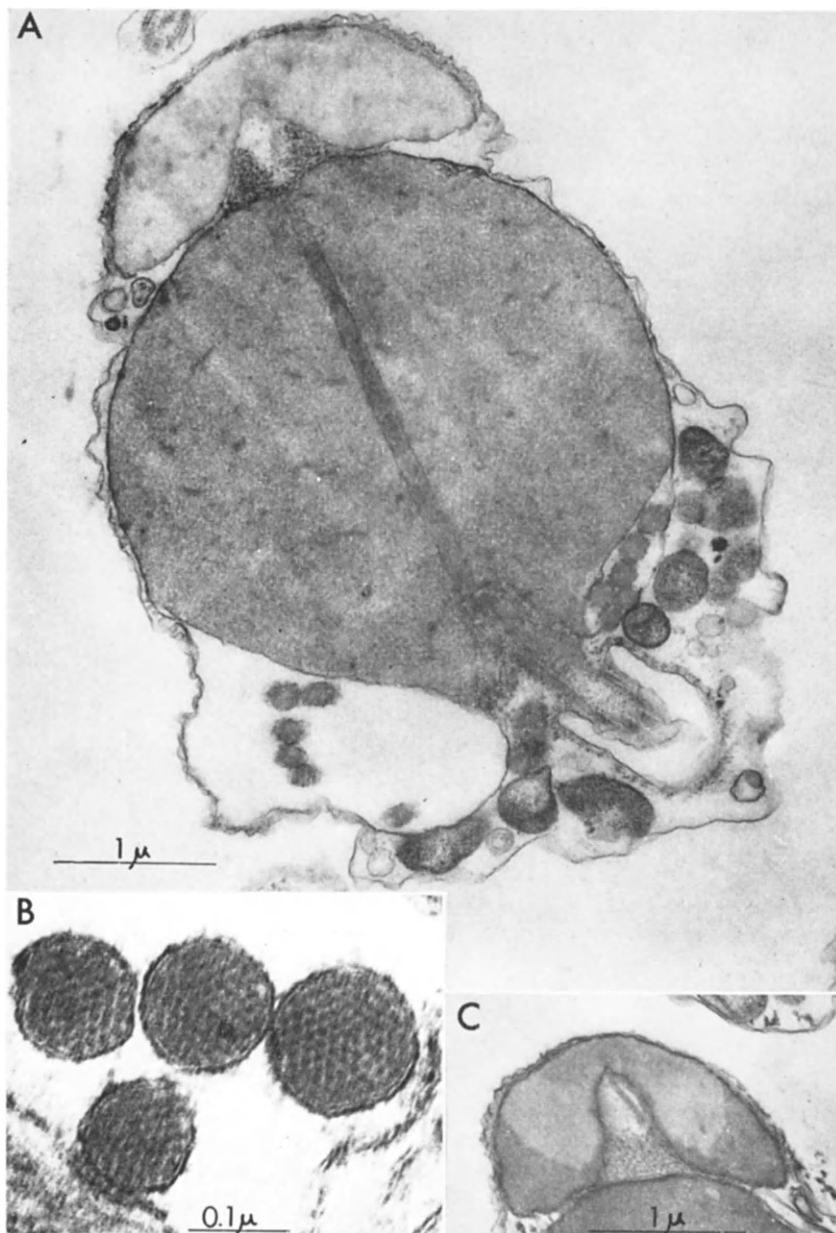


FIG. 12. Sagittally sectioned spermatozoon of *Xiphosura polyphemus* (André, 1963). (A, C) See explanation of Fig. 11A. (B) Cross section of coiled rod, showing component 80 Å fibrils. Magnification: (A) and (C) $\times 12,000$; (B) $\times 75,000$.

and Fawcett, 1956). From this we know that the late spermatid acrosome consists of an acrosomal vesicle uniformly filled with particulate material. This forms a conical cap which covers a perforatorium and extends over the anterior part of the elongate nucleus. Presumably it is somewhat more condensed in the mature spermatozoon.

The presence of a perforatorium in the salamander spermatozoon can confidently be inferred from its external structure as observed with the electron microscope in whole mounts (Dan, unpublished). The slender apex of this very large sperm cell has a sharply barbed tip which must be made of resistant material, since it undergoes no perceptible deformation during fixation and vacuum desiccation.

In the birds, also, the presence in the mature spermatozoon of a simple acrosomal cap covering a perforatorium is deduced from the appearance in the late spermatid of a fibrous rod within the invagination lumen of a conical acrosomal vesicle (Nagano, 1962).

Thin-section study of mammalian spermatozoa began with the observations of Clermont *et al.* (1955) on the rat. These workers reported that the apical part of the curved sperm head (Fig. 5S) includes a sharply pointed, three-pronged perforatorium which is distinct from the acrosome. They interpreted this structure, which they found to be the part of the cell most resistant to alkaline hydrolysis, as an outgrowth or thickening of the nuclear membrane.

A recent report by Pikó and Tyler (1964) describing the structure of the rat epididymal spermatozoon represents the perforatorium as consisting of densely packed fibrous material separate from the nuclear envelope. This confirms the finding of Burgos and Fawcett (1956) that the perforatorium in *Bufo* arises contiguous to, but distinct from, the nuclear envelope. Most of the finely granular contents of the rat acrosomal vesicle are accumulated in a thick crest along the convex edge of the nucleus and perforatorium; the flattened periphery of the acrosomal sac covers the sides of the perforatorium and about three-fifths of the nucleus. The concave surface of the perforatorium is covered by the plasma membrane only.

The acrosomal cap of the rabbit spermatozoon is thickened along the anterior edge of the nucleus and thins to cover about two-thirds of its flat surfaces (Fig. 5T). According to Hadek (1963a,b), a thin layer of cytoplasm, continuous with the postnuclear cap, separates the acrosomal cap from the nuclear membrane. At the anterior extremity of this layer, along the apical edge of the nucleus, there is found a crest of material, sharply triangular in sagittal section, which is in immediate contact with the inner surface of the acrosomal membrane. Hadek

(1963a,b) called this the "apical body," following Blom and Birch-Andersen (1961); who also reported the presence of an "apical body" in the bull spermatozoon. In this case, however, the term is applied to the whole anterior edge of the acrosomal vesicle and refers to the part often called the "acrosome" by observers of mammalian spermatozoa. A certain amount of differentiation is visible in this region, and it seems not unlikely that higher resolution will disclose some perforatoriumlike structure there, similar to that of the rabbit spermatozoon.

V. Fine-Structure Studies of Acrosomal Changes

A. INVERTEBRATES

The major changes which together make up the acrosome reaction as it is seen in invertebrate spermatozoa are the opening and removal of the membranes and layers forming the superficial parts of the intact organelle, the dissipation of the lytic material when such is present, and the formation of an acrosomal process from precursor materials located in the interior of the acrosome. When an acrosomal vesicle is present, a more or less deeply invaginated posterior portion of its membrane is extended to become the covering of the process. Within this covering other materials from different parts of the acrosomal complex usually form fibrous supporting elements, which may be wholly or partially preformed in the intact acrosome or organized at the time of reaction. The spermatozoa of sea urchins and starfishes lack an acrosomal vesicle; the membrane of the acrosomal process is formed from laminate or dispersed material.

The course of acrosomal process formation has been followed step by step in five animals: the annelid *Hydroides*, the enteropneust *Saccoglossus*, the mussel *Mytilus*, the sea urchin *Pseudocentrotus*, and the starfish *Asterias*. The fine structure of the reacted acrosome has also been observed in the annelid *Nereis*, the horseshoe crab *Xiphosura*, and the crabs *Callinectes* and *Menippe*. The first two examples are described in full in Chapter 7; the others will be reported here. These accounts will represent a considerable condensation of the original descriptions, to which the reader is referred for detailed evidence and discussion.

1. *Mytilus edulis* (Niiijima and Dan, 1965b)

The apex of the acrosome opens and the membrane of the acrosomal vesicle (a) fuses, at the rim of the opening, with the plasma membrane (Fig. 13A,B). The outer wall of the acrosome, consisting of these fused membranes and the material (d) lining the inside of the vesicle, is turned outward and reflected back toward the nucleus. This process

is believed to be brought about by rapid swelling of the lining material prior to its dissolution; some of this material precedes the edge of the membrane complex as it is carried toward the base of the acrosome (Fig. 13B, d').

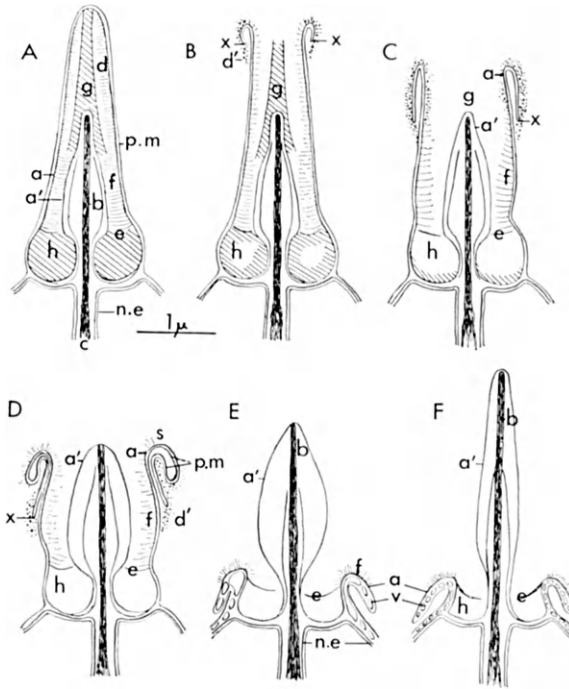


FIG. 13. Mode of reaction of *Mytilus* acrosome. Stages (A-F) were found in spermatozoa fixed 1 second after addition to egg suspension. (A) Intact acrosome. (B) Acrosome opened at apex, acrosomal and plasma membranes fused at (x), turned outward. Acrosomal vesicle is slightly swollen apically, and material at (g) and (h) is beginning to dissipate. (C) Increased swelling of the acrosomal vesicle; material at (g) and (h) has practically dissipated. (D) Secondary reflexion of the outer membranes (s) and swelling of the invagination (acrosomal process) membrane (a'). (E) The outer membranes are completely removed; the rod (b) has begun to elongate; the apposed surfaces of the plasma membrane vesiculate (v) under the acrosomal membrane. (d') Dissolving material of lining layer (d). For other components, see Fig. 9.

When the height of the acrosome has been reduced to about half, a secondary reflexion of the membrane complex occurs, although its fused edge continues to move toward, and finally over, the anterolateral part of the nucleus. By this stage all the particulate material of the apical and basal lumina (g) and (h) has disappeared (Fig. 13D), exposing the precursor of the acrosomal process (a'). The thick region of the lining layer (f) is next reverted, and the acrosomal process

expands somewhat and elongates (Fig. 13E,F). All these stages are found in material osmium-fixed 1 second after mixing eggs and sperm.

In supernumerary spermatozoa which have reacted in close contact with the egg surface, the length of the process (ca. 3μ) is less than that of the intact acrosome, and only slightly greater than that of the vesicular invagination (ca. 2.5μ). When the reaction is induced in freely suspended spermatozoa, however, the process may attain a length of 10–14 μ . The apical end of the rod forming its axis is always found in contact with the inside of the membrane at the tip of the process, and in long processes the membrane closely invests the surface of the rod.

2. *Pseudocentrotus depressus* (Dan *et al.*, 1964)

When suspended spermatozoa are mixed with a dilute seawater solution of the jelly surrounding their eggs, a thin layer of osmiophilic substance (d), which caps the acrosomal granule under the plasma membrane, expands and disintegrates, removing this apical section of the plasma membrane and exposing the surface of the acrosomal granule (Fig. 14A,B); the material (i) at the sides of the acrosome also loses its dense appearance. The acrosomal lumen is distended, exerting pressure against the outer side of the bowl-shaped complex of layered materials (e), (f), (g), (h) which pushes it forward (Fig. 14C). The three layers at the center of the complex form a lens-shaped mass, which then yields to the internal pressure and bulges in the apical direction, giving rise to a blind tubule at the center of the acrosomal granule (Fig. 14D, p). The basal part of this tubule incorporates the layer (e), which forms a flared connection between the main part of the process and the intact plasma membrane at (x-x).

The fibrous material in the acrosomal lumen is organized into a slender, somewhat irregular, fiber (c') which extends through the axis of the tubule to its tip. The granule substance spreads over the surface of the process as it elongates, maintaining a layer of fairly uniform thickness. Formation of the acrosomal process to this extent is found in spermatozoa fixed 1 second after exposure to jelly solution (Fig. 14E).

The longest process observed in such fixations has a length of 0.17 μ ; during the next 7 seconds it reaches 0.3 μ , its membrane appears somewhat thicker, and the axial fiber increases in diameter and regularity of structure (Fig. 14F). At this time the granule substance is still spread over the whole surface of the process, and its quantity has not perceptibly diminished, but by 20–30 seconds very little of it remains except around the base of the process. The axial fiber continues to elongate to a final length of about 0.45 μ at 60 seconds, stretching its covering membrane so that the whole apical part of the sperm head

becomes more pointed (Fig. 14G). The granule substance is completely dissipated at this stage.

Since these changes, as they occur in natural fertilization, presumably take place while the spermatozoon is rapidly swimming through the jelly coat of the egg, there is little doubt that the acrosomal process makes contact with the egg surface immediately after its formation, and

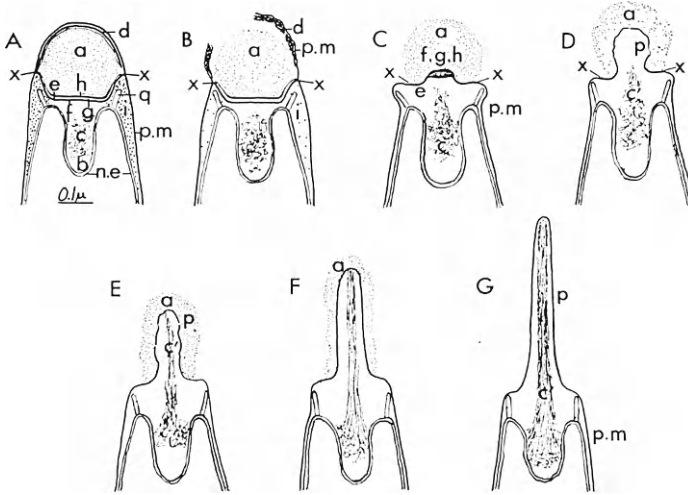


FIG. 14. Course of the acrosome reaction in sea urchin spermatozoon (*P. depressus*). Stages (A–E) were found in spermatozoa fixed 1 second after treatment with seawater solution of jelly coat material; (F) 8 seconds; (G) 60 seconds. (A) Intact acrosome (see Fig. 6 and text). (B) Beginning of acrosome reaction: the material of layer (d) swells and disintegrates, removing the plasma membrane to the ring (x-x). The material (i) loses electron opacity. (C) The process-membrane precursor complex (e, f, g, h) is pushed forward by the increase in the contents of the acrosomal lumen; (f, g, h) form a lens-shaped mass. (D) The mass of (f, g, h) material gives rise to a new process membrane (p) at the inner rim of (e); (e) is continuous with the plasma membrane around (x-x). The fiber precursor (c) extends into the axis of process (c'). (E) The process elongates; (c') is organized into a slender fiber. The substance of the granule (a) spreads over the surface of the process membrane (p). (F) (8 seconds) The process is elongated and the granule substance is spread evenly over its surface. (G) (60 seconds) The process length is ca. $0.45\ \mu$; the granule substance has completely dissipated.

that fusion of its membrane with the egg plasma membrane (see Chap. 7) takes place within about 2 seconds after the beginning of the acrosome reaction. The changes observed in the acrosomal process after this time, therefore, probably have meaning only as they may offer suggestions with respect to the nature of the chemical reactions underlying them.

3. *Asterias amurensis* (Dan and Hagiwara, in press)

As in the sea urchin acrosome reaction, the corresponding changes in the starfish spermatozoon which lead to the first appearance of the acrosomal process take place within 1 second. In this animal, however, the acrosomal process continues to function for at least 2 minutes—the period required for the sperm head to traverse the dense jelly layer and reach the egg surface.

When a suspension of *Asterias* spermatozoa is treated with dissolved jelly substance, the first change is seen at the anterior face of the acrosome (Fig. 15A,B), where the osmiophilic layer (c) is found to have sunk a short distance below the margin of the surrounding band (e), carrying with it the overlying plasma membrane (p.m'). The latter is thus severed around the circle described by the inner edge of (e), and together with the circular plate of (c) disintegrates into transitory vesicles and membranous debris. The granule (b) disappears (Fig. 15C, b'). The (a) material retracts centrifugally to form a spongy layer (a') which becomes amalgamated with the inner surface of its bowl-shaped bounding layer (d), and extends past its rim, over the inner side and anterior edge of the (e) band and onto the anterolateral part of the nucleus (Fig. 15D).

While this is going on, the double-layered disk (d') begins to bulge forward to form the acrosomal process, and randomly oriented fibrous material beneath it moves to the upper part of the "subdepression." From its anterior side a slender strand of closely aligned fibrils (f') extends in the axis of the elongating process. At 1 second the process reaches slightly beyond the original surface of the acrosome (Fig. 15E,F). Observation of process length is complicated by various factors, such as agglutination of reacting spermatozoa and increased probability of damage to longer processes, so that it is difficult to obtain a reliable measure of the rate of process elongation; the greatest length observed in a 4-second fixation was 14 μ .

In sectioned material fixed at 4 seconds, the spongy (a') material is found to have condensed into a membrane continuous at its periphery with the plasma membrane at the sides of the nucleus, and centrally with the process membrane. At the base of the process, this membrane is being drawn out of the nuclear depression (Fig. 15G), apparently by the continued elongation of the fibrous shaft. At 4 seconds this retains its original diameter of about 50 m μ , but during the next 4 seconds its diameter is more than doubled (Fig. 15H). At about this time the nucleus rounds up and the nuclear depression is obliterated; as a

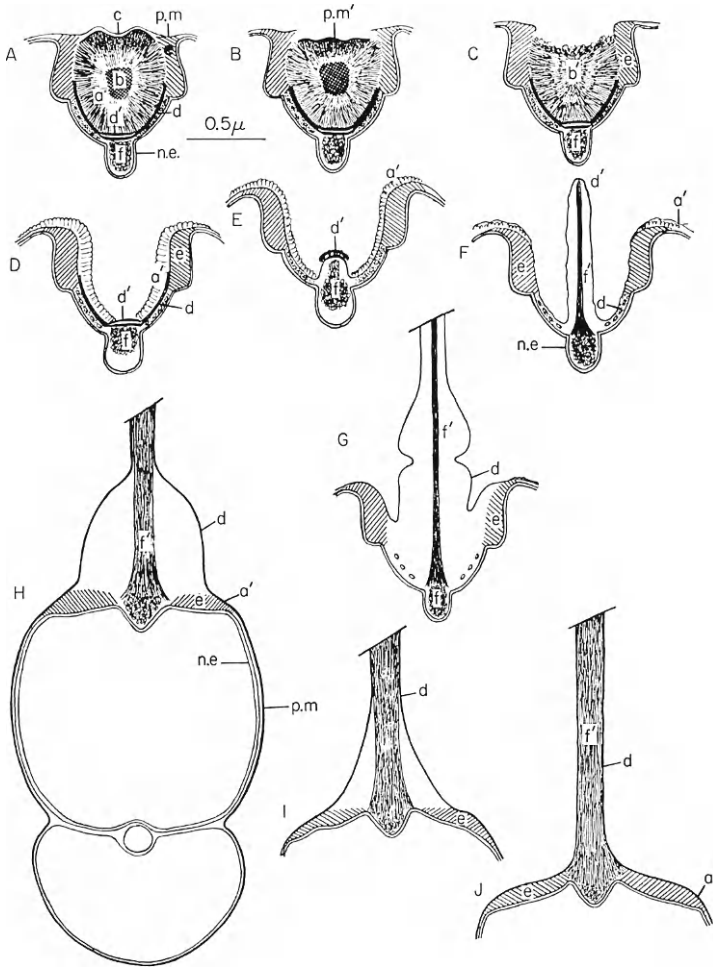


FIG. 15. Acrosome reaction of starfish spermatozoon (*A. amurensis*). Stages (A–F) were found in spermatozoa fixed 1 second after treatment with jelly solution; (G) 4 seconds; (H) 8 seconds; (I) 20 seconds; (J) 60 seconds. (A) The intact acrosome (see text). (B) Layer (c) has sunk into the acrosomal depression, severing the plasma membrane (p.m') at the inner edge of the band (e). (C) The granule (b') is missing; layer (c) and the severed plasma membrane are disintegrating. (D) Material (a) is retracted centrifugally onto the surface of (d) and outward over the rim of band (e). Layers of process-membrane precursor (d') are beginning to bulge forward. (E) The subdepression is distended; the fiber-precursor (f) is giving rise to the fibrous shaft (f') under the expanding process membrane (d'). (F) The most advanced stage found in 1-second fixations. The process is elongating with the smooth membrane (d'); (a') has retracted against layer (d) and the inner surface of (e) and forms a spongy layer over the anterior part of the nucleus. (G) (4 seconds) Elongation of the fibrous shaft pulls the process

consequence, the (e) band is changed into a flat plate with a central perforation encircling the base of the process shaft.

At 20 seconds (Fig. 15I) the process membrane is further drawn toward the shaft, and at 60 seconds (Fig. 15J) it is found in immediate contact with the plate of (e) material at the anterior surface of the sperm head, and the whole extent of the shaft except its base, which fits into a small crater derived from the subdepression.

4. *Nereis japonica* (Takashima and Takashima, 1963) and *N. limbata* (Fallon and Austin, 1964)

The reaction of the *Nereis* acrosome results in fusion of the plasma and outer acrosomal membranes following their opening at the sperm apex, dispersal of the vesicle contents, and complete extrusion of the axial rod to the outside of the nucleus, under the inner membrane of the acrosomal vesicle. The length of the rod is increased by about 60%, to about 4μ , in the reacted acrosome (*N. japonica*).

5. *Callinectes sapidus* and *Menippe mercenaria* (Brown, 1966)

Thin-sectioned crab spermatozoa, the acrosomes of which have reacted either in contact with the egg surface or in suspension (Fig. 16A,B), show that the center of the apical cap opens, with the invaginated part of the acrosomal membrane (central tubule) expelled through this opening, pulling the rest of the acrosomal membrane after it. This process turns the whole acrosome inside out and gives rise to a "spike" with a pointed, clearly differentiated apical component (Fig. 17). The negative PAS reaction of this apical structure identifies it with the central tubule (invaginated acrosomal membrane) of the intact acrosome.

The shaft of the spike is a tube formed by the everted acrosomal membrane. The now-basal edge of this membrane is attached to the periphery of the opened apical cap, which in entering spermatozoa is fused with the rim of a small aperture in the egg chorion. Its anterior extremity is identified in the reacted condition by the presence of the dense collarlike element which originally surrounded the base of the central tubule. The large mass of material which made up the major

membrane out of the acrosomal depression. (H) (8 seconds) The rounding up of the nucleus has obliterated the acrosomal depression and changed the band of (e) material into a perforated plate. The diameter of the fibrous shaft has increased. A single, continuous outer membrane extends from the sides of the nucleus (p.m) over (e) material (a') to the surface of the shaft (d). (I) (20 seconds) The slack in the membrane is taken up by continued elongation of the shaft (?). (J) (60 seconds) The surface membrane is in contact with all underlying structures.

component of the acrosomal contents is turned inside out around the shaft of the spike. Sections of entering spermatozoa indicate that this mass of material is everted as it passes through the opening in the chorion; it subsequently expands between the chorion and the egg

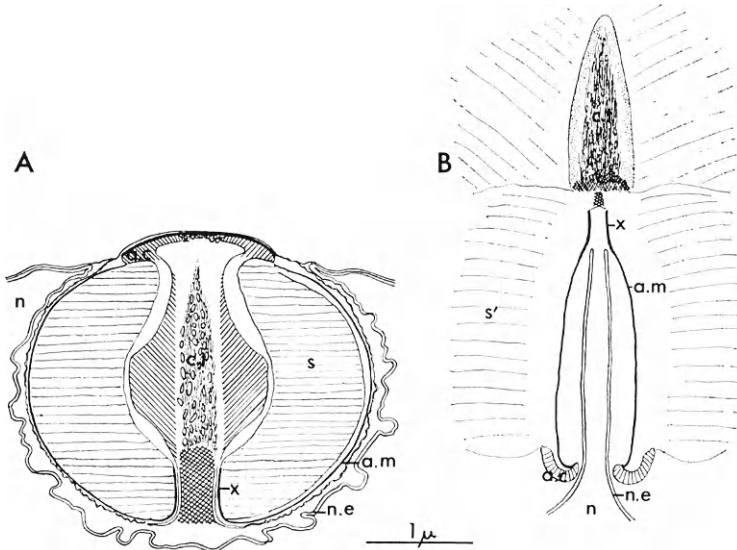


FIG. 16. Acrosome reaction in the crab (After Brown, 1966). (A) Sagittal section of the intact acrosome (*Callinectes sapidus*, cf. Fig. 10). (B) "Spike" formed by the reaction of the acrosome at the surface of the egg chorion. The perforated apical cap is fused with the chorion around the opening through which the acrosomal contents are everted. Swelling of the latter (s') has pushed back the egg plasma membrane. (a.c) Apical cap. (a.m) Acrosomal membrane. (c.t) Central tubule (invaginated part of the acrosomal membrane). (n) Nuclear substance. (n.e) Nuclear envelope. (s) Major component of the acrosomal vesicle. (s') Expanded acrosomal component. (x) Collar of dense material surrounding the base of the membrane of the central tubule.

plasma membrane, creating a space around the shaft of the acrosomal spike. Within the tube making up this shaft, laminated membranes extending into the nucleus form a funnel through which the nuclear contents apparently move toward the egg.

6. *Xiphosura polyphemus* (André, 1963)

What the author regards as an acrosome reaction comparable to those seen in other invertebrates takes place when spermatozoa of the horseshoe crab are suspended in seawater between slide and cover glass (Fig. 18). The acrosomal vesicle opens, the usual fusion between the

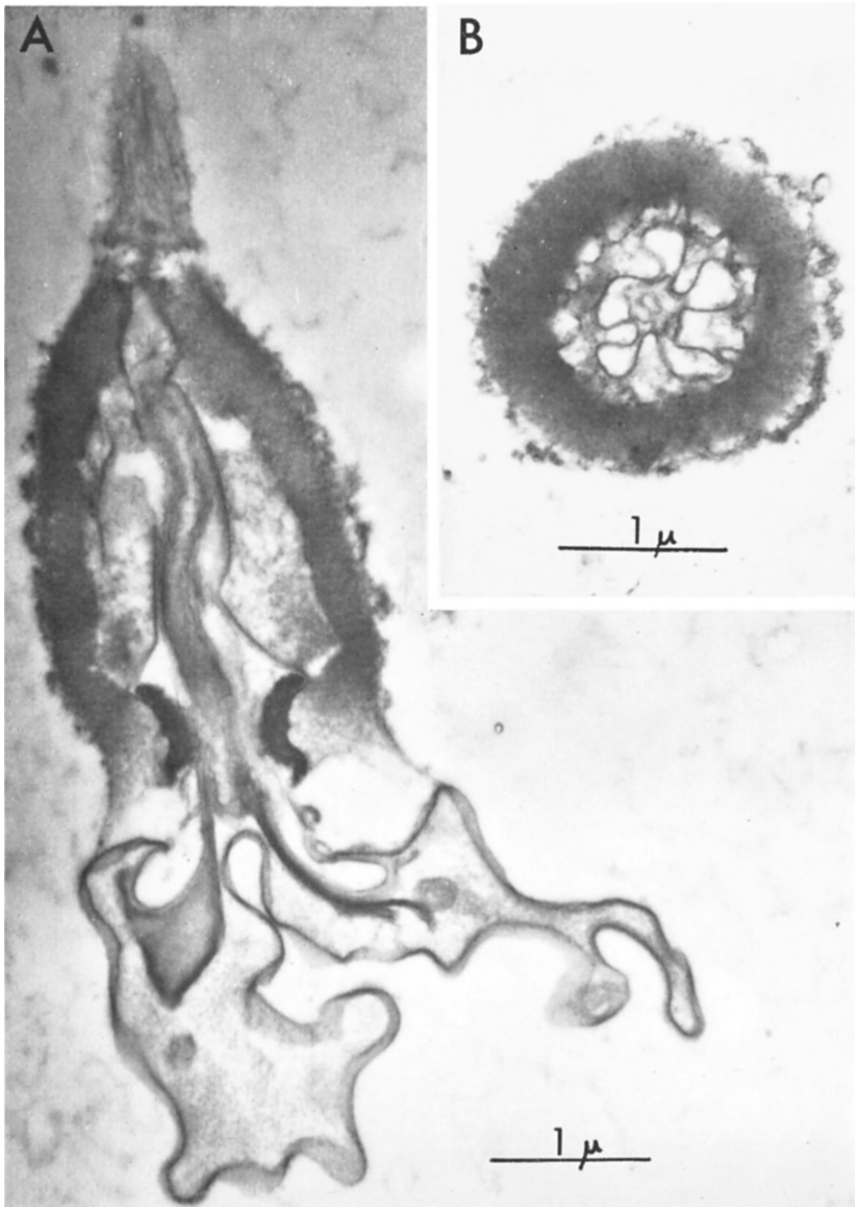


FIG. 17. (A) Longitudinal section of *Callinectes* spermatozoon which reacted in suspension. The contents of the acrosomal vesicle are precipitated by the action of the fixative (cf. Fig. 16B). (B) Transverse section through reacted *Callinectes* spermatozoon. (Brown, 1966.)

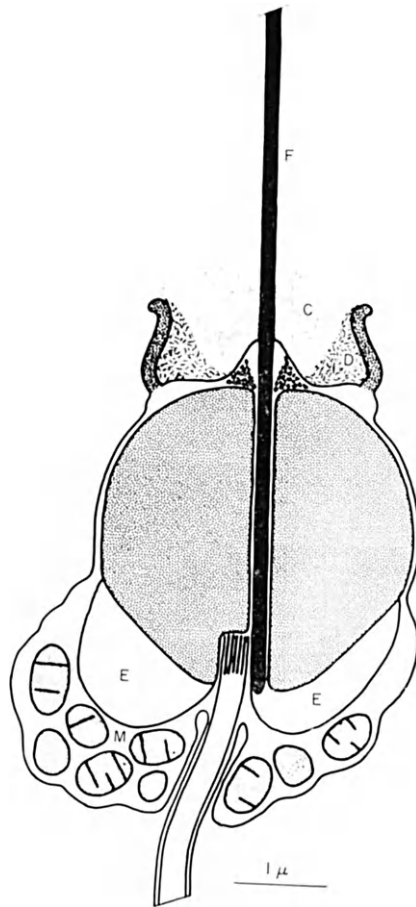


FIG. 18. Acrosome reaction in spermatozoon of *Xiphosura polyphemus* (André, 1963). (C) Hyaline component. (D) Dense component, of acrosomal vesicle. (E) Perinuclear space containing coiled filament before reaction. (F) Proximal part of acrosomal filament. (M) Mitochondria.

outer membrane of the acrosomal vesicle and the plasma membrane apparently takes place, and the elongate, fibrous organelle, originally found extending through the axis of the nucleus and coiled around its base, is abruptly expelled in the form of a straight, rigid rod about $50\ \mu$ in length. It would be very interesting to know whether the inner acrosomal membrane somehow stretches to cover the whole length of this rod, as it is found to do in other invertebrates.

B. VERTEBRATES

The simpler composition of the acrosome that characterizes the higher vertebrates apparently reflects the shift to internal fertilization, with its corollary of a favorable environment for the meeting of the gametes. This logic fails to apply, however, to the protective coverings of the eggs, which in the mammals are still more impregnable than those of their predecessors, even lacking the micropyle found as far along in evolution as the fishes. One result of this seemingly haphazard arrangement is that the golden hamster spermatozoon, after dissolving its way through the follicle cell layer, is observed to spend 3–4 minutes in penetrating the zona pellucida to arrive at the egg surface (Yanagimachi, unpublished observation), whereas the sea urchin spermatozoon reaches its goal in 2 seconds (Rothschild and Swann, 1952).

The circumstance that the reproductive tract of the female is the site of whatever changes the mammalian spermatozoon undergoes as it approaches the egg has hitherto hampered investigation of these changes. Recent success in securing fertilization of mammalian eggs *in vitro*, however, paves the way for more accurately controlled experimentation which should facilitate observation of mammalian acrosome behavior.

Earlier work on rodent spermatozoa (see Vol. II, Chap. 8) has demonstrated that the structure and properties of the acrosome are affected during its passage through the female reproductive tract so that its attachment to the sperm head becomes less firm. Hyaluronidase released from the loosened acrosome attacks the intercellular cement (hyaluronic acid) and enables the spermatozoon to pass through the layer of follicle cells (corona radiata). Detachment of the acrosome exposes the perforatorium, which the spermatozoon is believed to use in making its way through the mammalian equivalent of the vitelline coat, the zona pellucida. Austin and Bishop (1958) have proposed that a second enzyme, specific for the zona substance, is associated with the perforatorium. Pikó (1964), on the other hand, believes that the rodent acrosome contains two components: one "located along the anterior and dorsal edge of the acrosome is thought to be hyaluronidase," while a "deeper located component could be a hypothetical 'zona lysin.'"

Moricard (1960) observed thin sections of rabbit spermatozoa fixed within "tunnels" which are formed in the zona pellucida by the penetrating sperm cells. He reported (1964) that no trace of the acrosome lies ahead of the nucleus, although there is a "comma-shaped" structure which might be a perforatorium ("en partie perforatorium des classiques"). This may well be the structure called the "apical body" by Hadek (1963a,b), although Hadek believes that it more closely re-

sembles the invertebrate acrosome than the rat perforatorium. However, his diagrams illustrating rabbit spermatozoa which have passed through the zona are very similar to diagrams of sagittally sectioned rat spermatozoa (see Vol. II, Chap. 8) fixed at the same stage of penetration (Pikó and Tyler, 1964). These authors report that the acrosome of spermatozoa which have just entered the zona is open and empty; the outer acrosomal membrane is fused with the plasma membrane to form small vesicles.

Yanagimachi (1964), observing living golden hamster spermatozoa at the surface of the zona pellucida, reported that "The tip of the perforatorium bends as the head of the spermatozoon moves, suggesting that this structure is not rigid, but rather flexible." Phase-contrast micrographs of several living golden hamster spermatozoa, attached to the zona about the time of penetration into the ovum, show the acrosome lifted or missing. In these cells, the tip of the exposed perforatorium may maintain its usual shape or be curled either inward or outward (Yanagimachi and Chang, 1964).

VI. Lysins

A. ISOLATION AND CHARACTERIZATION OF VITELLINE-COAT LYSINS

The search for a lytic substance in the sperm cell at first aimed to find an enzyme which would dissolve a superficial layer of the egg cytoplasm and thereby activate the egg; this masked the meaning of Hibbard's (1928) discovery that *Discoglossus* spermatozoa contain an agent which attacks the jelly coat of the eggs. In 1939, Tyler extracted from the spermatozoa of the keyhole limpet (*Megathura*) a lysin which was found to dissolve the egg membrane without exerting any other effect on the egg. Krauss (1950) subsequently made an exhaustive study of this lysin and the conditions under which it is released, and Berg (1950) found a similar substance in seawater extracts of the frozen-thawed spermatozoa of *Mytilus*. Tyler (1948) concluded that such lysins are instrumental in enabling the individual spermatozoon to penetrate through the membrane barriers to the surface of the egg proper.

The *Mytilus* lysin has been isolated by Hauschka (1963) and purified to the extent that it showed a single peak sedimenting at 3 S, with a minor component sedimenting slightly faster. It failed to exert a proteolytic action on serum albumin, although trypsin has an effect similar to that of the lysin on the vitelline coat substance. Hauschka (1964) further reported that the vitelline coat resists lysis by lysozyme, whereas it is dissolved by pronase; it is also dissipated by pH above 10.5, distilled

water (8 washes), prolonged treatment with 1 M NaCl, cysteine (0.2 M, 0.5 M NaCl, pH 8.2), 25% formamide, and 5 M urea. It is not attacked by a number of common organic solvents. Treatment with antiegg serum protects the vitelline coat against lysis by the purified lysin from sperm as well as by other enzymes, but the fact that it also makes it resistant to dissolution by formamide suggests that the serum produces a stronger binding within the vitelline coat material rather than acting as a blocking agent against the attacking enzymes.

Kigawa (in preparation) separated a lytic substance from frozen-thawed testes of the archeogastropod *Tegula pfeifferi* by ammonium sulfate fractionation of the seawater extract. Using turbidimetry to measure the capacity of the lysin to attack isolated egg membranes of this species, she found that it has the following characteristics: (a) it is heat-labile; (b) it is a globulin which dissolves most readily at approximately the ionic strength of seawater; (c) its optimum pH is 8.25; and (d) it is inhibited by sulfhydryl reagents such as PCMB and cystine.

According to Haino and Kigawa (1966), the egg membrane of *Tegula* which forms the substrate of this lysin is an acid glycoprotein containing the substances (% of) as shown in the following tabulation.

protein	33.1 %
glucosamine	6.7
galactosamine	16.7
fucose	11.2
galactose	1.4
sulfate	9.3
acetyl	8.5
metals (chiefly Mg)	2.9

Its constituent amino acids include large amounts of serine, threonine, proline, and oxyproline, and small amounts of basic amino acids.

Haino (1965) isolated a soluble product from the 60,000 g supernatant derived from digestion of the *Tegula* egg membrane by the acrosomal lysin. She found that the digestion product has reducing ends and fails to show N-terminal groups. This result makes it possible to state conclusively that the *Tegula* acrosomal lysin possesses at least polysaccharose activity.

B. LOCALIZATION IN ACROSOME

Utilizing the fact that the *Mytilus* acrosome can be induced to react by treating it with calcium-rich seawater, Wada *et al.* (1956) determined that the supernatant from reacted sperm suspensions includes a com-

ponent which disperses the vitelline coat of the egg; no such activity is found in a control supernatant of intact spermatozoa. Since the only observable effect on the spermatozoa of the calcium treatment is the change in the acrosome, this result is interpreted as showing that the lytic substance is located in the intact acrosome and released into the medium when the acrosome reacts.

A thin-section study of *Mytilus* spermatozoa (Nijima and Dan, 1965b) shows that particulate material (Fig. 13A-D, g,h), located in the base and the apical part of the acrosomal vesicle, disappears within 1 second after the initiation of the acrosome reaction. This rapid dispersion in seawater, the location of the material in the central lumen of the acrosomal vesicle, its homogeneously particulate appearance after osmium fixation, and the fact that no role in connection with the mechanics of the acrosome reaction can be assigned to it point to this substance as the *Mytilus* vitelline-coat lysin, and the homologue of the "acrosomal granule" found in *Hydroides* (see Chap. 7) and sea urchin spermatozoa.

C. EFFECT ON EGG SURFACE

It has since been demonstrated (Humphreys, 1962; Dan, 1962) by electron microscopy of *Mytilus* eggs thin-sectioned after exposure to this lysin that the only effect of the lysin on the egg surface is to dissolve the rather thick (ca. $0.5\ \mu$) layer of substance making up the vitelline coat of the egg. No significant change could be detected in either the layer of very fine fibrils ("jelly coat") surrounding the vitelline coat or the plasma membrane of the microvilli extending through it.

A comparable lysis of the major component of the vitelline envelope protecting the *Hydroides* egg has been demonstrated in thin-sectioned material by Colwin *et al.* (1957). Further studies have shown (Colwin and Colwin, 1958, 1960a,b) that the lytic activity found in seawater extracts of frozen-thawed *Hydroides* sperm suspensions is destroyed by trypsin and chymotrypsin and by boiling. The active substance is non-dialyzable and is precipitated by about 60% saturation with ammonium sulfate. The authors conclude that the substance is probably a protein, presumably enzymic in nature. When *Hydroides* eggs are exposed to such extracts, the thin outer layer of the vitelline envelope is not attacked directly, but is caused to expand by the liquifaction of the thick middle layer. A thin inner layer is not affected by the lysin.

Thin sections of *Hydroides* spermatozoa (Colwin and Colwin, 1961 a,b) reveal the presence in the acrosomal vesicle of a mass of particulate material, which rapidly diminishes after the acrosome opens and disappears within a few seconds. This behavior suggests that the particulate material is the source of the lytic activity.

D. HYALURONIDASE

The rather general presence of hyaluronic acid as a component of intercellular cementing substances in various tissues led to some confusion in defining the role of hyaluronidase in fertilization after its discovery in mammalian testis extracts. In 1948, Austin advanced the suggestion that each spermatozoon carries a small amount of the enzyme, with which it dissolves the cement between the follicle cells of the corona radiata and makes its way to the surface of the zona pellucida. He further showed (1960) experimentally that 22 U/ml of hyaluronidase are present in the supernatant of a sperm suspension treated with digitonin to disrupt the acrosomes, in contrast to 2.4 U/ml in a control suspension treated with dilute formaldehyde, which fixes the acrosomes *in situ*. This result corroborates the conclusion of Wada *et al.* (1956) that the lytic agent detected in sperm extracts is located in the acrosome and released when the acrosomal vesicle is disrupted.

E. "ZONA LYSIN"

Acrosomes isolated with anionic detergents from ram, bull, and rabbit spermatozoa have been extracted with ethanol to give a lipoglycoprotein complex (Srivastava *et al.*, 1965) which disperses the follicle cells of rabbit ova and in some cases also dissolves the zona pellucida. The liberation of amino groups when this substance is incubated alone indicates that it possesses proteolytic activity in addition to its demonstrated lysis of hyaluronic acid. Whether the acrosomal isolate included the perforatoria of the sperm cells is not specified, but it is possible that these structures may have been detached by the detergents and a lytic agent extracted from them which attacks the zona material.

VII. General Considerations

A. TRIGGER MECHANISM

In all five of the cases in which the early steps in the acrosome reaction have been closely followed, a particular, apical part of the acrosomal complex is found to undergo the first change when the spermatozoon makes contact with the surface of an egg, or is treated with jelly solution. In every instance this is a part where the sperm plasma membrane is in close contact with the acrosomal membrane (*Hydroides*, *Saccoglossus*, *Mytilus*) or with a layer of osmiophilic substance (sea urchin, starfish). A similar osmiophilic layer also forms the outermost part of the acrosomal vesicle in decapod spermatozoa (see Section III).

The strong osmiophilia of this layer in the crab spermatozoon (Fig.

10) led Yasuzumi (1960) to suggest that it may consist of a lipid. Moses (1961) found that in crayfish spermatozoa it fails to stain with either fast green or the PAS method. In the sea urchin it is thinner but more osmiophilic than the plasma membrane which covers it.

The echinoderm spermatozoa are favorable objects for studying this "trigger layer," because their acrosomes respond to the stimulus of the chemically definable substance making up the jelly coat of the egg (Tyler, 1956). As described earlier (Section V,A), the first change observed in the sea urchin spermatozoon after exposure to jelly solution occurs in the osmiophilic layer which surrounds the apical half of the acrosomal granule. This layer thickens irregularly, expands, and within a fraction of a second it has broken and disappeared, together with the section of plasma membrane overlying it. One interesting aspect of this phenomenon is that the layer in question is located beneath the plasma membrane, where it would presumably be protected from any direct influence of the large mucopolysaccharide molecules of the jelly solution. This line of reasoning points to the possibility that the primary effect of the jelly substance may be to cause a change in the permeability characteristics of the sperm plasma membrane.

The situation in spermatozoa with a large acrosomal vesicle is most clearly evident in *Hydroides* (Colwin and Colwin, 1961a,b). The trigger mechanism in this case removes a small, circular segment of the acrosomal and plasma membranes; around the rim of the opening the edges of the severed membranes fuse. The authors suggest that a natural "fracture line" may be present in this location: i.e., some special characteristic of either the acrosomal or plasma membrane, or both, which results in instability in the presence of the egg envelope. The evidence from echinoderm and decapod spermatozoa, as well as the general concept that the various elements which play an active role in the acrosome reaction are all derived from the proacrosomal granule (vesicle) of the spermatid, suggest that such specialization is likely to occur in the acrosomal, rather than in the plasma, membrane.

B. PROCESS MEMBRANE

In spermatozoa with a prominent acrosomal vesicle, the establishment of continuity at the sperm apex between the originally separate acrosomal and plasma membranes doubles the area of surplus membrane which has to be removed to expose the process rudiment. In *Mytilus* (Fig. 13), this two-layered outer wall of the opened acrosome is turned inside out and stretched as it is carried backward to cover the anterolateral part of the nucleus, with the inner surface of the acrosomal membrane facing outward. The two apposed layers of plasma membrane

caught under the acrosomal membrane break up into small vesicles, and the free edge of the acrosomal membrane fuses with the plasma membrane around the sides of the nucleus. The result is a smoothly continuous, mosaic membrane enveloping the sperm cell and extending anteriorly as the surface of the acrosomal process.

Although the echinoderms lack an acrosomal vesicle, the same result (establishment of a mosaic membrane made up of plasma membrane plus the acrosomal process membrane) is achieved by a different series of changes. In the sea urchin, the membrane segment of acrosomal origin is newly formed (see Section V) from laminate precursor material. As this material is transformed into the process membrane, it becomes structurally continuous with the edge of intact plasma membrane girdling the acrosomal granule [Fig. 14A-C (x-x)]; after 2 seconds it is impossible to recognize exactly where this junction took place.

The membrane forming the base of the starfish acrosomal process (Fig. 15C-G) is derived from material which flows out of the acrosomal depression during the first 2 seconds of reaction and condenses into a membranous sheet within about 5 seconds. In spermatozoa fixed at 8 seconds this membrane is found to be continuous with the plasma membrane at the sides of the nucleus.

Precisely defining the membrane of the acrosomal process is not a simple matter (see Colwin and Colwin, 1963a, 1964). Even at this stage of investigation, while the mode of its most important function—that of fusing with the plasma membrane of the egg—remains unknown, the process membrane is found to have a number of unusual characteristics. As has been described above, it may be derived from a portion of the acrosomal membrane, turned inside out, or formed *de novo* from non-membranous precursors in the course of the acrosome reaction. In the former case it is capable in some species of an extreme degree of rapid expansion; the source of the supplementary membrane-forming material is not evident. The membrane can fuse edge-to-edge along “natural fracture lines,” with the plasma membrane surrounding it. At the sides of the nucleus, it may supersede a portion of the plasma membrane, fuse with the remainder, and presumably function as plasma membrane in this region.

It may also be suggested that the whole plasma membrane of most invertebrate spermatozoa is in a special category, since it seems nowhere to serve as the outer boundary of a mass of cytoplasm in the usual sense. The implications of this quasiautonomous nature of the sperm membranes constitute a challenging problem which will require for its resolution further investigation of both the fine structure and the physiological activities of these membranes.

C. PERFORATORIUM

Although there is an obvious need to clarify the content of the term "perforatorium" (see Colwin and Colwin, 1963a), it will remain difficult to do this until the function of this structure is more certainly known. If, as Austin and Bishop (1958) have suggested, the perforatorium is found to have associated with it a "zona lysin," this property will differentiate it from the various rods, fibrous cores, etc., which are recognized in invertebrate (and probably in lower vertebrate) acrosomal processes. There seems little reason to doubt that the role of the latter is in general that of a supporting element, serving to bring about elongation of the process and provide enough rigidity to ensure firm contact between the process membrane and the egg plasma membrane. Whatever their role, such fibrous cores are always covered by the process membrane, whereas the mammalian perforatorium should probably be exposed if it is to carry on lytic activity.

As has been outlined above (Section V), a number of investigators are currently studying the condition of rodent spermatozoa as they penetrate the egg envelopes (Moricard, 1960, 1964; Hadek, 1963a,b; Dickmann, 1964; Dickmann and Dziuk, 1964; Pikó, 1964; Pikó and Tyler, 1964; Yanagimachi, 1964; Yanagimachi and Chang, 1964). Whether the perforatorium is completely exposed by the opening of the acrosomal cap is not specifically stated in these studies. Hadek's (1963a) earlier diagram of the rabbit spermatozoon shows the "apical body" attached to the outside of the "cytoplasmic layer" in the last stage of removal of the acrosomal membranes, and he refers to the "unsheathing" of the apical body. In his later paper (1963b), however, he figured this structure enclosed within the inner acrosomal membrane. Demonstration that the perforatorium of spermatozoa penetrating the zona extends outside the inner acrosomal membrane would be a point in support of the idea that the perforatorium performs a lytic function.

Such a lytic capacity associated with the perforatorium would provide one possible way for the mammalian spermatozoon to cope with the problem it faces, of successively dissolving two protective coats composed of chemically different substances. It has been convincingly demonstrated that disruption of the acrosomal cap releases one enzyme, specific for the substance of the outer coat. If a zona lysin exists as, or is firmly bound to, the fibrous elements observed in the perforatorium, it would be appropriately located to attack the inner coat after removal of the acrosomal membranes. Such a stable lysin would also be expected to act more slowly than a soluble enzyme and require to be brought into direct contact with its substrate, as in the case of the molluskan crystalline

style. As described by Gray (1958), the type of flagellar activity characteristic of the bull spermatozoon would impart a small-scale back-and-forth displacement in the plane of its flat surfaces to the anterior edge of the paddle-shaped head, in addition to the strong forward-propelling component. This would appear to be the most efficient arrangement for enabling a slowly acting lysin, located along this advancing edge, to open a passage through the dense zona material.

As circumstantial evidence in support of this mechanism, it may be pointed out that the path of a mammalian spermatozoon penetrating the zona is repeatedly described as a uniformly narrow "slit" or "tunnel," 1-2 μ in diameter, indicating that any lysis which occurs is strictly localized. On the other hand, such a track is always observed; this argues against a purely mechanical pushing aside of the zona material. To solve the problem unequivocally, it will be necessary to demonstrate the presence of a zona-dissolving agent in isolated perforatoria.

REFERENCES

- Afzelius, B. A. (1955). The fine structure of the sea urchin spermatozoa as revealed by the electron microscope. *Z. Zellforsch.* **42**, 134.
- Afzelius, B. A., and Murray, A. (1957). The acrosomal reaction of spermatozoa during fertilization or treatment with egg water. *Exptl. Cell Res.* **12**, 325.
- André, J. (1963). Existence d'un axe nucléaire dans le spermatozoïde de la Limule; ses relations avec la réaction acrosomienne. *J. Microscopie* **2**, 21.
- Austin, C. R. (1948). Function of hyaluronidase in fertilization. *Nature* **162**, 63.
- Austin, C. R. (1960). Capacitation and the release of hyaluronidase from spermatozoa. *J. Reprod. Fertility* **3**, 310.
- Austin, C. R., and Bishop, M. W. H. (1958). Role of the rodent acrosome and perforatorium in fertilization. *Proc. Roy. Soc. (London), Ser. B* **149**, 241.
- Bawa, S. R. (1964). Electron microscopy study of spermiogenesis in a fire-brat insect, *Thermobia domestica* Pack. I. Mature spermatozoon. *J. Cell. Biol.* **23**, 431.
- Benda, C. (1887). Untersuchungen über den Bau des funktionirenden Samenkanälchens einiger Säugethiere und Folgerungen für die Spermatogenese dieser Wirbelthierklasse. *Arch. mikr. Anat.* **30**, 49.
- Berg, W. E. (1950). Lytic effects of sperm extracts on the eggs of *Mytilus edulis*. *Biol. Bull.* **98**, 128.
- Bernstein, M. H. (1962). Normal and reactive morphology of sea urchin spermatozoa. *Exptl. Cell Res.* **27**, 197.
- Blom, E., and Birch-Andersen, A. (1961). An "apical body" in the galea capita of the normal bull sperm. *Nature* **190**, 1127.
- Bowen, R. H. (1924). On the acrosome of the animal sperm. *Anat. Record* **28**, 1.
- Brown, G. G. (1966). Ultrastructural studies of sperm morphology and sperm-egg interaction in the decapod *Callinectes sapidus*. *J. Ultrastruct. Res.* **14**, 425.
- Burgos, M. H., and Fawcett, D. W. (1955). Studies of the fine structure of the mammalian testis. I. Differentiation of the spermatids in the cat (*Felis domestica*). *J. Biophys. Biochem. Cytol.* **1**, 287.

- Burgos, M. H., and Fawcett, D. W. (1956). An electron microscopic study of spermatid differentiation in the toad, *Bufo arenarum* Hensel. *J. Biophys. Biochem. Cytol.* **2**, 223.
- Clermont, Y., Einberg, E., Leblond, C. P., and Wagner, S. (1955). The perforatorium—an extension of the nuclear membrane of the rat spermatozoon. *Anat. Record* **121**, 1.
- Clermont, Y., and Leblond, C. P. (1955). Spermiogenesis of man, monkey, ram and other mammals as shown by the "periodic acid-Schiff" technique. *Am. J. Anat.* **96**, 299.
- Collier, J. R. (1959). The effect of homologous fertilizin on the sperm of *Strongylocentrotus purpuratus*. *Acta Embryol. Morphol. Exptl.* **2**, 163.
- Colwin, A. L., and Colwin, L. H. (1955a). Sperm entry and the acrosome filament (*Holothuria atra* and *Asterias amurensis*). *J. Morphol.* **97**, 543.
- Colwin, A. L., and Colwin, L. H. (1955b). Concerning the spermatozoon and fertilization in the egg of *Sabellaria vulgaris*. *Biol. Bull.* **109**, 357.
- Colwin, L. H., and Colwin, A. L. (1954). Sperm penetration and the fertilization cone in the egg of *Saccoglossus kowalevskii* (Enteropneusta). *J. Morphol.* **95**, 351.
- Colwin, L. H., and Colwin, A. L. (1955c). Some factors related to sperm entry in two species of *Asterias*. *Biol. Bull.* **109**, 357.
- Colwin, L. H., and Colwin, A. L. (1955d). The spermatozoon and sperm entry in the egg of the holothurian, *Thyone briareus*. *Biol. Bull.* **109**, 357.
- Colwin, A. L., and Colwin, L. H. (1958). Some characteristics of the egg membrane lytic agent derived from sperm extracts of *Hydroides hexagonus*. *Biol. Bull.* **115**, 348.
- Colwin, L. H., and Colwin, A. L. (1960a). Formation of sperm entry holes in the vitelline membrane of *Hydroides hexagonus* (Annelida) and evidence of their lytic origin. *J. Biophys. Biochem. Cytol.* **7**, 315.
- Colwin, A. L., and Colwin, L. H. (1960b). Egg membrane lytic activity of sperm extract and its significance in relation to sperm entry in *Hydroides hexagonus* (Annelida). *J. Biophys. Biochem. Cytol.* **7**, 321.
- Colwin, A. L., and Colwin, L. H. (1961a). Fine structure of the spermatozoon of *Hydroides hexagonus* (Annelida), with special reference to the acrosomal region. *J. Biophys. Biochem. Cytol.* **10**, 211.
- Colwin, A. L., and Colwin, L. H. (1961b). Changes in the spermatozoon during fertilization in *Hydroides hexagonus* (Annelida). II. Incorporation with the egg. *J. Biophys. Biochem. Cytol.* **10**, 255.
- Colwin, L. H., and Colwin, A. L. (1961c). Changes in the spermatozoon during fertilization in *Hydroides hexagonus* (Annelida). I. Passage of the acrosomal region through the vitelline membrane. *J. Biophys. Biochem. Cytol.* **10**, 231.
- Colwin, A. L., and Colwin, L. H. (1963a). Role of the gamete membranes in fertilization in *Saccoglossus kowalevskii* (Enteropneusta). I. The acrosomal region and its changes in early stages of fertilization. *J. Cell Biol.* **19**, 477.
- Colwin, L. H., and Colwin, A. L. (1963b). Role of the gamete membranes in fertilization in *Saccoglossus kowalevskii* (Enteropneusta). II. Zygote formation by membrane fusion. *J. Cell Biol.* **19**, 501.
- Colwin, A. L., and Colwin, L. H. (1964). Role of the gamete membranes in fertilization. In "Cellular Membranes in Development." (M. Locke, ed.), p. 233. Academic Press, New York.
- Colwin, A. L., Colwin, L. H., and Philpott, D. E. (1957). Electron microscope studies of early stages of sperm penetration in *Hydroides hexagonus* (Annelida)

- and *Saccoglossus kowalevskii* (Enteropneusta). *J. Biophys Biochem. Cytol.* **3**, 489.
- Dan, J. (1952). Studies on the acrosome. I. Reaction to egg-water and other stimuli. *Biol. Bull.* **103**, 54.
- Dan, J. (1954). Studies on the acrosome. II. Acrosome reaction in starfish spermatozoa. *Biol. Bull.* **107**, 203.
- Dan, J. (1956). The acrosome reaction. *Intern. Rev. Cytol.* **5**, 365.
- Dan, J. (1958). The acrosome reaction. In "Experiments in Developmental Physiology" (K. Dan and T. Yamada, eds.). p. 1. Bai Fu Kan, Tokyo (in Japanese).
- Dan, J. (1962). The vitelline coat of the *Mytilus* egg. I. Normal structure and effect of acrosomal lysin. *Biol. Bull.* **123**, 531.
- Dan, J., and Hagiwara, Y. (1967). Studies on the acrosome. IX. Course of acrosome reaction in the starfish. *J. Ultrastruct. Res.* (in press).
- Dan, J., Ohori, Y., and Kushida, H. (1964). Studies on the acrosome. VII. Formation of the acrosomal process in sea urchin spermatozoa. *J. Ultrastruct. Res.* **11**, 508.
- Dan, J., and Wada, S. K. (1955). Studies on the acrosome. IV. The acrosome reaction in some bivalve spermatozoa. *Biol. Bull.* **109**, 40.
- Dettlaff, T. A. (1958). The couplings of the gametes in the absence of Ca ions in Acipenseridae. *Compt. Rend. Acad. Sci. U.R.S.S.* **120**, 1165 (in Russian).
- Dettlaff, T. A. (1959). The role of calcium ions in the activation of the eggs of the Salmonids. *Zh. Obshch. Biol.* **20**, 184 (in Russian).
- Dettlaff, T. A. (1960). An attempt of repeated insemination of eggs of Acipenseridae fish in media devoid of calcium, and in water. *Compt. Rend. Acad. Sci. U.R.S.S.* **130**, 708 (in Russian).
- Dettlaff, T. A., and Ginsburg, A. S. (1963). Acrosome reaction in sturgeons and the role of Ca-ions in sperm-egg association. *Compt. Rend. Acad. Sci. U.R.S.S.* **153**, 1461 (in Russian).
- Dickmann, Z. (1964). The passage of spermatozoa through and into the zona pellucida of the rabbit egg. *J. Exptl. Biol.* **41**, 177.
- Dickmann, Z., and Dziuk, P. J. (1964). Sperm penetration of the zona pellucida of the pig egg. *J. Exptl. Biol.* **41**, 603.
- Fallon, J. F., and Austin, C. R. (1963). Fine structure of *Nereis limbata* spermatozoa. *Biol. Bull.* **125**, 362.
- Fallon, J. F., and Austin, C. R. (1964). A fine-structure study of the activation reactions of *Nereis limbata* gametes. *Biol. Bull.* **127**, 369.
- Galtsoff, P. S., and Philpott, D. E. (1960). Ultrastructure of the spermatozoon of the oyster, *Crassostrea virginica*. *J. Ultrastruct. Res.* **3**, 241.
- Ginsburg, A. S. (1963). Sperm-egg association and its relationship to the activation of the egg in salmonid fishes. *J. Embryol. Exptl. Morphol.* **11**, 13.
- Gray, J. (1958). The movement of the spermatozoa of the bull. *J. Exptl. Biol.* **35**, 96.
- Hadek, R. (1963a). Submicroscopic changes in the penetrating spermatozoon of the rabbit. *J. Ultrastruct. Res.* **8**, 161.
- Hadek, R. (1963b). Study on the fine structure of the sperm head. *J. Ultrastruct. Res.* **9**, 110.
- Hagiwara, Y., Dan, J., and Saito, A. (1967). Studies on the acrosome. VIII. The intact starfish acrosome. *J. Ultrastruct. Res.* (in press).
- Haino, K. (1965). Isolation of soluble product resulting from digestion of egg-membrane by egg-membrane lysin. *Seikagaku* **37**, 691 (in Japanese).

- Haino, K., and Kigawa, M. (1966). Studies on the egg-membrane lysin of *Tegula pfeifferi*: isolation and chemical analysis of the egg membrane. *Exptl. Cell Res.* **42**, 625.
- Hartmann, M., Schartau, O., and Wallenfels, K. (1940). Untersuchungen über die Befruchtungsstoffe der Seeigel. II. *Biol. Zentr.* **60**, 398.
- Hartree, E. F., and Srivastava, P. N. (1965). Chemical composition of the acrosomes of ram spermatozoa. *J. Reprod. Fertility* **9**, 47.
- Hathaway, R. R., and Hartree, E. F. (1963). Observations on the mammalian acrosome: experimental removal of acrosomes from ram and bull spermatozoa. *J. Reprod. Fertility* **5**, 225.
- Hauschka, S. D. (1963). Purification and characterization of *Mytilus* egg membrane lysin from sperm. *Biol. Bull.* **125**, 363.
- Hauschka, S. D. (1964). Hyaline membrane lysis in *Mytilus edulis* eggs. *Biol. Bull.* **127**, 373.
- Hibbard, H. (1928). Contribution à l'étude de l'ovogénèse, de la fécondation et de l'histogénèse chez *Discoglossus pictus* Otth. *Arch. Biol.* **38**, 251.
- Horstmann, E. (1961). Elektronmikroskopische Untersuchungen zur Spermiogénese beim Menschen. *Z. Zellforsch.* **54**, 68.
- Humphreys, W. J. (1962). Electron microscope studies on eggs of *Mytilus edulis*. *J. Ultrastruct. Res.* **7**, 467.
- Kaye, J. S. (1962). Acrosome formation in the house cricket. *J. Cell Biol.* **12**, 411.
- Kille, R. A. (1960). Fertilization of the lamprey egg. *Exptl. Cell Res.*, **20**, 12.
- Krauss, M. (1950). Lytic agents of the sperm of some marine animals. I. The egg membrane lysin from sperm of the giant keyhole limpet *Megathura crenulata*. *J. Exptl. Zool.* **114**, 239.
- Lenhossek, M. von (1898). Untersuchungen über Spermatogenese. *Arch. mikr. Anat.* **51**, 215.
- Leuchtenberger, C., and Schrader, F. (1950). The chemical nature of the acrosome in the male germ cells. *Proc. Natl. Acad. Sci. U. S.* **36**, 677.
- Lillie, F. R. (1912). The production of sperm iso-agglutinin by ova. *Science* **36**, 527.
- Lillie, F. R. (1919). "Problems of Fertilization." Univ. of Chicago Press, Chicago, Illinois.
- Loeb, J. (1901). Experiments on artificial parthenogenesis in annelids (*Chaetopterus*) and the nature of the process of fertilization. *Am. J. Physiol.* **4**, 423.
- Loeb, J. (1915). On the nature of the conditions which determine or prevent the entrance of the spermatozoon into the egg. *Am. Naturalist* **49**, 257.
- Metz, C. B., and Morrill, J. B. (1955). Formation of acrosome filaments in response to treatment of sperm with fertilizin in *Asterias* and *Nereis*. *Biol. Bull.* **109**, 349.
- Meves, F. (1915). Über den Befruchtungsvorgang bei der Miesmuschel (*Mytilus edulis* L.). *Arch. Mikrobiol.* **87**, 47.
- Moriber, L. C. (1956). A cytochemical study of hemipteran spermatogenesis. *J. Morphol* **99**, 271.
- Moricard, R. (1960). Observations de microscopie électronique sur des modifications acrosomiques lors de la pénétration spermatique dans l'oeuf des mammifères. *Compt. Rend. Soc. Biol.* **154**, 2187.
- Moricard, R. (1964). Fécondation et différenciations ultrastructurales intratubaire des spermatozoïdes provenant du canal déférent. *Proc. 5th Intern. Cong. on Animal Reproduction and Artificial Insemination, Trento, 1964*.
- Moser, F. (1939). Studies on a cortical layer response to stimulating agents in the *Arbacia* egg. II. Response to chemical and physical agents. *J. Exptl. Zool.* **80**, 447.

- Moses, M. J. (1961). Spermatogenesis in the crayfish (*Procambarus clarkii*). II. Description of stages. *J. Biophys. Biochem. Cytol.* **10**, 301.
- Nagano, T. (1962). Observations on the fine structure of the developing spermatid in the domestic chicken. *J. Cell Biol.* **14**, 193.
- Niijima, L., and Dan, J. (1965a). The acrosome reaction in *Mytilus edulis*. I. Fine structure of the intact acrosome. *J. Cell Biol.* **25**, 243.
- Niijima, L., and Dan, J. (1965b). The acrosome reaction in *Mytilus edulis*. II. Stages in the reaction, observed in supernumerary and calcium-treated spermatozoa. *J. Cell Biol.* **25**, 249.
- Onuma, H. (1963). Studies on the acrosomic system of spermatozoa of domestic animals. IV. The acrosomic system in developing spermatids of rodents, with special reference to that of domestic animals. *Bull. Natl. Inst. Animal Husbandry* **2**, 225 (in Japanese).
- Onuma, H., and Nishikawa, Y. (1963). Studies on the acrosomic system of spermatozoa of domestic animals. I. Cytochemical nature of the PAS positive material in the acrosomic system of boar spermatids. *Bull. Natl. Inst. Animal Husbandry* **1**, 125 (in Japanese).
- Pasteels, J. J., and de Harven, E. (1962). Etude au microscope électronique du spermatozoïde d'un mollusque bivalve, *Barnea candida*. *Arch. Biol. (Liège)* **73**, 445.
- Piatigorsky, J., and Austin, C. R. (1962). Relationship of fertilizin to the acrosome reaction in *Arbacia*. *Biol. Bull.* **123**, 473.
- Pikó, L. (1964). Mechanism of sperm penetration in the rat and the Chinese hamster based on fine structural studies. *Proc. 5th Intern. Conf. on Animal Reproduction and Artificial Insemination, Trento, 1964*.
- Pikó, L., and Tyler, A. (1964). Fine structural studies of sperm penetration in the rat. *Proc. 5th Intern. Conf. on Animal Reproduction and Artificial Insemination, Trento, 1964*, p. 373.
- Pollister, A. W. (1930). Cytoplasmic phenomena in the spermatogenesis of *Gerris*. *J. Morphol.* **49**, 455.
- Porte, A., and Follenius, E. (1960). La spermiogénèse chez *Lebistes reticulatus*. Etude au microscope électronique. *Bull. Soc. Zool. France* **85**, 82.
- Reger, J. F. (1963). Spermiogenesis in the tick, *Amblyomma dissimili*, as revealed by electron microscopy. *J. Ultrastruct. Res.* **8**, 607.
- Reger, J. F. (1964). The fine structure of spermatozoa from the isopod *Asellus militaris*. *J. Ultrastruct. Res.* **11**, 181.
- Rothschild, Lord, and Swann, M. M. (1952). The fertilization reaction in the sea urchin. The block to polyspermy. *J. Exptl. Biol.* **29**, 469.
- Rothschild, Lord, and Tyler A. (1955). Acrosomal filaments in spermatozoa. *Exptl. Cell Res. Suppl.* **3**, 304.
- Sakai, Y. T. (1961). Method for removal of chorion and fertilization of the naked egg in *Oryzias latipes*. *Embryologia (Nagoya)* **5**, 357.
- Srivastava, P. N., Adams, C. E., and Hartree, E. F. (1965). Enzymatic action of lipoglycoprotein preparations from sperm acrosomes on rabbit ova. *Nature* **205**, 498.
- Takashima, R., and Takashima, Y. (1963). Electron microscope studies on the fine structure of *Nereis* spermatozoa before and after the acrosomal reaction. *Tokushima J. Exptl. Med.* **10**, 117.
- Tyler, A. (1939). Extraction of an egg membrane lysin from sperm of the giant keyhole limpet (*Megathura crenulata*). *Proc. Natl. Acad. Sci. U. S.* **25**, 317.

- Tyler, A. (1948). Fertilization and immunity. *Physiol. Rev.* **28**, 180.
- Tyler, A. (1953). Prolongation of life-span of sea urchin spermatozoa, and improvement of the fertilization-reaction, by treatment of spermatozoa and eggs with metal-chelating agents (amino acids, Versene, DEDTC, oxine, cupron). *Biol. Bull.* **104**, 224.
- Tyler, A. (1956). Physico-chemical properties of the fertilizins of the sea urchin *Arbacia punctulata* and the sand dollar *Echinarachnius parma*. *Exptl. Cell Res.* **10**, 377.
- Wada, S. K., Collier, J. R., and Dan, J. (1956). Studies on the acrosome. V. An egg-membrane lysin from the acrosomes of *Mytilus edulis* spermatozoa. *Exptl. Cell Res.* **10**, 168.
- Waldeyer, W. (1870). "Eistock und Ei." Leipzig.
- Wigglesworth, V. B. (1953). "The Principles of Insect Physiology," 5th ed. Methuen, London.
- Wintrebert, P. (1929). La digestion de l'enveloppe tubaire interne de l'oeuf par des ferments issus des spermatozoïdes, et de l'ovule, chez *Discoglossus pictus* Otth. *Compt. Rend.* **188**, 97.
- Yanagimachi, R. (1964). Sperm penetration into hamster egg in vitro (1). *Proc. 5th Intern. Conf. on Animal Reproduction and Artificial Insemination, Trento, 1964*, p. 321.
- Yanagimachi, R., and Chang, M. C. (1964). In vitro fertilization of golden hamster ova. *J. Exptl. Zool.* **156**, 361.
- Yasuzumi, G. (1960). Spermatogenesis in animals as revealed by electron microscopy. VII. Spermatid differentiation in the crab, *Eriocheir japonicus* J. *Biophys. Biochem. Cytol.* **7**, 73.
- Yasuzumi, G., Kaye, G. I., Pappas, G. D., Yamamoto, Y., and Tsubo, I. (1961). Nuclear and cytoplasmic differentiation in developing sperm of the crayfish, *Cambaroides japonicus*. *Z. Zellforsch. mikr. Anat.* **53**, 141.

CHAPTER 7

Membrane Fusion in Relation to Sperm-Egg Association*

Laura Hunter Colwin and Arthur L. Colwin

DEPARTMENT OF BIOLOGY, QUEENS COLLEGE OF THE CITY UNIVERSITY OF
NEW YORK, FLUSHING, NEW YORK

I. Introduction	295
II. Gametic Association	296
A. Hydroides (Annelida) and Saccoglossus (Hemichordata)	296
B. Comparative Aspects of Association (Vertebrate and Invertebrate Phyla)	319
III. Discussion	346
A. Manner of Zygote Formation	346
B. Some Implications of Gametic Contact during Fertilization	348
C. Perforatorium and Periacrosomal Material	349
D. Perforatorium or Core and Inward Movement of Sperm Organelles	351
E. Acrosomal Region and Its Relation to Egg Activation	352
F. The Mechanism of Membrane Fusion	353
References	359

I. Introduction

The expression "membrane fusion" is here taken to mean that initially unconnected membranes meet, coalesce, and open up over a limited area, with the result that they become one continuous membrane (Fig. 50). This kind of continuity is established during the very early part of fertilization, and it is particularly with this part that the present chapter will deal.

Most of the information pertaining to this topic has been derived from studies of the ultrastructural morphology of the gametes as it changes during the events of sperm-egg association. Electron microscopy

*Part of the work reported here was supported by National Institutes of Health research grants GM-04948 and HD-00007.

has given new insight into some of these events, and as a result certain concepts which had seemed acceptable according to the evidence from light microscopy must now be revised. A case in point is the use of the word "membrane" in this chapter. At the surface of the egg two structures in particular have been known as membranes. The inner one of these structures is the plasma membrane, which in all known instances bounds the surface of the egg cytoplasm. Ultrastructurally it is a "unit membrane" (Robertson, 1961), in the sense that in certain circumstances in cross section it appears to be trilaminar as do various cytomembranes with which we shall be concerned. In mammalian eggs the plasma membrane has usually been called the vitelline membrane. By contrast, in invertebrate eggs the other, outer, structure (a possible counterpart of the mammalian zona pellucida) has been called the vitelline membrane. This structure is very different from species to species, and in some species it even fails to occur. Recently some authors have used expressions such as "vitelline coat" (Dan, 1962) or "vitelline envelope" (Colwin and Colwin, 1963a,b, 1964) to designate this outer structure. In the present chapter it will be called "envelope" or "capsule" rather than "membrane," and the term "membrane" will be reserved to designate only the plasma membrane or cytomembranes.

In the process of fertilization the spermatozoon undergoes an extensive series of changes. As the ultrastructure has become known it has become possible to relate these changes to precise components of the spermatozoon and especially to the acrosomal region, which, of course, includes the acrosome. The expression "sperm activation" has been used in connection with this series of changes (Colwin and Colwin, 1963a) because these changes in many ways parallel and are complementary to the activation reaction of the egg. This sperm activity, closely related to sperm-egg association, is quite distinct from other sperm activities such as those related to motility, which are concerned rather with stages preceding the encounter of the gametes. Since "sperm activation" is intended to be a more inclusive expression than "acrosome reaction" (Dan, Chap. 6), it will be used consistently in the present chapter, even in some cases in which the expression "acrosome reaction" would be equally appropriate.

II. Gametic Association

A. HYDROIDES (ANNELIDA) AND SACCOGLOSSUS (HEMICHORDATA)

1. Introduction

The events of sperm-egg association will be presented in detail for *Hydroides* and *Saccoglossus*. These two forms were selected because

they have yielded firm evidence which justifies the drawing of certain general conclusions.

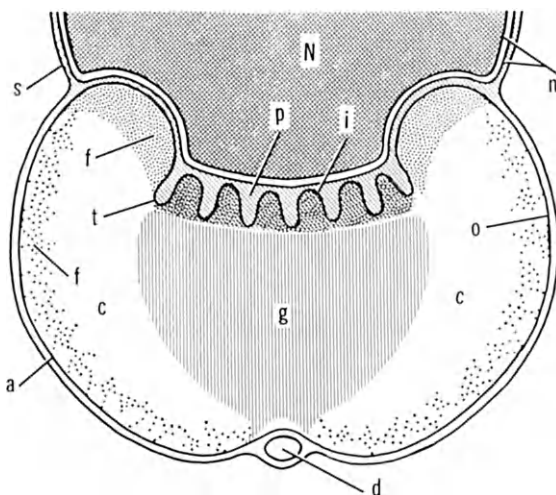
For fine-structure studies in these as well as in other species it is very difficult to find thin sections of the uniting gametes, and even more difficult to find them in orientations favorable for interpretation. Since the production of a fertilization cone is a widespread occurrence at gametic union, it might be supposed that developing cones would be ideal for such studies. If the cone region could be identified in the embedding block, then the fertilization site and hence the uniting gametes could be appropriately oriented for sectioning. *Saccoglossus* would be admirable for this procedure, since the early cone is easily located (Colwin and Colwin, 1954). However, as will be seen below, the presence of a fertilization cone in itself indicates that the two gametes are already united, and consequently such an approach is useless for studying phenomena leading to gametic union.

Another avenue of approach is the use of polyspermy to increase the likelihood of finding suitable sections and orientations. Eggs of *Hydroides* and *Saccoglossus* have been deliberately made polyspermic for such a purpose (Colwin *et al.*, 1957). But can polyspermic material accurately reflect normal events of sperm-egg association? There are reasons for supposing that it can. To begin with, major changes in the spermatozoon culminate in acrosomal tubule formation. Since such changes in some species may also occur spontaneously or be induced experimentally entirely in the absence of any eggs (reviewed by Dan, 1956; Colwin and Colwin, 1957b), these changes cannot be peculiar to polyspermy. To continue, extensive studies of living eggs of *Hydroides* and *Saccoglossus* are available (Colwin and Colwin, 1954, 1960b,c; Colwin *et al.*, 1957). These studies show that, except for the size of the cone and the length of time the cone persists, monospermic and polyspermic eggs appear to be alike during early stages. Therefore, it is probably during subsequent stages, and presumably in connection with mitosis, that polyspermy affects zygote behavior. It would be desirable, of course, to know the ultrastructure of gametic union in monospermic eggs, but studies are not yet available that are as detailed as those of *Hydroides* and *Saccoglossus*. Insofar as studies of monospermic eggs go, the observations parallel the following accounts of *Hydroides* and *Saccoglossus*, which are based on polyspermic eggs. For the very early events of syngamy, with which we are concerned here, this material almost certainly does reveal the normal picture.

2. *Hydroides*

a. *Capsule and Plasma Membrane of Egg* (Figs. 5 and 6). The egg is enclosed in a thick (3–4 μ) nonmembranous vitelline capsule or

envelope (the vitelline "membrane" of light microscopy) composed of three layers: a thin outer border layer consisting mainly of a sheet of small, hexagonally packed spherical bodies, a thick middle layer comprising the bulk of the envelope, and a thin inner border layer. The materials of the middle and inner layers are amorphous or feltlike in arrangement. Beneath this envelope is the living egg cell, bounded by its plasma membrane. Microvilli, which are projections of the cytoplasm together with the plasma membrane, extend to but not through the outer border layer (Colwin and Colwin, 1960c, 1961b).



All figures except Figs. 1, 5, 17, 18, and 47-50 are electron micrographs. The magnifications given for electron micrographs are only approximate. (a) Acrosomal membrane or originally acrosomal membrane. (c) Cavity of acrosomal vesicle. (cg) Cortical granule. (d) Apical vesicle. (e) Egg plasma membrane or, in zygote, part derived therefrom. (f) Fertilization cone. (f) Fine granular material lining acrosomal membrane. (g) Acrosomal granule. (i) Inner (adnuclear or juxtannuclear) portion of acrosomal membrane; in mammals, "inner membrane." (k) Acrosomal remnant serving as a marker for identifying the sperm plasma membrane. (m) Vitelline "membrane" or egg envelope or egg capsule. (mi) Inner border layer or inner part of egg capsule. (mm) Middle layer of egg capsule. (mo) Outer border layer or outer portion of egg capsule. (N) Nucleus. (n) Nuclear envelope or membrane components of nuclear envelope. (o) Outer portion of acrosomal membrane; in mammals, "outer membrane." (p) Periacrosomal material. (pc) Fibrous "core" derived from periacrosomal material. (pp) Perforatorium. (s) Sperm plasma membrane or, in zygote, part derived therefrom. (t) Acrosomal tubule, or invagination (or indentation) in juxtannuclear portion of inner acrosomal membrane. (v) Egg microvillus.

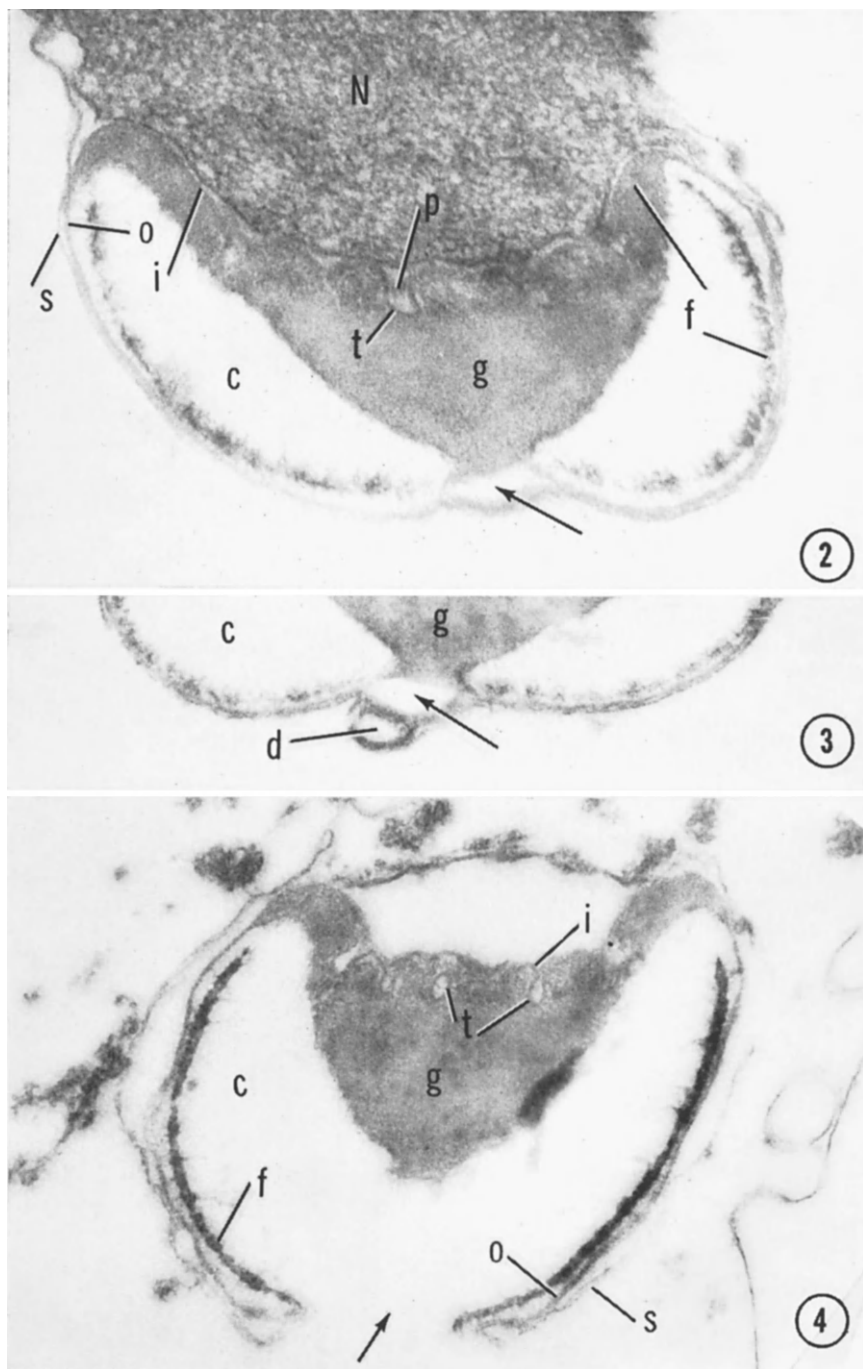
FIG. 1. *Hydroides*. Diagram of unactivated acrosomal region; median longitudinal section. The acrosome is a vesicle bounded by the acrosomal membrane (a)

b. Composition of Acrosomal Region (Figs. 1–4). Although four principal regions may be recognized in the spermatozoon, namely, acrosomal, nuclear, mitochondrial, and flagellar, only the acrosomal region is primarily involved in the beginning events of fertilization, and this region alone will be described in some detail (Colwin and Colwin, 1961a). The acrosomal region covers the anterior end of the nucleus and constitutes the apical part of the sperm head (Fig. 1). This region includes (a) the acrosome, (b) a small amount of periacrosomal (i.e., extraacrosomal) material located primarily between the acrosome and the anterior end of the nuclear envelope, (c) a small membrane-bounded apical vesicle, and (d) that part of the sperm plasma membrane which overlies these structures.

The acrosome is a vesicle bounded by the acrosomal membrane, whose adnuclear or juxtannuclear portion is depressed into a number of short tubular invaginations which contain much of the periacrosomal material (Fig. 2). Within the cavity of the acrosome fine granular material lines the membrane except at the apex. The axial part of the cavity is spanned by a large acrosomal granule which apically makes a limited contact with the acrosomal membrane; essentially, the granule does not touch that apical part of the acrosomal membrane which lacks the finely granular lining.

The apical vesicle and those parts of the acrosomal and sperm plasma membranes which enclose it constitute a lid at the apex of the acrosomal region (Fig. 3). The part of the acrosomal membrane involved in the lid is that part which lacks the finely granular lining and which at the same time is not in contact with the apex of the acrosomal granule. Encircling this lid there appears to be a natural fracture line, or rim of dehiscence. Under appropriate circumstances the lid is shed, and thereafter the apex remains open. In effect, the region dehisces. Examples of dehiscence are occasionally seen in preparations of frozen-thawed sperm (Fig. 4) or even in seawater suspensions of sperm. In these, the acrosomal region is open and open only at the apex, as serial sections of many specimens have shown. Around the rim or lip of this orifice the

whose inner or juxtannuclear portion (i) has a number of short tubular invaginations (t). The outer portion of the acrosomal membrane (o) lies very close to the sperm plasma membrane (s). The cavity (c) side of the acrosomal membrane is lined with fine granular material (f) except at its apex, where the acrosomal granule (g) makes limited contact with the acrosomal membrane. Periacrosomal material (p) is confined almost exclusively to the space between the inner acrosomal membrane and the nuclear envelope (n). Note the apical vesicle (d). (From Colwin and Colwin, 1961a.)



plasma and acrosomal membranes are found to be joined, that is, to have undergone membrane fusion, in such a way that they form one continuous membrane. Dehiscence strikingly marks the beginning of sperm activation.

c. Sequence of Events Culminating in Zygote Formation (Figs. 5-16). A diagram of this sequence (Colwin and Colwin, 1961b,c) is shown in Fig. 5. The initial gametic contact takes place between the sperm plasma membrane and the nonmembranous egg capsule (Fig. 6). When these meet, the acrosomal region dehisces and its apex somehow interrupts the outer border layer of the envelope. As mentioned above, following dehiscence the acrosomal and sperm plasma membranes are in continuity at the open apex (Fig. 7). Hence the acrosomal membrane is an inserted segment of the sperm plasma membrane, and consequently the sperm plasma membrane is now a mosaic. These parts remain connected and hereafter act as one continuous membrane.

After dehiscing, the spermatozoon, with the acrosomal region leading, progresses through the rest of the egg envelope (Figs. 8 and 9). As it advances the following events take place. (a) The acrosomal granule separates from the base of the acrosomal vesicle and rapidly diminishes in size and disappears; correspondingly, the part of the capsule in front of the granule also disappears. It is known that sperm extract can lyse away the principal substance of the egg capsule in this species, and very probably the acrosomal granule is the source of capsule lysin or lysins (Colwin *et al.*, 1957; Colwin and Colwin, 1958, 1960b,c, 1961b). At any rate, the disappearing part of the capsule is not the egg plasma

FIG. 2. *Hydroides*. Unactivated acrosomal region, nearly median longitudinal section. The acrosomal membrane consists of one continuous membrane of which the outer (o) portion may be distinguished from the inner (i) or juxtanuclear portion. In a number of places the inner portion is indented in the form of tubular invaginations (t) which contain periacrosomal material (p). Except at the apex, the acrosomal membrane is lined with fine granular material (f). At the arrow tip is the space where the apex of the acrosomal granule (g) is separated from the acrosomal membrane. (c) Cavity of acrosomal vesicle. Magnification: $\times 84,000$. (From Colwin and Colwin, 1961a.)

FIG. 3. *Hydroides*. Median longitudinal section through that part of the acrosomal region which contains the apical vesicle (d). The arrow tip lies in the space where the apex of the acrosomal granule (g) is separated from the acrosomal membrane. Magnification: $\times 60,000$. (From Colwin and Colwin, 1961a.)

FIG. 4. *Hydroides*. Nearly median longitudinal section through the acrosomal region of a frozen-thawed specimen with a dehiscent acrosomal apex. At the arrow tip is the opening around the rim of which the acrosomal membrane (o) is continuous with the sperm plasma membrane (s). Magnification: $\times 61,000$. (From Colwin and Colwin, 1961a.)

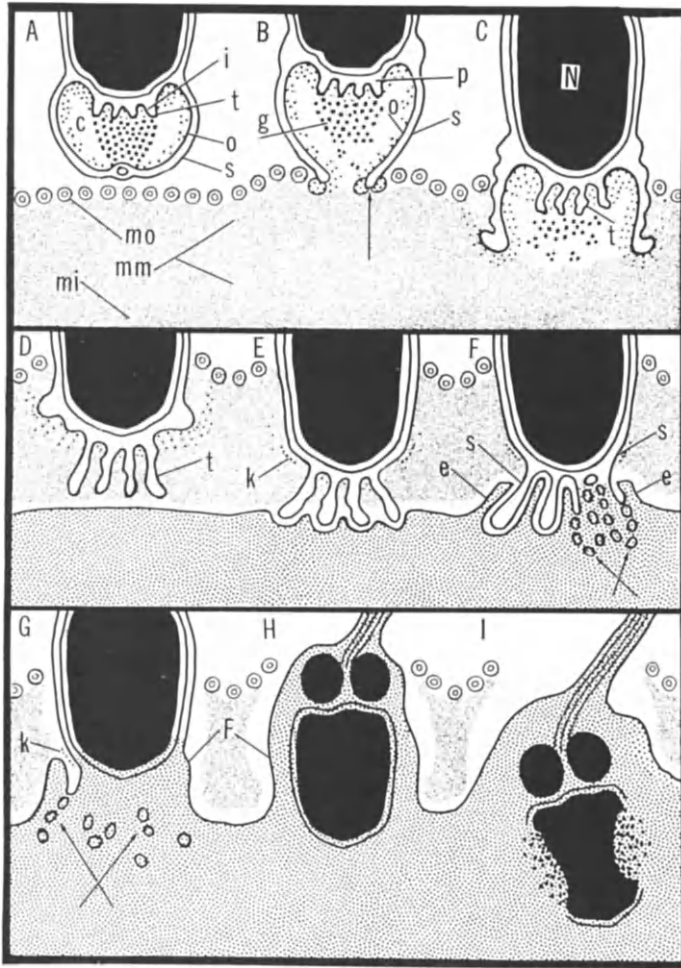
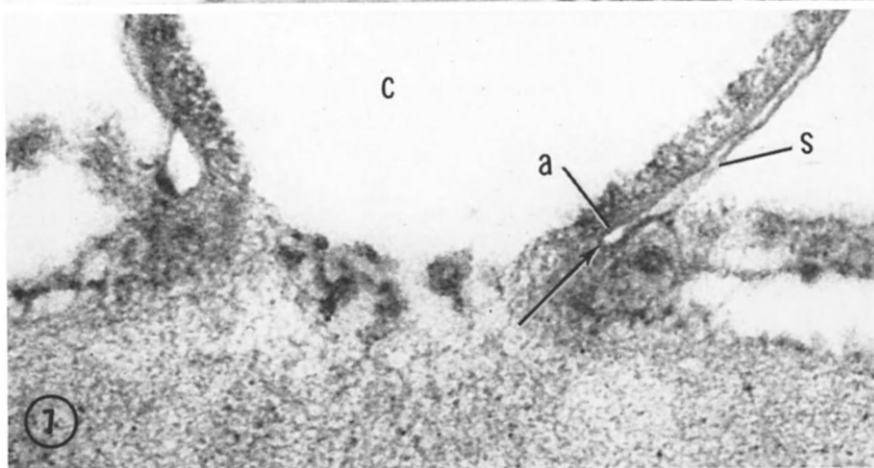
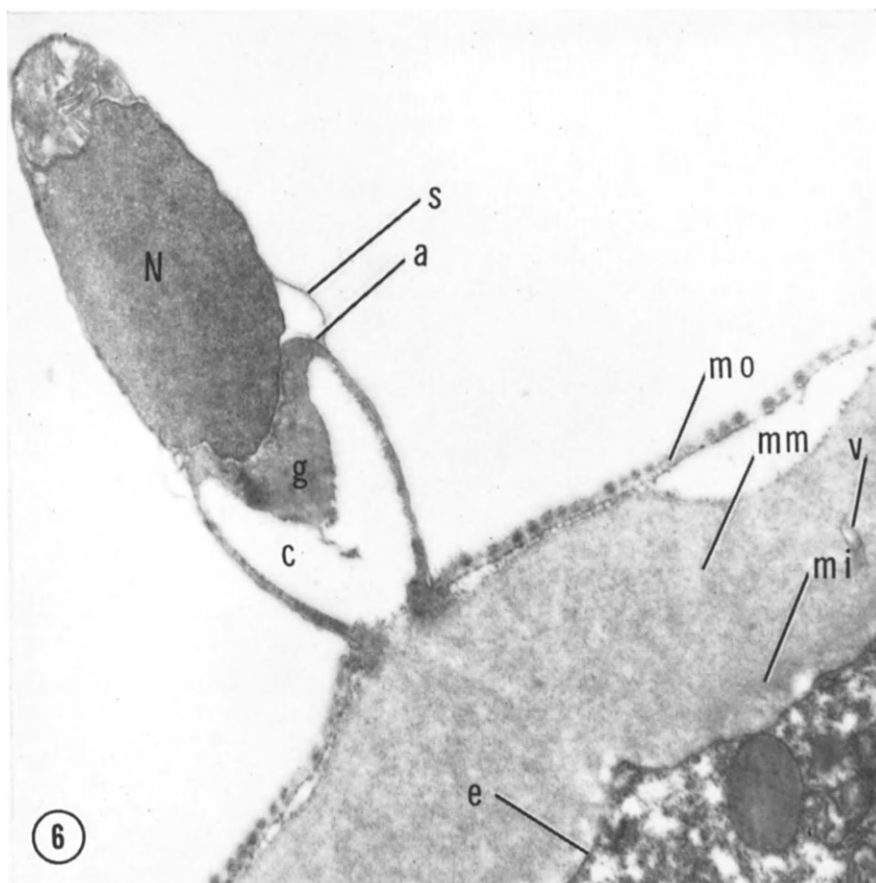


FIG. 5. *Hydroides*. Diagrams of principal stages of sperm-egg association. All diagrams are not to the same scale. (A) Unactivated spermatozoon at about the time of initial contact with the egg envelope. (B) Activated spermatozoon soon after initial contact; dehiscence of the apex has occurred; the arrow points to the rim around which the mosaically inserted acrosomal membrane (o) joins the sperm plasma membrane (s) of which it is now a continuous part. (C) and (D) Successive stages of eversion of the wall of the acrosomal vesicle as the sperm apex moves through the egg envelope. Note the disappearance of the acrosomal granule and the elongation of the acrosomal tubules. (E) Eversion has been completed; the acrosomal tubules have made their initial contact with the egg plasma membrane and interdigitation of the two gamete plasma membranes has started. (F) The left part of this figure shows a later stage of interdigitation at the stage of an early fertilization cone; the right part of the figure shows a still later stage, in which continuity between the sperm (s) and egg (e) plasma membranes has been

membrane but only its nonmembranous envelope. (b) The entire acrosomal membrane, together with its associated granular lining, becomes everted through the gradually enlarging orifice which was first opened by dehiscence. All the contents of the acrosome come to lie completely outside the sperm cell. (c) While the eversion is taking place the short tubular invaginations of the juxtannuclear (i.e., inner) part of the acrosomal membrane lengthen into acrosomal tubules. These, it should be noted, contain periacrosomal and not acrosomal material. And since their walls are in fact acrosomal membrane which has been turned inside out, the tubular surface is a newly exposed surface (Colwin and Colwin, 1961c, 1964). This tuft of tubules is now the most anterior part of the sperm plasma membrane, and this surface is the first sperm surface to encounter the egg plasma membrane.

Soon the encounter takes place, and for the first time the plasma membranes of the two gametes come into contact (Figs. 10–12). The acrosomal tubules indent the surface of the egg, and in this vicinity a fertilization cone begins to rise. As the cone is elevated the egg surface and the acrosomal tubules mutually interdigitate. The indentations and protuberances of the egg surface have no particular relation to and should not be confused with the pre-existing microvilli which in this species play no particular role in fertilization. Next, these interdigitations of apposed sperm and egg plasma membranes disappear and are replaced by many small membranous vesicles, while at the same time the plasma membranes of the two gametes are found to have fused to become one continuous zygote plasma membrane (Figs. 5 and 13–15). The time from insemination to membrane fusion is less than 9 seconds. The newly established zygote membrane consists mostly of egg plasma membrane, but it has a mosaically inserted segment of sperm plasma membrane. The approximate line where the two segments joined continues to be recognizable for some time following fusion, because it is marked by a granular remnant of the acrosomal lining which remains associated with the sperm plasma membrane (Figs. 13 and 15).

established. Note that vesicles (at crossed arrows) are present now in the region formerly occupied by the interdigitations. (G) A very early zygote; the mosaic composition of the zygote plasma membrane is evident; the plasma membranes of the two former gametes are in continuity. The crossed arrows point to vesicles in the region formerly occupied by the interdigitations. (H) Young zygote with the profile of both gametes, since the sperm parts still protrude into the egg envelope and beyond; note that the egg cytoplasm has intruded between the former sperm plasma membrane and the internal sperm organelles and extends into the base of the flagellum. (I) Young zygote in which the internal sperm organelles lie further within the egg cytoplasm than in (H).



For some time the profile of the now single-celled individual will retain the appearance of two gametes in close contact (Fig. 14). Nevertheless, the internal elements of the formerly separated cells begin to mingle. The egg cytoplasm at first intrudes between the sperm nucleus and that part of the zygote membrane which was formerly sperm plasma membrane. Later this cytoplasm penetrates to the vicinity of the sperm mitochondria, and finally it enters even the flagellum (Fig. 16). As more and more egg cytoplasm penetrates beneath the former sperm plasma membrane, the fertilization cone enlarges. Meanwhile the nucleus and other internal sperm organelles move or are moved into the body of the egg. The shape of the zygote finally becomes spherical when, at last, the fertilization cone retreats.

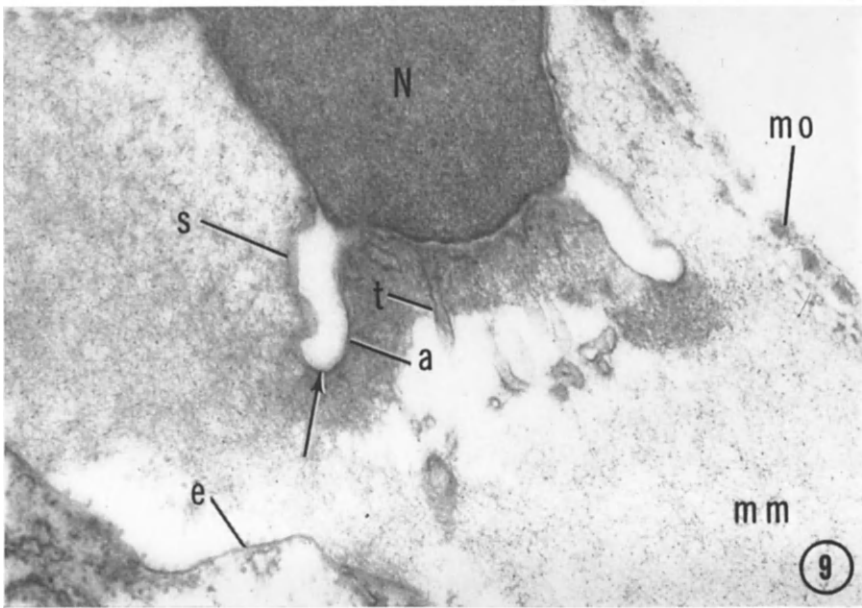
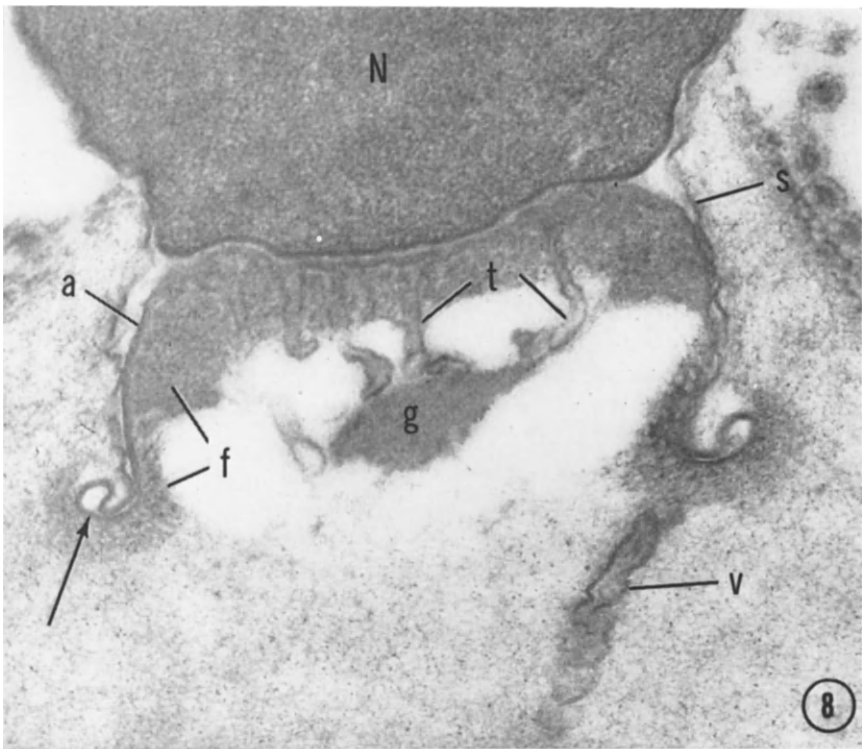
3. *Saccoglossus*

a. Capsule and Plasma Membrane of Egg (Figs. 20 and 26) (Colwin and Colwin, 1963a). Two layers of amorphous or feltlike material comprise a thick (ca. 7μ) nonmembranous capsule which surrounds the egg. The inner layer, which is narrower and denser, is the functional vitelline envelope and later becomes modified to form the fertilization "membrane" (envelope). Inside the capsule the living egg cytoplasm has as its boundary the plasma membrane. Microvilli bounded by this membrane extend across a shallow perivitelline space. Although the microvilli touch the vitelline envelope, they do not project into it.

b. Composition of Acrosomal Region (Figs. 17 and 19) (Colwin and Colwin, 1963a). As in *Hydroides*, only the acrosomal region is extensively involved in the early events of fertilization. This region has proportions different from those in *Hydroides*, but in *Saccoglossus* too it covers the anterior end of the sperm nucleus, constitutes the apical part of the sperm head, and is bounded externally by part of the sperm plasma membrane. The acrosome occupies an axial position and is invested by a large amount of periacrosomal material. Although some peri-

FIG. 6. *Hydroides*. Stage of initial contact. Where the sperm plasma membrane meets the outer part of the egg envelope (mo), the apex of the acrosomal region undergoes dehiscence, thus the cavity of the acrosomal vesicle (c) opens to the outside. The egg plasma membrane (e) is still separated from the sperm cell by the bulk of the egg envelope (mo), (mm), (mi). (Relationships at the sperm apex are shown at a higher magnification in Fig. 7.) Magnification: $\times 39,000$. (Includes portion shown at a higher magnification in Colwin and Colwin, 1961b.)

FIG. 7. *Hydroides*. Stage of initial contact. Detail of specimen similar to that in Fig. 6. The acrosomal membrane (a) is now an inserted mosaic section in the sperm plasma membrane (s) with which it is in continuity around the rim of dehiscence (arrow). Magnification: $\times 109,000$. (Includes portion from Colwin and Colwin, 1961b.)



acrosomal material lies between the acrosome and the nuclear envelope, most of it lies between the acrosome and plasma membrane.

The acrosome is a vesicle whose boundary or wall is the acrosomal membrane. One shallow invagination indents the center of the adnuclear or juxtannuclear portion of this membrane. The vesicle contains a large acrosomal granule and is lined except at the apex by a thin layer of fine granular material. Where this layer is absent, an apical space separates the acrosomal granule from the acrosomal membrane which in this region closely adjoins the plasma membrane. Here at the apex the plasma membrane is usually smooth, whereas the acrosomal membrane may be smooth, wrinkled, or even vesiculated. This is the site at which the acrosomal region opens when it dehisces, at sperm activation.

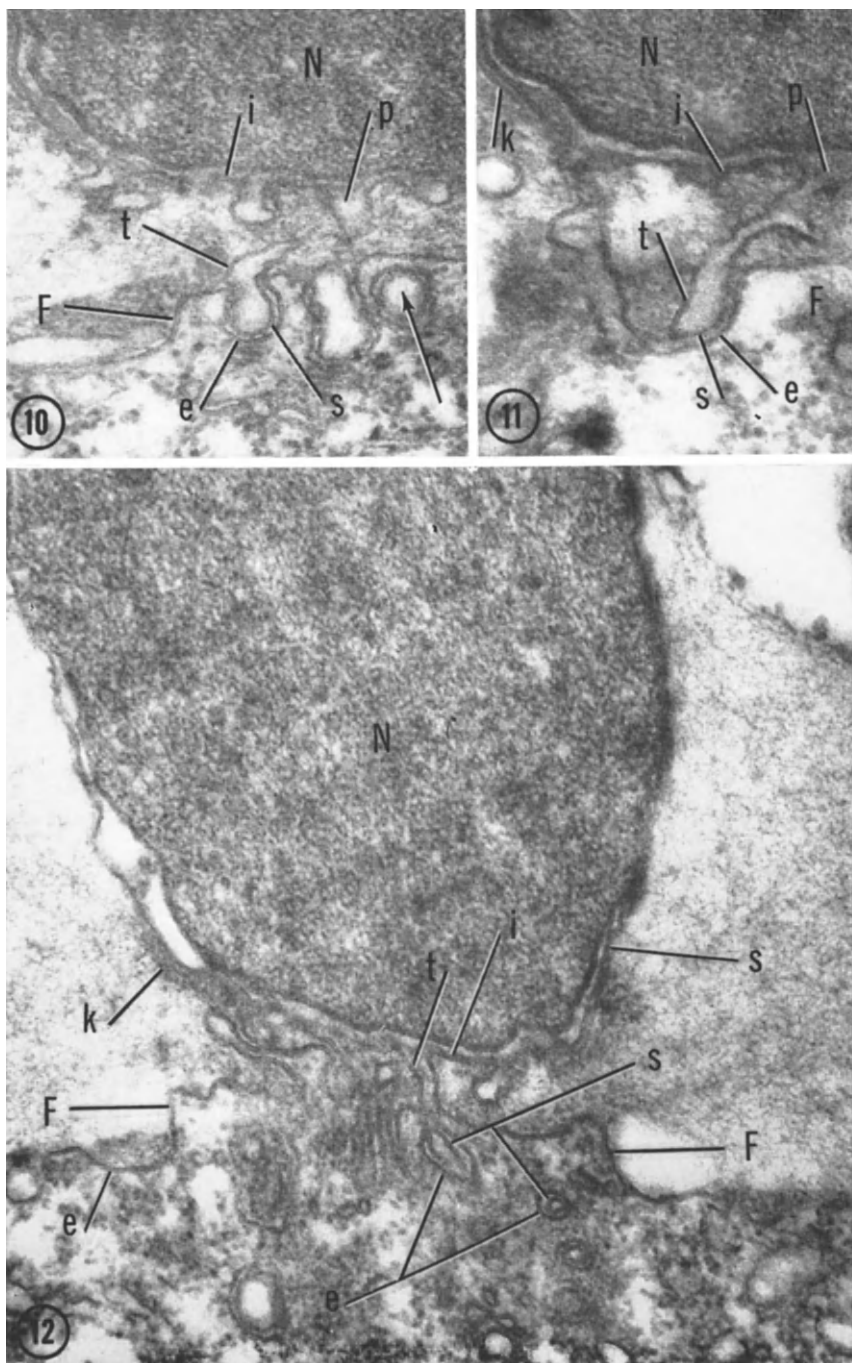
c. Sequence of Events Culminating in Zygote Formation (Figs. 18 and 20–35) A diagram of this sequence (Colwin and Colwin, 1963a,b) is shown in Fig. 18. The first contact is between the sperm plasma membrane and the nonmembranous egg capsule (Fig. 20). Soon the acrosomal region dehisces, and since the two membranes of the region are then in continuity around the rim of dehiscence, the sperm plasma membrane is a mosaic in which the acrosomal membrane is an inserted segment (Figs. 21–23). Henceforth the joined segments function as one continuous membrane. Little vesicles and curved fragments of membrane occasionally are found near the orifice of dehiscence (Fig. 21), and presumably these were cast off when the membranes fused.

Dehiscence exposes the acrosomal granule. The granule comes into contact with the egg capsule, disintegrates and disappears, and simultaneously a small part of the capsule also disappears. At the juxtannuclear or inner end of the acrosome the initially shallow invagination of the acrosomal membrane deepens and becomes a long acrosomal tubule (Figs. 23 and 27) which extends all the way through the capsule and meets the egg plasma membrane (Fig. 26). This is the first contact between the two plasma membranes. Inasmuch as the tubule is acrosomal membrane which has been turned inside out, the tubular surface

FIGS. 8–9. *Hydroides*. Stages showing the eversion and modification of the acrosomal region during the progression of the sperm head through the egg envelope. At the arrows, note the continuity of the acrosomal membrane (a) with adjacent parts of the sperm plasma membrane (s) of which it is now a mosaically inserted part.

FIG. 8. Early stage. Note the partly lengthened tubular invaginations (t) of the acrosomal membrane and the reduced size of the acrosomal granule (g). Magnification: $\times 75,500$. (From Colwin and Colwin, 1961b.)

FIG. 9. Later stage than in Fig. 8; the acrosomal tubules (t) are longer and the acrosomal granule has disappeared. Magnification: $\times 58,000$. (From Colwin and Colwin, 1961b.)



participating in this contact is a newly exposed surface. The whole of the acrosome finally everts, and the effect of this eversion is to lengthen the acrosomal tubule (Figs. 24 and 25). As a consequence of eversion all the contents of the acrosome come to lie completely outside the sperm cell, and all the periacrosomal material comes to lie completely inside the acrosomal tubule (Figs. 21–25).

The acrosomal tubule can reach the egg plasma membrane within 7 seconds from the time of insemination. Almost at once the two membranes fuse and become continuous, and in this way the first cell of the new individual is established (Figs. 28 and 29). The internal elements of the two gametes begin to intermix and a fertilization cone begins to rise.

At the inception of the zygote the mosaic composition of its plasma membrane is easily recognized. This is because the initial shape of the zygote is that of both gametes; indeed, it appears still to be an ovum and a spermatozoon linked together by an acrosomal tubule, and as long as the sperm profile protudes, it can be identified as such; accordingly, that part of the zygote plasma membrane which is of sperm origin can be distinguished from the egg-contributed part. Figs. 30–33 are of specimens still young enough to permit one to distinguish the two parts. In each case it is clear that there is uninterrupted continuity between sperm- and egg-contributed parts of the membrane. Later the zygote becomes spherical, and since no marker has been recognized, it has not been possible beyond this stage to distinguish one portion of this mosaic membrane from the other.

While the shape is changing, the following events take place. Within the zygote plasma membrane, the apical part of the sperm nucleus lengthens to become a very slender projection whose tip soon reaches the egg cytoplasm in the fertilization cone (Figs. 27–30). The nuclear

FIGS. 10–11. *Hydroides*. Serial sections 1 and 4, respectively, of the early fertilization cone (F) at the stage of contact of the sperm plasma (s) and the egg plasma (e) membranes. Sections are through the area in which the acrosomal tubules (t) have indented the plasma membrane of the egg (e). At this stage the distinction between the sperm plasma membrane (s) and the egg plasma membrane (e) is readily discernible. Thus in Fig. 10, at the arrow, the configuration of two concentric rings is composed of an inner ring of sperm plasma membrane and an outer ring of egg plasma membrane. In Fig. 11 an acrosomal remnant (k) shows clearly. Magnification: Fig. 10, $\times 93,000$; Fig. 11, $\times 108,000$. (Includes portions from Colwin and Colwin, 1961c.)

FIG. 12. *Hydroides*. Stage of early fertilization cone. Note interdigitations of the acrosomal tubules (t) with the egg plasma membrane (e) of the fertilization cone (F). The double concentric rings are composed of the gamete plasma membranes arranged in the manner described in Fig. 10. The acrosomal remnant material is shown at (k). Magnification: $\times 82,000$. (From Colwin and Colwin, 1961c).

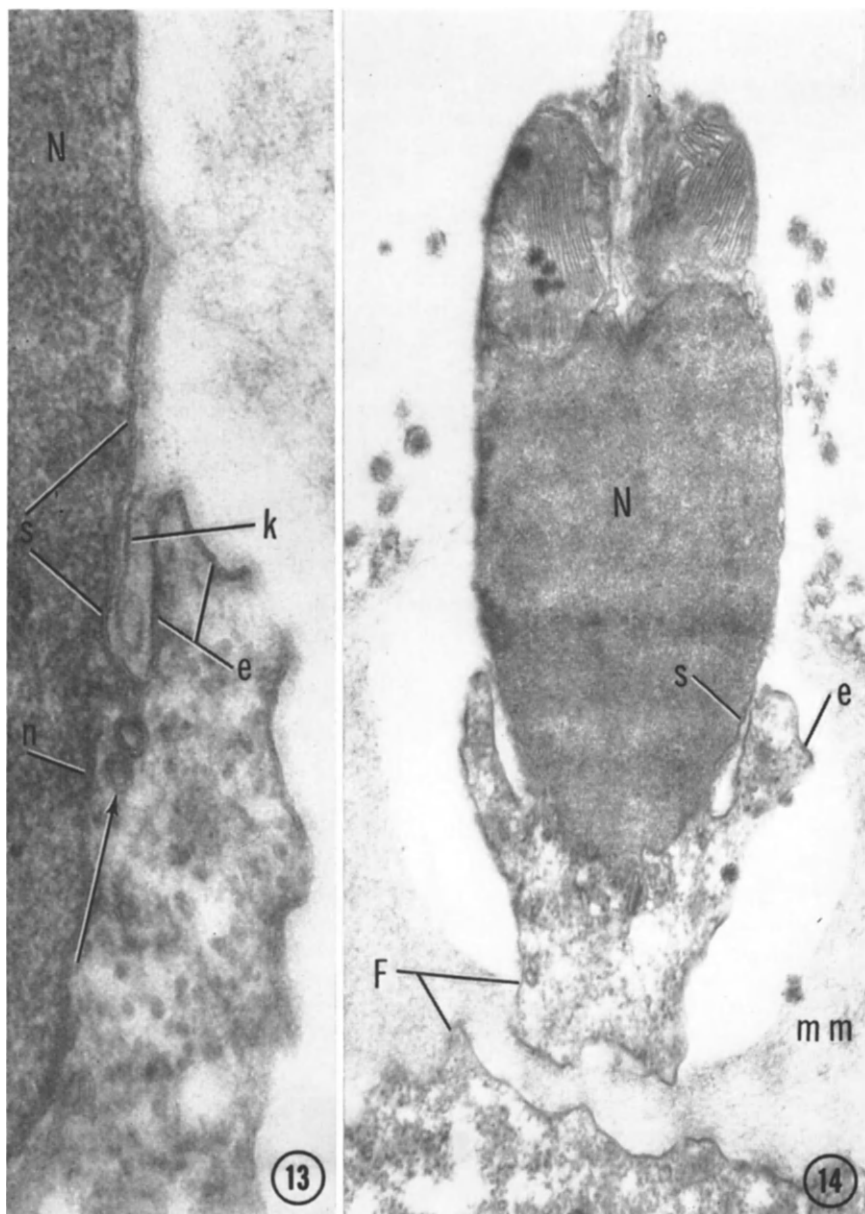


FIG. 13-14. *Hydroides*. Serial sections 1 and 5, respectively, of an early zygote, soon after fusion of the gamete plasma membranes. FIG. 14 shows the over-all relationship at the fertilization cone (F) and the continuity of the sperm plasma membrane (s) with the egg plasma membrane (e). FIG. 13 shows at a higher mag-

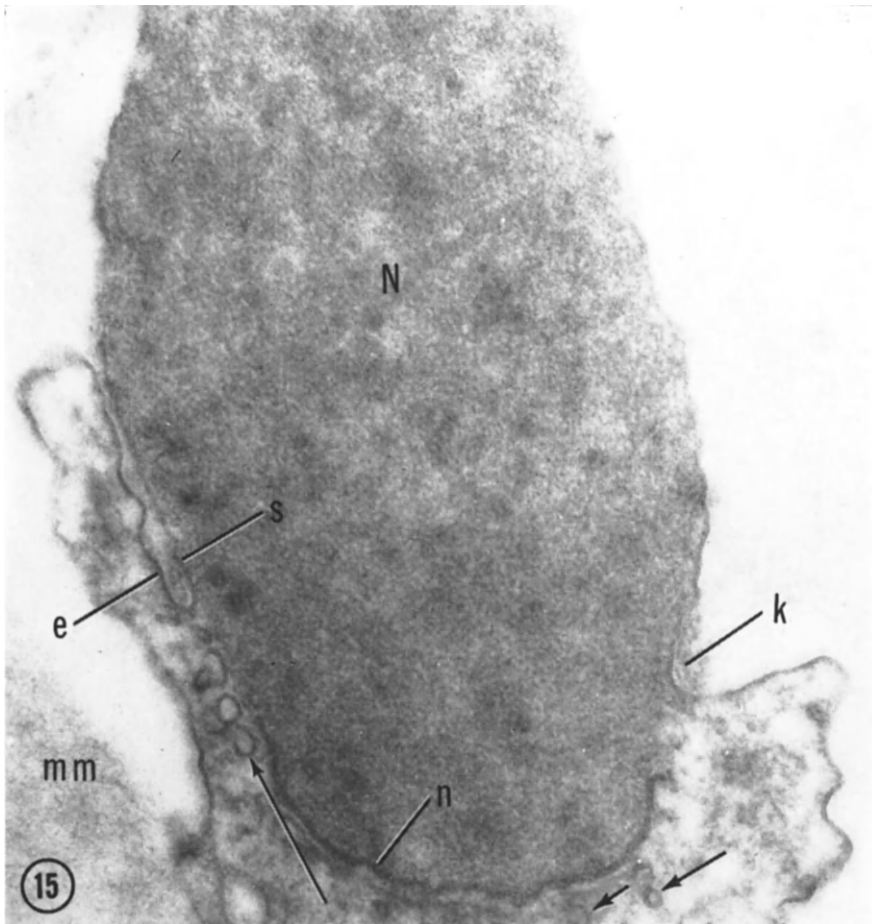


FIG. 15. *Hydroides*. Young zygote soon after gamete membrane fusion. In the zygote plasma membrane note the continuity between the sperm-contributed part (s) and the egg-contributed part (e). The arrow on the left points to a row of vesicles immediately beneath the site of continuity between the gamete plasma membranes. The arrows on the right point to vesicles where the gamete membranes formerly were interdigitated. Magnification: $\times 84,000$. (From Colwin and Colwin, 1961c.)

nification the region where the sperm plasma membrane (s) is continuous with the egg plasma membrane (e). The acrosomal remnant (k) serves as a marker to indicate the approximate level at which the two gamete plasma membranes have fused. The arrow points to a row of vesicles immediately beneath the site of fusion of the gamete membranes. Magnification: Fig. 13, $\times 58,000$; Fig. 14, $\times 130,000$. (From Colwin and Colwin, 1961c.)

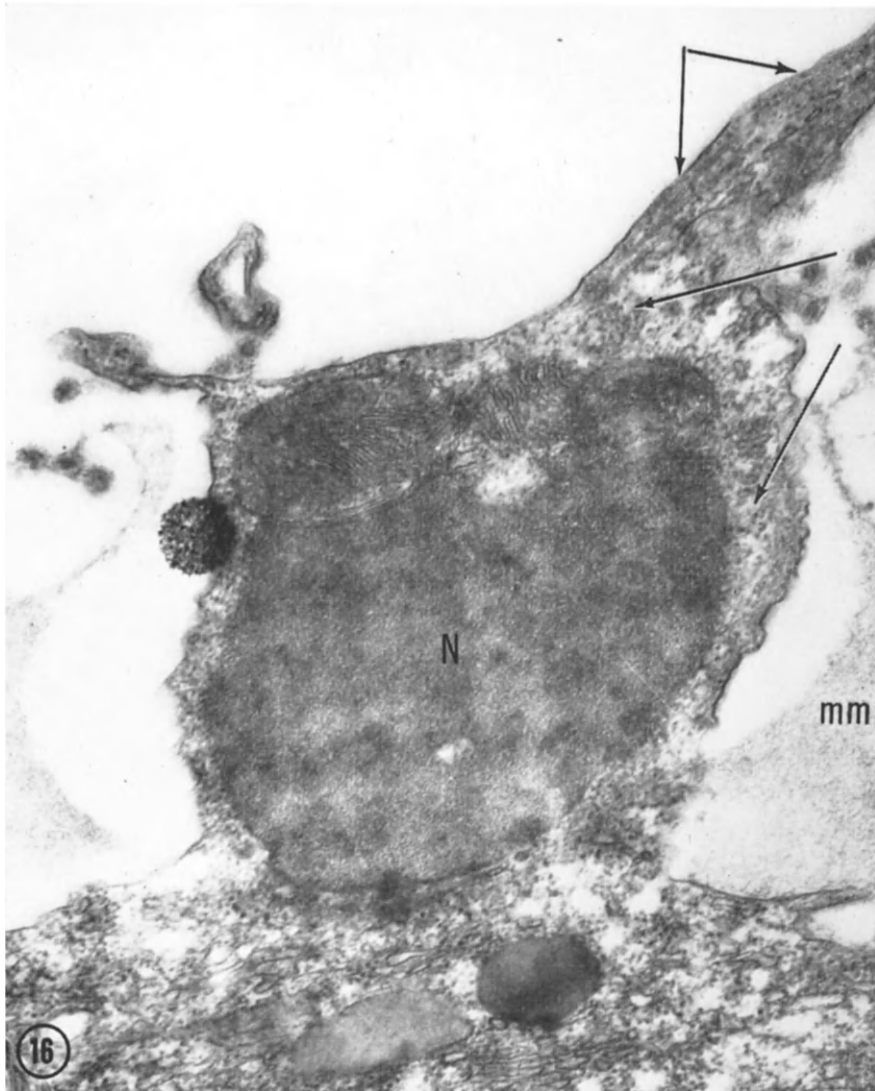


FIG. 16. *Hydroides*. Zygote at later stage than in Figs. 13–15, but still showing the profile of both gametes, since the part containing the internal sperm structures still protrudes through the egg envelope. The arrow tips lie in the egg cytoplasm, which has intruded between the internal sperm structures and the former sperm plasma membrane. The egg cytoplasm extends at least into the base of the flagellum (double-headed arrows) of the former sperm cell. Magnification: $\times 61,000$. (From Colwin and Colwin, 1961c.)

projection thickens until the entire nucleus becomes more or less the shape of a cigar (Fig. 31). The egg cytoplasm moving outward begins to surround the sperm nucleus, i.e., it penetrates between the sperm nucleus and the sperm-contributed part of the zygote plasma membrane (Figs. 32 and 33). Eventually the nucleus moves in below the level of the egg capsule and comes to lie entirely within the egg cytoplasm (Fig. 34). The mitochondria, centrioles, and axial structures of the

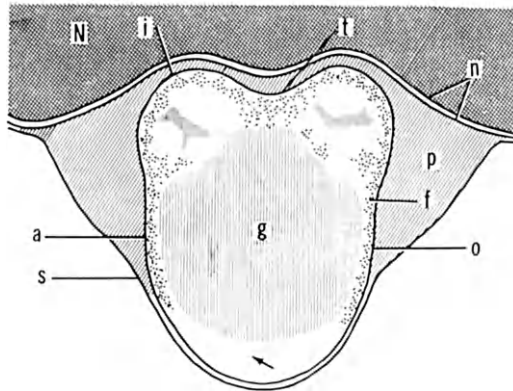


FIG. 17. *Saccoglossus*. Diagram of unactivated acrosomal region: median longitudinal section. The acrosomal vesicle is bounded by the acrosomal membrane (a) of which the inner or juxtannuclear portion (i) has a shallow indentation (t). The large amount of periacrosomal material (p) lies chiefly between the outer portion of the acrosomal membrane (o) and the sperm plasma membrane (s), but some of it lies between the inner acrosomal membrane (i) and the nuclear envelope (n). Within the acrosomal vesicle fine granular material (f) lines the acrosomal membrane, except at the apex where an apical space (arrow) separates the acrosomal membrane from the acrosomal granule (g). (From Colwin and Colwin, 1963a.)

flagellum, all of which, like the nucleus, are internal sperm organelles, remain associated with the nucleus and they too enter the egg cytoplasm. For a while the fertilization cone enlarges, but it finally recedes.

A prominent feature of the acrosomal tubule is the early formation within it of a fiberlike "core" (Figs. 24-26 and 28). The core appears coincidentally with marked changes in the periacrosomal material and presumably is formed from some of this material. The core extends the length of the acrosomal tubule and appears to precede the nucleus into the egg cytoplasm (Fig. 35).

4. The *Hydroides-Saccoglossus* Pattern

a. Acrosomal Region and Changes Preliminary to Zygote Formation.
In both species the sperm cell passes through a nonmembranous egg

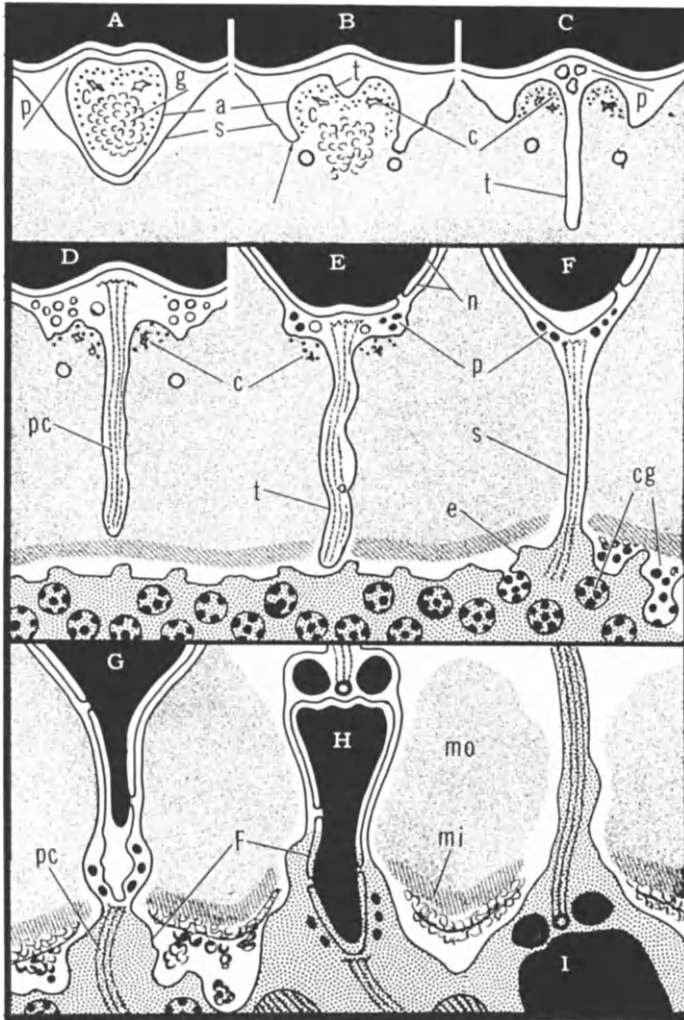


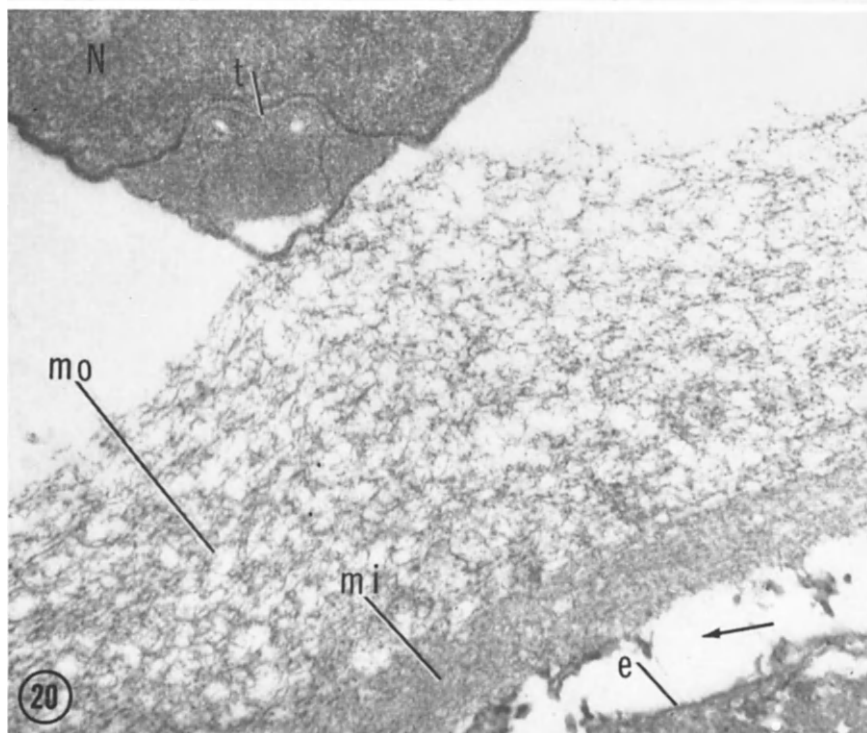
FIG. 18. *Saccoglossus*. Diagrams of successive stages of sperm-egg association leading to the establishment of the zygote. (A) Unactivated acrosomal region at initial contact with the outer layer of the egg envelope. (B) Acrosomal region soon after activation; acrosomal (a) and sperm (s) plasma membranes are continuous at the arrow; the cavity (c) of the acrosomal vesicle is open and the acrosomal granule (g) is exposed to the exterior. (C) The acrosomal tubule (t) undergoes initial rapid lengthening; since eversion of the acrosomal vesicle is not yet extensive, the relationship of the contents of the cavity (c) to the exterior is evident. (D) Eversion of the acrosomal vesicle has progressed; the acrosomal tubule (t) has lengthened and has reached the inner layer of the egg envelope; a core (pc) of periacrosomal material lies within the tubule cavity, which is continuous with the cavity containing other periacrosomal material. (E) The acrosomal

capsule before meeting the egg plasma membrane, and this passage involves major changes in the acrosomal region. In both species the region includes periacrosomal material as well as an acrosome and is bounded externally by part of the sperm plasma membrane. Each acrosome is a membrane-bounded vesicle whose wall, the acrosomal membrane, is lined by fine granular material and whose cavity contains a large acrosomal granule. The juxtannuclear or inner portion of the acrosomal membrane is indented in both species, the single depression in *Saccoglossus* having as a counterpart the group of depressions in *Hydroides*. Although there is a difference in the amount and distribution of periacrosomal material in the two species, the difference is one of detail and is not fundamental; in both species some of this material lies between the nucleus and the acrosome.

Even though the apical vesicle of *Hydroides* has no clear counterpart in *Saccoglossus*, in both species an area at the apex of the spermatozoon has the following distinctive characteristics: absence of the granular lining of the acrosomal membrane and absence of contact between the acrosomal granule and this membrane. These characteristics seem to be related to the phenomenon of dehiscence, because the fracture line along which the apex opens when the spermatozoon is activated encircles precisely this region.

The preliminaries to zygote formation in the two species may be summarized as follows: (a) Soon after initial contact with the egg capsule the sperm apex dehisces, and around the rim of the opening the acrosomal and sperm plasma membranes are seen to be continuous, with the acrosomal membrane now an inserted mosaic section of the sperm plasma membrane. Associated with dehiscence there appears to be some loss of apical membrane, presumably of both acrosomal and plasma membrane origin. (b) The acrosomal granule disappears, as does also that part of the egg capsule which was exposed to it. (c) There is acrosomal tubule formation. The single juxtannuclear indentation of

tubule (t) has penetrated the inner egg envelope and is almost in contact with the egg plasma membrane (e). (F) Young zygote soon after the tip of the acrosomal tubule has opened and the tubule contents have begun to mingle with the egg cytoplasm. The mosaic origin of the zygote plasma membrane from sperm (s) and egg (e) plasma membranes is apparent; at (cg) are shown both a closed and an open cortical granule. (G, H) Two successively later stages of the zygote, which still show the profile of both gametes; note the elongation of the nucleus as it progresses toward the fertilization cone (F); note the intrusion of egg cytoplasm between the former sperm plasma membrane and the sperm organelles. (I) Most of the sperm organelles are well within the egg outline, but flagellar fibers surrounded by some egg cytoplasm still protrude; the fertilization cone is receding. (From Colwin and Colwin, 1963a,b.)



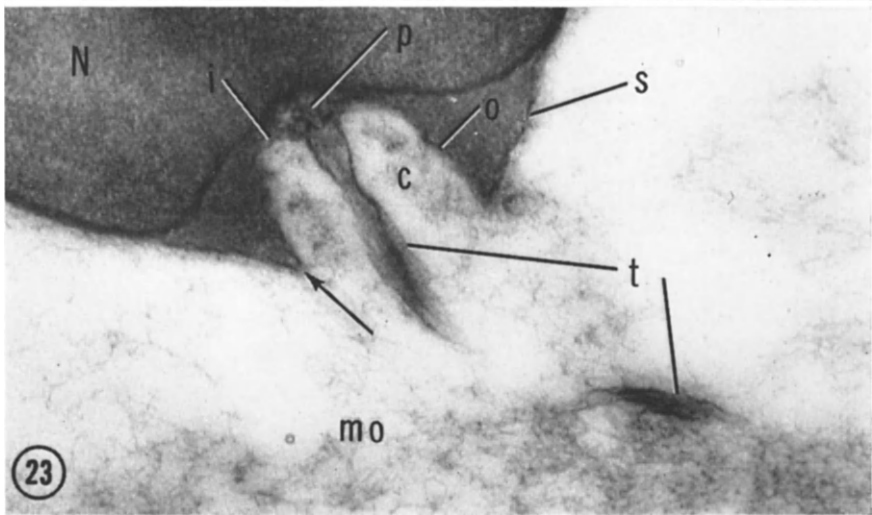
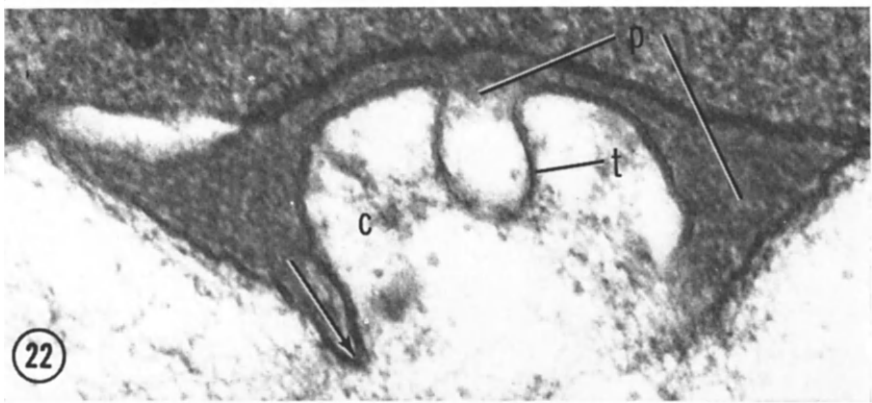
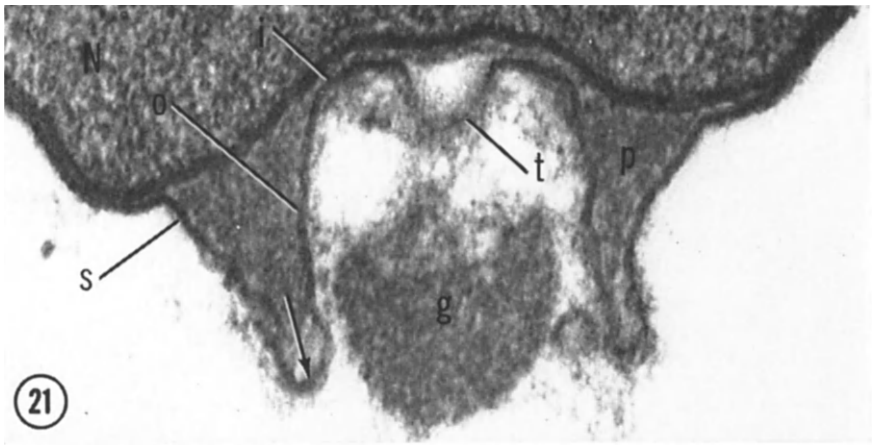
the acrosomal membrane in *Saccoglossus* lengthens to form a single tubule, and in *Hydroides* equivalent but multiple indentations lengthen to form a tuft of tubules; in both species, associated with this lengthening is the complete eversion of the acrosomal vesicle. (d) Having traversed the egg capsule, the tubule or tuft of tubules meets the egg plasma membrane.

b. Zygote Formation by Gamete Membrane Fusion. The above-mentioned preliminaries culminate in the establishment of the zygote, and this process too takes place in essentially the same way in both species. The acrosomal tubule or tuft of tubules meets the egg plasma membrane. This is the initial contact between the plasma membranes of the two gametes. Soon the two membranes fuse to become one continuous but mosaic membrane, and thus the zygote plasma membrane is established. This fusion permits the contents of the formerly separate cells to intermingle. The egg cytoplasm gradually intrudes between the sperm-contributed part of the common plasma membrane and the sperm internal organelles. All the contents of the two gametes finally intermingle. This process simply cannot begin, however, until the zygote has had its inception in membrane fusion.

c. Significance of the Hydroides-Saccoglossus Pattern. In all fundamental respects these two dissimilar species are strikingly similar with regard to the beginnings of fertilization. Not only is the structural arrangement essentially the same but the corresponding structures function in the same manner. In the present context, then, function is closely correlated with structure. Since the same pattern of structure and function has been found to occur in two species from such very different phyla, it has been suggested (Colwin and Colwin, 1963a,b) that this particular pattern would be a fundamental one and would have widespread occurrence. A judgment as to whether or not the *Hydroides*-

FIG. 19. *Saccoglossus*. Median section of acrosomal region. Note the broad shallow invagination or indentation (t) of the inner (i) or juxtannuclear portion of the acrosomal membrane. A large amount of periacrosomal material (p) lies between the outer (o) portion of the acrosomal membrane and the sperm plasma membrane (s); this is continuous with the periacrosomal material (p), which lies between the inner acrosomal membrane (i) and the membranes of the nuclear envelope (n). At the apex the acrosomal membrane is close to the plasma membrane, but is separated from the acrosomal granule (g) by apical space (arrow). Magnification: $\times 108,000$. (From Colwin and Colwin, 1963a.)

FIG. 20. *Saccoglossus*. Sperm-egg association; stage of initial contact of the sperm plasma membrane and the egg envelope (mo) before apical dehiscence occurs. The outer (mo) and inner (mi) portions of the egg envelope overlie the egg plasma membrane (e); microvilli do not project into the egg envelope. The arrow lies in the very narrow perivitelline space. Magnification: $\times 44,000$. (From Colwin and Colwin, 1963a.)



Saccoglossus pattern of fertilization actually is fundamental can be reached only after equivalent information is available from other phyla. But even in the absence of full information let us conjecture that if some aspects of structural arrangement or of behavior are like those in the *Hydroides-Saccoglossus* pattern, then, by extrapolation, other aspects will also follow this pattern, and in this context let us examine the growing body of information that is becoming available from other phyla.

B. COMPARATIVE ASPECTS OF ASSOCIATION (VERTEBRATE AND INVERTEBRATE PHYLA)

1. Introduction

From the accounts in the preceding section it is evident that study at the ultrastructural level is needed in order to obtain the details required to compare various groups. The groups to be considered, then, are mainly those in which electron microscopy has provided knowledge of the structure of the acrosomal region or of the events at the beginning of fertilization, or of both. It should be emphasized that the interpretations to be given are not necessarily those of the original authors.

2. Groups with Acrosome

a. Echinoderms. i. Sea urchins. (a). Acrosomal region and changes. The ultrastructure is known from a number of studies. In *Arbacia* (Bernstein, 1962; Franklin, 1965) (Fig. 36), *Lytechinus* (Franklin,

FIGS. 21–23. *Saccoglossus*. Acrosomal regions of successive stages of sperm-egg association, following the stage shown in Fig. 20. The arrows point to the rim of dehiscence.

FIG. 21. The acrosomal region soon after dehiscence. At the juxtanuclear end the invagination (t) of the inner portion of the acrosomal membrane (i) begins to elongate into a tubule. The acrosomal membrane (o) and the sperm plasma membrane (s) are now one continuous membrane whose mosaically joined parts meet at the rim of dehiscence. The exposed acrosomal granule (g) is still present.

FIG. 22. The acrosomal tubule (t) has slightly lengthened; the acrosomal granule has disappeared. Note that the cavity of the acrosomal vesicle (c) is open to the outside.

FIG. 23. A stage soon after the initial elongation of the acrosomal tubule. Two portions of the proximal part of the acrosomal tubule (t) are shown. Most of the original acrosomal membrane (i) and (o) still occupies its original position, indicating that at this stage very little, if any, of this membrane was used in lengthening the tubule. The periacrosomal material (p) at the base of the tubule appears altered. Note the continuity of the former cavity of the acrosomal vesicle (c) with the outside. Magnification: Fig. 21, $\times 121,000$; Fig. 22, $\times 121,000$; Fig. 23, $\times 90,000$. (From Colwin and Colwin, 1963c.)

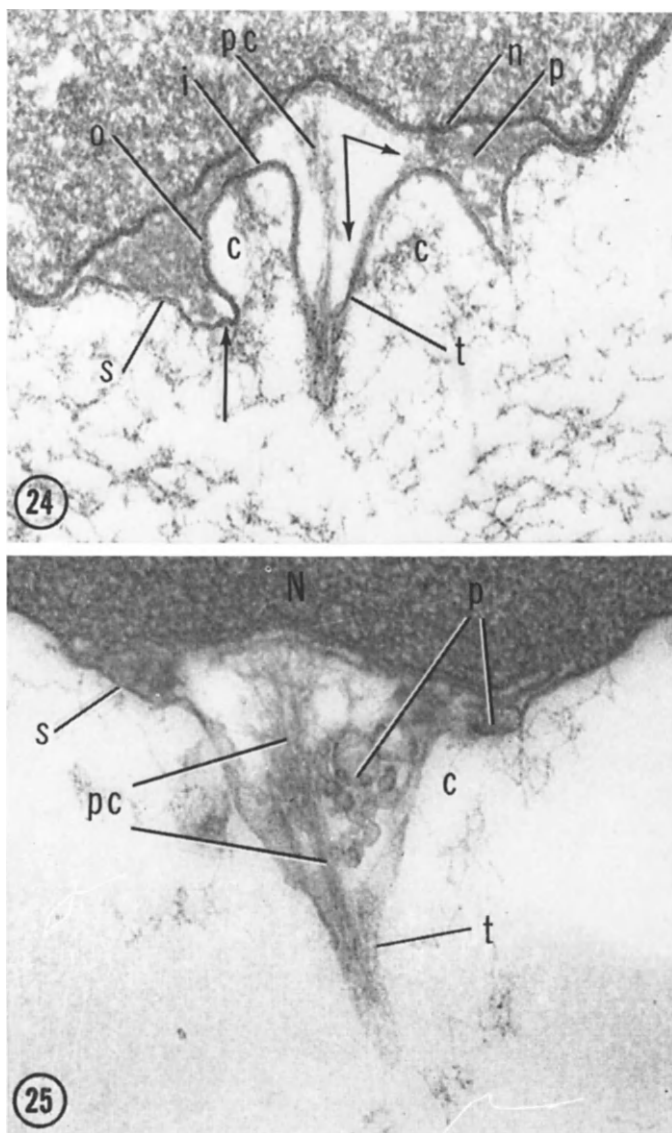


FIG. 24. *Saccoglossus*. Acrosomal region, following initial tubular elongation, at the stage when the acrosomal vesicle begins eversion; only the basal part of the tubule (t) is shown. Note that the periacrosomal cavity (within which lies the periacrosomal material [p]) is confluent with the cavity of the tubule, as indicated by the double-headed arrows. A fibrous core (pc) of periacrosomal material extends into the tubule. The inner membrane (i) of the everting acrosomal vesicle has separated further from the nuclear envelope (n). The contents of the cavity of the acrosomal vesicle (c) are now more obviously on the outside. The lower-left arrow points to the approximate site at which the acrosomal membrane (o) has

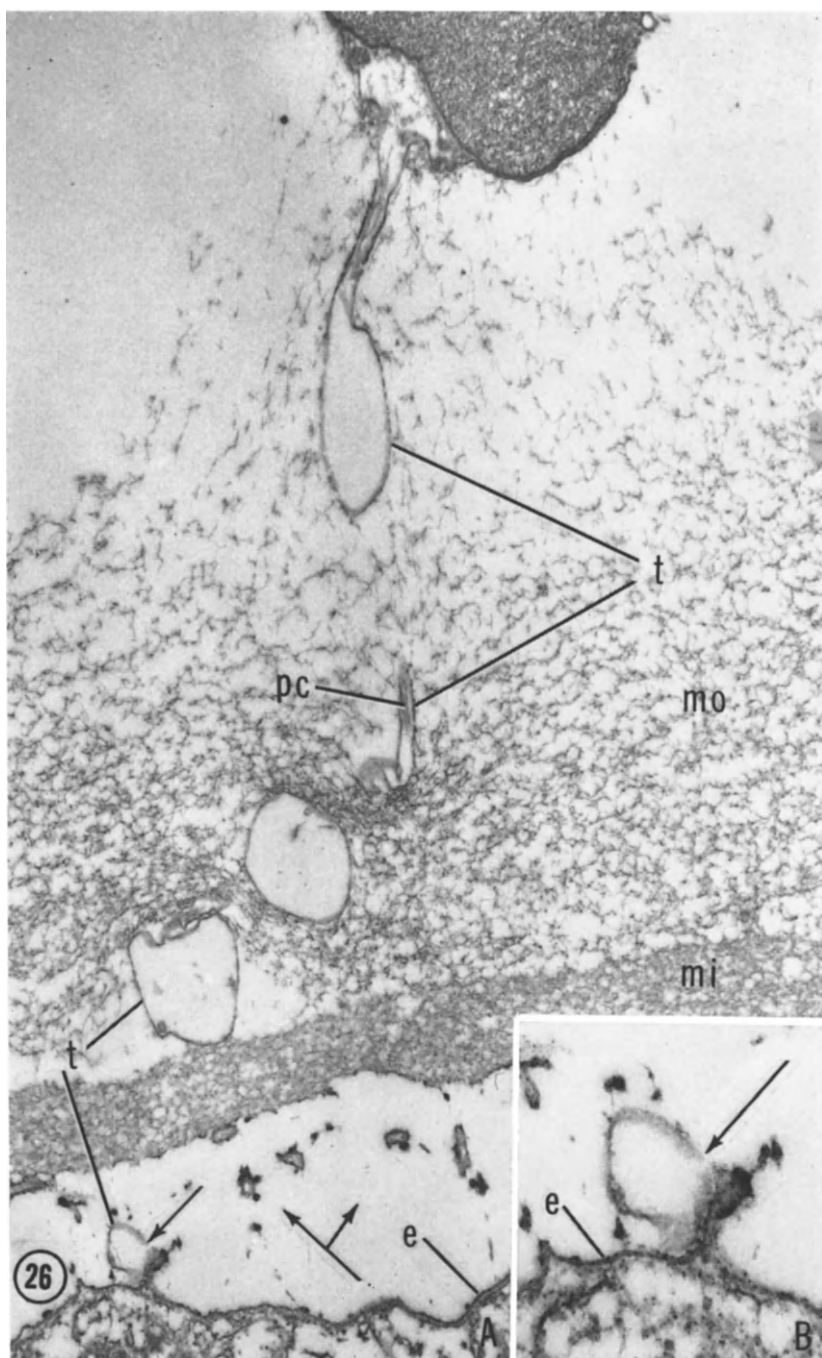
1965), and *Paracentrotus* (Pasteels, 1965) (Fig. 39), the acrosome is a membrane-bounded vesicle whose cavity contains an acrosomal granule. Earlier electron micrographs of Afzelius (1955) might be interpreted as showing a membrane-bounded acrosome also in *Strongylocentrotus*. However, Dan *et al.* (1964) reported that in *Pseudocentrotus* the acrosomal granule is not membrane-bounded. Whether this lack of a membrane around the acrosomal granule reflects a species difference or a difference due to techniques of preparation remains unresolved. Franklin (1965) interprets some of his observations as showing tubular invaginations of the inner (i.e., juxta- or adnuclear) portion of the acrosomal membrane in *Arbacia* and the presence of an apical vesicle in *Lytechinus*. In many if not all of these species there is periacrosomal material between the acrosome and nucleus.

Following activation of the spermatozoon either by egg water or by the presence of eggs, the acrosomal region is highly modified, and a single short process which is obviously tubular and which contains somewhat fibrous material is found at the tip of the spermatozoon (Figs. 37 and 40). Afzelius (1956a), Afzelius and Murray (1957), Takashima and Takashima (1960), Dan *et al.* (1962), Bernstein (1962), Franklin (1965), and Pasteels (1965, and personal communication, 1964) have shown thin sections of this process. Although none of these studies shows how the process arises, the structural evidence suggests, in the terminology of Colwin and Colwin (1963a, 1964), that the following events occur: dehiscence; exposure and at least partial dissipation of the acrosomal granule; transformation of the sperm plasma membrane into a mosaic by means of insertion of the acrosomal membrane; and eversion of the acrosomal membrane, whose juxtannuclear portion elongates to form the acrosomal tubule. Dan *et al.* (1964) consider that the acrosomal tubule or process in *Pseudocentrotus* arises in an entirely different way, namely, from "precursors of acrosomal process membrane"; however, the electron micrographs offered in support of this view indicate rather that the acrosomal tubule is formed in the manner suggested above.

In what we would call the acrosomal tubule, Afzelius (1956a), Dan *et al.* (1964), and Franklin (1965) described fibrillar material, which in our terminology is periacrosomal material.

been inserted as a mosaic segment into the sperm plasma membrane (s). Magnification: $\times 71,000$. (From Colwin and Colwin, 1963a.)

FIG. 25. *Saccoglossus*. Acrosomal region at the stage when the acrosomal vesicle has been completely everted. The entire periacrosomal cavity is now part of the cavity of the acrosomal tubule (t). A fibrous core (pc) extends into the tubule from the nuclear apex. Other periacrosomal material (p) in the form of large and small vesicles is also present in the periacrosomal cavity (cavity of the tubule). Magnification: $\times 72,000$. (From Colwin and Colwin, 1963a.)



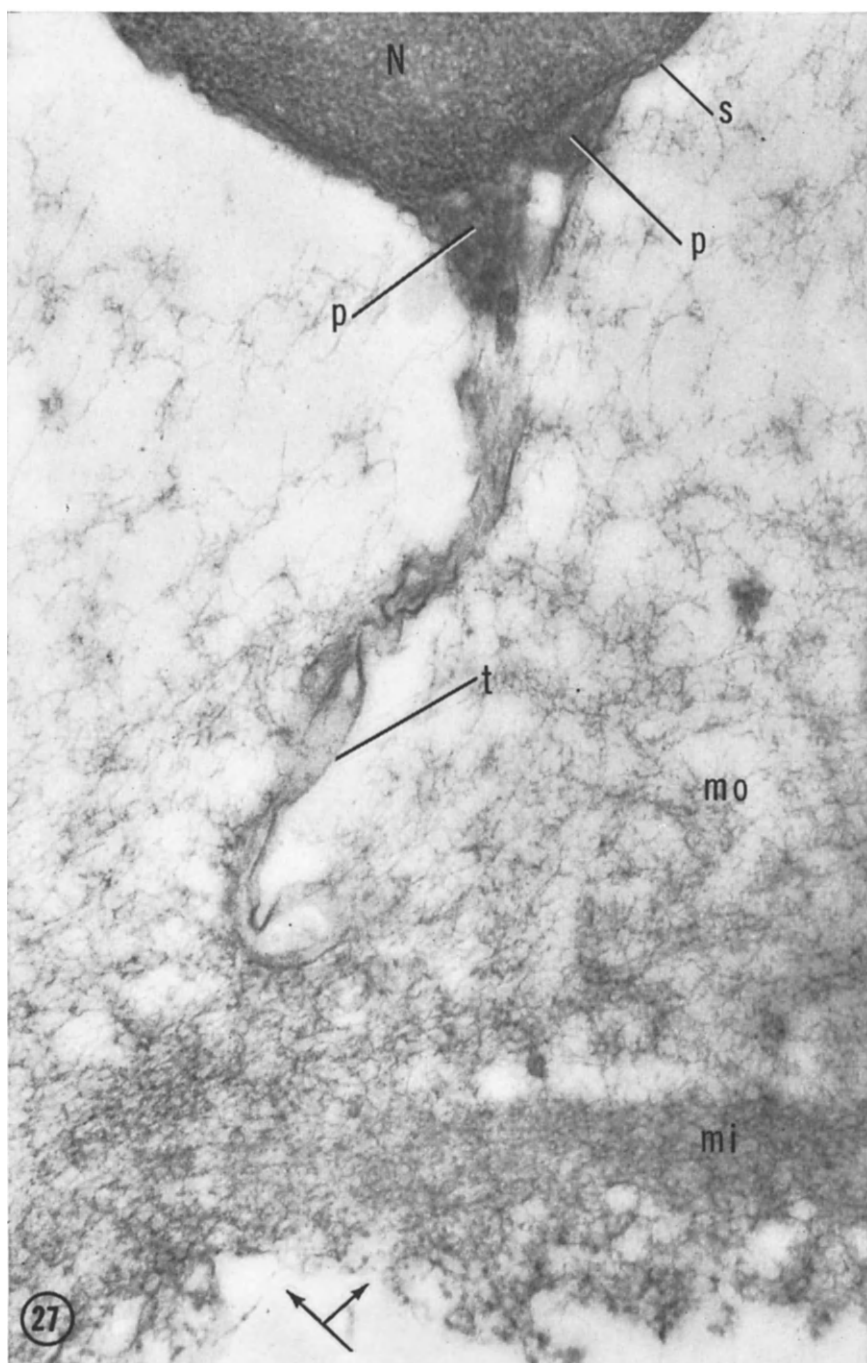
Before any part of the spermatozoon can come into contact with the egg plasma membrane it must pass through the egg jelly and the vitelline envelope. It is not known how this passage is accomplished, but since the acrosomal granule is at least partially expended before the gamete membranes meet (Afzelius, 1956a; Franklin, 1965), it seems likely that the granule may play some role in this passage.

(b). *Zygote formation*. It was previously postulated (Colwin and Colwin, 1963a, 1964) that in echinoderms zygote formation would take place according to the *Hydroides-Saccoglossus* pattern. Franklin (1965) has now found that in *Arbacia* the acrosomal tubule does indeed fuse with the egg plasma membrane to establish continuity of membranes between the two gametes (Fig. 38). This membrane fusion will take place with the mature egg as well as with the oocyte. A later stage of sperm association with the oocyte of *Brissopsis* is figured by Lönning (1964). We interpret the figure to indicate that in this species too the sperm and egg plasma membranes fuse and become continuous. In *Lytechinus* (Franklin, 1965), as in *Brissopsis* and *Arbacia*, the sperm nucleus evidently enters the egg cytoplasm without the sperm plasma membrane.

(c). *Conclusion*. In sea urchins, then, a substantial body of evidence indicates a pattern of sperm-egg association that is essentially the same as in *Hydroides* and *Saccoglossus* (cf. Fig. 49C with Fig. 49A and B).

ii. *Starfish*. (a) *Acrosomal region and changes*. Dan (1954, 1960) and Dan *et al.* (1962) described the fine structure in *Asterias*. Although a membrane-bounded acrosomal vesicle with a contained acrosomal granule was not reported, a dense central mass and associated structures are shown, and these we interpret to be an acrosomal granule partly filling a much larger and membrane-bounded acrosomal vesicle. Material that we should call periacrosomal material lies outside what presumably is the acrosomal membrane. Although a lytic agent has been considered unnecessary in starfish (Dan, 1960), if an acrosomal granule is present it might be expected to play some lytic role (Colwin and Colwin, 1961b).

FIG. 26. *Saccoglossus*. (A) Stage of initial contact between the sperm plasma membrane and the egg plasma membrane. At the arrow, the tip of the acrosomal tubule (t), which is sperm plasma membrane, meets the egg plasma membrane (e). Several segments of the long acrosomal tubule (t) are shown; the portion at the arrow has penetrated both layers of the egg envelope (mo), (mi). The acrosomal vesicle has not yet fully everted. Within the tubule is some periacrosomal core (pc). The double-headed arrow lies in the somewhat enlarged perivitelline space. (B) Enlarged detail of the region of contact of the gamete plasma membranes shown near the arrow in (A). Magnification: (A), $\times 30,000$; (B) $\times 69,000$. (From Colwin and Colwin, 1963a.)



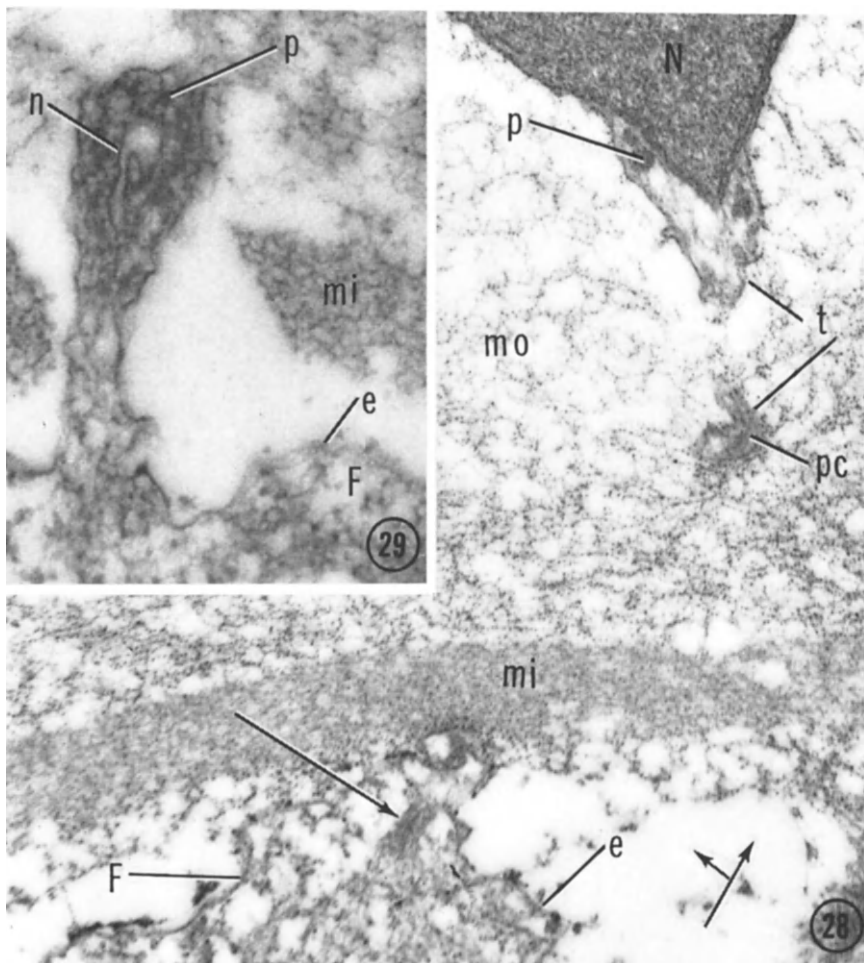
In several species a long acrosomal process appears following artificial sperm activation (Dan, 1954) or during association with the egg (Colwin and Colwin, 1955, 1956). Dan *et al.* (1962), after exposing spermatozoa to egg jelly solution, found that the acrosomal region was much modified, with a long process projecting at the tip. As seen in their electron micrographs of thin sections, two stages of elongation of this process strikingly resemble stages of elongation of the acrosomal tubule of *Saccoglossus*, as shown in Figs. 24 and 25. The process in the starfish certainly has the appearance of being tubular and, as Dan (1960) and Dan *et al.* (1962) have indicated, it has fibrous elements. From this ultrastructural evidence we proposed (Colwin and Colwin, 1963b, 1964) that at activation in starfish spermatozoa, as in the *Hydroides-Saccoglossus* pattern, the acrosomal and sperm plasma membranes form a single mosaic continuum, the acrosomal granule is expelled, and the entire acrosomal membrane turned inside out. Moreover, we proposed that the juxtannuclear part of the acrosomal membrane (which perhaps is already indented prior to sperm activation) by lengthening becomes the acrosomal tubule, and that the tubule contains fibrous periacrosomal material (Fig. 49C).

(b). *Zygote formation.* At present the ultrastructure of zygote formation is not known. From our interpretation of the ultrastructural evidence as to the preliminaries, however, and from the fact that the picture of gamete junction as observed in living material of *Asterias* (Colwin and Colwin, 1955, 1956) is remarkably similar to that in living material in *Saccoglossus* (Colwin and Colwin, 1954), electron microscopy may reasonably be expected to show that the acrosomal tubule forms a continuum with the egg plasma membrane.

(c). *Conclusion.* In starfish, too, there is evidence of a pattern of sperm-egg association similar to the *Hydroides-Saccoglossus* pattern; gamete membrane fusion is probably the means of zygote formation (cf. Fig. 49C with Figs. 49A and B).

iii. *Holothurians.* There are no fine-structure studies for this group, but in a number of species (Colwin and Colwin, 1955, 1956, and unpublished) the acrosomal region during sperm activation produces a long process, the "acrosome filament." Since this process is probably an acrosomal tubule, the holothurians can be expected to follow the

FIG. 27. *Saccoglossus*. Young zygote in an early stage of elongation of the nucleus. The apex of the nucleus is preceded by periacrosomal material (p) in the acrosomal tubule (t). The double-headed arrow lies in the perivitelline space. The outer (mo) and inner (mi) portions of the egg capsule are indicated. Magnification: $\times 61,000$. (From Colwin and Colwin, 1963b.)



FIGS. 28-29. *Saccoglossus*. Portions of young zygotes in stages during which they still retain the profile of the two gametes even though they are bounded by a single continuous cell membrane. These figures are of successively later stages of nuclear elongation and progression through the region of the egg capsule. Clumps of periacrosomal material (p) remain near the apex of the nucleus. (From Colwin and Colwin, 1963b.)

FIG. 28. Three parts of one zygote. The upper part shows the apex of the elongating sperm nucleus protruding into the acrosomal tubule; the middle part shows a small distal part of the tubule (t); and the lower part shows the fertilization cone (F) protruding into the perivitelline space (double-headed arrow). The arrow on the left points to fibrous material, presumed to be the core, which lies in the fertilization cone. Magnification: $\times 49,000$.

FIG. 29. A stage slightly later than in Fig. 28. The sperm nuclear apex, whose outer membrane (n), only, is shown here, is now near the region where the acrosomal tubule meets the fertilization cone (F). Magnification: $\times 61,000$.

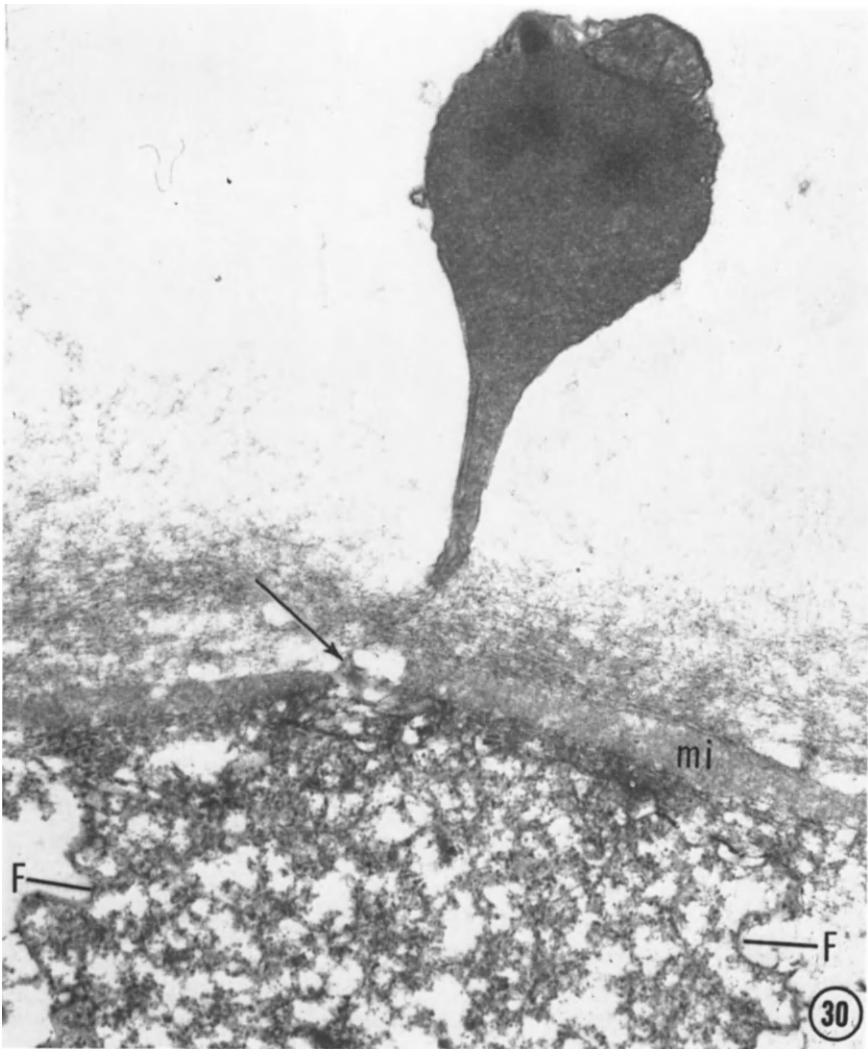


FIG. 30. *Saccoglossus*. Zygote at a stage later than in Fig. 28. The apex of the sperm nucleus is now a slender projection extending toward the fertilization cone (F). The arrow points to part of the coiled acrosomal tubule just outside the region in which it breaches the inner egg envelope (mi). Magnification: $\times 24,000$. (From Colwin and Colwin, 1963b.)

Hydroides-Saccoglossus pattern. Further comments on the holothurians will be found in the next paragraph.

iv. *Reinterpretation of older studies of sperm-egg association in echinoderms.* From the time of Fol's classical description of fertilization (1877, 1878-1879), the initiation of fertilization in starfish has provoked

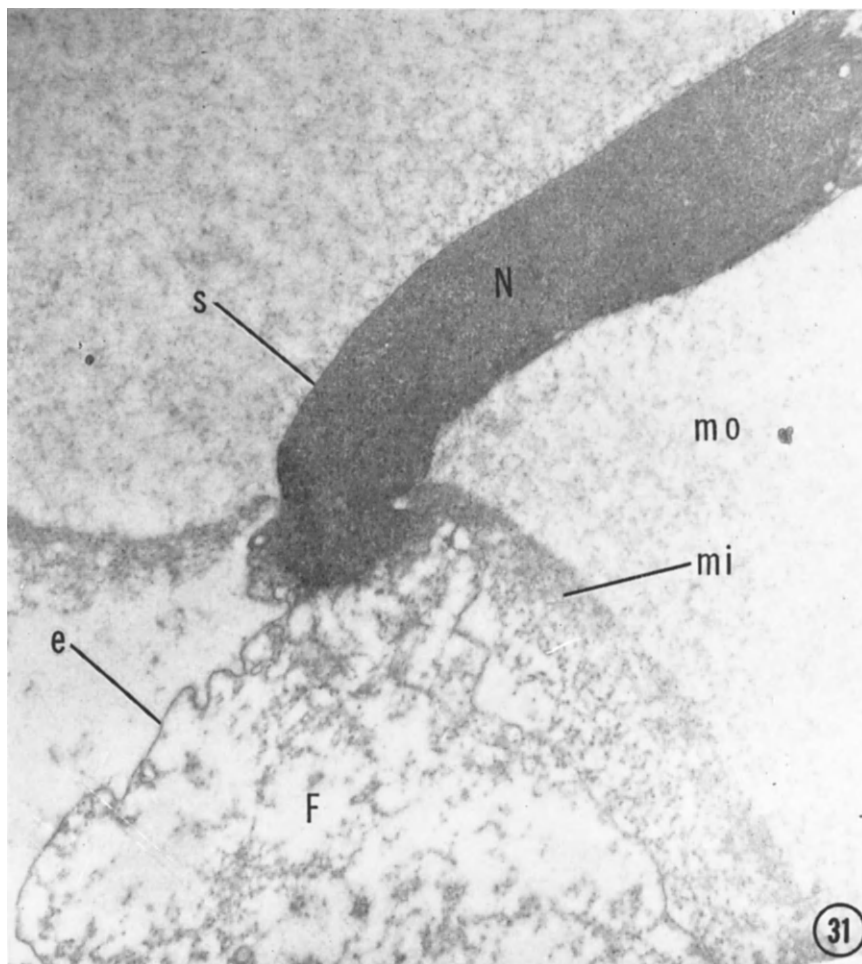
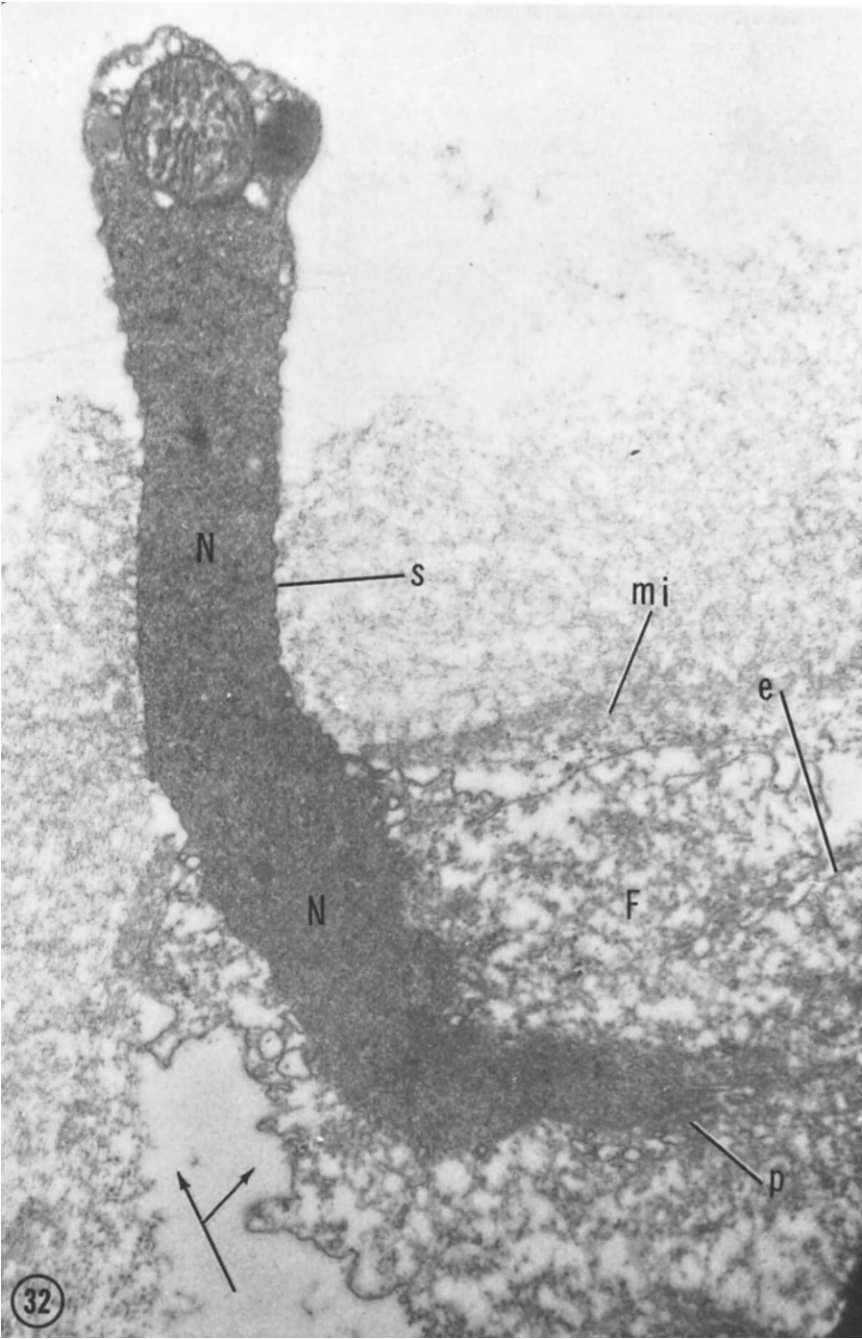


FIG. 31. *Saccoglossus*. Zygote at the stage in which the somewhat cigar-shaped sperm nucleus (N) meets the egg cytoplasm at the tip of the fertilization cone (F). The continuity between that part of the zygote membrane which was originally sperm plasma membrane (s) and that part which was originally egg plasma membrane (e) is quite evident. Magnification: $\times 29,000$. (From Colwin and Colwin, 1963b.)

a substantial body of speculation regarding not only echinoderms but other species as well, based on studies by light microscopy (reviewed in Colwin and Colwin, 1955, 1957b). When studies of living material in holothurians and starfish revealed new aspects of sperm-egg association (Colwin and Colwin, 1955, 1956) the older speculations were re-examined and new conclusions drawn. It appeared in living specimens

that a rodlike acrosome filament was the most anterior sperm structure, that it entered the egg, and that the other sperm organelles moved into the egg following this rod, and it was suggested that this manner of sperm-egg association occurred also in other species. In the absence of any evidence to the contrary, the old notion that the intact sperm cell enters the egg was taken for granted. Today the evidence from electron microscopy makes this notion untenable for a number of species, and the question arises as to whether it is valid for any species. For example, the original interpretation of events observed in living specimens of *Holothuria atra* (Colwin and Colwin, 1955) should probably be reinterpreted as follows (Fig. 47; cf. lower figs. with upper figs.). The apical sperm structure which first approaches the egg plasma membrane is not a rodlike "filament" but a tubule, and what first enters the egg is only a core of periacrosomal material from within this tubule. The sperm plasma membrane of the tubule fuses with the egg plasma membrane before this rodlike core enters. Since all the remaining sperm parts also become divested of their plasma membrane as they enter the egg cytoplasm, it is not an intact sperm cell but only the internal components of the sperm cell that would enter the egg. This interpretation, already established for certain sea urchins, would probably apply also to the events observable in living material in other holothurians and in starfishes.

b. Mollusks. i. Acrosomal region and changes. Aspects of the fine structure have been described for the bivalves *Mytilus* by Dan (1956), *Crassostrea* by Galtsoff and Philpott (1960), and *Barnea* by Pasteels and de Harven (1962) and Pasteels (1965), and the electron micrographs and diagrams of the several authors reveal a fairly consistent design of construction. Using *Barnea* as an example (Fig. 49D, a), in the terminology of the *Hydroides-Saccoglossus* pattern, there is clearly a membrane-bounded acrosomal vesicle whose juxtannuclear portion is deeply indented in the form of a tubule. Periacrosomal material, some of it fibrous, occupies this indentation and extends to the apex of the nucleus. Within the acrosomal vesicle is disposed electron-dense material, perhaps corresponding in part to the acrosomal granule. Although lysis of the egg capsule has not been described for *Barnea*, it occurs in *Mytilus* (Dan, 1962) and, in both species, at least part of the contents of the acrosomal vesicle would be a probable source of a capsule lysin. In *Barnea* the spermatozoa are activated in the presence of eggs and undergo a series of changes which may be interpreted as follows (Fig. 49D, a-d). The acrosomal region dehisces, and around the newly formed apical opening the acrosomal and plasma membranes then constitute one continuous membrane. The contents of the acrosomal vesicle are thus placed entirely outside the sperm cell, while the invagination of the



juxtannuclear or inner portion of the acrosomal membrane becomes the definitive acrosomal tubule. Nijima and Dan (1965a,b) have recently described a similar series of changes in *Mytilus*. In both *Mytilus* and *Barnea*, then, these changes follow the general plan of the *Hydroides-Saccoglossus* pattern (cf. Fig. 49D with Figs. 49A and B).

ii. *Zygote formation*. There is ultrastructural evidence to show that in *Barnea* (Pasteels, 1965) the zygote plasma membrane is a continuum composed of egg and sperm plasma membranes (Fig. 41). An earlier electron micrograph by Rebhun (1962) suggests that in the bivalve *Spisula*, too, the zygote is formed by gamete membrane fusion. The behavior at zygote formation, then, also parallels the *Hydroides-Saccoglossus* pattern.

c. *Other Invertebrate Phyla*. For the following groups the essential information is so limited that they cannot be dealt with extensively. However, these groups merit some comment.

i. *Annelids*. Lillie's classical studies on fertilization in *Nereis* (1912a,b) deserve to be supplemented by fine-structure studies if the events of sperm-egg association are to be properly compared with those in other species. Such studies are beginning to appear. Metz and Morrill (1955) reported that the *Nereis* sperm tip changes and gives rise to a filament when it is exposed to fertilizin treatment. Fallon and Austin (1964) indicated that in activated spermatozoa of *Nereis* the outer (part of the) acrosomal membrane fuses with the sperm plasma membrane and that the inner (part of the) acrosomal membrane makes contact and fuses with the egg plasma membrane. In *Sabellaria* Pasteels (personal communication, 1964) showed an acrosomal region containing a membrane-bounded acrosomal vesicle (Fig. 42). In the vesicle is much dense material, some of which might be equivalent to an acrosomal granule. The juxtannuclear region of the vesicle is so deeply indented that it has the form of a tubule. Outside the acrosome is periacrosomal material, most of it occupying this acrosomal tubule.

ii. *Echiuroids*. Fine-structure studies of the acrosomal region during the early stages of sperm-egg association are not available. However, in electron micrographs of later stages by Tyler *et al.* (1964) the egg of *Urechis* can be seen to be surrounded by a vitelline envelope of

FIG. 32. *Saccoglossus*. Zygote at the stage in which the anterior half of the sperm nucleus is surrounded by egg cytoplasm in the fertilization cone (F). The sperm (s) and egg (e) parts of the zygote plasma membrane are continuous. At the apex of the nucleus, in the cone, persisting periacrosomal material (p) can be seen. The double-headed arrow lies in the perivitelline space. Magnification: $\times 30,000$. (From Colwin and Colwin, 1963b.)

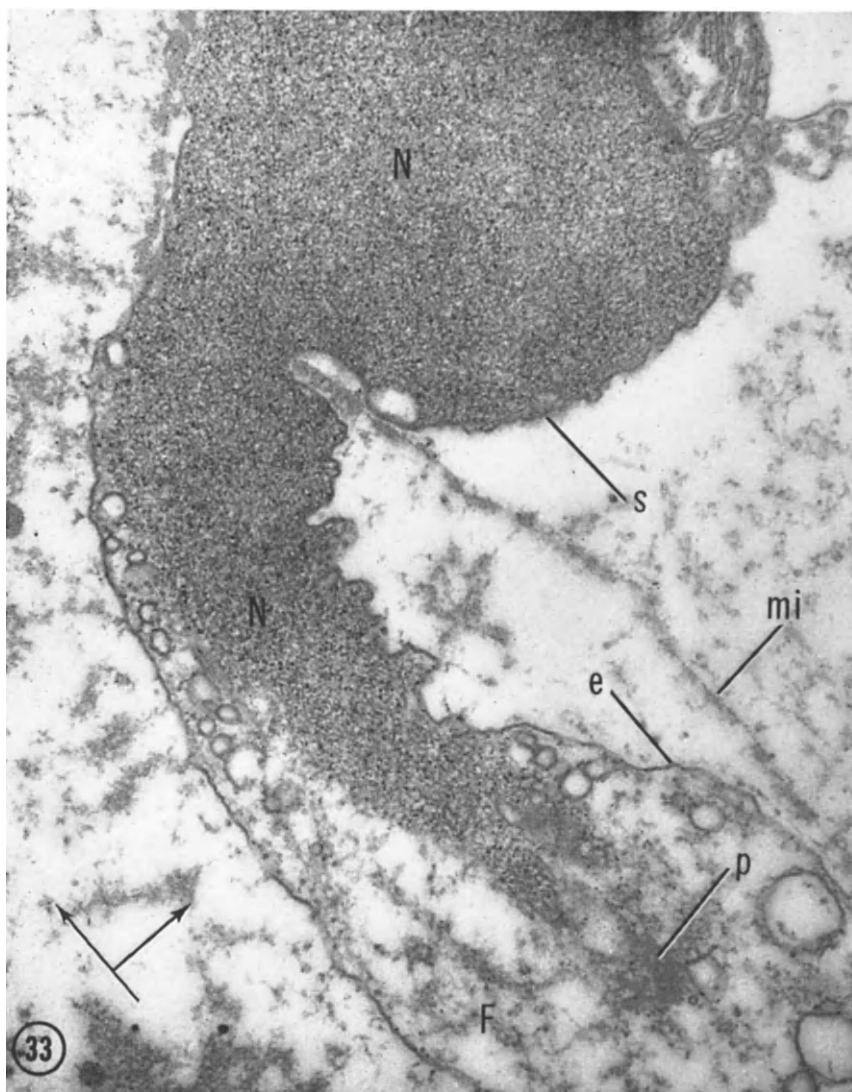


FIG. 33. *Saccoglossus*. Zygote at a stage later than in Fig. 32. Note the continuity of the zygote plasma membrane, which in one region (s) encloses the sperm nucleus and in another region (e) overlies the egg cytoplasm. The apical part of the nucleus mingles with the egg cytoplasm in the fertilization cone (F). Periacrosomal material (p) is still identifiable. The double-headed arrow lies in the perivitelline space. Magnification: $\times 53,000$. (From Colwin and Colwin, 1963b.)

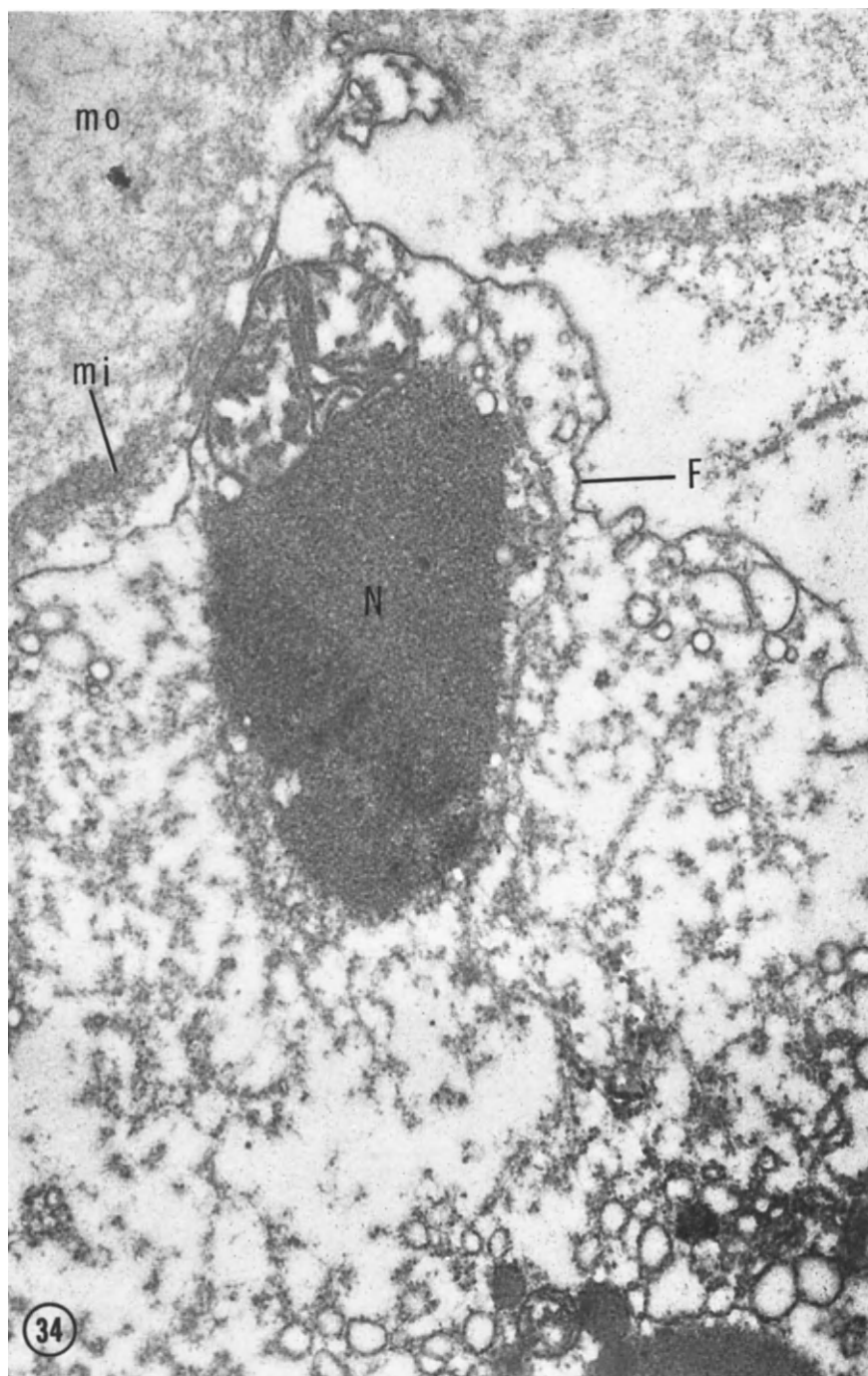


FIG. 34. *Saccoglossus*. Zygote showing the sperm nucleus and a mitochondrion in the cytoplasm of the fertilization cone (F). Magnification: $\times 32,000$.

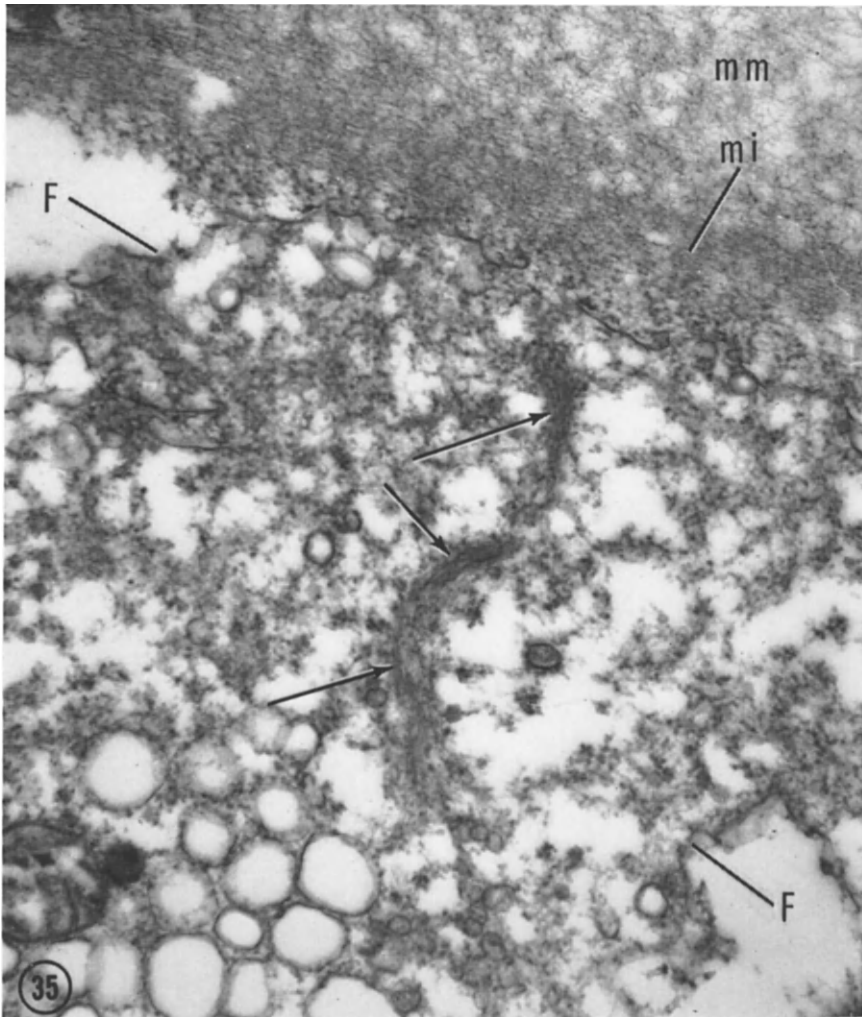


FIG. 35. *Saccoglossus*. Fertilization cone (F). The arrows point to curved segments of fibrous corelike material which, it is suggested, is the periacrosomal core that has entered the fertilization cone through the acrosomal tubule. Magnification: $\times 37,000$. (From Colwin and Colwin, 1963b.)

considerable thickness, and a well-defined hole has remained in this envelope after passage of the spermatozoon. Accordingly, it seems likely that the spermatozoon of *Urechis* possesses an acrosomal granule with lytic capabilities, and presumably this granule would lie within a membrane-bounded vesicle. It might also be supposed that acrosomal changes would result in tubule formation, since one of the specimens

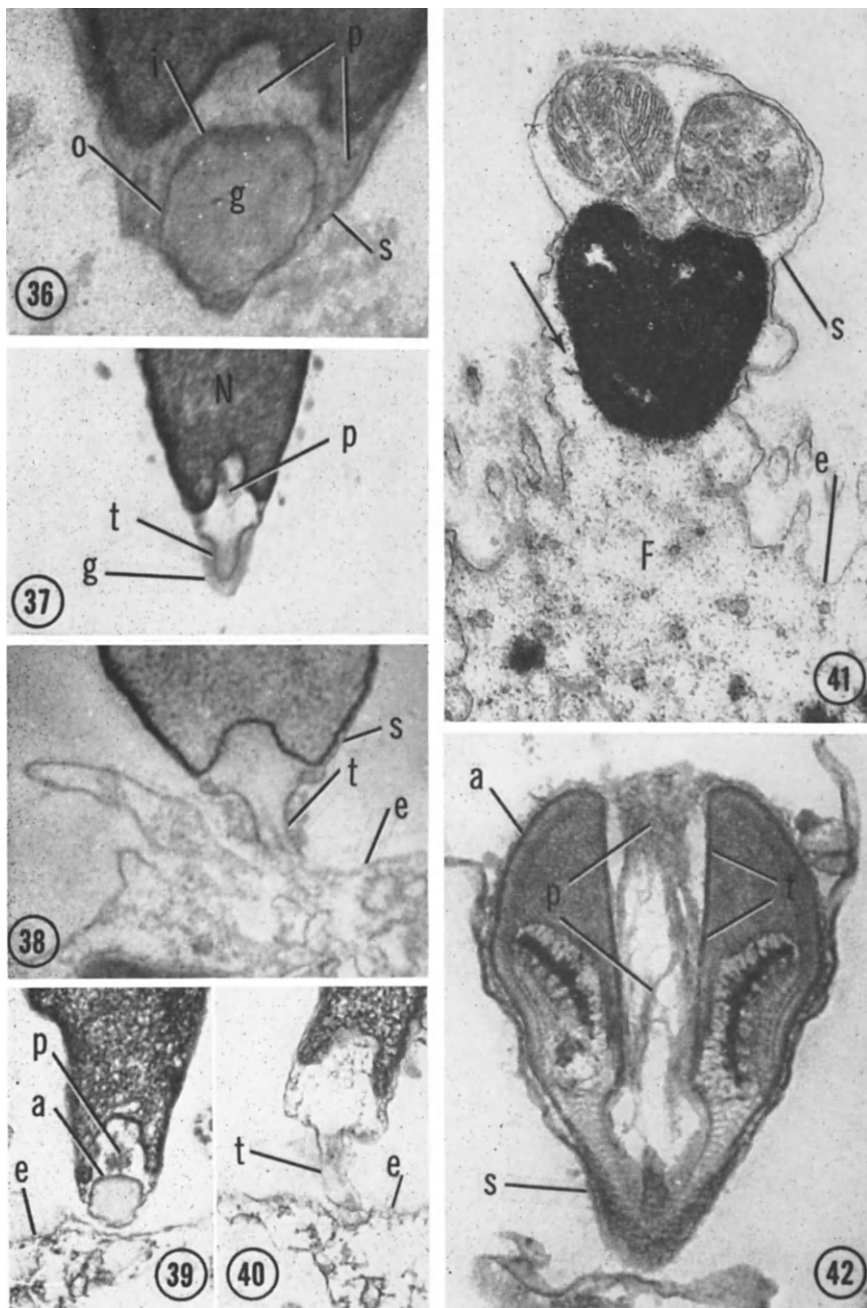
shows a funnel-like connection, the appearance of which suggests that it was originally an acrosomal tubule containing fibrous material or "core." In the same specimen it is evident that the sperm and egg plasma membranes are one continuous membrane, although the main body of the spermatozoon is still well outside the egg capsule. From the above evidence we conclude that the zygote plasma membrane is established by gamete membrane fusion. Thus in *Urechis*, as far as information is available, the events of sperm-egg association appear to follow the *Hydroides-Saccoglossus* pattern.

iii. *Arthropods*. Moses (1961) and Yasuzumi *et al.* (1961) described the developing acrosomal region for two species of crayfish, respectively. For each is described a membranous acrosomal vesicle deeply invaginated at its juxtannuclear end. No acrosomal granule is described, but in both species the vesicle contains dense material. From light microscopy, it has long been known that decapod spermatozoa "explode." Koltzoff (1906), Binford (1913), Fasten (1921), and especially Bloch (1933, 1935) have related the eversion of part of the "capsule" to fertilization. In the house cricket Kaye (1962) described the development of the acrosomal region. In our interpretation the region contains an acrosomal vesicle of which the membrane at the juxtannuclear end has the form of a deep and broad tubular indentation, while outside the acrosomal vesicle lies periacrosomal material, some of which extends into the indentation. In the aberrant spermatozoon of the tick (Reger, 1963) the structural homologies related to fertilization require clarification.

iv. *Ascidians*. Much remains to be learned about the fine structure of the spermatozoon and of changes related to the early events of fertilization. In *Ascidia nigra* (Ursprung, personal communication, 1964) the evidence clearly indicates that the zygote is established by fusion of the sperm and egg plasma membranes (Fig. 44).

v. *Conclusion*. To the limited extent that ultrastructural information is available, the events of fertilization in at least some representatives of these four phyla can be said to follow the *Hydroides-Saccoglossus* pattern.

d. *Mammals*. i. *Acrosomal region and changes* (Fig. 49E). The acrosome (also termed "head cap," "acrosome cap," or "galea capitis") of several species is a membrane-bounded vesicle. This has been established at the fine-structure level for the cat (Burgos and Fawcett, 1955), for man (Ånberg, 1957; Fawcett, 1958; Horstmann, 1961), for the rabbit (Hadek, 1963b; Bedford, 1964), for the bull (Saacke and Almquist, 1964), and for the boar (Nicander and Bane, 1962). Within this vesicle is electron-dense material which is or derives from the acrosomal granule.



FIGS. 36-42. From various authors; the labels and interpretations are not necessarily those of the original authors.

In vertebrates the juxtannuclear or inner portion of the acrosomal membrane is commonly called the inner membrane and the remaining or outer portion is called the outer membrane. It should be emphasized that these are not separate membranes, but simply different regions of one continuous membrane. In the above-mentioned mammals the inner or juxtannuclear part of this membrane closely surrounds the tapering apical part of the nucleus and, in effect, has the form of a large tubular invagination. Small amounts of what we should call periacrosomal material, in some species recognizable as a perforatorium (see Section III,C, p. 349), lie between this part of the acrosomal membrane and the nucleus.

Saacke and Almquist (1964) reported that in ejaculated bull sperm, centrifuged at 4°C to form loose pellets before fixation, specimens were found in which a large portion of the outer membrane is lost by vesiculation (Fig. 46), leaving only a posterior portion, the "equatorial segment." It seems not improbable, however, that more than the outer membrane is involved in this vesiculation. It may be recalled that *Hydroides* spermatozoa, even in the absence of eggs, occasionally undergo "spontaneous" dehiscence, shedding from the apex of the acrosomal region a lid which includes a certain amount of sperm plasma

FIGS. 36–38. Sea urchin *Arbacia punctulata*. (From Franklin, 1965, relabeled.)

FIG. 36. Unactivated acrosomal region; the acrosomal granule (g) is bounded by an acrosomal membrane (i) and (o); a relatively large amount of periacrosomal material (p) is present. Magnification: $\times 101,000$.

FIG. 37. Activated acrosomal region shows an acrosomal tubule whose cavity is continuous with the periacrosomal cavity; within the continuous cavity lies the periacrosomal material (p), some of which is fibrous. Magnification: $\times 34,500$.

FIG. 38. Early zygote showing continuity of former sperm (s, t) and egg (e) plasma membranes to form the zygote plasma membrane. Magnification: $\times 49,500$.

FIGS. 39–40. Sea urchin *Paracentrotus lividus*. (From Pasteels, 1965, labels added.)

FIG. 39. An intact acrosomal region within which lies periacrosomal material (p) and an acrosomal granule which is bounded by the acrosomal membrane (a).

FIG. 40. An activated acrosomal region; note the acrosomal tubule (t) which appears very close to the egg plasma membrane (e).

FIG. 41. A young zygote of the bivalve *Barnea candida*. Note the continuity of the former sperm (s) and egg (e) plasma membranes to form the continuous zygote plasma membrane. At the arrow on the left note that the egg cytoplasm from the fertilization cone (F) has intruded between the former sperm plasma membrane and the sperm nucleus. (From Pasteels, 1965; labels added.)

FIG. 42. An unactivated acrosomal region of the annelid *Sabellaria*. Note that the inner portion of the acrosomal membrane (a) is deeply indented in the form of a tubule (t). Considerable periacrosomal material (p) lies within this tubule. (Pasteels, personal communication, 1964; labels added.)

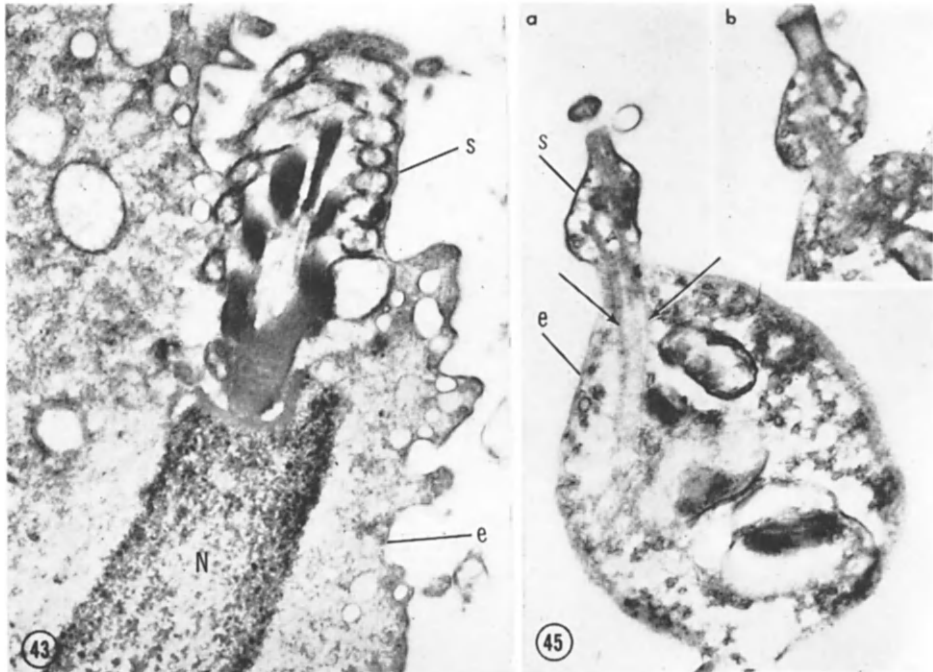


FIG. 43. Early zygote of the rat, *Rattus rattus*, relabeled. Note the continuity of the former sperm plasma membrane (s) with the former egg plasma membrane (e) to form the zygote plasma membrane. The internal sperm organelles (nucleus, etc.) which have entered the egg cytoplasm are divested of their plasma membrane. (From Szollosi and Ris, 1961.)

FIG. 44. Early zygote of the ascidian *Ascidia nigra*, labels added. The profile appears to be that of the two original gametes, but note that the sperm plasma membrane (s) is continuous with the egg plasma membrane (e), thus forming the zygote plasma membrane. (From Ursprung, personal communication, 1964.)

FIG. 45. a and b. Two successive sections of a young zygote of the alga *Prasiola stipitata*, labels added. The plasma membrane of the former male gamete (s) is continuous with the plasma membrane of the former female gamete (e). At the arrow tips the flagellar fibers of the male gamete, divested of their plasma membrane, lie in the cytoplasm of the female gamete. (From Manton and Friedmann, 1960.)

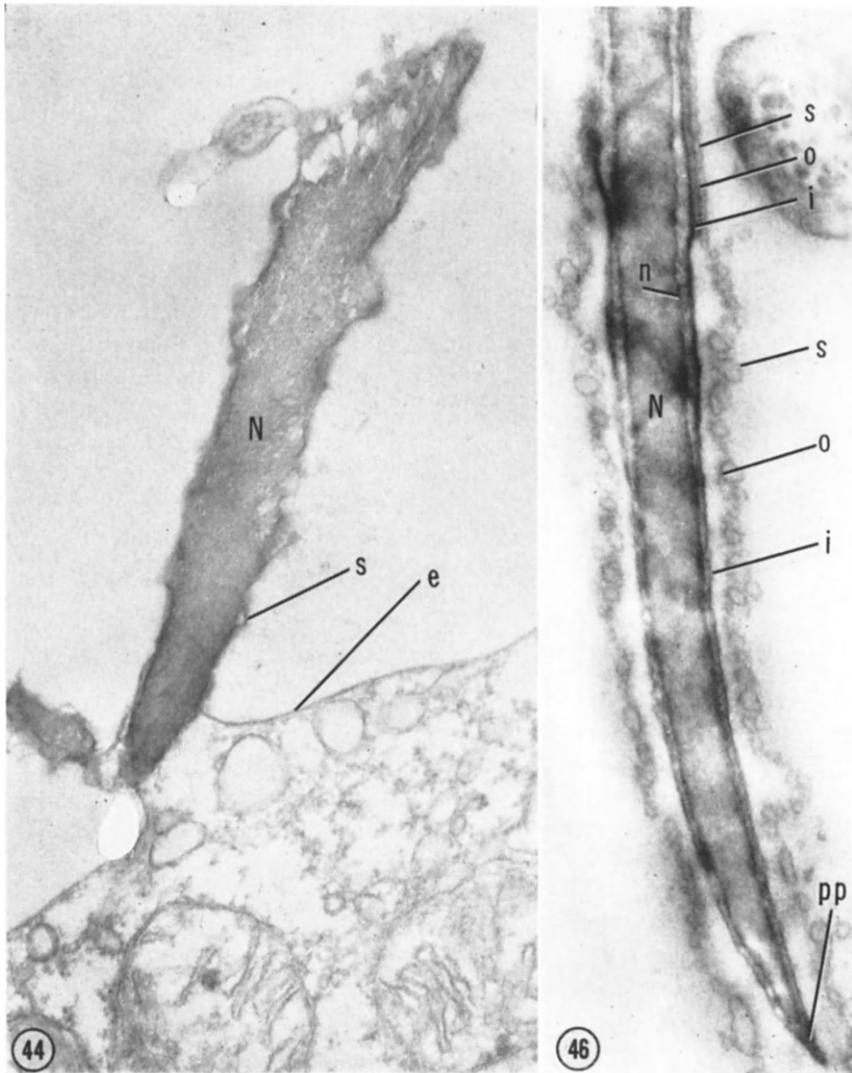


FIG. 46. Head of bull spermatozoon from semen. It is suggested that the vesicles around the sperm tip are composed of outer acrosomal membrane (o) together with the overlying sperm plasma membrane (s). At the level where vesiculation ceases, it is suggested, acrosomal and plasma membranes fuse to form a continuous membrane. Thus after vesiculation the sperm apex is covered by the inner acrosomal membrane (i). It is probable that a perforatorium (pp) lies immediately between the nuclear envelope (n) and the apex of the inner acrosomal membrane (i). Note that the inner membrane (i) has the form of a tubule; it is suggested that this is the equivalent of the acrosomal tubule. (cf. Fig. 49E, bb.) (From Saacke and Almquist, 1964; relabeled.)

membrane and acrosomal membrane plus a small apical vesicle. We suggested (Colwin and Colwin, 1965) that spontaneous dehiscence occurs also in bull sperm, and since dehiscence involves some loss of plasma membrane as well as acrosomal membrane from the apex, we suggested that in the bull too there is loss of plasma membrane as well as acrosomal membrane. Hence, the vesicles which Saacke and Almquist (1964) observed (Fig. 46) might consist not solely of outer membrane but of outer membrane together with the overlying plasma membrane (Fig. 49E, bb). The site at which vesiculation ceases would be the orifice of dehiscence, and at this site the remaining or inner part of the acrosomal membrane would be fused with the unshed part of the plasma membrane to form a continuous (mosaically constituted) membrane. As a consequence of dehiscence, then, the sperm apex would be closer to the apical part of the sperm nucleus, and the newly exposed inner or juxtannuclear part of the acrosomal membrane would be the apical part of the sperm plasma membrane. This membrane would be the equivalent of an acrosomal tubule and might be expected to be the part of the sperm plasma membrane to fuse with the egg plasma membrane at fertilization.

In another study with semen from normal bulls, Blom (1963) observed numerous spermatozoa from which the galea capitis had "disappeared." Numerous specimens of the galea capitis which were free in this semen were shaped like "bathing caps." Blom presented a theory for the possible function of the galea capitis in the fertilization process. He speculated that when the spermatozoon has traveled through the female genital tract and has approached close to the egg, the sperm plasma membrane is dissolved or broken and the galea is loosened and breaks off, thus "the sharp edge of the nucleus is free and is ready to penetrate the ovum." Our interpretation (Colwin and Colwin, 1965) is that the disappearance of the galea represents a loss of the outer membrane of the acrosome and not a loss of the entire acrosome. In this we are in agreement with Saacke and Almquist (1964); but we have suggested, in addition, that the plasma membrane overlying this outer membrane is also shed (Fig. 49E, b). We consider that the "bathing cap" would have an inner and outer membrane structure composed not of the inner and outer membranes of the galea, as Blom (1963) proposed, but of the outer membrane of the galea plus that part of the plasma membrane which overlies it in the intact spermatozoon. The "sharp front edge of the nucleus" to which Blom referred (and which resembles the apical body or perforatorium of the rabbit) would be covered by the unshed inner membrane, and where the "bathing cap" detaches, that is, at the rim of dehiscence, this membrane would join and be continuous

with the plasma membrane in which it would constitute a mosaically inserted portion. Some part of this plasma membrane would have to fuse with the egg plasma membrane before the nucleus could penetrate the ovum.

These suggestions as to bull spermatozoa may also be made regarding rabbit spermatozoa. Austin (1963) in electron microscopic studies on the rabbit found that the penetrating spermatozoon does not lose its entire acrosome, as earlier studies with light microscopy in a number of species had seemed to indicate (Austin and Bishop, 1958a,b), but that instead the inner membrane remains about the anterior part of the sperm nucleus. Bedford (1964) found occasional ejaculate and uterine spermatozoa which were evidently losing the outer membrane while retaining the inner one plus the apical cone (i.e., perforatorium; see Section III,C, p. 349). We suggest that the overlying anterior part of the sperm plasma membrane is shed together with the outer (part of the acrosomal) membrane and that the inner (part of the acrosomal) membrane fuses with the remaining posterior part of the sperm plasma membrane to form a continuum around a rim of dehiscence. We suggest that this is the arrangement of membranes on the tips of the zona-penetrating spermatozoa also observed in the rabbit by Moricard (1960) and Hadek (1963a,b) by electron microscopy, in the rabbit and pig by Dickmann (1964) and Dickmann and Dziuk (1964), who studied living material, as well as in the hamster, which Yanagimachi and Chang (1964) described from living material. In every case the changes can be considered to be counterparts of the changes which occur during dehiscence and eversion in *Hydroides* and *Saccoglossus*.

Our interpretations of the evidence available from the aforesaid mammals find further support in observations by Pikó and Tyler (1964a) regarding the rat. According to their report, in spermatozoa which have penetrated the zona, "The acrosome is open . . . and its contents are missing." Also "...the outer acrosomal membrane is...joined to the plasma membrane over a considerable distance and a vesiculation of the two membranes is observed in the region of junction." And "the exposed inner acrosome membrane and the sperm's plasma membrane . . . form a continuous sheet." These observations strikingly resemble those reported earlier for *Hydroides* and *Saccoglossus* (Colwin and Colwin, 1960a, 1961b,c, 1963a,b) and strengthen our view that the *Hydroides-Saccoglossus* pattern obtains also in mammals.

In the rat and certain other rodents a slit is left in the zona as the spermatozoon passes through this envelope. Austin (1951) and Austin and Bishop (1958b), who demonstrated the slit, suggested that this hole might be caused by a lysin coming from the spermatozoon. We suggest

that the acrosome would be a likely source of the putative zona lysin and that this lysin would be released at dehiscence.

ii. *Zygote formation.* Thus far the rat is the only mammal in which anything has been reported regarding the ultrastructure of this phase of fertilization. The first report was by Szollosi and Ris (1961). They demonstrated that the plasma membrane of the spermatozoon fuses with the egg plasma membrane to form a single continuous zygote plasma membrane (Fig. 43). More recently Pikó and Tyler (1964b) similarly reported that in the early zygote of the rat the plasma membrane is mosaically composed of the gamete plasma membranes. We interpret this evidence from two separate investigations to indicate that in this mammal the zygote membrane is established in the same way as in the *Hydroides-Saccoglossus* pattern.

iii. *Conclusions regarding sperm-egg association in mammals.* From our interpretation of the available evidence the over-all plan of sperm-egg association in mammals appears to be the same as in the *Hydroides-Saccoglossus* pattern (cf. Fig. 49A and B with Fig. 49E). The basic structure of the acrosomal region is the same and the membranes seem to behave in the same way. Dehiscence occurs and the acrosomal vesicle opens, releasing its contents. It seems likely that a zona (envelope) lysin is among these contents. Although mammalian spermatozoa shed relatively more extensive amounts of membrane than do spermatozoa of *Hydroides* or *Saccoglossus*, nevertheless these portions similarly are lost from a strictly circumscribed apical area delimited by a rim of dehiscence; similarly, too, this rim is the site at which the unshed part of the acrosomal membrane is in continuity with, and inserted as a mosaic part in, the remaining unshed part of the sperm plasma membrane. Then later, the sperm plasma membrane fuses with the egg plasma membrane to establish the zygote membrane, which consequently is also a mosaic plasma membrane.

The membrane at the sperm apex at the time of zygote formation is the newly exposed inner or juxtannuclear part of the acrosomal membrane and is the counterpart of an acrosomal tubule. We suggest, therefore, that it is this part of the membrane which makes contact and fuses with the egg plasma membrane.

In our view a spermatozoon which becomes activated, as shown, for example, by apical dehiscence, in situations other than in contact with the egg envelope would lose its ability to fertilize an egg. Such a spermatozoon would no longer possess the machinery for penetrating the egg envelopes, and so the plasma membranes of the gametes could never meet and consequently could never fuse. Perhaps, among other reasons, it is because these changes occur to so many spermatozoa while in the

genital tract of at least some mammals that so many spermatozoa are "needed" for successful fertilization in these mammals. This is not to suggest that other changes as well, for example, those constituting "capacitation" (Austin, 1951; Chang, 1951) may not be required for the ultimate "success" of a spermatozoon.

e. Other Vertebrates. The light microscope study by Kille (1960), substantiating observations and a suggestion of Ballowitz (1905), indicated that in the cyclostome *Lampetra* a "very fine filament" is projected from the tip of the sperm head and becomes the first sperm part to come into contact with the egg. The "filament," according to Kille, "plays an essential part in getting the spermatozoon into the egg cytoplasm." In making this passage the spermatozoon crosses a plug of nonmembranous material outside the egg proper. Tentatively the "filament" may be interpreted as an acrosomal tubule which would play the same role as in the *Hydroides-Saccoglossus* pattern. In an electron microscope study of whole mounted spermatozoa of two species of sturgeon (Acipenseridae), Dettlaff and Ginsburg (1963) reported that a long and sometimes branching acrosome "filament" is formed when the spermatozoon is activated. Presumably this filament too in thin sections will prove to be tubular.

Ultrastructural studies of spermatogenesis in the toad (Burgos and Fawcett, 1956) and in the chicken (Nagano, 1962) have shown that a membrane-bounded acrosomal vesicle forms which contains a dense acrosomal granule. When this vesicle transforms into a head cap, the inner or juxtannuclear part of the acrosomal membrane becomes deeply indented and may be considered to have the shape of a tubular invagination. Within this invagination lies the perforatorium, which, in our terminology, consists of periacrosomal material.

This limited information indicates that the pattern in these other vertebrates is also in harmony with the *Hydroides-Saccoglossus* pattern.

3. Groups without Acrosome

The acrosome is reported to be absent in certain species, for example, in isopods (Reger, 1964) and in teleost fishes. Moreover, algae which liberate naked gametes and ciliated protozoa which undergo conjugation are acrosomeless. Since in species with an acrosome it is the acrosome which enables the plasma membrane of the male gamete to come into contact with that of the female gamete (see Section III,E, p. 352), it should follow that in species without an acrosome there might be some other machinery pertaining to one or both of the gametes which would still enable their respective plasma membranes to make contact.

a. *Teleosts*. Mainly through light microscope studies, but with some electron microscopy, as, for example, that of Porte and Follenius (1960) and of Ginsburg (1963), it has been reported that no acrosome occurs in the spermatozoon in a number of teleosts (briefly reviewed by Ginsburg). In at least some of these the egg capsule or chorion is penetrated by one or more minute "holes," or micropyles, whose terminal diameter is about that of the spermatozoon. This relationship is shown in Fig. 48, from Ginsburg (1963). It has been suggested (Colwin and Colwin, 1964) that such a micropyle might permit the sperm plasma

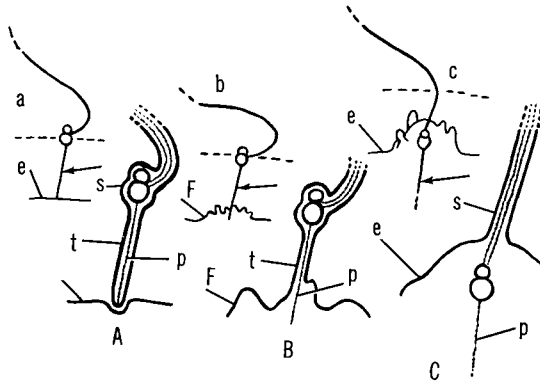


FIG. 47. A reinterpretation of sperm-egg association in *Holothuria atra*. (a-c): copies of the diagrams used by Colwin and Colwin (1955) to represent observations of "sperm entry" in living specimens of *H. atra*. (A-C): reinterpretations which, it is suggested, an electron microscopic study would support. In (a)-(c) the arrow tips point to the "acrosome filaments." At the level of electron microscopy, it is suggested, (a) would appear as in (A), that is, the "acrosome filament" would really be the periacrosomal core within an acrosomal tubule; (b) would appear as in (B), that is, it is already a young zygote as a result of fusion of the sperm and egg plasma membranes, and the tip of the periacrosomal core would be in the cytoplasm of the fertilization cone (F); and (c) would appear as in (C), that is, the internal sperm organelles divested of their plasma membrane would lie free in the egg cytoplasm.

membrane to have direct access to the egg plasma membrane, and that in this circumstance the functional necessity of an acrosome as a prelude to gamete membrane fusion would be eliminated. In a specimen such as the one represented in Fig. 48 one might speculate, too, on the basis of the *Hydroides-Saccoglossus* pattern, that the plasma membranes of the egg and the fertilizing sperm cell had already fused to become a continuum, and that it was the sperm nucleus, rather than sperm cell, which had begun to intermingle with egg cytoplasm.

b. *Algae*. In the green alga *Prasiola*, Manton and Friedmann (1960) showed that the sexual plants liberate gametes which are naked proto-

plasts bounded only by their own plasma membranes. At the onset of syngamy the tip of one flagellum of the male gamete meets the egg, and in this region the plasma membranes of the two gametes coalesce to become a continuum, the zygote plasma membrane. Thin sections show that this membrane extends as an unbroken line around the zygote. As the detailed description and electron micrographs (Fig. 45) indicate,

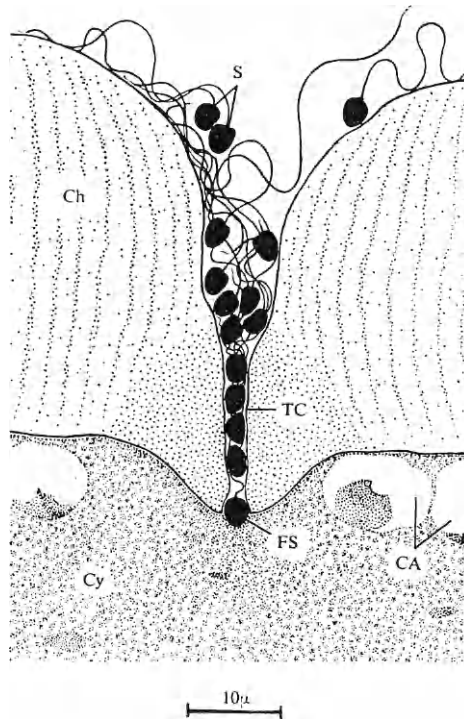


FIG. 48. A schematic representation of a section of a trout egg after insemination. (CA) Cortical alveoli. (Ch) Chorion. (Cy) Cytoplasm. (FS) Fertilizing spermatozoon. (S) Spermatozoa. (TC) Terminal canal of micropyle. (From Ginsburg, 1963; original labels.)

the entire flagellum does not become submerged or engulfed in the egg cytoplasm; instead, it is merely the flagellar axis, minus its plasma membrane, which enters the egg cytoplasm. All other internal organelles of both gametes also commingle once the zygote membrane is established by the gamete membrane fusion.

c. Ciliates. In electron micrographs of *Tetrahymena* (Elliott and Tremor, 1958), the plasma membranes of a pair of conjugating cells may be interpreted as being fused into a single continuum. Schneider (1960, 1963) and André and Vivier (1962) studied such fusion more extensively

in *Paramecium*. In each case there is a particular and limited area within which the plasma membranes of the two cells fuse at several sites, and at these sites the internal cytoplasm and organelles can intermingle between the two cells.

d. Conclusion. Although in these groups the gamete plasma membranes are brought into contact by preliminary steps which lack the machinery of an acrosomal region, nevertheless the zygote is still initiated by means of gamete membrane fusion. In this respect these acrosomeless groups exhibit the *Hydroides-Saccoglossus* pattern.

4. General Conclusions Regarding Comparative Aspects of Gametic Association. In echinoderms, mollusks, and vertebrates, considerable ultrastructural evidence is available as to the early events of fertilization, and all of it reveals a remarkable similarity to the pattern found in an annelid and a hemichord (*Hydroides* and *Saccoglossus*). Moreover, the same pattern is again indicated by the scantier evidence available from several other phyla. It is striking that the evidence from so many phyla points to the same pattern. In view of these considerations, and in the absence of evidence to the contrary, we conclude that the pattern of acrosomal preliminaries followed by gamete membrane fusion which was first described for *Hydroides* and *Saccoglossus* is a fundamental pattern. In addition, in view of the evidence as to fertilization in acrosomeless species, we further conclude that the acrosomal preliminaries are an ancillary process and that the most fundamental step in this pattern of fertilization is the initiation of the zygote by means of gamete membrane fusion.

III. Discussion

A. MANNER OF ZYGOTE FORMATION

The zoological literature antedating electron microscopy contains the notion that when the egg proper is reached the sperm cell or, in some cases the sperm cell minus its flagellum, "penetrates" or "enters" the egg or is "engulfed" or "swallowed" by the egg. Loeb (1917), for example, examined the concept of phagocytosis as a possible mechanism in fertilization. This notion is implicit in the terminology of older textbooks, and indeed it is not uncommon even today to find the sperm cell described as being "captured" by the egg or illustrated as being taken in by means of phagocytosis, much as a smaller protozoan might be engulfed by an amoeba, or a particle by a phagocyte. The concept was formalized by Tyler (1959, 1960, 1962) in a hypothesis proposing "Specific Pinocytosis," or phagocytosis, "as a possible Sperm-Engulfing Process." In

short, it has been assumed that the sperm cell enters bodily into the egg cell. It follows from this assumption that the spermatozoon as a whole unit with its plasma membrane intact, would be completely surrounded by a vesicle formed of egg plasma membrane and would be transported into the egg cytoplasm in that condition.

The results from electron microscopy, reviewed in Section II, do not support this assumption. The evidence from *Hydroides* and *Saccoglossus*, as well as from a number of other forms ranging from the rat (Szollosi and Ris, 1961) to an alga (Manton and Friedmann, 1960), demonstrates that the sperm and egg plasma membranes initially fuse to become the continuous mosaic plasma membrane of the zygote, and that the nucleus and other internal sperm components subsequently enter the egg cytoplasm divested of their plasma membrane. The ultimate fate of the sperm membrane is still unknown, but this is really irrelevant to the manner in which the zygote is established. Since the intact sperm cell does not bodily enter the egg cytoplasm, the concept of engulfment, or phagocytosis, would seem to be untenable. However, Pikó and Tyler (1964b) still favor this concept, although they too now regard the egg and sperm plasma membranes of the rat as fusing to form a mosaic.

Other evidence suggests that if a cell with its plasma membrane intact should come to lie within an egg it could not initiate the egg's development or contribute its nucleus to subsequent activities of the egg. Hiramoto (1962), using several species of sea urchin, injected intact sperm cells into eggs and found that these spermatozoa never activated the eggs even though, subsequently, these same eggs were fertilizable after normal insemination. He reported that the injected spermatozoa continued to retain their plasma membranes after being surrounded by the egg cytoplasm. Apparently, then, a sperm cell undivested of its plasma membrane does not activate an egg. In other pertinent experiments Briggs and King (1953), during their early nuclear transplantation work, found that intact blastula cells implanted into enucleated frog eggs did not cause the eggs to develop. Only if the plasma membrane of the injected blastula cell were ruptured to expose the nucleus would the egg be activated. Apparently, then, a cell must be divested of its plasma membrane if its nucleus is to cause the egg to develop.

Another aspect of the manner in which the zygote is formed concerns the microvilli of the egg. Tyler (1962) postulated an important role for these structures. There is no evidence, however, that the microvilli of *Hydroides*, which do indeed project into the egg capsule, play any particular role in fertilization. In *Saccoglossus* the microvilli do not project into the egg capsule at any time during fertilization and

consequently could never play a role in this process. This evidence suggests that microvilli may be unessential to fertilization in other species as well.

(For further discussion of the manner of zygote formation, see Colwin and Colwin, 1963b, 1964.)

B. SOME IMPLICATIONS OF GAMETIC CONTACT DURING FERTILIZATION

It seems probable, although it is by no means certain, that the initial contact resulting in adhesion between a spermatozoon and an egg envelope is of a molecular binding type. Whether such adhesion is the result of a fertilizin-antifertilizin reaction, a concept postulated by Lillie (1919) and extensively developed by Tyler (1949) and his associates, still remains to be established. However, in view of present knowledge about the details of the events at the beginning of fertilization in *Hydroides*, *Saccoglossus*, and a number of other species, it seems improbable that all of the events of fertilization from the initial adhesion of the spermatozoon to the establishment of the zygote would be mediated by the one fertilizin-antifertilizin interaction postulated by Tyler (1959, 1960, 1961, 1962, 1963). More probably several specific biochemical interactions would be involved (Colwin and Colwin, 1963b, 1964).

The initial contact and adhesion of the two gametes in the hemichordate, *Saccoglossus*, and indeed between the gametes of a great many animal species, is between two structurally dissimilar surfaces. On the one hand it is the sperm plasma membrane and on the other hand it is the egg capsule, and the latter is nonmembranous in the fine-structure sense. In some ways this coming together of membranous and non-membranous surfaces resembles the highly specific initial contact and adhesion of phage and bacterial wall (reviewed by Tolmach, 1957), and perhaps similarly this gametic contact may involve a highly specific molecular adhesion. However, the final zygote-establishing contact of the two gametes is between two other surfaces, surfaces which have been newly exposed: one surface is that of the acrosomal tubule, which is now the apical part of the sperm plasma membrane, but which formerly bounded and faced into the cavity of the acrosome; the other surface is that of the egg plasma membrane, which originally was covered by the egg capsule. It seems likely that for this contact too, as for the initial one, some degree of chemical specificity would be involved at the surfaces, but it seems very unlikely that the same molecular specificities (e.g., fertilizin-antifertilizin) would be involved in two contact situations which are so dissimilar. In this connection it is significant to note what may be a comparable set of situations: whereas a certain phage will

adhere to the cell wall of the intact *Bacillus megaterium*, it will not adhere to the naked protoplast (Tolmach, 1957). These two surfaces, one a cell wall and the other a plasma membrane, present different contact situations in which the phage reacts in quite different ways. There are valid reasons for supposing, then, that the initial and final contacts of the gametes would be biochemically different.

In an evolutionary sense one might expect that the initial contact between spermatozoon and egg would be the most highly specific event of association, since failure of the two gametes to adhere at this juncture would abolish any further relationship between them. A requirement of a highly specific initial contact between the gametes would minimize or completely prevent interspecific crosses. Perhaps the final contact between the membranes of both gametes, however, need not be so highly specific, since interspecific crossing would already have been guarded against. This type of conjecture lends itself to experimental exploitation, since it is possible to study this aspect of fertilization by specific and interspecific crosses of intact and denuded eggs. Spermatozoa of *Saccoglossus* can unite with denuded eggs and indeed with denuded blastomeres (Colwin and Colwin, 1957a).

C. PERFORATORIUM AND PERIACROSOMAL MATERIAL

It has recently become clear that the perforatorium and the acrosome are different structures and that both lie within the acrosomal region. But in the older literature, for example in papers of Lillie (1912a,b) or Mèves (1915), and even in contemporary studies which are based entirely on light microscopy, the word "perforatorium" cannot always be taken to imply fine discrimination between the perforatorium or acrosome or acrosomal region.

On the basis of recent, especially electron microscopic, studies the perforatorium should be regarded as part of the periacrosomal material, that is, as material lying within the extraacrosomal part of the acrosomal region (Colwin and Colwin, 1963a, 1964). The amount of periacrosomal material differs from species to species, and its distribution differs, too. So far as is known, however, some of this material always lies between the nuclear envelope and the juxtannuclear end of the acrosomal membrane, and this is the same location in which the perforatorium is found. The perforatorium is generally a rodlike, and perhaps fibrous, structure, variously modified and closely associated with, although distinctly separate from, the acrosome.

In mammalian literature the term "apical body," as employed, for example, by Bedford (1964) and Hadek (1963b), has been used to designate material which, from its position, appears to be homologous

with the perforatorium. However, "apical body" has also been used to designate an entirely different and unrelated structure, namely, an enlarged part of the apex of the acrosome, as employed, for example, by Blom (1963) and Saacke and Almquist (1964). In the present chapter the term "perforatorium" is used in place of "apical body," but only to indicate the apical body in the sense used by Bedford and by Hadek.

The perforatorium (Fig. 49E) has been reported in a number of vertebrate groups. In the toad it is a dense fibrous and hook-shaped structure lying between the nuclear envelope and the juxtanuclear part of the membrane of the head cap or acrosome (Burgos and Fawcett, 1955). In the chicken Nagano (1962) interprets as a perforatorium a dense elongate, and perhaps fibrous, structure extending from a cave in the nuclear envelope into an invagination of the juxtanuclear part of the acrosomal membrane. A perforatorium has been described for a number of mammals including the rat (Clermont *et al.*, 1955; Austin and Bishop, 1958a,b). In thin section studies of rabbit spermatozoa (Hadek, 1963a,b; Bedford, 1964), both before and after sperm activation, material can be seen in the position homologous to that of a perforatorium, at the apex. There is probably such a "perforatorium" also in the boar (Nicander and Bane, 1962) as well as in the bull, ram, dog, and guinea pig (Bane and Nicander, 1963). Even in spermatids of the cat and of man in which Burgos and Fawcett (1955) and Fawcett (1958) have shown that a fibrous perforatorium does not occur, it can nevertheless be seen in thin sections (Fawcett, 1958; Horstmann, 1961) that the homologous sites contain at least small amounts of nonacrosomal substance. In vertebrates, then, an extraacrosomal body or extraacrosomal material occupies a position between the acrosome and the nuclear envelope and, in our terminology, in activated spermatozoa it would occupy the lumen of the acrosomal tubule. Dickmann (1964) in living material of the rabbit described a "hair-like structure" which protrudes from the front end of the sperm head into the zona of the egg, and Dickmann and Dziuk (1964) described a "sperm penetration filament" in the live pig zona. We suggest that these may be equivalents of the perforatorium covered by the inner acrosomal membrane.

Among invertebrates there are also structures which might be considered to be perforatoria (cf. Fig. 49B-D with Fig. 49E). For example, in several mollusks (Dan, 1956; Galtsoff and Philpott, 1960; Pasteels and de Harven, 1962) and in the annelid *Sabellaria* (Fig. 42) (Pasteels, personal communication, 1964) the adnuclear part of the acrosomal membrane appears as a tubular invagination. Extending into this axial invagination is a rodlike arrangement of fibrous periacrosomal material which could be the counterpart of a perforatorium. Again, in the

house cricket dense periacrosomal material which occupies a deep indentation of the acrosomal vesicle has been considered (Kaye, 1962) to be a possible homologue of the perforatorium of the toad (Burgos and Fawcett, 1955).

In other invertebrates no perforatorium as such is found before sperm activation, but what seems to be an equivalent structure is found following sperm activation. Thus in activated spermatozoa of *Saccoglossus* (Colwin and Colwin, 1963a) a long fibrous "core" extends the length of the acrosomal tubule, and in sea urchins (Afzelius, 1956a; Dan *et al.*, 1962; Franklin, 1965), and in our opinion in starfish (Dan, 1960; Dan *et al.*, 1962) too, there is a fibrous shaft within an acrosomal tubule. In these forms the core or fibrous shaft which is found after sperm activation is longer than the entire acrosomal region before activation and appears to arise from periacrosomal material in the juxtannuclear area.

It is clear, then, that in some invertebrate species, as in some vertebrates, fiberlike periacrosomal material is present before activation, whereas in other invertebrates a fibrous structure is produced only as a consequence of activation. In either case the structure might be considered to be a perforatorium (Colwin and Colwin, 1963a). One wonders whether those vertebrate spermatozoa which initially lack a fibrous perforatorium, as in the cat and man, also form such a fibrous structure following activation. A possible function of perforatorium or core may now be considered.

D. PERFORATORIUM OR CORE AND INWARD MOVEMENT OF SPERM ORGANELLES

It is not known how the internal sperm organelles such as the nucleus or mitochondria are moved inward once the zygote plasma membrane has been established. The old idea that a filament between sperm cell and egg would contract and in this way draw the two cells together (Tyler, 1948; Monroy, 1948) has been untenable for some time (Colwin and Colwin, 1955). Moreover, the notion that contraction or recession of the fertilization cone would move the spermatozoon inward is also untenable; even by light microscopy the sperm structures can be seen to move inward at the same time that cytoplasm in the cone is moving outward (Colwin and Colwin, 1955, 1956). Now electron microscopy of sperm-egg association suggests a new line of speculation. Since the core or perforatorium is contained within the sperm plasma membrane (i.e., within the cavity of the acrosomal tubule), and since the fusion of this membrane with the egg plasma membrane at fertilization brings the contained structure into direct contact with the egg cytoplasm, perhaps the core (Colwin and Colwin, 1963b) or the perforatorium subsequently

draws or facilitates the drawing of the remaining internal sperm organelles more deeply into the egg cytoplasm. The actual function of either perforatorium or core has not yet been established. For another possible function of the core, see the following section.

E. ACROSOMAL REGION AND ITS RELATION TO EGG ACTIVATION

Although the literature dealing with the development and structure of the acrosomal region is large in volume, only a few of the studies have dealt with the ultrastructure of the region and fewer still with the behavior and function of this region in fertilization. In this latter connection there is pressing need for extensive investigation.

It has been a recurrent view that the acrosome might activate the egg. Because of its origin from the Golgi apparatus, Bowen (1924) suggested that the acrosome be regarded as a secretory product perhaps composed of enzymelike material. He speculated that the function of the acrosome might relate to release of this product from the sperm cell into the egg cytoplasm and that the acrosome might activate the egg. As to how the spermatozoon becomes associated with the egg, Bowen simply stated that the spermatozoon appeared to be "swallowed" by the egg. But since the acrosomal region and its changes in relation to sperm-egg association are now known in ultrastructural detail for *Hydroides* and *Saccoglossus*, and since the available information from other phyla indicates that other species with an acrosome have essentially the same ultrastructural pattern, these views of Bowen are no longer tenable.

The spermatozoon is not "swallowed": it does not even reach the egg plasma membrane with its acrosome intact. Instead, the acrosome goes into action, everts, and empties its entire contents outside the sperm cell before the egg plasma membrane is met (Fig. 49). In performing this action the acrosome as such disappears. Very probably the content of the acrosome, or much of the content, is expended in lytically removing the local portion of the egg capsule that serves as a barrier between the egg and sperm plasma membranes. Thus the role of the acrosome is that it provides the machinery which enables the plasma membrane of the male gamete to meet that of the female gamete (Colwin and Colwin, 1961b, 1963a). It is possible, too, that some of the acrosomal content might help to mediate the fusion of these membranes after they meet. In any case, since these membranes ultimately fuse to become one continuous zygote plasma membrane, the intact sperm cell does not enter the egg but, rather, releases its remaining contents to join the similarly released egg contents within the now common membrane. The sperm content which first comes into contact with the egg cytoplasm is periacrosomal material from within the acrosomal

tubule and not acrosomal material from within the acrosome. Consequently, if any substances in the acrosomal region are responsible for egg activation after being introduced into the egg (Bowen, 1924; Monroy, 1948; Runnstrom, 1949), they should be sought in the periacrosomal and not the acrosomal material. Certainly as soon as the membrane of the acrosomal tubule fuses with the egg plasma membrane the egg is activated. Therefore, it does seem possible that periacrosomal material might activate the egg.

There is no evidence as to which part, if any, of the periacrosomal material might be involved in egg activation. In *Saccoglossus* there is a fibrous core, together with other materials, and perhaps one or another of these activates the egg by chemical or mechanical means (Colwin and Colwin, 1963b, 1964). Fibrous cores and presumably other materials as well are present in the acrosomal tubules of a number of phyla. For another possible function of the core see the preceding Section (D).

F. THE MECHANISM OF MEMBRANE FUSION

The mechanism which underlies membrane fusion has yet to be explained. The phenomenon must stem at least in part from the nature of the plasma membrane, and even though the concept of this membrane may have originated as long ago as 1855 (Smith, 1962), no cellular membrane is thoroughly understood today. For example, even though electron microscopy is now producing improved images of membrane ultrastructure (see, e.g., Brandt, 1962; Fawcett, 1962, 1963; Fernández-Morán, 1962; Robertson, 1964; Sjöstrand, 1963; Tormey, 1964), many details remain to be clarified. And although biochemistry and biophysics have led to a broad concept of molecular arrangement within cell membranes (Danielli, 1962; Davson, 1962), many theoretical considerations are still being evaluated against this useful concept as well as against the ultrastructural image (see, e.g., Finean, 1962; Kavanau, 1964; Razin *et al.*, 1965; Stoeckenius, 1962; Tobias *et al.*, 1962). These obstacles must be overcome in order to resolve fully the problem of membrane fusion. The following considerations may have some significance for the ultimate solution of this problem.

At least three different events related to sperm-egg association should be considered with respect to membrane fusion. These are (a) the inception of the zygote, when the plasma membranes of the two gametes become the single continuous zygote plasma membrane (Fig. 50, d-f); (b) sperm activation in species with an acrosome, when the acrosomal and sperm plasma membranes are found to be one continuous membrane (Fig. 50, a-c); and (c) egg activation in species having cortical granules or alveoli, when the membranes of these structures are found to be

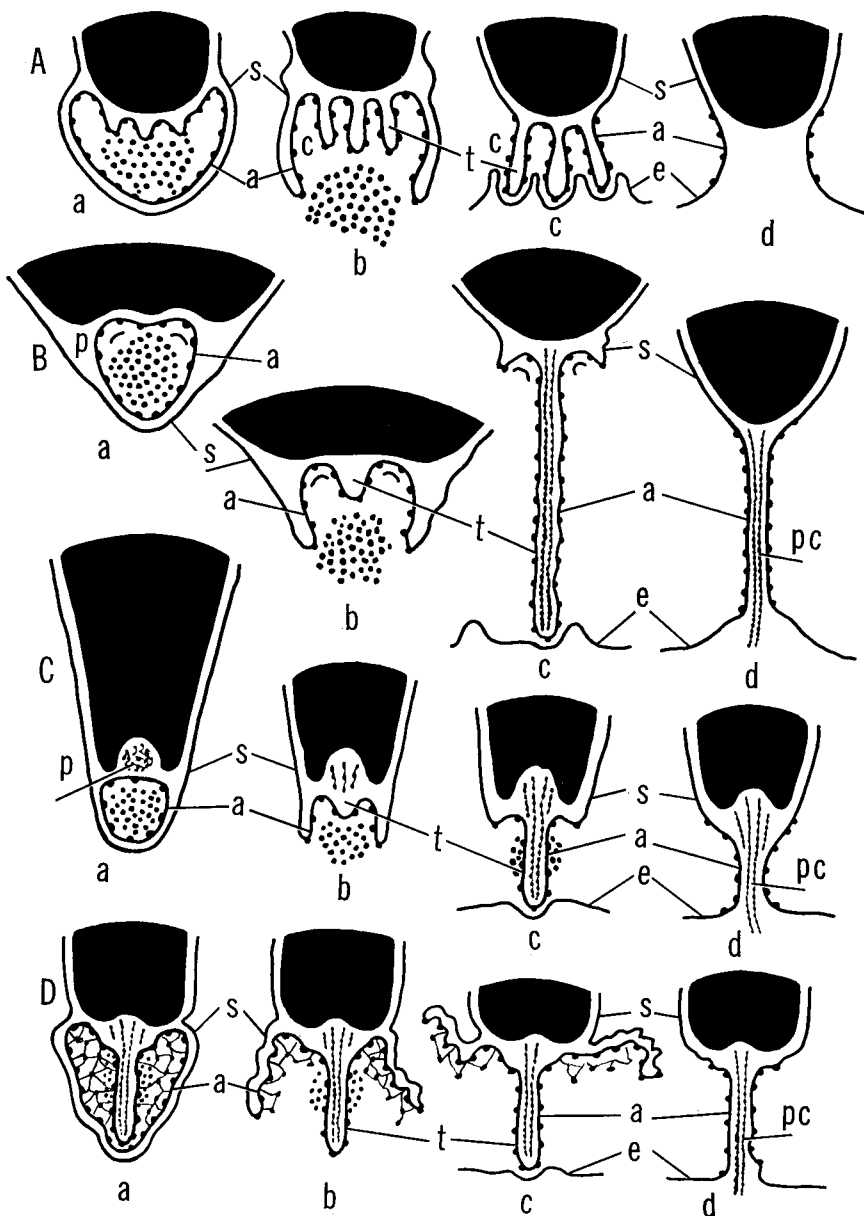
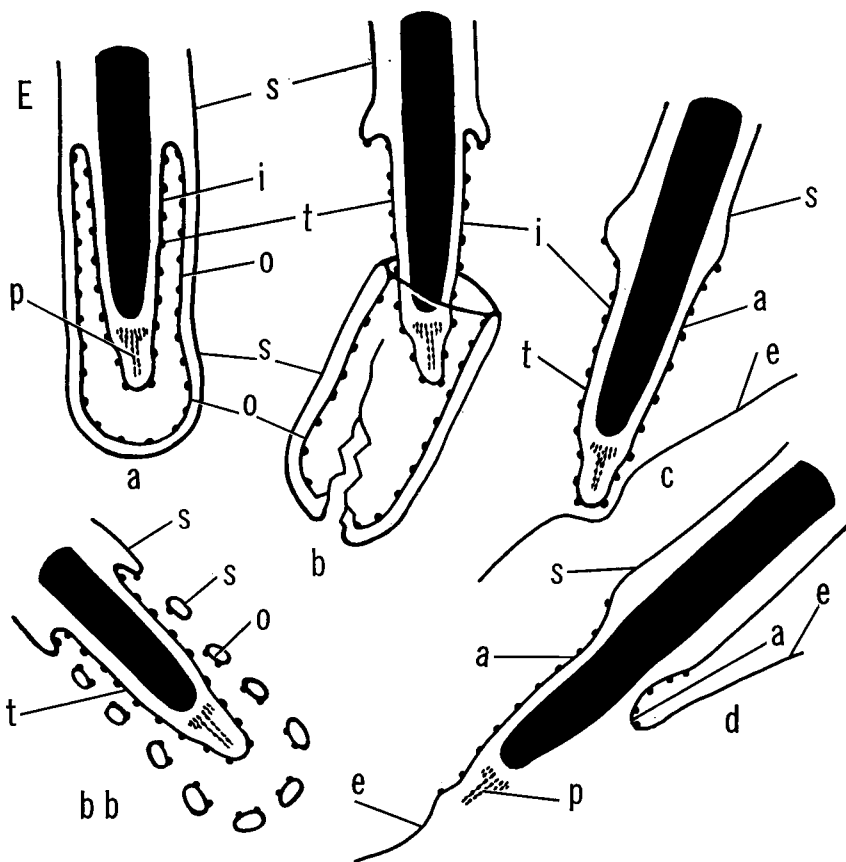


FIG. 49. A diagram comparing the *Hydroides-Saccoglossus* pattern of the early events of fertilization with known and/or proposed events in other phyla. (A) *Hydroides*. (B) *Saccoglossus*. (C) Echinoderms (sea urchin, starfish). (D) Mollusks (*Barnea*, *Mytilus*). (E) Mammals (rat, rabbit, bull). The acrosomal membrane (a, o, i) is represented by a dot-bearing line; the sperm (s) and egg (e) plasma membranes are represented by solid lines. In (A-E) the first figure (a) shows the intact acrosomal region; the second figure (b, or bb) shows the acrosomal region after dehiscence at the apex and establishment of continuity between acrosomal and sperm



plasma membranes; (c) shows the definitive acrosomal tubule during an advanced stage of eversion of the acrosomal vesicle; and (d) shows the zygote plasma membrane which is a mosaic formed by fusion of sperm (s, a) and egg (e) plasma membranes. The continuity of the cavity of the acrosomal tubule with the periacrosomal region is evident from the position of the periacrosomal core or perforatorium (PC, or p). In (E) the figures (b) and (bb) show loss of acrosomal (o) and sperm plasma (s) membrane at the sperm apex by alternative methods, namely, loss of galea capitis (b) or vesiculation (bb); in either case, at a posterior level continuity is established between the inner acrosomal (i) and sperm plasma (s) membranes, and this level marks the equivalent of a rim of dehiscence; the exposed inner membrane (i) is the equivalent of an acrosomal tubule, and as shown in figures (c) and (d) this is the part of the sperm plasma membrane (i, t, a) which fuses with the egg plasma membrane (e). [(A) from Colwin and Colwin, 1961a,b,c. (B) from Colwin and Colwin, 1963a,b. (C) based in part on suggestions in Colwin and Colwin, 1963a, 1964; in part on electron micrographs and diagrams by Franklin, 1965; and in part on the electron micrographs of Pasteels shown in Figs. 39 and 40. (D), (a) modified from a diagram by Pasteels and de Harven, 1962; (b) modified from a diagram by Pasteels, personal communication, 1964; (c) and (d) probable later stages, proposed in text. (E) scheme of events proposed by Colwin and Colwin, 1965, including their interpretation of observations (b) by Blom, 1963, and (bb) by Saacke and Almquist, 1964. (For further explanation, see text.)]

inserted parts of the egg plasma membrane (Fig. 50, g-i; Fig. 18F) (see Chap. 8; Afzelius, 1956b; Endo, 1961). Following each of these three events there is a single continuous membrane composed of segments which before the event appeared to be two separate membranes.

At the inception of the zygote the membranes which fuse are, of course, from different cells, and we know that before the event the two membranes are completely separate. These circumstances obtain also

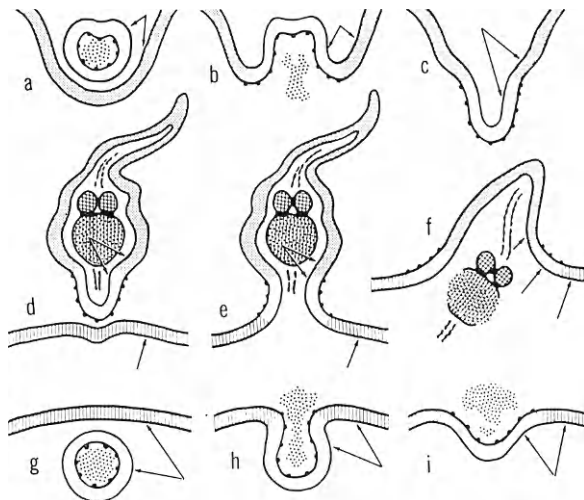


FIG. 50. Diagram of membrane relationships in three situations related to fertilization. (a-c) Successive stages in sperm activation. The acrosomal membrane (unshaded) becomes a mosaically inserted part of the sperm plasma membrane (shaded). (d-f) Successive stages in zygote formation. The sperm plasma membrane meets and fuses with the egg plasma membrane (shaded with parallel lines) to form a continuous zygote plasma membrane. The initial meeting is between the external (rather than cytoplasmic) surfaces of the fusing membranes. (g-i) Successive stages in egg activation. The cortical granular membrane (unshaded) becomes a mosaically inserted part of the egg plasma membrane.

Arrows indicate membrane surfaces facing the cytoplasm. The dot-bearing lines indicate membrane surfaces initially facing into the acrosomal vesicle (a-f) or into the cortical granule (g-i).

during the fusion of nuclei in plant fertilization (Jensen, 1964) and during the temporary fusion of the plasma membranes of the two mates during conjugation in ciliates. Schneider (1963, 1965) examined this fusion in *Paramecium* and weighed a theoretical model of the plasma membrane (cf. Davson, 1962) against the observed ultrastructural morphology of the membrane under these and other circumstances. His micrographs and interpretation suggest that vesiculation occurs between the confronting plasma membranes. There is evidence that such vesi-

culation occurs also in *Hydroides* (Colwin and Colwin, 1961c). The ultrastructure of these membranes recalls the images of vesiculation between the two membranes of the nuclear envelope which Moses (1960) showed and which have since been shown in *Hydroides* and *Saccoglossus* as well as in other forms. Could the forces that mediate vesiculation (and fusion) between two membranes within a single cell be the same as those that mediate fusion between the plasma membranes of two different gametic cells? The occurrence of membrane fusion at this juncture in fertilization would seem to offer a wide field for further exploration of membrane fusion by means of sophisticated techniques. An interesting starting point might be to compare gametic fusion with macrophage-lymphocyte fusion (Schoenberg *et al.*, 1964) and with the cell fusion which circumstantial evidence so strongly suggests in tissue cultures of embryonic muscle cells (Capers, 1960; Cooper and Konigsburg, 1960) and of certain giant cells (Barsky and Cornefert, 1960).

In contrast with the circumstances of fusion when the gametes meet, the membranes which are found to be in continuity following sperm activation and egg activation are in each case membranes of a single cell. Moreover, just before the event of activation the membrane of the granule (whether acrosomal or cortical) is so close to its respective plasma membrane that the question arises as to whether the process of fusion might already have begun. That is, might this process begin earlier and remain arrested until the time of activation? Different degrees of what might be called partial fusion between plasma membranes of certain kinds of cells have been described, for example, by Farquhar and Palade (1963), Robertson (1964), and Wiener *et al.*, (1964). Although the cytology of gametogenesis would seem to indicate that the granules certainly at one time are not connected with the plasma membrane, further ultrastructural studies will probably be needed in order to find out just when the fusion process begins. The extrusion of granular material during activation brings to mind the extrusion of granular material in various kinds of secretory cells of animals, as described, for example, by Palade (1959), Farquhar (1961), Fujita (1963), and Goldberg and Green (1964), and in plant cells (Mollenhauer *et al.*, 1961). Comparative study of these seemingly different circumstances might yield further insight into the basic mechanism of membrane fusion.

The surfaces which meet when the membranes fuse during fertilization may now be considered. (a) At the inception of the zygote the external rather than the cytoplasmic sides of the plasma membranes are the first to be involved (Fig. 50, d-f). These are somewhat specialized exteriors. In species with an acrosome the part of the sperm membrane which engages in fusion is a newly exposed surface that only recently

faced into the acrosomal vesicle. In species with vitelline envelopes the meeting of plasma membranes takes place beneath the envelope, so that that part of the egg plasma membrane which meets the sperm plasma membrane is in a sense also a newly exposed surface. In algae the gametes are recently shed cells, so that both membranes are newly exposed ones. In ciliates the site of fusion is at a newly modified paroral location, and it is probable that here too the parts of the plasma membrane that begin the fusion are newly exposed exterior surfaces. In each case the result of membrane fusion is that the cytoplasmic contents of the two cells come into confluence. (*b & c*) At sperm activation and at egg activation it is the cytoplasmic sides of the respective membranes which initially meet, i.e., the cytoplasmic side of the acrosomal membrane meets the cytoplasmic side of the sperm plasma membrane (Fig. 50, a-c), and the cytoplasmic side of the membrane of the cortical granule meets the cytoplasmic side of the egg plasma membrane (Fig. 50, g-i). In both cases, following fusion, a surface which formerly faced the interior of a membrane-bounded vesicle subsequently is part of the external surface of the plasma membrane. Incidentally, as a consequence of this fusion, the content of the vesicle is conducted out of the cell without ever coming into contact with the cytoplasm (see Bennett, 1956).

In all three of these events of fertilization, material which has been membrane bounded reaches beyond its membrane unaccompanied by any visible discontinuity of membrane. Since, as outlined above, either an external or a cytoplasmic surface can accomplish the initial steps of membrane fusion, perhaps the mechanism of this fusion involves properties common to both surfaces.

NOTE ADDED IN PROOF

Since this chapter was written a number of papers have appeared, of which the following are selected for brief comment. The terminology to be used is the same as in the chapter and is not necessarily that of the original authors. The papers cited are listed below among the other References.

Mollusks

Bourcart *et al.* (1965), in *Mytilus perna*, show an unactivated acrosomal region whose ultrastructure resembles that in the *Hydroides-Saccoglossus* pattern more closely than hitherto described for bivalves. The internal organization of the acrosomal vesicle, as these authors indicate, resembles that in *Hydroides*. As in other mollusks, however, some of the axial periacrosomal material is filamentous (although more highly structured than hitherto described). This material, which we homologize with a perforatorium, is outside the acrosomal vesicle; we interpret it as being contents of the future acrosomal tubule. Following sperm activation, the acrosomal tubule would have as its wall the adnuclear part of the acrosomal membrane.

Arthropods

The fine structure of the acrosomal region in relation to sperm activation has been studied in *Limulus*, by André (1965), and in several decapods by Pochon-Masson (1965a,b); Brown (1965) has considered this structure in relation to decapod fertilization. In *Limulus* a structure, which appears to us to be homologous with the perforatorium and with the fibrous periacrosomal material in the axial region in bivalve mollusks, is highly specialized; aside from this, however, the acrosomal region as seen in the electron micrographs looks and behaves remarkably like this region as described above for the *Hydroides-Saccoglossus* pattern. In both *Limulus* and the decapods, the events of fertilization require further clarification.

Tandler and Moriber (1966) describe a very highly specialized acrosomal region in the water-strider, *Gerris*. Although the role of the acrosome is not elucidated, these observations reveal nothing to indicate that the general plan of sperm-egg association might necessarily differ from the *Hydroides-Saccoglossus* pattern.

Lamprey

The specialized acrosomal region in *Lampetra planeri* described by Follenius (1965) contains a well-defined axial fibril and a conical body which we equate with the perforatorium and other periacrosomal material. We suggest that following sperm activation these structures would be contained within the acrosomal tubule. However, the acrosomal vesicle in this species requires further clarification.

Mammals

In the observations of Dickmann (1965), Hopsu and Arstila (1965), Nicander and Bane (1966), and Yanagimachi (1966), covering a number of species, we find additional evidence in support of our views expressed above. Blom and Birch-Andersen (1965) have redefined galea capitis; for mammals, they postulate a series of events which, in several respects, resembles the series we propose in this chapter.

Fawcett and Ito (1965), in an ultrastructural study of bat spermatozoa, postulate no role in fertilization for an apical body, occupant of the subacrosomal space between the nucleus and the acrosomal vesicle. There appears to be no evidence thus far, however, which rules out the possibility that extra or periacrosomal material which occupies this space might, as we have suggested, have some role in fertilization.

General

Pasteels (1965a) has reviewed the comparative aspects of the fine structure of fertilization. This substantial analysis complements his paper, also in 1965, cited in the above chapter. It should be noted that in certain interpretations we differ somewhat from Pasteels.

Tyler (1965), in a consideration of the biology and chemistry of fertilization, has rounded out the account of fertilization in *Urechis* with new micrographs. The added information from these suggests that sperm-egg association in this echiuroid does indeed follow the *Hydroides-Saccoglossus* pattern.

REFERENCES

- Afzelius, B. A. (1955). The fine structure of the sea urchin spermatozoa as revealed by the electron microscope. *Z. Zellforsch. Mikroskop. Anat.* **42**, 134-148.

- Afzelius, B. A. (1956a). The acrosomal reaction of the sea urchin spermatozoon. *Proc. Conf. Electron Microscopy, Stockholm, 1956*, pp. 167-169.
- Afzelius, B. A. (1956b). The ultrastructure of the cortical granules and their products in the sea urchin egg as studied with the electron microscope. *Exptl. Cell Res.* **10**, 257-285.
- Afzelius, B. A., and Murray, A. (1957). The acrosomal reaction of spermatozoa during fertilization or treatment with egg water. *Exptl. Cell Res.* **12**, 325-337.
- Ånberg, A. (1957). The ultrastructure of the human spermatozoon. *Acta Obst. Gynecol. Scand.* **36**, Suppl. 2, 1-133.
- André, J. (1965). A propos d'une leçon sur la Limule. *Ann. Fac. Sci. Univ. Clermont* No. 26, 27-38.
- André, J., and Vivier, E. (1962). Quelques aspects ultrastructuraux de l'échange micronucléaire lors de la conjugaison chez *Paramecium caudatum*. *J. Ultrastruct. Res.* **6**, 390-406.
- Austin, C. R. (1951). Observations on the penetration of the sperm into the mammalian egg. *Australian J. Sci. Res., Ser. B* **4**, 581-596.
- Austin, C. R. (1963). Acrosome loss from the rabbit spermatozoon in relation to entry into the egg. *J. Reprod. Fertility* **6**, 313-314.
- Austin, C. R., and Bishop, M. W. H. (1958a). Some features of the acrosome and perforatorium in mammalian spermatozoa. *Proc. Roy. Soc. (London), Ser. B* **149**, 234-240.
- Austin, C. R., and Bishop, M. W. H. (1958b). Role of the rodent acrosome and perforatorium in fertilization. *Proc. Roy. Soc. (London), Ser. B* **149**, 241-248.
- Ballowitz, E. (1905). Über die Spermien des Flussneunauges (*Petromyzon fluviatilis* L.) und ihre merkwürdige Kopfborste. *Arch. Mikros. Anat.* **65**, 96-120.
- Bane, A., and Nicander, L. (1963). The structure and formation of the perforatorium in mammalian sperm. *Intern. J. Fertility*, **8**, No. 4 865-866.
- Barsky, G., and Cornefert, F. (1960). Mise en évidence, en cultures de la souche cellulaire HeLa de l'infection ectromélique latente chez la souris. *Ann. Inst. Pasteur* **98**, 112-118.
- Bedford, J. M. (1964). Fine structure of the sperm head in ejaculate and uterine spermatozoa of the rabbit. *J. Reprod. Fertility* **7**, 221-228.
- Bennett, H. S. (1956). The concepts of membrane flow and membrane vesiculation as mechanisms for active transport and ion pumping. *J. Biophys. Biochem. Cytol.* **2**, No. 4, Suppl. 99-103.
- Bernstein, M. H. (1962). Normal and reactive morphology of sea urchin spermatozoa. *Exptl. Cell Res.* **27**, 197-209.
- Binford, R. (1913). The germ cells and the process of fertilization in the crab, *Menippe Mercenaria*. *J. Morphol.* **24**, 147-201.
- Bloch, F. (1933). Fécondation et maturation de l'oeuf chez un Pagure (*Diogenes pugilator* Roux). *Compt. Rend. Acad. Sci. Paris* **196**, 429-431.
- Bloch, F. (1935). Contributions à l'étude des gamètes et de la fécondation chez les crustacés décapodes. *Trav. Sta. Zool. Wimereux* **12**, 183-279.
- Blom, E. (1963). The galea capitis and apical body in bull sperm and the fertilization process. *Intern. J. Fertility* **8**, 447-452.
- Blom, E., and Birch-Andersen, A. (1965). The ultrastructure of the bull sperm. II. The sperm head. *Nord. Veterinarmed.* **17**, 193-212.
- Bourcart, C., Lavallard, P., and Lubet, P. (1965). Ultrastructure du spermatozoïde de la Moule (*Mytilus perna* von Ihering). *Compt. Rend. Acad. Sci. Paris* **260**, 5096-5112.

- Bowen, R. H. (1924). On the acrosome of the animal sperm. *Anat. Record* **28**, 1-13.
- Brandt, P. W. (1962). A consideration of the extraneous coats on the plasma membrane. *Circulation* **26**, 1075-1104.
- Briggs, R., and King, T. J. (1953). Factors affecting the transplantability of nuclei of frog embryonic cells. *J. Exptl. Zool.* **122**, 485-505.
- Brown, G. G. (1965). Ultrastructural studies of sperm morphology and sperm-egg interaction in the decapod *Callinectes sapidus*. *J. Ultrastruct. Res.* **14**, 425-440.
- Burgos, M. H., and Fawcett, D. W. (1955). Studies on the fine structure of the mammalian testis. I. Differentiation of the spermatids in the cat (*Felis domestica*). *J. Biophys. Biochem. Cytol.* **1**, 287-300.
- Burgos, M. H., and Fawcett, D. W. (1956). An electron microscope study of spermatid differentiation in the toad, *Bufo arenarum* Hensel. *J. Biophys. Biochem. Cytol.* **2**, 223-240.
- Capers, C. R. (1960). Multinucleation of skeletal muscle *in vitro*. *J. Biophys. Biochem. Cytol.* **7**, 559-566.
- Chang, M. C. (1951). Fertilizing capacity of spermatozoa deposited into Fallopian tubes. *Nature* **168**, 697-698.
- Clermont, Y., Einberg, E., LeBlond, C. P., and Wagner, S. (1955). The perforatorium, an extension of the nuclear membrane of the rat spermatozoon. *Anat. Record* **121**, 1-12.
- Colwin, L. H., and Colwin, A. L. (1954). Sperm penetration and the fertilization cone in the egg of *Saccoglossus kowalevskii* (Enteropneusta). *J. Morphol.* **95**, 351-372.
- Colwin, A. L., and Colwin, L. H. (1955). Sperm entry and the acrosome filament (*Holothuria atra* and *Asterias amurensis*). *J. Morphol.* **97**, 543-568.
- Colwin, L. H., and Colwin, A. L. (1956). The acrosome filament and sperm entry in *Thyone briareus* (Holothuria) and *Asterias*. *Biol. Bull.* **110**, 243-257.
- Colwin, L. H., and Colwin, A. L. (1957a). Observations of sperm entry during refertilization in *Saccoglossus kowalevskii* (Enteropneusta). *Biol. Bull.* **113**, 341.
- Colwin, A. L., and Colwin, L. H. (1957b). Morphology of fertilization: acrosome filament formation and sperm entry. In "The Beginnings of Embryonic Development" (A. Tyler, R. C. von Borstel, and C. B. Metz, eds.) pp. 135-168. Am. Assoc. Advanc. Sci., Washington, D.C.
- Colwin, A. L., and Colwin, L. H. (1958). Some characterization of the egg membrane lytic agent derived from sperm extracts of *Hydroides hexagonus*. *Biol. Bull.* **115**, 348.
- Colwin, A. L., and Colwin, L. H. (1960a). Fine structure studies of fertilization with special reference to the role of the acrosomal region of the spermatozoon during penetration of the egg (*Hydroides hexagonus*—Annelida). In Symposium on the Germ Cells and Earliest Stages of Development, pp. 220-222. Institut Intern. d'Embryologie and Fondazione A. Baselli, Istituto Lombardo, Milan.
- Colwin, L. H., and Colwin, A. L. (1960b). Formation of sperm entry holes in the vitelline membrane of *Hydroides hexagonus* (Annelida) and evidence of their lytic origin. *J. Biophys. Biochem. Cytol.* **7**, 315-320.
- Colwin, A. L., and Colwin, L. H. (1960c). Egg membrane lytic activity of sperm extract and its significance in relation to sperm entry in *Hydroides hexagonus* (Annelida). *J. Biophys. Biochem. Cytol.* **7**, 321-328.
- Colwin, A. L., and Colwin, L. H. (1961a). Fine structure of the spermatozoon of *Hydroides hexagonus* (Annelida), with special reference to the acrosomal region. *J. Biophys. Biochem. Cytol.* **10**, 211-230.

- Colwin, L. H., and Colwin, A. L. (1961b). Changes in the spermatozoon during fertilization in *Hydroides hexagonus* (Annelida). I. Passage of the acrosomal region through the vitelline membrane. *J. Biophys. Biochem. Cytol.* **10**, 231-254.
- Colwin, A. L., and Colwin, L. H. (1961c). Changes in the spermatozoon during fertilization in *Hydroides hexagonus* (Annelida). II. Incorporation with the egg. *J. Biophys. Biochem. Cytol.* **10**, 255-274.
- Colwin, A. L., and Colwin, L. H. (1963a). Role of the gamete membranes in fertilization in *Saccoglossus kowalevskii* (Enteropneusta). I. The acrosomal region and its changes in early stages of fertilization. *J. Cell Biol.* **19**, 477-500.
- Colwin, L. H., and Colwin, A. L. (1963b). Role of the gamete membranes in fertilization in *Saccoglossus kowalevskii* (Enteropneusta). II. Zygote formation by gamete membrane fusion. *J. Cell Biol.* **19**, 501-518.
- Colwin, A. L., and Colwin, L. H. (1964). Role of the gamete membranes in fertilization. In "Cellular Membranes in Development" (M. Locke, ed.), 22nd Symp. Soc. Study Dev. Growth, pp. 233-279. Academic Press, New York.
- Colwin, A. L., and Colwin, L. H. (1965). Suggested similarity between early events of fertilization in certain mammals and invertebrates. *Anat. Record.* **151**, 447.
- Colwin, A. L., Colwin, L. H., and Philpott, D. E. (1957). Electron microscope studies of early stages of sperm penetration in *Hydroides hexagonus* (Annelida) and *Saccoglossus kowalevskii* (Enteropneusta). *J. Biophys. Biochem. Cytol.* **3**, 489-502.
- Cooper, W. G., and Konigsberg, I. R. (1960). Dynamics of myogenesis *in vitro*. *Anat. Record* **140**, 195-205.
- Dan, J. C. (1954). Studies on the acrosome. II. Acrosome reaction in starfish spermatozoa. *Biol. Bull.* **107**, 203-218.
- Dan, J. C. (1956). The acrosome reaction. *Intern. Rev. Cytol.* **5**, 365-393.
- Dan, J. C. (1960). Studies on the acrosome. VI. Fine structure of the starfish acrosome. *Exptl. Cell Res.* **19**, 13-28.
- Dan, J. C. (1962). The vitelline coat of the *Mytilus* egg. I. Normal structure and effect of acrosomal lysin. *Biol. Bull.* **123**, 531-541.
- Dan, J. C., Kushida, H., and Ohori, Y. (1962). Formation of the acrosomal process in echinoderm spermatozoa. *Proc. 5th Intern. Congr. Electron Microscopy, Philadelphia, 1961* (S. Breese, ed.) **2**, YY-12. Academic Press, New York.
- Dan, J. C., Ohori, Y., and Kushida, H. (1964). Studies on the acrosome. VII. Formation of the acrosomal process in sea urchin spermatozoa. *J. Ultrastruct. Res.* **11**, 508-524.
- Danielli, J. F. (1962). Structure of the cell surface. *Circulation* **26**, 1163-1166.
- Davson, H. (1962). Growth of the concept of the paucimolecular membrane. *Circulation* **26**, 1022-1037.
- Detlaff, T. A., and Ginsburg, A. S. (1963). Acrosome reaction in sturgeons and the role of Ca-ions in sperm-egg association. *Compt. Rend. Acad. Sci. URSS* **153**, 1461-1464 (in Russian).
- Dickmann, Z. (1964). The passage of spermatozoa through and into the zona pellucida of the rabbit egg. *J. Exptl. Biol.* **41**, 177-182.
- Dickmann, Z. (1965). Sperm penetration into and through the zona pellucida of the mammalian egg. In "Ciba Foundation Symposium on Preimplantation Stages of Pregnancy" (G. E. W. Wolstenholme and M. O'Connor, eds.), pp. 169-178. J. and A. Churchill Ltd., London.
- Dickmann, Z., and Dziuk, P. J. (1964). Sperm penetration of the zona pellucida of the pig egg. *J. Exptl. Biol.* **41**, 603-608.

- Endo, Y. (1961). Changes in the cortical layer of sea urchin eggs at fertilization as studied with the electron microscope. *Exptl. Cell Res.* **25**, 383-397.
- Elliott, A. M., and Tremor, J. W. (1958). The fine structure of the pellicle in the contact area of conjugating *Tetrahymena pyriformis*. *J. Biophys. Biochem. Cytol.* **4**, 839-840.
- Fallon, J. F., and Austin, C. R. (1964). A fine-structure study of the activation reactions of *Nereis limbata* gametes. *Biol. Bull.* **127**, 369-
- Farquhar, M. G. (1961). Origin and fate of secretory granules in cells of the anterior pituitary gland. *Trans. N. Y. Acad. Sci.* **23**, 346-351.
- Farquhar, M. G., and Palade, G. E. (1963). Junctional complexes in various epithelia. *J. Cell Biol.* **17**, 375-412.
- Fasten, N. (1921). The explosion of the spermatozoa of the crab *Lophopanpoeus bellus* (Stimpson) Rathbun. *Biol. Bull.* **41**, 288-300.
- Fawcett, D. W. (1958). The structure of the mammalian spermatozoon. *Intern. Rev. Cytol.* **7**, 195-234.
- Fawcett, D. W. (1962). Physiologically significant specializations of the cell surface. *Circulation* **26**, 1105-1125.
- Fawcett, D. W. (1963). Morphological and functional variations in the endoplasmic reticulum. *J. Cell Biol.* **19**, 80A.
- Fawcett, D. W., and Ito, S. (1965). The fine structure of bat spermatozoa. *Am. J. Anat.* **116**, 567-610.
- Fernández-Morán, H. (1962). Cell membrane ultrastructure. *Circulation* **26**, 1039-1065.
- Finian, J. B. (1962). The nature and stability of the plasma membrane. *Circulation* **26**, 1151-1162.
- Fol, H. (1877). Sur le commencement de l'hénogénie chez divers animaux. *Arch. Zool. Exptl. Gén.* **6**, 145-169.
- Fol, H. (1878-1879). Recherches sur la fécondation et le commencement de l'hénogénie chez divers animaux. *Mem. Soc. Phys. Hist. Nat. (Geneva)* **26**, 89-397.
- Follenius, E. (1965). Particularités de Structures des Spermatozoïdes de *Lampetra planeri*. Étude au Microscope Électronique. *J. Ultrastruct. Res.* **13**, 459-468.
- Franklin, L. (1965). Morphology of gamete membrane fusion and of sperm entry into oocytes of the sea urchin. *J. Cell. Biol.* **25** (No. 2, Part 1), 81-100.
- Fujita, H. (1963). Electron microscopic studies on the thyroid gland of domestic fowl, with special reference to the mode of secretion and the occurrence of a central flagellum in the follicular cell. *Z. Zellforsch. Mikroskop. Anat.* **60**, 615-632.
- Galtsoff, P. S., and Philpott, D. E. (1960). Ultrastructure of the spermatozoon of the oyster, *Crassostrea virginica*. *J. Ultrastruct. Res.* **3**, 241-253.
- Ginsburg, A. S. (1963). Sperm-egg association and its relationship to the activation of the egg in salmonid fishes. *J. Embryol. Exptl. Morphol.* **11**, 13-33.
- Goldberg, B., and Green, H. (1964). An analysis of collagen secretion by established mouse fibroblast lines. *J. Cell. Biol.* **22**, 227-258.
- Hadek, R. (1963a). Submicroscopic changes in the penetrating spermatozoon of the rabbit. *J. Ultrastruct. Res.* **8**, 161-169.
- Hadek, R. (1963b). Study on the fine structure of rabbit sperm head. *J. Ultrastruct. Res.* **9**, 110-122.
- Hiramoto, Y. (1962). Microinjection of the live spermatozoa into sea urchin eggs. *Exptl. Cell Res.* **27**, 416-426.

- Hopsu, V. K., and Arstila, A. U. (1965). Development of the acrosomic system of the spermatozoon in the Norwegian lemming (*Lemmus lemmus*). *Z. Zellforsch. Mikroskop. Anat.* **65**, 562-572.
- Horstmann, E. (1961). Elektronenmikroskopische Untersuchungen zur Spermiogonogenese beim Menschen. *Z. Zellforsch. Mikroskop. Anat.* **54**, 68-89.
- Jensen, W. A. (1964). Observations on the fusion of nuclei in plants. *J. Cell Biol.* **23**, 669-672.
- Kavanau, J. L. (1964). The role of membranes in intracellular transport in 11th *Intern. Congr. Cell Biol. Excerpta Med., Intern. Congr. Ser. No. 77*, p. 24.
- Kaye, J. S. (1962). Acrosome formation in the house cricket. *J. Cell Biol.* **12**, 411-431.
- Kille, R. A. (1960). Fertilization of the lamprey egg. *Exptl. Cell Res.* **20**, 12-27.
- Koltzoff, N. K. (1906). Studien über die Gestalt der Zelle. I. Untersuchungen über die Spermien der Decapoden, als Einleitung in das Problem der Zellengestalt. *Arch. Mikros. Anat.* **67**, 364-572.
- Lillie, F. R. (1912a). Studies of fertilization in *Nereis*. I. The cortical changes in the egg. II. Partial fertilization. *J. Morphol.* **22**, 361-393.
- Lillie, F. R. (1912b). Studies of fertilization in *Nereis*. III. The morphology of the normal fertilization of *Nereis*. IV. The fertilizing powers of portions of the spermatozoon. *J. Exptl. Zool.* **12**, 413-478.
- Lillie, F. R. (1919). "Problems of Fertilization." Univ. of Chicago Press, Chicago, Illinois.
- Loeb, J. (1917). Fécondation et Phagocytose. *An. l'Inst. Pasteur* **31**, 437-441.
- Lönning, S. (1964). Studies of the ultrastructure of sea urchin oocytes and the changes induced by insemination. *Sarsia* **15**, 9-15.
- Manton, I., and Friedmann, I. (1960). Gametes, fertilization and zygote development in *Prasiola stipitata* Suhr. II. Electron microscopy. *Nova Hedwigia* **1**, 443-462.
- Metz, C. B., and Morrill, J. B., Jr. (1955). Formation of acrosome filaments in response to treatment of sperm with fertilizin in *Asterias* and *Nereis*. *Biol. Bull.* **109**, 349.
- Mèves, F. (1915). Ueber den Befruchtungsvorgang bei der Meismuschel (*Mytilus edulis* L.). *Arch. Mikroskop. Anat.* **87**, Abt. 2, 47-62.
- Mollenhauer, H. H., Whaley, W. G., and Leech, J. H. (1961). A function of the Golgi apparatus in outer rootcap cells. *J. Ultrastruct. Res.* **5**, 193-200.
- Monroy, A. (1948). A preliminary approach to the physiology of fertilization in *Pomatoceros triqueter* L. *Arkiv Zool.* **40A**, No. 21, 1-9.
- Moricard, R. (1960). Observations de microscopie électronique sur des modifications acrosomiques lors de la pénétration spermatique dans l'oeuf des mammifères. *Comp. Rend. Soc. Biol.* **154**, 2187-2189.
- Moses, M. J. (1960). Breakdown and reformation of the nuclear envelope at cell division. *Proc. 4th Intern. Congr. on Electron Microscopy, Berlin, 1958*, **2**, 230-233. Springer, Berlin.
- Moses, M. J. (1961). Spermiogenesis in the crayfish (*Procambarus clarkii*). II. Description of stages. *J. Biophys. Biochem. Cytol.* **10**, 301-333.
- Nagano, T. (1962). Observations on the fine structure of the developing spermatid in the domestic chicken. *J. Cell Biol.* **14**, 193-205.
- Nicander, L., and Bane, A. (1962). Fine structure of boar spermatozoa. *Z. Zellforsch. Mikroskop. Anat.* **57**, 390-405.

- Nicander, L., and Bane, A. (1966). Fine structure of the sperm head in some mammals, with particular reference to the acrosome and the subacrosomal substance. *Z. Zellforsch. Mikroskop. Anat.* **72**, 496-515.
- Niijima, L., and Dan, J. (1965a). The acrosome reaction in *Mytilus edulis*. I. Fine structure of the intact acrosome. *J. Cell Biol.* **25**, No. 2 (pt. 1), 243-248.
- Niijima, L., and Dan, J. (1965b). The acrosome reaction in *Mytilus edulis*. II. Stages in the reaction, observed in supernumerary and calcium-treated spermatozoa. *J. Cell Biol.* **25**, No. 2 (pt. 1), 249-259.
- Palade, G. E. (1959). Functional changes in the structure of cell components. In "Subcellular Particles" (T. Hayashi, ed.), pp. 64-83. Ronald Press, New York.
- Pasteels, J. J. (1965). Aspects structuraux de la fécondation vue au microscope électronique. *Arch. Biol. (Liège)* **76**, 463-509.
- Pasteels, J. J. (1965a). La Fécondation étudiée au microscope électronique. Étude comparative. *Bull. Soc. Zool. France* **90**, 195-224.
- Pasteels, J. J., and de Harven, E. (1962). Etude au microscope électronique du spermatozoïde d'un mollusque bivalve, *Barnea candida*. *Arch. Biol. (Liège)* **73**, 445-463.
- Pikó, L., and Tyler, A. (1964a). Ultrastructure of the acrosome and the early events of sperm penetration in the rat. *Am. Zool.* **4**, 287.
- Pikó, L., and Tyler, A. (1964b). Fine structural studies of sperm penetration in the rat. *Proc. 5th Intern. Congr. on Animal Reproduction, Trento* **2**, 372-377.
- Pochon-Masson, J. (1965a). Schéma général du spermatozoïde vésiculaire des Décapodes. *Compt. Rend. Acad. Sci. Paris* **260**, 5093-5099.
- Pochon-Masson, J. (1965b). Schéma du spermatozoïde dévaginé de *Homarus vulgaris* (Décapode Macroure). *Compt. Rend. Acad. Sci. Paris*, **260**, 5352-5058.
- Porte, A., and Follenius, E. (1960). La spermiogénèse chez *Lebistes reticulatus*. Etude au microscope électronique. *Bull. Soc. Zool. France* **85**, 82-88.
- Razin, S., Morowitz, H. J., and Terry, T. M. (1965). Membrane subunits of *Mycoplasma laidlawii* and their assembly to membranelike structures. *Proc. Natl. Acad. Sci. U.S.A.* **54**, 219-225.
- Rebhun, L. I. (1962). Electron microscope studies on the vitelline membrane of the surf clam, *Spisula solidissima*. *J. Ultrastruct. Res.* **6**, 107-122.
- Reger, J. F. (1963). Spermiogenesis in the tick, *Amblyomma dissimili*, as revealed by electron microscopy. *J. Ultrastruct. Res.* **8**, 607-621.
- Reger, J. F. (1964). The fine structure of spermatozoa from the isopod, *Asellus militaris* (Hay). *J. Ultrastruct. Res.* **11**, 181-192.
- Robertson, J. D. (1961). Ultrastructure of excitable membranes and the crayfish median giant synapse. *Ann. N. Y. Acad. Sci.* **94**, 339-389.
- Robertson, J. D. (1964). Unit membranes: a review with recent new studies of experimental alterations and a new subunit structure in synaptic membranes. In "Cellular Membranes in Development" (M. Locke, ed.), pp. 1-81.
- Runnström, J., (1949). The mechanism of fertilization in metazoa. *Advan. Enzymol.* **9**, 241-327.
- Saacke, R. G., and Almquist, J. O. (1964). Ultrastructure of bovine spermatozoa. I. The head of normal, ejaculated sperm. *Am. J. Anat.* **115**, 143-162.
- Schneider, L. (1960). Die Auflösung und Neubildung der Zellmembran bei der Konjugation von *Paramecium*. *Naturwissenschaften* **23**, 543-544.

- Schneider, L. (1963). Elektronenmikroskopische Untersuchungen der Konjugation von *Paramecium*. I. Die Auflösung und Neubildung der Zellmembran bei den Konjuganten. (Zugleich ein Beitrag zur Morphogenese cytoplasmatischer Membranen.) *Protoplasma* **56**, 109–140.
- Schneider, L. (1965). Morphogenese und Dynamik cytoplasmatischer Membranen. *Zool. Anz., Suppl.* **28**, 243–272.
- Schoenberg, M. D., Mumaw, V. R., Moore, R. D., and Weisberger, A. S. (1964). Cytoplasmic interaction between macrophages and lymphocytic cells in antibody synthesis. *Science* **143**, 964–965.
- Sjöstrand, F. S. (1963). A comparison of plasma membrane, cytomembranes, and mitochondrial membrane elements with respect to ultrastructural features. *J. Ultrastruct. Res.* **9**, 561–580.
- Smith, H. W. (1962). The plasma membrane, with notes on the history of botany. *Circulation* **26**, 987–1012.
- Stoeckenius, W. (1962). Structure of the plasma membrane. *Circulation* **26**, 1066–1069.
- Szollasi, D., and Ris, H. (1961). Observations on sperm penetration in the rat. *J. Biophys. Biochem. Cytol.* **10**, 275–283.
- Takashima, R., and Takashima, Y. (1960). Electron microscopical observations on the fertilization phenomenon of sea urchins with special reference to the acrosome filament. *Tokushima J. Exptl. Med.* **6**, 334–340.
- Tandler, B., and Moriber, L. G. (1966). Microtubular structures associated with the acrosome during spermiogenesis in the Water-Strider, *Gerris remigis* (Say). *J. Ultrastruct. Res.* **14**, 391–404.
- Tobias, J. M., Agin, D. P., and Pawlowski, R. (1962). The excitable system in the cell surface. *Circulation* **26**, 1145–1150.
- Tolmach, L. J. (1957). Attachment and penetration of cells by viruses. *Advan. Virus Res.* **4**, 63–110.
- Tormey, J. McD. (1964). Differences in membrane configuration between osmium tetroxide-fixed and glutaraldehyde-fixed ciliary epithelium. *J. Cell Biol.* **23**, 658–664.
- Tyler, A. (1948). Fertilization and immunity. *Physiol. Rev.* **28**, 180–219.
- Tyler, A. (1949). Properties of fertilizin and related substances of eggs and sperm of marine animals. *Am. Naturalist* **83**, 195–219.
- Tyler, A. (1959). Some immunobiological experiments on fertilization and early development in sea urchins. *Exptl. Cell Res. Suppl.* **7**, 183–199.
- Tyler, A. (1960). Introductory remarks on theories of fertilization. In "Symposium on the Germ Cells and Earliest Stages of Development, pp. 155–174. Institut Intern. d'Embryologie and Fondazione A. Baselli, Istituto Lombardo, Milano.
- Tyler, A. (1961). The Fourth Oliver Bird Lecture, Approaches to the control of fertility based on immunological phenomena. *J. Reprod. Fertility* **2**, 473–506.
- Tyler, A. (1962). Antisyngamic and antidevelopmental effects of antibodies. *Proc. Conf. Immuno-Reprod., La Jolla, California, Sept., 1962*. pp. 117–126. Population Council, New York.
- Tyler, A. (1963). The manipulations of macromolecular substances during fertilization and early development of animal eggs. *Am. Zoologist* **3**, 109–126.
- Tyler, A. et al. (1964). As reported in Studies on fertilization and early development. *Eng. Sci. Mag.* **27**, 17–20. Calif. Inst. Technology, Pasadena, California.
- Tyler, A. (1965). The biology and chemistry of fertilization. *Am. Naturalist* **99**, 309–334.

- Wiener, J., Spiro, D., and Loewenstein, W. R. (1964). Studies on an epithelial (gland) cell junction. II. Surface structure. *J. Cell Biol.* **22**, 587-598.
- Yanagimachi, R. (1966). Time and process of sperm penetration into hamster ova *in vivo* and *in vitro*. *J. Reprod. Fertility* **11**, 359-370.
- Yanagimachi, R., and Chang, M. C. (1964). *In vitro* fertilization of golden hamster ova. *J. Exptl. Zool.* **156**, 361-376.
- Yasuzumi, G., Kaye, G. I., Pappas, G. D., Yamamoto, H., and Tsubo, I. (1961). Nuclear and cytoplasmic differentiation in developing sperm of the crayfish, *Cambaroides japonicus*. *Z. Zellforsch. Mikroskop. Anat.* **53**, 141-158.

CHAPTER 8

The Activation of the Egg

Alberto Monroy and Albert Tyler†*

INSTITUTE OF COMPARATIVE ANATOMY, UNIVERSITY OF PALERMO,
AND RESEARCH UNIT FOR MOLECULAR EMBRYOLOGY,
NATIONAL COUNCIL OF RESEARCH, PALERMO, ITALY;
AND DIVISION OF BIOLOGY, CALIFORNIA INSTITUTE OF
TECHNOLOGY, PASADENA, CALIFORNIA

I. Introduction	369
II. Metabolic Aspects of Oogenesis and Maturation	371
A. Breakdown of the Germinal Vesicle	371
B. Growth of the Oocyte	375
C. Relative Inertness of the Mature Unfertilized Egg	376
D. Cytoplasmic DNA	377
III. Relation of Activation to Fertilization	379
A. Artificial Parthenogenesis	379
B. Fertilization of Fertilized Eggs	380
IV. Respiratory Metabolism of the Fertilized Egg	381
A. Quantitative Changes	381
B. Qualitative Changes	383
V. RNA and Protein Synthesis	389
A. Demonstration of Stimulation of Protein Synthesis Provided by Fertilization	389
B. Basis for the Relative Inertness of the Unfertilized Egg	390
C. Capacitation of the Ribosomes of the Unfertilized Egg	396
D. Protein Synthesis in Nonechinoid Species	399
E. Onset of RNA Synthesis	399
VI. Activation and Changes in Protein Structure	402
References	403

I. Introduction

Before becoming fertilizable a terminal oogonium undergoes two principal processes. One is the tremendous growth of the cell, which is

* Original work of this author has been supported in part by grants of the Rockefeller Foundation and of the National Institutes of Health (GM-06211).

† Original work of this author reported in this article and preparation of the article was supported by grants of the National Institutes of Health (GM-12777) and the National Science Foundation (GB-28).

mainly due to the accumulation within its cytoplasm of reserve materials, including yolk. The second process, referred to as maturation, or meiosis, comprises the series of events starting with synapsis of homologous chromosomes and ending with a halving of the number of chromosomes. The growth process generally begins after the occurrence of chromosomal synapsis. In most organisms it is completed within the ovary. There are, however, a number of species, particularly among the annelids, in which the early oocytes, usually with one or more attached nurse cells, are liberated into the coelom, where they accomplish the greater part of their growth. Attached nurse cells degenerate toward the end of the growth period. In the geophyrean *Urechis caupo* the oocytes are liberated into the hemocoel, without nurse cells, at the start of the growth period and complete their growth as free-floating cells.

In most species of animals meiosis is arrested at a more or less advanced stage and is completed only upon shedding or fertilization of the egg. Sea urchins are one of the few groups of animals in which the female gamete goes through the entire maturation process within the ovary and is shed as a haploid cell. In the starfish, on the contrary, as long as the egg is in the ovary it remains in the germinal vesicle stage, but as soon as it is shed into the seawater maturation proceeds to completion. Eggs of many other animals (e.g., the annelid *Nereis limbata*, the geophyrean *Urechis caupo*, the mollusk *Spisula solidissima*) are shed in the germinal vesicle stage, but maturation does not proceed until after fertilization. Finally, in the eggs of many groups of animals the maturation divisions begin, but then stop, in the metaphase of either the first (e.g., *Ciona*, *Phallusia*, *Chaetopterus*) or the second (e.g., *Rana*, *Oryzias*) maturation division.

These facts indicate that the blocking mechanism that operates after the oocyte has reached its full size in the ovary may intervene at any stage of the maturation divisions or after its completion. Also, in some species the process of shedding may temporarily release the egg from the inhibition, as in *Chaetopterus*, in which the eggs are shed in the germinal vesicle stage, but proceed to the stage of the metaphase of the first polar division before being blocked again, and as in the starfish, in which the shed oocytes complete their maturation divisions in the seawater and again are blocked as ootids. The evidence that will be presented indicates that one of the most interesting features of maturation is the metabolic block that sets in during the course of this process. This inhibition is removed when the egg is fertilized or parthenogenetically activated. The understanding of oogenesis and maturation is thus a necessary prerequisite to the understanding of the metabolic events of fertilization. Moreover, recent work has shown that the early stages of

embryonic development, up to the onset of gastrulation, are indeed controlled by the "information" produced and stored during oogenesis.

II. Metabolic Aspects of Oogenesis and Maturation

A. BREAKDOWN OF THE GERMINAL VESICLE

As noted above, the process of maturation technically begins after a terminal oogonial division, the products of which are termed primary oocytes. In these cells the nucleus becomes characteristically enlarged and the chromosomes first spin out into long fine filaments (leptonema) and then conjugate in pairs (synapsis). They also probably replicate at this time or earlier. Growth of the oocyte occurs in this stage, and no further progress with regard to the reduction divisions occurs until full growth is attained. At that time the nucleus has become very large and is usually termed a germinal vesicle. Its large size is due mainly to its high content of nuclear sap. It usually contains one or more large nucleoli. It has been shown that the nuclear sap of the germinal vesicle of the oocyte of the newt *Triturus viridescens* contains as much DNA as the chromosomes, but that part of this DNA seems to be in the nucleoli (Izawa *et al.*, 1963). Furthermore, it has been shown that the Na^+ and K^+ content of the nucleus of oocytes of *Rana pipiens* is 3.2 and 2.4 times greater than that of the cytoplasm, respectively (Naora *et al.*, 1962).

These observations, although thus far limited to amphibian oocytes, are certainly of great interest, since they show that when the germinal vesicle breaks down at the onset of the meiotic divisions the cytoplasm is mixed with the content of the nucleoplasm, the composition of which is much different from that of the former. The nucleoli also disappear, and it is their disappearance that is in general the first visible indication of the ensuing breakdown of the germinal vesicle.

Concerning the question of the effect of the admixture, no chemical data are available as yet. Some old experiments of Delage (1901) suggest that this intermingling may indeed be very important, since his results showed that when an oocyte of *Asterias* is cut into two fragments before the breakdown of the germinal vesicle, only the nucleated part is able to respond to fertilization. On the other hand, if the operation is performed after the breakdown of the germinal vesicle, then both fragments respond to fertilization. These observations were confirmed by Wilson (1903) and Yatsu (1905) in *Cerebratulus* and by Costello (1940) in *Nereis*. In the experiments of Yatsu, formation of cytasters was obtained in the cytoplasm of nonnucleated egg fragments following treatment with calcium chloride only when they were derived from

oocytes in which the membrane of the germinal vesicle had already broken down.

These results are of importance, as they indicate that the breakdown of the germinal vesicle coincides with the initiation of a series of changes which are connected with the ability of the egg cytoplasm to respond to the activating stimuli. These changes were denoted by Delage (1901) as cytoplasmic maturation. Whether the breakdown of the germinal vesicle, with the subsequent mixing of the nucleoplasm with the cytoplasm, is the initiator of the changes leading to cytoplasmic maturation or, rather, whether the germinal vesicle breaks down as a response to the attainment of cytoplasmic maturation is unknown. It should, of course, be noted that in many groups of animals the egg is fertilized, and capable also of artificial activation, while in the stage with an intact germinal vesicle. This is not in disaccord with the results of the experiments that have been cited. It simply means that the ability to incorporate the sperm, or respond initially to artificial agents, is not dependent upon previous admixture of the contents of the germinal vesicle with that of the cytoplasm. However, further progress might well be dependent upon changes initiated by such admixture.

TABLE I
PERCENT CLEAVAGE OF EGGS OF THE TOADS *Bufo bufo asiaticus*^a AND *B. viridis*^a
AFTER TRANSPLANTATION OF NUCLEI FROM BLASTULAS OR EARLY GASTRULAS
INTO OOCYTES WITH OR WITHOUT THE GERMINAL VESICLE (GV) OR A PART
OF THE KARYOPLASM AND CYTOPLASM^b

Stage of host oocyte at time of nuclear transplant						
GV or karyoplasm + cytoplasm	Initial with intact GV	Before GV dissolu- tion (inertial progress to matura- tion by a few)	GV breaks down (ovulation onset)	GV breaks down (in- ertial prog- ress to matura- tion present)	After GV dissolu- tion	Control pricked after matura- tion
Present	70.6	74.6	67.7	—	—	9.5
Removed	1.0	7.3	—	53	63.2	—

^a The authors do not state how much of the data in this table is derived from one of the two species and how much from the other.

^b Dettlaff *et al.*, 1964.

Some recent experiments (Dettlaff *et al.*, 1964) with eggs of toads (*Bufo bufo asiaticus* and *B. viridis*) provide further evidence of an influence of the contents of the germinal vesicle on the ability of the egg

to develop. In these experiments nuclei of blastula cells were transplanted into oocytes from which the germinal vesicle had been removed, the procedures being essentially those now well developed for amphibian eggs (see Briggs and King, 1960). Such eggs fail to cleave, whereas the controls that are enucleated after dissolution of the germinal vesicle and receive transplanted blastula nuclei do so with the typical high frequency. Data pertaining to these and other experiments are given in Table I. Additional experiments that were made consisted of the injection of karyoplasm and cytoplasm, taken from oocytes during or after the dissolution of the germinal vesicle membrane, into oocytes at the initial stage. This causes the oocyte to "mature," and in a sense substitutes for the action of gonadotropic hormones which can induce maturation (and ovulation) of oocytes from pieces of ovary *in vitro* in amphibia (Tchou-Su and Wang Yu-lan, 1959).

Since in most species of animals the breakdown of the germinal vesicle occurs before or during release of the egg from the ovary, the process is not readily amenable to metabolic study. Also, the ensuing meiotic divisions are ordinarily not available for study apart from those species in which these divisions overlap fertilization. However, in groups of animals in which germinal vesicle breakdown normally occurs in the ovary, one often finds at the beginning and at the end of the spawning season a considerable number of eggs that are shed in the germinal vesicle stage; or eggs in this stage may be obtained by maceration of the excised ovary during the spawning season. In many instances such eggs can start and complete their maturation divisions in seawater or other external medium. Thus, respiration studies have been made on oocytes of sea urchins and of fish during their maturation while kept continuously in a microrespirometer (Lindahl and Holter, 1941; Nakano, 1953). In the case of both forms, it has been shown that the rate of oxygen consumption is high during the period of growth of the oocyte, increasing steadily during this time. As soon as maturation begins (i.e., coincident with the breakdown of the germinal vesicle), a sudden drop in oxygen consumption is observed (Fig. 1). This, however, does not necessarily imply that the rate of oxygen consumption in the mature egg is at a subnormal level; it shows only that the breakdown of the germinal vesicle marks the end of a metabolic phase characterized by high respiratory rate and the onset of a new phase with a considerably lower respiratory rate. Indeed, in the sea urchin egg fertilization is immediately followed by an increase in oxygen consumption, and hence it may be inferred that the respiration of the egg at the time of fertilization was below the rate necessary to meet the requirements of cell division. On the other hand, in the fish egg no such increase occurs at

fertilization, and, in fact, the rate of oxygen consumption begins to increase only at the blastula stage.

It will become clearer when the metabolic changes occurring at fertilization are discussed that changes of oxygen consumption, taken alone, are a rather poor criterion by which to evaluate metabolism.

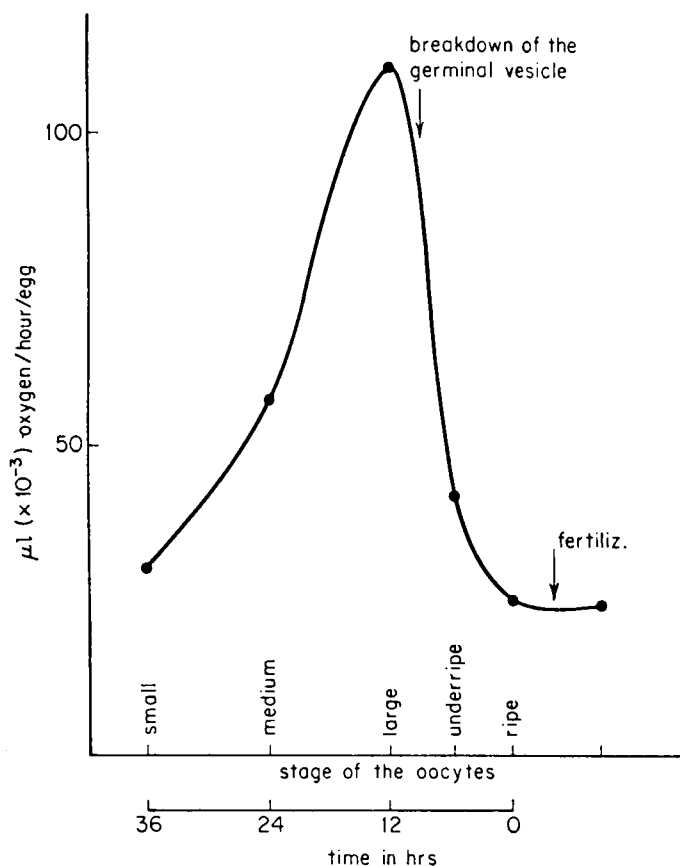


FIG. 1. Rate of oxygen consumption in the course of maturation and following fertilization in the egg of *Oryzias latipes*. (From Monroy, 1965, calculated from data of Nakano, 1953.)

They acquire value only when integrated with the study of the energy-yielding reactions.

In the fish *Oryzias latipes*, increase in incorporation of ^{32}P during oogenesis closely parallels the increase in respiration. However, incorporation into the acid-soluble P-fraction decreases, while incorporation into nucleic acids increases (Tsusaka and Nakano, 1965),

reflecting the active synthesis of nucleic acid during oogenesis, a subject to be discussed presently. It has been well established that there is synthesis of RNA during growth of the oocyte (see Brachet, 1960; Ficq, 1964; Esper, 1965).

B. GROWTH OF THE OOCYTE

In an earlier review (Tyler, 1955) of evidence obtained by autoradiographic, immunologic, and other methods it was concluded that much of the macromolecular material of the growing oocyte is manufactured in other cells of the body (nurse cells, liver, etc.) and supplied to the oocyte as a finished product. As a current illustration, one may refer to experiments on the effect of estrogen on the production of the yolk proteins lipovitellin and phosvitin in the fowl. These proteins are synthesized in the liver, in response to estrogen released by the hen's ovary, and transferred to the growing oocyte. The same proteins can also be made in the liver of a rooster upon injection of estrogen, thus adding to the evidence that the oocyte is not their site of manufacture (Schjeide and Urist, 1956). Apart from their interest in the present context the experiments (Carlsen et al., 1964) on the estrogen stimulation of synthesis of these proteins add interesting information concerning the mode of action; namely, that the stimulated liver cells show a great increase in the transfer RNA's for the amino acid serine. This amino acid comprises almost half of the residues of the phosvitin.

The growing oocyte does also synthesize some proteins endogenously, but this process takes place mainly in the early oocytes (Ficq, 1955). There have been no detailed quantitative studies on the basis of which one could specify the stage of growth of the oocyte at which protein synthesis is shut off. Probably the stage differs for the various proteins, but in any case it seems clear that endogenous synthesis of most of the egg proteins decreases as oogenesis proceeds, and probably stops a considerable period of time before full growth is attained. Studies (Piatigorsky *et al.*, 1966) with isolated oocytes that are occasionally shed in large numbers in sea urchins have shown these to be capable of active protein- and RNA-synthesis, both of which must be endogenous in this situation.

Generally, in animals the synthesis of RNA continues actively throughout oogenesis, although it is retarded in the later stages. Experiments with oocytes of frogs and starfish (see Ficq, 1964) have shown that there is a high molecular RNA that is synthesized in the nucleus during most of the growth period. By use of labeled 5-methylcytosine, a constituent of transfer RNA, as a precursor it appears that the new RNA that is found in the cytoplasm of the growing oocyte is of this

type. Concomitant experiments with labeled cytidine show it to be selectively incorporated into RNA of the nucleolus. This RNA is evidently ribosomal (see Edström *et al.*, 1961), and its synthesis continues throughout the growth period.

Similar results have been obtained in other animals by various investigators. For example, in the toad *Xenopus laevis* it has been shown that oogenesis is characterized by the active synthesis of considerable amounts of ribosomal RNA (Brown and Littna, 1964a,b; Davidson *et al.*, 1964). In particular, in the lampbrush chromosomes, which are supposed to be in a state of intense genetic activity, 90 percent or more of the RNA synthesized is of the ribosomal type (Davidson *et al.*, 1964). At the onset of the maturation divisions this synthesis ceases, although a small rate of synthesis of sRNA and of heterogeneous RNA (mRNA?) can still be detected. No further synthesis of ribosomal RNA takes place until the gastrula stage, whereas synthesis of heterogeneous, DNA-like RNA is found during cleavage stages (Brown and Littna, 1964a). These results indicate that until gastrulation no new ribosomes are manufactured by the embryo (i.e., all protein synthesis is carried out on the ribosomes which were synthesized during oogenesis). Consistent with this finding are those which indicate that definitive nucleoli first appear at the time of gastrulation and that a requirement for Mg^{++} appears at this time. An anucleolate mutant can, however, develop until early swimming stages, which is evidently when the lack of new ribosomes becomes critical.

C. RELATIVE INERTNESS OF THE MATURE UNFERTILIZED EGG

As noted above, the progress of oogenesis is accompanied by a decline in the ability of the egg to incorporate labeled amino acids into its proteins (see Ficq, 1964; Monroy and Maggio, 1964). Early experiments with sea urchins (Hultin, 1950, 1952; Hoberman, *et al.*, 1952) showed very little, if any, incorporation of labeled precursors into protein in the mature unfertilized egg. This is not due to lack of penetration of the labeled amino acids into the ripe eggs; indeed, the labeled amino acids do enter the pool of low molecular weight substances, but fail to be incorporated into proteins (Nakano and Monroy, 1958). Also, cell-free systems prepared from unfertilized eggs show this inertness with respect to incorporation of amino acids into protein (Hultin and Bergstrand, 1960). These experiments prove that in the mature unfertilized egg there is a block somewhere along the chain of reactions leading from amino acids to proteins.

In experiments with amphibian eggs, radioactive amino acids were injected into the cytoplasm of the mature egg to overcome the impermeability of the surface coat, but even so no incorporation occurred (Brachet, 1964).

The amphibian oocyte is shed and fertilized at the metaphase of its second maturation division. However, there does not appear to be any correlation between inhibition of protein synthesis and the stage of nuclear maturation reached at the time of fertilization. For example, a similar inhibited condition has been found in the eggs of starfish and of *Spisula*, which are shed in the germinal vesicle stage (Monroy and Tolis, 1964). Also, it is possible that the cessation of protein synthesis may occur some time earlier in the oocyte.

Actually, all types of eggs thus far examined, no matter at which stage of maturation they are fertilizable, appear to suffer from the same inhibition of their protein-synthesizing machinery. The following hypothesis may be considered to explain this phenomenon (Monroy and Tolis, 1964; Monroy, 1965).

In the course of oogenesis inhibitors build up in the oocyte, and these, upon reaching a certain threshold concentration, block the progress of maturation. The stage at which such a critical concentration is attained is different in different species, and hence the stage at which maturation is arrested within the ovary should indicate the moment when the inhibitors have reached threshold concentration.

How the inhibition is brought about in the maturing egg is still obscure. The inhibition may, in fact, depend on the formation of specific enzyme inhibitors, or it may be due to the upsetting of the equilibrium of key metabolic reactions, either as a result of the inhibition of an enzymatic intermediate step or of the excess production of a metabolite. On the other hand, it may be brought about by the formation of allosteric effectors; or, finally, it may depend on the alteration of the steric configuration of some proteins, whether endowed with enzymic activity or not. As far as we can judge from the data available at present, these possibilities are not mutually exclusive, and indeed it appears more and more likely that they may all converge in bringing about the inhibited condition of the unfertilized egg.

D. CYTOPLASMIC DNA

During the growth of the oocyte there is no evidence of incorporation of specific precursors (labeled thymidine) into DNA, either in the nucleus or in the cytoplasm. There is, however, evidence that nuclear

material and even whole cells may be incorporated into the growing oocyte in many species of animals (see Tyler, 1955). In addition, there have been a number of reports of the occurrence of DNA in the cytoplasm of the oocyte in amounts that far exceed that present in the germinal vesicle (see Brachet, 1962; Haggis, 1964; Monroy, 1965; Tyler and Tyler, 1966a).

The significance of the cytoplasmic DNA is still somewhat obscure; in fact, the exact amount and properties have been subject to uncertainty. This is mainly because of the great excess of cytoplasmic materials, and especially of substances (polysaccharides and ribonucleic acids) that interfere with the determinations.

In some more recent experiments (Pikó and Tyler, 1965) an attempt was made to circumvent some of the difficulties by use of CsCl-buoyant density centrifugation on sea urchin egg homogenates prepared in a direct manner (Kay, 1964). The determinations for the sea urchins *Lytechinus pictus* and *Strongylocentrotus purpuratus* give values of not more than 13 and 8 times the haploid amount, respectively. Since determinations made on the isolated nuclei (Hinegardner, 1961) give the haploid amount, as determined on sperm, the excess must be cytoplasmic. Values of this same order have been obtained in another species of sea urchin by a fluorometric method (Baltus *et al.*, 1965). These values are considerably less than those reported in many of the earlier experiments. It is even possible that it may all be mitochondrial. For example, if one assumes 0.05% for the mitochondrial content of DNA and uses a value of 8.4% (Shaver, 1956) for the volume occupied by mitochondria in an egg ($6.7 \times 10^5 \mu^3$) of *Lytechinus*, this yields 28 per gram, which is more than enough to account for the experimentally determined values.

Experiments with ^{14}C -labeled actinomycin (Brachet and Ficq, 1965), which binds to DNA, have enabled a preliminary localization to be made of the cytoplasmic DNA of amphibian oocytes. In these experiments the labeled actinomycin has been found associated with organelles that sediment at low centrifugal forces, which do not affect the distribution of the label in the germinal vesicle. The nucleoli failed to bind significant amounts of the labeled actinomycin.

That cytoplasmic DNA may have some function appears from the results of two recent investigations. In one of these (Baltus *et al.*, 1965) it was reported that artificially activated nonnucleate fragments of sea urchin eggs incorporate uridine into RNA, and this incorporation is inhibited by treatment with actinomycin-D. In the other investigation (Shmerling, 1965) it was reported that cytoplasmic DNA, extracted from sturgeon oocytes, can act as a primer for DNA and for RNA synthesis.

III. Relation of Activation to Fertilization

A. ARTIFICIAL PARTHENOGENESIS

From the time of the discovery of artificial parthenogenesis at the turn of the century many investigators have hoped to find, by investigation of physical and chemical activating agents of various sorts, the particular agent that the sperm employs to induce activation of the egg. This hope has not, as yet, been realized. The subject has not been actively pursued in recent years, and most of the pertinent investigations are found in older reviews (see Loeb, 1913; Morgan, 1927; Dalcq, 1928; Tyler, 1941).

There have been no consistent, substantiated demonstrations of the extraction from sperm of a specific substance capable of regularly inducing activation of the egg. Among various agents that have been tested for activating action, some special interest attaches to the use of antibodies directed against materials of sea urchin eggs. It has been reported (Perlmann, 1959; Baxandall *et al.*, 1964) that development can be initiated by treatment of the eggs with rabbit antisera prepared against homogenates of the unfertilized eggs. The antisera are reported to be active after absorption with various egg materials other than those which may contain what has been termed the "activation antigen." The latter might be considered an inhibiting agent whose neutralization by specific antibody permits development to proceed. Attempts have been made (Tyler, 1959, 1963; Tyler *et al.*, 1961) to repeat these findings with sea urchin eggs, but without success, no changes typical of artificial parthenogenesis having been obtained.

On the other hand, one may interfere with progress of development quite readily by means of specific antisera. For this action it has been discovered (Tyler and Brookbank, 1956; Tyler, 1959, 1963) that antibodies prepared against purified jelly coat substance (fertilizin) are highly effective. The suggested interpretation of this inhibition is that fertilizin, or a substance bearing similar antigenic groups, is a constituent of the plasma membrane which thus reacts with, and is disrupted by, the antibodies. In fact, antisera prepared against whole egg homogenates and absorbed with fertilizin lose most, if not all, of their anti-developmental action, despite a high content of precipitating antibodies directed against internal constituents of the egg (Tyler, 1963). It appears that for the inhibiting action antibodies must be present that are directed against a constituent of the plasma membrane. At the same time, it appears that the particular antigenic groups against which the antibodies are directed may be common to other substances in the sea

urchin egg. Thus, it has been found (Brookbank and Yonge, 1964) that ribosomal preparations from unfertilized eggs can absorb the cleavage-blocking activity from antisera prepared in rabbits against purified fertilizins of *Lytechinus* or of *Arbacia*.

In these experiments there is as yet no evidence that the antibodies intervene directly in the machinery of activation and development. Their inhibiting action may simply be due to an effect on the egg's plasma membrane that adversely affects permeability properties. However, the possibility of more direct action is not excluded, and, in any case, specific antibodies may prove of further value with cell-free systems to aid in identification of changes that occur upon activation.

The blocking of fertilization itself has been accomplished in many experiments (see Metz, 1961; Metz *et al.*, 1964; Shaver and Barch, 1960; Shivers and Metz, 1962; Tyler, 1963, 1964) with antisera prepared against egg materials, and particularly the surface constituents, fertilizin and antifertilizin of egg and sperm, respectively. However, this effect is evidently a result of failure of the gametes to adhere to each other because of the presence of antibody on one or the other of these receptor substances. So far there is no evidence that these antibodies interfere with any particular parts of the activation process itself.

B. FERTILIZATION OF FERTILIZED EGGS

An interruption of the activation process was achieved a number of years ago in eggs of *Urechis caupo* by treatment with slightly acidified (pH 7) seawater within 3 minutes after insemination. The spermatozoon completed its entrance into the treated eggs, a process requiring about 4 minutes in this species, but the elevation of the vitelline membrane and other visible changes did not ensue. When returned to normal seawater within about 15 minutes, these eggs could resume development, but after that time they would not unless they were refertilized or activated parthenogenetically (Tyler and Schultz, 1932).

Refertilization has been achieved (Sugiyama, 1951) in sea urchins by another kind of treatment; namely, mechanical removal of the fertilization membrane and exposure of the eggs to calcium- and magnesium-free seawater. Eggs treated in this manner may be refertilized as late as the two-cell stage, or later. This kind of treatment can also be used for the fertilization of eggs that had been artificially activated (Ishida and Nakano, 1950; Nakano, 1954). A modified method (Hagström and Hagström, 1954a,b) consists in trypsin treatment to inhibit fertilization membrane formation, followed by treatment with calcium- or magnesium-free seawater, or both.

Refertilization without the use of calcium- and magnesium-free seawater was accomplished (Tyler *et al.*, 1956) in the sea urchins *Lytechinus pictus* and *L. variegatus* within a period of about $\frac{1}{2}$ hour after the initial fertilization. After this time the hyaline material that is formed on the surface of the plasma membrane evidently constitutes a barrier. However, its removal by calcium- and magnesium-free solutions permits refertilization at the later stages.

Except for the experiments with *Urechis*, the refertilization experiments do not involve an interruption of the activation processes at any stage. Development is in progress in these eggs when they are refertilized. The experiments add to the evidence of the separability of the processes concerned with attachment and engulfment of the sperm and those concerned with "turning on" the developmental machinery within the egg. The experiments also contribute to our understanding of the mechanism of sperm entry and establishment of the block to polyspermy, but these subjects are outside the scope of this chapter.

IV. Respiratory Metabolism of the Fertilized Egg

A. QUANTITATIVE CHANGES

The only egg for which, thus far, some detailed information is available concerning its metabolic conditions before and after fertilization is that of the sea urchin. This discussion will, therefore, be based mainly on the results obtained with this material. From what follows it will become clear that a comparative analysis involving several forms would be most illuminating and is indeed badly needed.

It was discovered by Warburg (1910) that in the sea urchin egg fertilization is immediately followed by an increase in oxygen consumption, and this result has been obtained by many subsequent investigators. However, in one series of investigations (Borei, 1948, 1949) it was reported that eggs that are fertilized immediately after being ovulated fail to show the rise, which is, then, the result of a decrease in rate of respiration that the unfertilized eggs undergo on standing. Earlier experiments (Tyler *et al.*, 1938) with another species, *Arbacia punctulata*, did not reveal this decrease, the rate remaining rather constant for periods up to several days as long as no appreciable bacterial growth occurred. In these experiments a rapid initial decrease could have been missed. However, in recent experiments (Yasumasu and Nakano, 1963; Ohnishi and Sugiyama, 1963; Horwitz, 1965) on four other species, *Pseudocentrotus depressus*, *Hemicentrotus pulcherrimus*, *Anthocidaris crassispina*, and *Arbacia punctulata*, starting very shortly after the eggs were shed, oxygen uptake was found to proceed at a

steady low rate and to show the typical large increase upon fertilization, regardless of just when fertilization occurred.

Actually, the respiratory change is more complex than was originally thought. Within a few seconds after sperm penetration there occurs a sudden burst of oxygen consumption (Laser and Rothschild, 1939; Ohnishi and Sugiyama, 1963) which lasts only 2-3 minutes. Then the respiratory rate returns almost to the prefertilization level (actually, it is always a little higher), and after a few minutes begins to increase again. The initial transient respiratory increase is coeval with the formation of the fertilization membrane and with the complex series of surface changes which, in the sea urchin egg, are known to accompany fertilization. These involve drastic changes in the structure of the egg surface, which are represented at the physiological and biochemical levels by changes in permeability (R. S. Lillie, 1916; Ishikawa, 1954; Whiteley and Chambers, 1960), by transient changes in the electrical characteristics of the egg surface (Tyler *et al.*, 1956; Hiramoto, 1959a,b), and by changes in the rate of exchange of potassium (Tyler and Monroy, 1959), and a few other properties (see Monroy, 1965; Tyler and Tyler, 1966b, for reviews).

Whether or not the first transient respiratory change is also linked to or is dependent on the surface changes is difficult to say. The only piece of circumstantial evidence is the finding that in the starfish egg, in which the rate of oxygen consumption does not change for a long time after fertilization (at least until the first cleavage), but in which surface changes similar to those of the sea urchin egg occur (Millonig and Bosco, 1964), fertilization is also accompanied by a transient outburst of oxygen consumption (Horwitz, 1965). The respiratory rate then reverts permanently to the prefertilization level. On the other hand, in the eggs of *Spisula* both the structure of the surface (Rebhun, 1962) and respiration remain unchanged at fertilization (Horwitz, 1965; and data in Table II).

Great emphasis was placed at one time on the respiratory changes of the egg at fertilization. Indeed, it was thought that the primary event of fertilization was the release of the egg from a condition of asphyxia. Starting particularly with the work of Whitaker (1933a,b), extensive comparative studies have, however, shown that the respiratory response to fertilization varies considerably among the eggs of various animal species. As indicated in Table III, in the eggs of some animals fertilization is accompanied by an increased respiration, in others no change is detectable, and in others even a temporary drop in respiration occurs. A correlation between the direction of the change and the stage of nuclear maturation at the time of fertilization does not seem to exist.

TABLE II
CHANGES IN RATE OF OXYGEN CONSUMPTION IN *Arbacia*, *Asterias*, AND *Spisula* EGGS
DURING THE FIRST 6 MINUTES AFTER FERTILIZATION^{a,b}

Species	1 ^c	2 ^d	3 ^e
<i>Arbacia</i>	1.0	2.85	1.75
<i>Asterias</i> (fertilized before first polar body extruded)	1.0	1.98	1.05
<i>Asterias</i> (fertilized after first polar body extruded)	1.0	1.85	0.99
<i>Spisula</i>	1.0	—	0.98

^a Minimum difference (Duncan) for significance: 5 % = 0.45, 1 % = 0.59; standard error = 0.133.

^b From data of Horwitz, 1965.

^c Theoretical ratios: (Rate of O₂ consumption of fertilized eggs)/(Rate of O₂ consumption of unfertilized eggs) expected if fertilization has no effect on the rates of oxygen consumption.

^d Ratio: Fertilized/Unfertilized during the time interval 0.5–1.5 minutes after fertilization.

^e Ratio: Fertilized/Unfertilized during the time interval 3–6 minutes after fertilization.

As already noted, direct measurements of the rate of oxygen consumption are not very informative. What is needed is information about the metabolic pathways underlying the respiratory changes, especially as related to energy-yielding reactions. Here again very little is known about such reactions in eggs of animals other than the sea urchin.

B. QUALITATIVE CHANGES

There has been uncertainty for some time as to whether or not the cytochrome system is involved in the respiration prior to fertilization. Early experiments (Runnström, 1930) showed an apparent inability of potassium cyanide and of carbon monoxide to inhibit the respiration of unfertilized sea urchin eggs. However, the apparent lack of inhibitory action of potassium cyanide was later shown (Robbie, 1946) to be due to its rapid absorption by the alkali in the respiration flasks. With regard to photo-reversible inhibition by carbon monoxide, this is also demonstrable (Rothschild, 1949; Rothschild and Tyler, 1958) when certain complicating factors are taken into account. One of these factors is a depression of the respiration of the unfertilized egg by the intense light which is used to reverse carbon monoxide inhibition. Another factor that was discovered (Black and Tyler, 1959a,b) in sea urchins and in the gephyrean worm *Urechis* is the ability of the unfertilized egg to oxidize the carbon monoxide itself. This property becomes progressively less significant after fertilization as the rate of respiration increases (Table IVa,b).

TABLE III
RATIO OF O₂ UPTAKE OF FERTILIZED (f) TO THAT OF UNFERTILIZED (u) EGGS^{a,b}

Stage of maturation at the time of fertilization	Organism	$\frac{-O_2 f}{-O_2 u}$	Author
Germinal vesicle	<i>Nereis succinea</i>	1.3	Barron, 1932
	<i>Macra lateralia</i>	1.8	Ballentine, 1940
	<i>Urechis caupo</i>	1.2	Tyler and Humason, 1937
	<i>Nereis limbata</i>	1.35-1.45	Whitaker, 1931c
	<i>Asterias glacialis</i>	1.0	Whitaker, 1933b
Metaphase of the first maturation division	<i>Cumingia tellinoides</i>	0.45	Whitaker, 1931b
	<i>Chaetopterus variopedatus</i>	0.53	Whitaker, 1933a; Brachet, 1938
	<i>Marthasterias glacialis</i>	1.0	Borei, 1948
	<i>Saxostrea commercialis</i>	1.0	Cleland, 1950
	<i>Sabellaria alveolata</i>	1.1	Fauré-Fremiet, 1922
	<i>Ciona intestinalis</i>	1.0	Holter and Zeuthen, 1944
	<i>Ciona intestinalis</i>	1.5	Tyler and Humason, 1937
	<i>Ciona intestinalis</i>	2.0	Lentini, 1961
	<i>Phallusia mamillata</i>	2.0	Lentini, 1961; Minganti, 1957
	<i>Rana platyrhina</i>	1.0	Zeuthen, 1944
	<i>Bufo bufo</i>	1.0	Stefanelli, 1938
Metaphase of the second maturation division	<i>Fundulus heteroclitus</i>	16.7	Boyd, 1928
	<i>Fundulus heteroclitus</i>	1.0	Philips, 1940
	<i>Oryzias latipes</i>	1.0	Nakano, 1953
	<i>Fucus vesiculosus</i>	1.9	Whitaker, 1931a
	<i>Strongylocentrotus purpuratus</i>	3.7	Tyler and Humason, 1937
	<i>Psammenechinus miliaris</i>	3.6	Borei, 1948
	<i>Paracentrotus lividus</i>	4.7	Brock <i>et al.</i> , 1938
	<i>Arbacia punctulata</i>	4.5	Ballentine, 1940
	<i>Dendroaster excentricus</i>	3.0	Tyler and Humason, 1937

^a The ratio of O₂ uptake, fertilized eggs (−O₂ f)/O₂ uptake, unfertilized eggs (−O₂ u) in various organisms is ordered in groups according to the stage of maturation when fertilization takes place.

^b From a table of Rothschild, 1956, with additions as in Monroy, 1965.

TABLE IVa
RATES OF RESPIRATION AND OF CO OXIDATION IN 80 % CO/O₂ IN LIGHT AND DARK
BY EGGS OF *Urechis caupo* AT EARLY AND LATER STAGES OF DEVELOPMENT^a

Experi- ment	Period of development (hours after fertilization)	Gas uptake ^b			Excess gas uptake ^b		CO oxidized from determinations of ¹³ C/ ¹² C by mass spectrometry ^b	
		In air (light)	In 80 % CO/O ₂		in 80 % CO/O ₂		Light	Dark
			Light	Dark	Light	Dark		
1	1-8	94	150	125	+56	+31	43.2	21.4
2	20-25	663	839	476	+176	-187	105.6	11.7
3	24-30	724	789	395	+65	-329	89.9	4.6
4	24-30	551	593	327	+42	-224	75.8	5.8
5	52-55.5	853	872	384	+19	-469	65.0	6.6
6	52-55.5	792	815	358	+23	-434	60.6	4.8
7	54-57.5	847	883	384	+36	-463	57.5	4.1

^a Black and Tyler, 1959a.

^b Measured in mm³/10⁶ eggs/hour.

TABLE IVb
RATES OF RESPIRATION AND OF CO OXIDATION IN LIGHT AND DARK BY EGGS OF
Strongylocentrotus purpuratus, UNFERTILIZED AND AT EARLY AND LATER
STAGES OF DEVELOPMENT^a

Experi- ment	Period of development (hours after fertilization)	Gas uptake ^b			Excess gas uptake ^b		CO oxidized from deter- minations of ¹³ C/ ¹² C by mass spectrometry ^b	
		In air (light)	In 80 % CO/O ₂		in 80 % CO/O ₂ ^b		Light	Dark
			Light	Dark	Light	Dark		
1	0 (u)	16.0	29.9	28.5	+13.5	+12.3	8.9	8.0
2	0 (u)	22.5	40.9	37.5	+18.4	+15.0	13.4	10.6
3	1-5	69.9	97.4	88.3	+27.5	+18.4	20.5	9.9
4	1-7	81.9	118.4	105.0	+36.5	+23.1	24.1	10.8
5	26-30	166.0	190.0	132.0	+24.0	-34.0	25.0	3.4
6	27.5-31.5	200	238	203	+38.0	+3.0	25.0	9.8
7	46-50	211	242	164	+31.0	-47.0	24.4	3.3
8	49-53	264	216	216	+57.0	-46.0	34.0	6.4

^a Black and Tyler, 1959a.

^b Measured in mm³/10⁶ eggs/hour.

Additional evidence that the oxidative processes are qualitatively similar in unfertilized and fertilized eggs is provided by the similarity of their temperature coefficients (Tyler and Humason, 1937; Borei and Lybing, 1949). To this may be added the evidence (Cleland and Roth-

schild, 1952a,b; Ycas, 1954) of the presence of most of the enzymes for the normal glycolytic and respiratory systems. Finally, the presence of a complete cytochrome system in the unfertilized and fertilized sea urchin eggs has been shown by the study of the difference spectra of

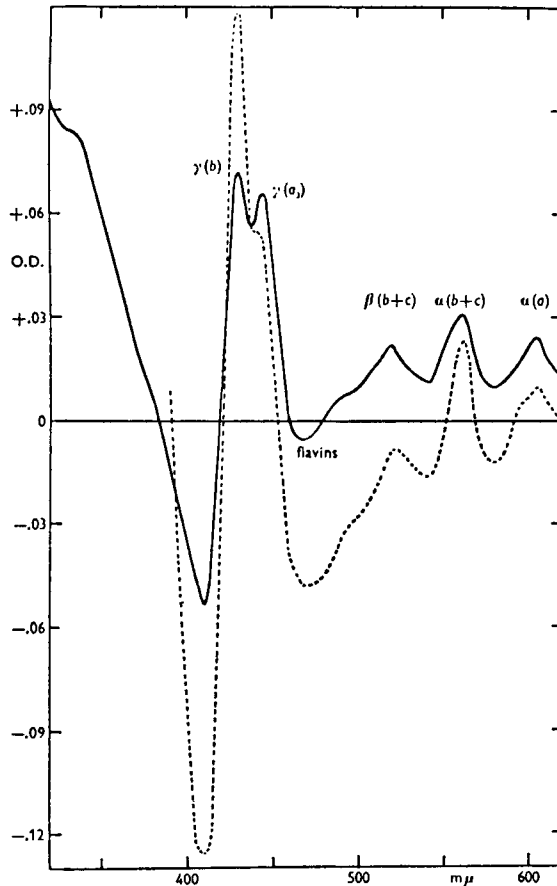


FIG. 2. Difference spectra (reduced minus oxidized) of a mitochondrial suspension from unfertilized eggs of *Sphaerechinus granularis*. Solid line: reduction with succinate; dotted line: reduction with dithionite. (From Maggio and Ghiretti-Magaldi, 1958.)

mitochondria suspensions (Maggio and Ghiretti-Magaldi, 1958) (Fig. 2). It appears, then, that the differences between fertilized and unfertilized eggs must involve factors other than change in respiratory pathway.

In the unfertilized sea urchin egg there is an unbalanced condition in the level of the adenine nucleotides, the relative concentration of ATP being high, whereas that of ADP is low. Within a few minutes after

fertilization the concentration of the former drops, while that of the latter increases, thus reestablishing the ratio normally found in all actively growing cells (Rossi *et al.*, 1965). As it is known that the concentration of the high energy phosphate acceptors is the regulatory factor in respiration (Lardy and Wellman, 1952), the low level of ADP may account for the low level of oxygen consumption of the unfertilized egg. Also, it has been known for some time that in the sea urchin egg within a few minutes after fertilization the breakdown of a polysaccharide fraction occurs (Örström and Lindberg, 1940; Monroy

TABLE V
CONCENTRATION OF GLUCOSE 6-PHOSPHATE (G-6-P), FRUCTOSE DIPHOSPHATE (F-dP),
AND TRIOSEPHOSPHATE (Tri.P) IN UNFERTILIZED AND NEWLY FERTILIZED EGGS
OF *Arbacia lixula* AND *Paracentrotus lividus*^{a,b}

		G-6-P	F-dP	Tri.P
<i>Arbacia</i>	(u)	0.053		
	(f) 5 min	0.260		
	(u)	0.055		
	(f) 10 min	0.470		
	(u)	0.076	0.003	0.007
	(f) 10 min	0.166	0.014	0.023
<i>Paracentrotus</i>	(u)		0.002	0.002
	(f) 5 min		0.039	0.043
	(u)	0.068		
	(f) 8 min	0.456		
	(u)	0.063		
	(f) 12 min	0.408		

^a Values are expressed as μ moles/100 mg of total N of eggs.

^b Monroy, 1965, from data of Aketa *et al.*, 1964.

and Vittorelli, 1960). At the same time, the concentration of the hexose phosphates, which is exceedingly low in the unfertilized egg, increases considerably (Table V) (Aketa *et al.*, 1964).

The very low level of hexose phosphates may be a limiting factor in the oxidative utilization of carbohydrates, and of respiration as a whole. This result has been interpreted as indicating that, in the unfertilized egg, the phosphorylase reaction is inhibited. This, indeed, is probably the most important step proceeding to glycogenolysis (Axelrod, 1960), and its activation upon fertilization should hence be one of the key reactions in the metabolic activation of the egg. The hexose monophosphate shunt, which was already known to be the main pathway of carbohydrate utilization in the sea urchin embryo (Lindberg and Ernster, 1948), may thus be activated immediately after fertilization. The synthesis of

TPNH is linked to the operation of the shunt, and, indeed, within 30-40 seconds after fertilization a three- to fivefold increase of TPNH has been observed. It is likely that this occurs at the expense of DPN, which at the same time undergoes a parallel decrease (Krane and Crane, 1960; 1963; Epel, 1964a). Simultaneous measurements of TPNH formation and respiration have shown that the increase of the former precedes by a short but significant time the beginning of the first transient increase of respiration. It has been suggested (Epel, 1964b) that the increased

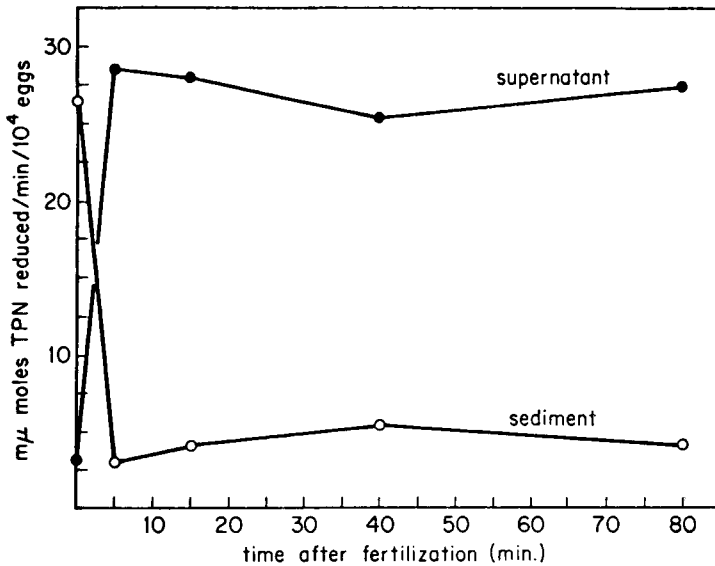


FIG. 3. Release of glucose 6-phosphate dehydrogenase from the insoluble into the soluble fraction upon fertilization in eggs of *Hemicentrotus pulcherrimus*. The sediment and supernatant from the homogenate were centrifuged at 24,000 g. (From Monroy, 1965, calculated from data of Isono, 1963.)

oxygen consumption may be accounted for as being due to an increase in ATP utilization for the synthesis of TPNH and TPN from DPN. It would, of course, be of interest to learn whether or not this synthesis occurs in eggs that show a decrease in O₂ uptake upon fertilization.

An increased activity of some enzymes connected with the function of the pentose phosphate cycle (G-6-P dehydrogenase, Isono, 1963; Isono, *et al.*, 1963; 6-phosphogluconate dehydrogenase, Backström, 1963), as well as of aldolase (Ishihara, 1957, 1958a,b), has been observed in sea urchin eggs soon after fertilization. The process whereby G-6-P dehydrogenase is activated is of some interest. In the unfertilized egg the enzyme is recovered from homogenates in a fraction sedimenting at

low speed; 5 minutes after fertilization it is found in the supernatant (Isono, 1963) (Fig. 3). A further interesting observation is that *in vitro* release of the enzyme from the bound form may be caused by increasing the ionic strength of the medium. In particular, the authors (Isono *et al.*, 1963) draw attention to the possible role of potassium, which, as will be discussed later, is released from a compartmentalized to an ionic condition immediately after fertilization.

These observations have some additional important general implications. They in fact suggest a process whereby inhibition and activation of enzymes may occur in the course of oogenesis and as a result of fertilization. Inhibition may indeed occur by a kind of compartmentalization, consisting in the binding of the enzymes to a cell structure (formation of lysosomelike structures?) and activation as a result of the breakdown of the compartment. The role of ions may be preeminent in this process.

V. RNA and Protein Synthesis

A. DEMONSTRATION OF STIMULATION OF PROTEIN SYNTHESIS PROVIDED BY FERTILIZATION

The inefficiency of the energy-yielding reactions in the unfertilized egg might be considered responsible for the blockage of all the energy-requiring processes. Among the latter one of the most important is certainly protein synthesis. Although it cannot be ruled out that the low metabolic condition of the unfertilized egg may play a role in inhibiting protein synthesis, there is now good evidence that the inhibition depends on some condition in the protein-synthesizing machinery itself.

As already mentioned, application of isotopic techniques to the problem of fertilization has shown that in the unfertilized egg the rate of incorporation of labeled precursors into the proteins is exceedingly small, and that fertilization is followed by an immediate increase of this rate (Hultin, 1950, 1952; Hoberman *et al.*, 1952; Nakano and Monroy, 1958). The same effect is obtained following parthenogenetic activation (Nakano *et al.*, 1958). That the sudden change in the rate of incorporation observed at fertilization does not depend on the greater access externally added amino acids may have to the cytoplasm of the egg is shown by the experiments already mentioned in which unfertilized eggs were loaded with radioactive amino acids, either by microinjection (Brachet, 1964) or by administration to the mature female (Nakano and Monroy, 1957). In both cases no incorporation into the proteins of unfertilized eggs was observed, but upon fertilization significant incorpora-

tion began within a few minutes (Nakano and Monroy, 1958; Monroy, 1960). These experiments prove that the inability of the unfertilized egg to carry out protein synthesis depends on some block residing within the egg itself. A priori, this inhibition may be at any place along the assembly line leading from the amino acids to the peptide chain. That the inhibition is not attributable to a block to entrance into the unfertilized egg is further illustrated in experiments (Mitchison and Cummins, 1966; Tyler *et al.*, 1966) in which uptake of amino acids as well as incorporation into protein were measured.

B. BASIS FOR THE RELATIVE INERTNESS OF THE UNFERTILIZED EGG

The amino acid-activating enzymes are present and functioning in the unfertilized egg (Scarano and Maggio, 1957). Although the activity is admittedly fairly low, it does not significantly increase until the blastula stage (Maggio and Catalano, 1963), when protein synthesis is already quite active. It therefore seems justified to assume that the activity of this system, however low, is sufficient to meet the requirements of the embryo for quite a while. Further evidence that this system is not responsible for the inability of the unfertilized egg to accomplish protein synthesis is furnished by the following experiments. Whole homogenates or ribosomes from unfertilized eggs exhibit very little if any ability to incorporate amino acids into proteins, and this situation is not changed by the addition of rat liver cell sap (Hultin and Bergstrand, 1960). Homogenates of fertilized eggs, on the contrary, are quite active in promoting such incorporation, and addition of cell sap from unfertilized eggs does not influence the result (Hultin, 1961). A similar result was obtained with the use of isolated ribosomes. Addition of supernatants from the fertilized eggs to unfertilized egg ribosomes was reported to be ineffective in stimulating incorporation of amino acids into proteins (Hultin, 1961). Ribosomes from unfertilized eggs were also unable to utilize sRNA-bound activated amino acids, which again were readily utilizable by ribosomes of fertilized eggs (Yasumasu and Koshihara, 1963).

These experiments strongly point to the ribosomes as being responsible for the lack of protein-synthesizing ability of the unfertilized egg. However, following the discovery that a synthetic RNA, polyuridylic acid (polyU), can stimulate incorporation of the amino acid phenylalanine into polypeptide in cell-free systems prepared from bacteria (Nirenberg and Matthaei, 1961), this was tested on cell-free systems prepared from unfertilized eggs. It was found (Tyler 1962, 1963; Nemer 1962; Nemer and Bard, 1963; Wilt and Hultin, 1962) that polyU stimulated phenylalanine incorporation into protein in the unfertilized

egg cell-free system. While differing in detail, these reports were in general accord in showing also that the magnitude of the polyU-stimulated incorporation was as great for the preparations from unfertilized eggs as for those from fertilized eggs and embryos (Table VI). These experiments showed, then, that the ribosomes of the unfertilized egg were not incompetent, at least as far as this synthetic mRNA was concerned. The results implied that the inactivity of the unfertilized egg was attributable to a lack of mRNA. This proposition was investigated in further experiments.

TABLE VI
INFLUENCE OF POLYURIDYLIC ACID (POLY U) ON INCORPORATION OF
 ^{14}C -L-PHENYLALANINE INTO PROTEIN WITH HOMOGENATES AND
SONICATES OF EGGS AND EMBRYOS OF *Lytechinus pictus*^{a,b}

Egg preparation	cpm ($-t_0$) ^c		Difference in cpm
	Without polyU	With polyU	
Homogenates:			
Unfertilized eggs	53, 56 Avg. 55	987, 916 Avg. 952	897
Blastulas (just hatching)	247, 261 Avg. 268	903, 891 Avg. 897	629
Sonicates:			
Unfertilized eggs	34, 40 Avg. 37	849, 888 Avg. 869	832
Blastula (just hatching)	123, 244 Avg. 124	1049, 1014 Avg. 1032	908

^a Incubation mixtures = 0.225 ml homogenate or sonicate (derived from 1.5×10^5 eggs in 0.01 M Tris, 0.01 M MgAc); 0.275 ml of Reaction Mixture (0.8 ml M/8 PEP; 0.1 ml of 0.0038 M ^{14}C -L-phenylalanine at 9.8 curies/mole; 0.1 ml M/10 ATP, with or without polyU at 0.08 M calculated as uridylic acid).

^b Tyler, 1963.

^c Blanks = 37, 40, 36, 34; t_0 's = 50, 73, 79, 67.

First the state of the ribosomes was examined. According to current evidence (see Rich *et al.*, 1963), it is not the single ribosomes that are the sites of protein synthesis in cells but assemblages of several that are held together by a strand of mRNA; the larger the strand, the more ribosomes are grouped in the "polysome" complex. Experiments with the sea urchins *Lytechinus pictus* and *Paracentrotus lividus* (Monroy and Tyler, 1963) showed that in the unfertilized egg the ribosomes are present almost exclusively as single units, whereas after fertilization, and with the progress of development, increasing numbers of polysomes appear (Table VII). Concomitant experiments with labeled amino acids

TABLE VII
APPROXIMATE POLYSOME/MONOSOME RATIOS AS RATIO OF SUM OF OD₂₆₀ READINGS IN POLYSOME REGION TO THAT IN THE MONOSOME REGION OF FRACTIONS COLLECTED AFTER SUCROSE-DENSITY GRADIENT CENTRIFUGATION OF HOMOGENATES OF SEA URCHIN EGGS AND EMBRYOS^a

<i>Lytechinus pictus</i>				<i>Paracentrotus lividus</i>			
Experi- ment No.	Stage	Treatment	Polys. Monos.	Exp. No.	Stage	Treatment	Polys. Monos.
2	Unfertilized	—	0.14	14	Unfertilized	—	0.27
	Fertilized (40 min.)	—	0.23		Fertilized (1 hr)	—	0.32
28 ^b	Unfertilized	—	0.15	21	Unfertilized	—	0.43
3	Unfertilized	—	°0.08, 0.11, +0.16		Fertilized (1 hr)	—	0.93
4	Early blastula	—	°0.44, 0.40, +0.53		Fertilized (1 hr)	RNase	0.59
24	Early blastula	—	+0.38, +0.42, +0.40	20	Two-cell	—	0.57
25	Hatching blastula	—	0.45, 0.45	15	Mes. blastula	—	1.02
11	Hatched blastula	—	0.45		Mes. blastula	RNase	0.52
	Hatched blastula	RNase	0.29	16	Mes. blastula	—	1.02
	Hatched blastula	DNase	0.46	17	Early gastrula	—	1.39
26 ^b	Hatched blastula	—	°0.68		Early gastrula	RNase	0.65
	Hatched blastula	RNase	°0.29	19	Early gastrula	—	1.55
	Hatched blastula	DNase	°0.76	23	Midgastrula	—	1.29
9	Mes. blastula	—	0.68				
12	Mes. blastula	—	0.88				
28 ^b	Mes. blastula	—	0.95				
	Mes. blastula	RNase	0.67				
29 ^b	Mes. blastula	—	0.95				
	Mes. blastula	RNase	0.63				
30 ^b	Prism larva	—	0.98				
	Prism larva	RNase	0.78				
	Prism larva	DNase	0.99				

^a Monroy and Tyler, 1963.

^b Data collected by Mr. J. Piatigorsky: (°) no deoxycholate; (+) 1.5% deoxycholate; 0.5% deoxycholate in all others.

showed protein synthesis to take place primarily in association with the polysomes. These experiments have been extended (Stafford *et al.*, 1964) to other species of sea urchins and provide further evidence of the importance of polysome formation in the increase in protein synthesis upon fertilization and development. They are consistent with the above-mentioned experiments which indicated that the inertness of the unfertilized egg is attributable to the fact that mRNA is not available to the ribosomes. This does not, however, necessarily mean that no mRNA is present in the mature unfertilized egg and that it is synthesized after fertilization. It could mean, alternatively, that mRNA is present but for some reason prevented from interacting with the ribosomes.

The decision between these alternative interpretations was provided by two kinds of experiments with sea urchin eggs. In one (Gross and Cousineau, 1963, 1964) a "chemical enucleation" was performed by treatment of the eggs with actinomycin D. Upon fertilization these eggs are able to carry out protein synthesis in spite of an almost complete abolition of RNA synthesis.

In the other type of experiment nonnucleated fragments were produced by the centrifugation method (see Harvey, 1956) and activated parthenogenetically. In one series (Tyler, 1963; Denny and Tyler, 1964) tests of amino acid incorporation into protein were performed with the intact nonnucleated fragments (and control nucleated cells) and with homogenate systems prepared from them, the measurements being done by scintillation counting (Table VIIIa,b). In the other series (Brachet, *et al.*, 1963) the experiments were done with the intact fragments and incorporation was followed autoradiographically. In both series the

TABLE VIIIa
INCORPORATION OF ^{14}C -L-VALINE INTO PROTEIN OF HOMOGENATES OF ARTIFICIALLY
ACTIVATED EGG FRAGMENTS OF *Strongylocentrotus purpuratus*^a

Fragment	Counts per minute (cpm) ^b		
	Untreated	Treated ^c	Activation (%)
Nonnucleated	19	98	50
	17	92	50
Nucleated	19	91	100
	($t_o = 45$)		
	14	94	100
	($t_o = 50$)		

^a Tyler, 1963.

^b 0.075 ml packed egg fragments; background = 32 cpm.

^c One minute in 5×10^{-3} M butyric acid in seawater.

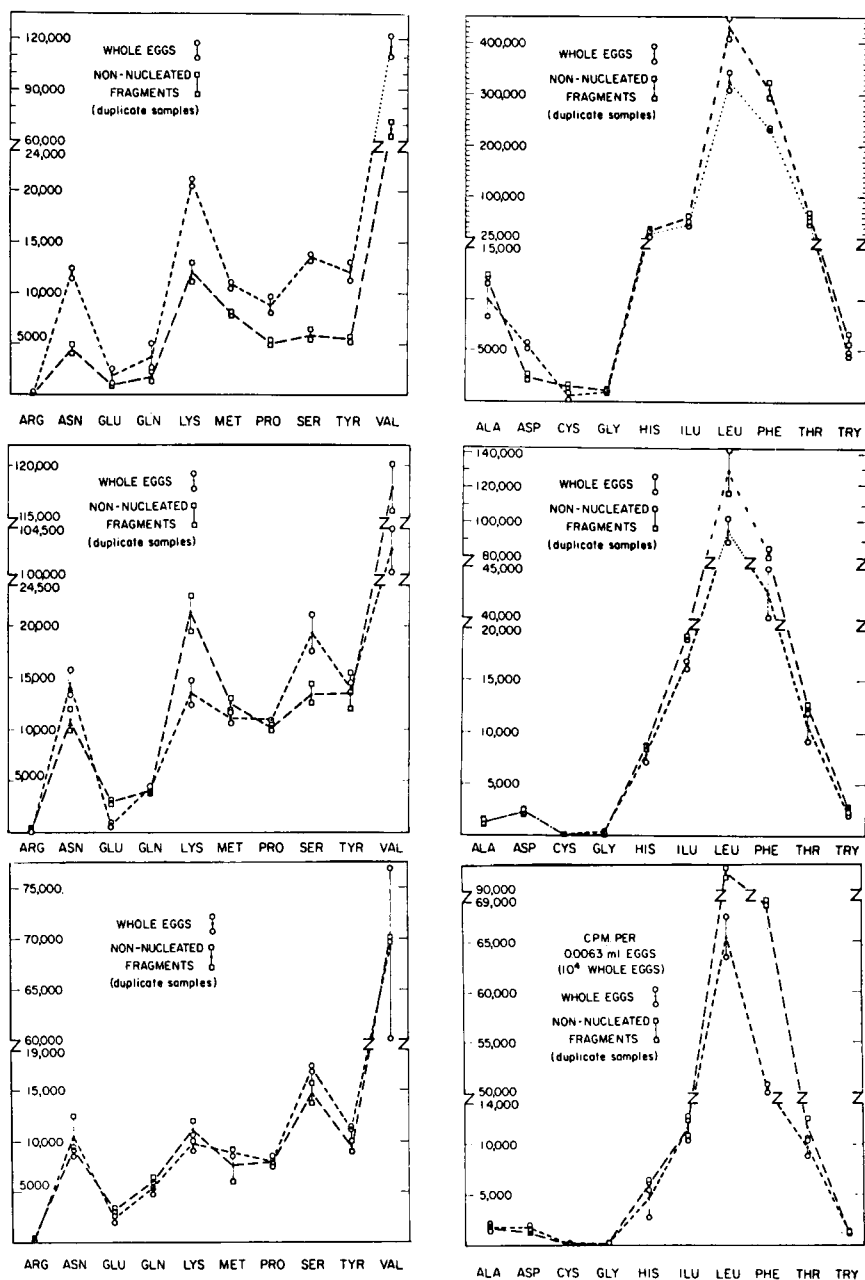


FIG. 4. Pattern of incorporation of ^{14}C -labeled amino acids by artificially activated (butyric acid) nonnucleated fragments and whole eggs of *Lytechinus*

TABLE VIIIb
EFFECT OF TREATMENT WITH BUTYRIC ACID (0.004 M, 1 MINUTE) ON INCORPORATION
OF ^{14}C -AMINO ACIDS BY INTACT NON-NUCLEATE FRAGMENTS OF EGGS OF
Lytechinus pictus^{a,b}

Expt.	Counts per minute (cpm)			Ratios	
	Untreated (U)	Treated (T)	Fertilized	T/U	F/U
1	25,650; 25,230	72,750; 72,300		2.8	
2	1,040; 920	3,340; 2,663		3.1	
3	5,861; 5,584	8,877; 8,669		1.5	
4	1,475; 1,281	3,384; 3,017		2.3	
5	9,540; 6,700	20,753; 17,467	19,348; 18,451	2.3	2.3
6	247; 220	788; 769	665; 663	3.3	2.8
7	1,005; 833	8,885; 7,526	8,969; 8,122	8.9	9.3
8	999; 879	4,627; 4,307	7,645; 7,404	4.8	8.0

^a Values for Experiment 1 are per milligram protein; for the other experiments quantities of egg fragments were not determined, but amounts were the same for (T) as for (U) samples in each experiment. Incubations were for 1/4 to 2 hours at 20°C in 50 mm³ of a 2.5 $\mu\text{C}/\text{ml}$ seawater solution of ^{14}C -valine/ml of egg-fragment suspension, except for experiment 5 in which ^{14}C -phenylalanine was used.

^b Denny and Tyler, 1964.

results show that the artificially activated nonnucleate fragment incorporates amino acids into protein as effectively as does the nucleate fragment. It is also as active in this respect as the fertilized whole egg during the early stages. Furthermore, when the experiments were extended to measurements of incorporation of all 20 of the "coded" amino acids the pattern of incorporation upon artificial activation of the nonnucleate part was found (Tyler, 1965, 1966) not to differ significantly from that of the fertilized, or artificially activated, whole egg (Fig. 4).

Other experiments (Baltus *et al.*, 1965) have shown that the activated nonnucleate fragment is capable of an RNA synthesis, presumably by transcription from cytoplasmic DNA. However, this synthesis is inhibited by actinomycin, and evidently that RNA is not involved in the bulk of the protein synthesis that occurs during early development. Further information along the same line is provided by experiments by Spiegel *et al.* (1965) and Terman and Gross (1965) showing that, electro-

pictus. In six sets of experiments 10 amino acids were tested in duplicate in each; 1-hour incubation, at 1/2 to 1 hour, in 0.4 $\mu\text{C}/\text{ml}$ at sp. act. 9.23 (Arg), 22.5 (Asn), 205 (Glu), 4.05 (Gln), 43.8 (Lys), 15.6 (Met), 7.1 (Pro), 1.7 (Ser), 11.54 (Tyr), 200 (Val), 4.24 (Ala), 22.5 (Asp), 7.70 (Cys), 5.25 (Gly), 22 (His), 6.16 (Ilu), 160 (Leu), 360 (Phe), 25.6 (Thr), 8.95 (Try). (From Tyler, 1965.)

phoretically, the pattern of soluble proteins synthesized (radioactively labeled) during early sea urchin development does not change significantly and is not markedly affected by actinomycin.

These results provide strong evidence for the presence of a "masked" mRNA in the unfertilized egg. The stimulation of protein synthesis upon fertilization, or artificial activation, is then attributable to an "unmasking" of this unavailable RNA. One suggestion, on the basis of ultrastructural observations on the fragments (Geuskens, 1965), is that the cortical vesicles may be the site of the masked mRNA. This would imply, however, that when the cortical vesicles open up upon fertilization or artificial activation some of the contents go into the egg, or that the presumed mRNA somehow gets back into the egg from the perivitelline space. While this is possible, it is not as yet supported by direct evidence.

Additional, even more direct, evidence that the unfertilized sea urchin egg contains RNA capable of acting as template RNA has been provided (Maggio *et al.*, 1964) by experiments with RNA extracts. Thus, it was found that the bulk RNA prepared from unfertilized eggs was able to stimulate *in vitro* incorporation of amino acids into proteins by ribosomes of rat liver. The same RNA preparations were active with ribosomes from sea urchin embryos. However, they failed to stimulate incorporation with ribosomes from unfertilized eggs. The conclusion was hence drawn that the ribosomes of the unfertilized egg must have some peculiarity that makes them unable to accept natural messenger RNA while still being able to react with synthetic polynucleotides. However, synthetic polynucleotides are known to have a much greater binding affinity for ribosomes than natural mRNA (Okamoto and Takanami, 1963). With the sea urchin homogenate system also polyU can inhibit incorporation of other amino acids while stimulating the incorporation of phenylalanine (Tyler, unpublished).

The experiments with the RNA extracts of unfertilized eggs show, then, that components of these preparations do exhibit mRNA activity when tested on appropriate systems (ribosomal preparations from rat liver or from sea urchin embryos and an excess of other necessary constituents of the cell-free system). Evidently the ribosomes of the unfertilized egg are somehow unable to cooperate with such "natural" mRNA's, although they are quite responsive to polyU.

C. CAPACITATION OF THE RIBOSOMES OF THE UNFERTILIZED EGG

Attempts have been made at removing the inhibition of the ribosomes of the unfertilized egg. Successful results have been obtained by treating such ribosomes with trypsin (Table IX). Removal of the inhibition is indicated by (a) the acquisition of the ability to be stimulated by

mRNA prepared from unfertilized or fertilized eggs or from rat liver nuclei; (b) a considerable increase in the capacity for stimulation by polyU; (c) the ability to carry out incorporation of amino acids in the absence of an exogenous source of RNA (Maggio *et al.*, 1965; Monroy *et al.*, 1965). This latter property is quite interesting. Maggio *et al.* (1964) tried to localize within the unfertilized egg the RNA fraction active in stimulating incorporation of amino acids by ribosomes. They found that the only active fraction was the one extracted from the ribosomal pellet. When this was further fractionated on a sucrose-density gradient, the activity was associated with the 28 S peak. One possibility is that the mRNA synthesized during oogenesis attaches to the ribosomes, but the complex is then rendered inoperative by the concurrent or previous attachment of a protein.

TABLE IX
EFFECT OF TREATMENT WITH TRYPSIN ON THE ABILITY OF RIBOSOMES FROM UNFERTILIZED EGGS TO INCORPORATE AMINO ACIDS INTO PROTEINS^{a,b}

Experiment No.	Amino acids (s)	Addition	cpm/mg Ribosomal RNA	
			Control	Trypsin treated
1	Phenylalanine	none	0	122
		PolyU	270	340
2	Phenylalanine	none	0	72
		PolyU	360	2200
	AH	none	25	260
		RNA (blastula)	0	475
3	AA	none	18	448
		RNA (u)	9	644

^a Ribosomes from unfertilized eggs of *Paracentrotus lividus* were incubated for 30 minutes in a mixture containing in 1.0 ml 25 μ M Tris buffer, pH 7.6; 4 μ M Mg acetate; 50 μ M KCl; 20 μ M mercaptoethanol; 4 μ M ATP; 0.5 μ M GTP; 10 μ M PEP; 10 μ g PEP-kinase; about 4 mg (estimated as proteins) of rat liver sap and the following additions as indicated: polyU or total RNA from unfertilized eggs [RNA (u)] or total RNA from blastula (RNA blastula). Radioactive amino acids: ¹⁴C-phenylalanine (1 μ c) or ¹⁴C-algal protein hydrolysate (AH) (1 μ c) or a mixture of ¹⁴C-phenylalanine, lysine, valine, glycine, and glutamic acid (AA) (1 μ c total). Trypsin treatment was with 100 μ g/ml for 30 minutes followed by washing of the ribosomes before incubation.

^b Monroy *et al.*, 1965.

Evidence for such mRNA-protein conjugates has recently been presented in experiments (Spirin *et al.*, 1964) with embryos of the teleostean fish *Misgurnus fossilis*. The evidence indicates that the mRNA leaves the nucleus as a complex with a protein, which has been termed

an "informosome." Upon establishing contact with the ribosomes, the mRNA is released. The possibility hence exists that during oogenesis, too, informosomes are formed and become attached to the ribosomes, but without the protein being released. The removal of the protein by trypsin treatment would then set free the mRNA.

That a protease may be the natural activator of protein synthesis in the egg in normal development is rendered not unlikely by the observations of Lundblad (1949, 1950). This author had in fact discovered the activation of proteolytic activity within 1 minute following fertilization. The significance of this finding had remained obscure; it had been suggested, for example, that these enzymes might be instrumental in the unmasking of important reactive groups of proteins (see Monroy, 1965). The present findings make it likely that the role of this proteolytic activity may be of very fundamental importance in the process of activation. Obviously, too, the proteolytic activity must be under additional controls, or of very specific types, so that it does not interfere with the concomitant protein synthesis.

The presence of particles made up of proteins and mRNA would also answer the question as to how the messenger is protected from destruction during its long stay in the cytoplasm. The possibility of an association of mRNA with a protein, the latter acting as a "messenger protector," has already been indicated by Vincent (1963).

If the ribosomes are incapacitated because they bear an mRNA that is conjugated with protein, and if the protein is removed upon fertilization, then one would expect to find a decrease in their sedimentability in the ultracentrifuge. Determinations that have been made (Ecker and Brookbank, 1963) on the ribosomes of unfertilized echinoid eggs give values of about 85 S, which is somewhat higher than is usually obtained with ribosomes of animal cells. In these experiments with the analytical ultracentrifuge there were no concomitant measurements on the fertilized egg material. The studies with the preparative machine by density-gradient methods (Monroy and Tyler, 1963; Stafford *et al.*, 1964) showed no significant difference.

A change in the compartmentalization of K^+ , as has been shown (Tyler and Monroy, 1959) to occur upon fertilization, or of other ions such as Mg^{++} , might also play a role in the activation process. These ions are known to affect protein synthesis in *in vitro* systems prepared from various kinds of cells, including sea urchin eggs (Hultin, 1961; Timourian and Denny, 1964; Molinaro and Hultin, 1965). However, there is not, as yet, sufficient evidence to assign any specific role to these or other ions in the activation process. It is a subject that would seem to merit further study.

D. PROTEIN SYNTHESIS IN NONECHINOID SPECIES

The study of the activation of protein synthesis in species whose eggs are fertilized before the onset or the completion of maturation is of great interest, as is the case in, for example, starfish and *Spisula* eggs. As already described, in these eggs no change in the rate of oxygen consumption occurs following fertilization. Furthermore, the fusion of the male with the female pronucleus does not occur until maturation has been completed. It was thus of interest to find out whether or not in these eggs activation of protein synthesis would immediately follow sperm penetration.

In starfish eggs the rate of uptake of radioactive amino acids and their incorporation into proteins is the same in unfertilized and fertilized eggs until after the formation of the second polar body, but once maturation is completed, the rate of incorporation declines in unfertilized eggs, whether it rapidly increases in fertilized ones (Monroy and Tolis, 1964). In this case, therefore, completion of maturation seems to be a necessary prerequisite for the activation of protein synthesis (Fig. 5). On the other hand, a different situation prevails in the eggs of *Spisula*. These eggs are also shed in the primary oocyte stage, but, unlike the starfish, maturation does not begin until the egg is fertilized. Shortly after sperm penetration, the germinal vesicle breaks down and a few minutes later, well before the formation of the first polar body, a very rapid increase in rate of uptake and incorporation of amino acids into proteins takes place (Monroy and Tolis, 1964) (Fig. 6). Here, therefore, the change in metabolic activity coincides with the onset rather than with the end of maturation. Of course, these observations are too limited to warrant any general conclusion and they need to be extended to the eggs of other animals. Thus far, it seems that sperm penetration is not in itself the sole cause of the activation of protein synthesis; some change in the egg itself must first occur, a change which somehow is related to the process of maturation. It would also be interesting to repeat on starfish and *Spisula* eggs the experiments with isolated ribosomes of the kind already described for the sea urchin.

E. ONSET OF RNA SYNTHESIS

The relative inertness of the ripe unfertilized sea urchin egg extends to an inability to incorporate significant amounts of precursors into RNA. However, whether or not this inability is intrinsic cannot be definitely specified at present, inasmuch as experiments (Piatigorsky and Whiteley, 1965) have shown that the unfertilized egg is relatively impermeable to the RNA precursors.

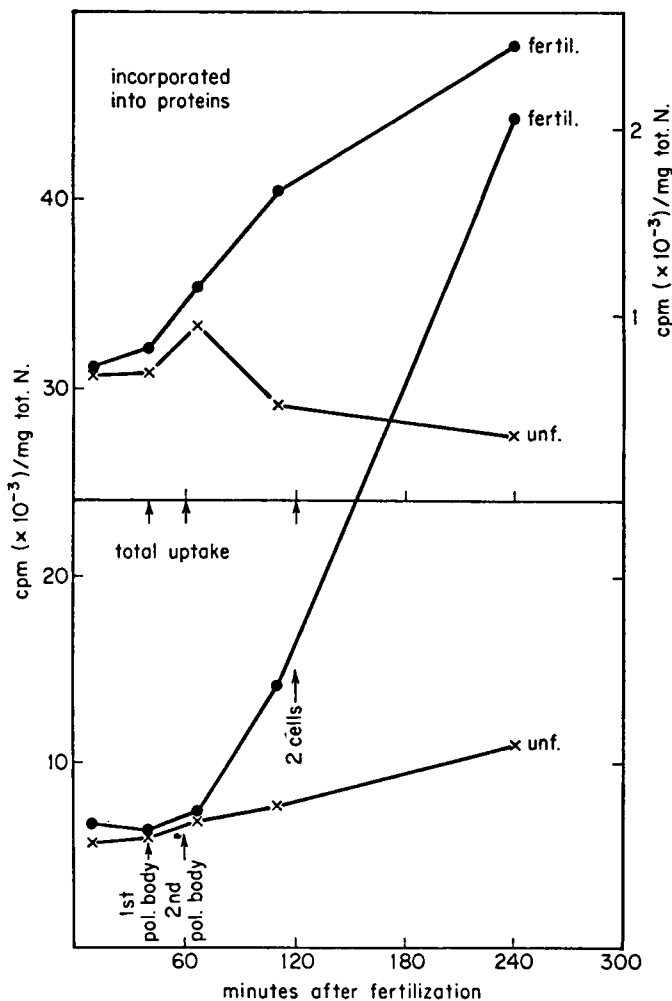


FIG. 5. Rate of entry and of incorporation into proteins of ^{35}S -methionine in unfertilized and fertilized eggs of *Asterias forbesi*. The eggs were pulse labeled for 5 minutes. Left ordinate: rate of entry. Right ordinate: incorporation into proteins. (From Monroy and Tolis, 1964.)

Following fertilization RNA synthesis becomes detectable. Various investigators differ with regard to how early synthesis is detected and the nature of the RNA that is formed. However, there is now fairly general accord that synthesis of RNA occurs during cleavage stages. The experiments have been done with sea urchins (Gross and Cousineau, 1963; Gross, 1964; Gross *et al.*, 1965; Nemer, 1963; Wilt, 1964; Comb and Brown, 1964; Glisin and Glisin, 1964; Baltus *et al.*, 1965; Whiteley

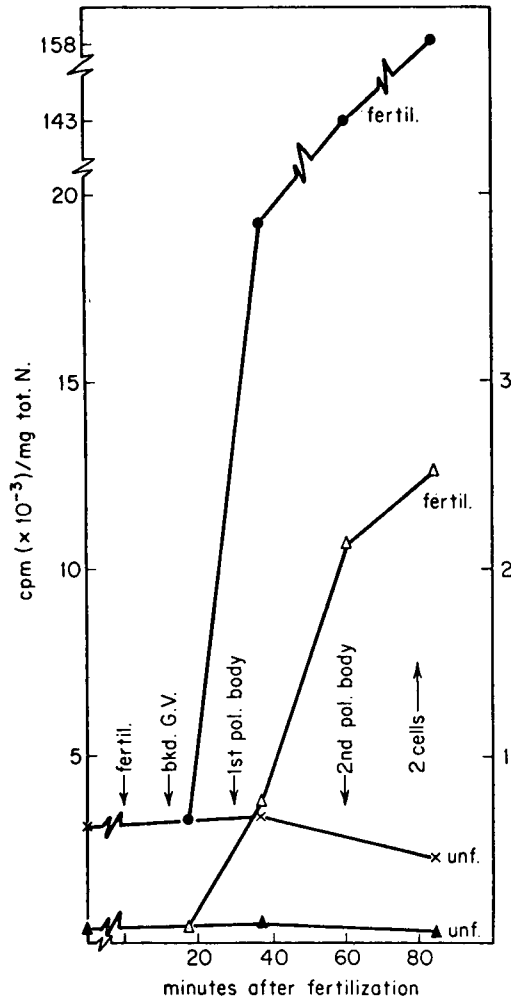


FIG. 6. Rate of entry ($\times$ — $\times$: unfertilized eggs; $\bullet$ — $\bullet$: fertilized eggs) and of incorporation into proteins ($\blacktriangle$ — $\blacktriangle$: unfertilized eggs; $\triangle$ — $\triangle$: fertilized eggs) following a 5-minute pulse with ^{14}C -labeled valine-1 in eggs of *Spisula solidissima*. Left ordinate: rate of entry. Right ordinate: rate of incorporation into proteins. (Bkd.G.V.): Breakdown of the germinal vesicle. (From Monroy and Tolis, 1964.)

et al., 1966); with fish (Spirin *et al.*, 1964); and with amphibians (Brown and Littna, 1964a,b; Decroly *et al.*, 1964). The RNA that is synthesized during cleavage appears to be messenger type, as evidenced in hybridization tests with DNA, by its relatively high adenine-uridine content, and by its sedimentation constant.

Ribosomal RNA is apparently not formed until the time of gastrulation, as has been mentioned above on the basis of observations on amphibian eggs. This is found to be correlated with the appearance of nucleoli in the nuclei also in sea urchin eggs (Cowden and Lehman, 1963). Recent studies of methylation of RNA in sea urchins (Comb, 1965) have shown that this also occurs at the time of gastrulation, the appropriate enzyme presumably being synthesized at that time.

Thus, although mRNA for the bulk of the synthesis that goes on in early development is present and becomes "unmasked" at the time of fertilization, new mRNA is also formed during the early stages. However, this new RNA itself is evidently "masked" at first and becomes activated only at some later time. The presence at an early stage of development of mRNA's that are to be used later appears to be a general feature of development. It has been proposed (Tyler, 1965) that this feature may account for the phenomenon of determination in embryonic development. Embryonic determination is a specification process whereby cells become fixed to their future fate. The occurrence of determination is detected usually by transplantation or explantation experiments. It generally takes place a considerable period of time in advance of actual differentiation of the tissue and without any microscopically visible change in the cells. Experiments with actinomycin on developing eggs of many species of animals have shown that, within the range of accuracy of the tests, the time at which the determination process occurs roughly coincides with the time at which insensitivity to this drug takes place. The actinomycin is known (Reich *et al.*, 1962; see Reich, 1964) to bind DNA and suppress mRNA synthesis.

VI. Activation and Changes in Protein Structure

The possibility that a change in the configuration of some egg proteins may be involved in the process of activation was suggested by the pioneering work of Mirsky (1936). In view of the findings cited in the preceding discussion, one might hypothesize that certain molecular configurations may characterize egg protein in the course of maturation and somehow be related to the inhibited condition of the mature egg. The changes in configuration occurring in conjunction with fertilization would thus reestablish the normal conditions.

The changes described in the sea urchin egg following fertilization (Mirsky, 1936; Monroy, 1950; Monroy and Monroy-Oddo, 1951; Ceas *et al.*, 1955; Giardina and Monroy, 1955; D'Amelio, 1955) involve primarily the solubility, the electrophoretic mobility, and the sensitivity to proteolytic enzymes and denaturing agents of some protein fractions. Similar changes have been observed in the fish egg (Hamano, 1957). It

has also been suggested that the hydrolytic cleavage of some proteins with release of terminal peptides may have an important role in these changes (see Monroy, 1957). This view is indirectly supported by the finding of an increase of the nonprotein amino nitrogen immediately following fertilization (Örström, 1941; Ricotta, 1956) and by the observations of Lundblad (1949, 1950), already mentioned. These results support the view originally expressed by Mirsky (1936) that the changes described for the proteins after fertilization may depend on the removal of the water shell around the protein molecules. This view is also consistent with the notion of volume changes and the disarrangement of the water lattice around the protein molecules which accompany proteolysis (see discussions in Linderström-Lang, 1952; and Klotz, 1958). The observation that the specific gravity of the egg increases after fertilization (from 1.068–1.088 to 1.091–1.095; Salzen, 1957) is also in accord with the above hypothesis.

The activation of a number of enzymes that follows fertilization might hence be accounted for by these changes. It may suffice to recall here the activation of such enzymes as trypsin, chymotrypsin, etc., which is known to be the result of the proteolytic cleavage of a terminal peptide.

REFERENCES

- Aketa, K., Bianchetti, R., Marré, E., and Monroy, A. (1964). Hexose monophosphate level as a limiting factor for respiration in unfertilized sea urchin eggs. *Biochim. Biophys. Acta* **86**, 211.
- Axelrod, R. (1960). Glycolysis. In "Metabolic Pathways" (D. M. Greenberg, ed.), Vol. I, pp. 97–128. Academic Press, New York.
- Bäckström, S. (1963). 6-Phosphogluconate dehydrogenase in sea urchin embryos. *Exptl. Cell Res.* **32**, 566.
- Ballentine, R. (1940). Analysis of the changes in respiratory activity accompanying the fertilization of marine eggs. *J. Cellular Comp. Physiol.* **15**, 217.
- Baltus, E., Quertier, J., Ficq, A., and Brachet, J. (1965). Biochemical studies of nucleate and anucleate fragments isolated from sea-urchin eggs. A comparison between fertilization and parthenogenetic activation. *Biochim. Biophys. Acta* **95**, 408.
- Barron, E. S. G. (1932). The effect of anaerobiosis on the eggs and sperm of sea urchin, starfish and *Nereis* and fertilization under anerobic conditions. *Biol. Bull.* **62**, 46.
- Baxandall, J., Perlmann, P., and Afzelius, B. A. (1964). Immuno-electron microscope analysis of the surface layers of the unfertilized sea urchin egg. I. Effects of the antisera on the cell ultrastructure. *J. Cell. Biol.* **23**, 609.
- Baxandall, J., Perlmann, P., and Afzelius, B. A. (1964). Immuno-electron microscope analysis of the surface layers of the unfertilized sea urchin egg. II. Localization of surface antigens. *J. Cell Biol.* **23**, 629.
- Black, R. E., and Tyler, A. (1959a). Effects of fertilization and development on the oxidation of carbon monoxide by eggs of *Strongylocentrotus* and *Urechis* as determined by use of C^{13} . *Biol. Bull.* **117**, 443.

- Black, R. E., and Tyler, A. (1959b). Cytochrome oxidase and oxidation of CO in eggs of the sea urchin *Strongylocentrotus purpuratus*. *Biol. Bull.* **117**, 454.
- Borei, H. (1948). Respiration of oocytes, unfertilized eggs and fertilized eggs from *Psammechinus* and *Asterias*. *Biol. Bull.* **95**, 124.
- Borei, H. (1949). Independence of post-fertilization respiration in the sea urchin egg from the level of respiration before fertilization. *Biol. Bull.* **96**, 117.
- Borei, H., and Lybing, S. (1949). Temperature coefficients of respiration in *Psammechinus* eggs. *Biol. Bull.* **96**, 107.
- Boyd, M. (1928). A comparison of the oxygen consumption of unfertilized and fertilized eggs of *Fundulus heteroclitus*. *Biol. Bull.* **55**, 92.
- Brachet, J. (1938). The oxygen consumption of artificially activated and fertilized *Chaetopterus* eggs. *Biol. Bull.* **74**, 93.
- Brachet, J. (1960). "The Biochemistry of Development." Pergamon Press, Oxford and London.
- Brachet, J. (1962). Nucleic acids in development. *J. Cellular Comp. Physiol., Suppl.* **1** **60**, 1.
- Brachet, J., and Ficq, A. (1965). Binding sites of ¹⁴C-actinomycin in amphibian oocytes and an autoradiography technique for the detection of cytoplasmic DNA. *Exptl. Cell Res.* **38**, 153.
- Brachet, J. (1964). Nouvelles observations sur le rôle des acides nucléiques dans la morphogénèse. *Symp. on Acidi Nucleici e loro Funzione*, pp. 288-315. Fondazione Baselli, Milan.
- Brachet, J., Ficq, A., and Tencer, R. (1963). Amino acid incorporation into proteins of nucleate fragments of sea urchin eggs: effect of parthenogenetic activation. *Exptl. Cell Res.* **32**, 168.
- Briggs, R., and King, T. J. (1960). Nuclear transplantation studies on the early gastrula (*Rana pipiens*). I. Nuclei of presumptive endoderm. *Develop. Biol.* **2**, 252.
- Brock, N., Druckrey, H., and Herken, H. (1938). Der Stoffwechsel des geschädigten Gewebes. III. Zugleich Beitrag zur Frage der Entwicklungserregung am Seeigellei. *Arch. Exp. Pathol. Pharmacol.* **188**, 451.
- Brookbank, J. W., and Yonge, J. G. (1964). Studies on the 85 S ribosome fraction of echinoid eggs and embryos. *Acta Embryol. Morphol. Exptl.* **7**, 296.
- Brown, D. D., and Littna, E. (1964a). RNA synthesis during development of *Xenopus laevis*, the South African clawed toad. *J. Mol. Biol.* **8**, 669.
- Brown, D. D., and Littna, E. (1964b). Variations in the synthesis of stable RNA's during oogenesis and development of *Xenopus laevis*. *J. Mol. Biol.* **8**, 688.
- Carlsen, E. N., Trelle, G. J., and Schjeide, O. A. (1964). Transfer ribonucleic acids. *Nature* **202**, 984.
- Ceas, M. P., Impellizzeri, M. A., and Monroy, A. (1955). The action of urea on some proteins of the unfertilized and fertilized sea urchin egg. *Exptl. Cell Res.* **9**, 366.
- Cleland, K. W. (1950). Respiration and cell division in developing oyster eggs. *Proc. Linnean Soc. N. S. Wales* **75**, 282.
- Cleland, K. W., and Rothschild, L. (1952a). The metabolism of the sea-urchin egg. Anaerobic breakdown of carbohydrate. *J. Exptl. Biol.* **29**, 285.
- Cleland, K. W., and Rothschild, L. (1952b). The metabolism of the sea-urchin egg. The oxidation of carbohydrate. *J. Exptl. Biol.* **29**, 416.
- Comb, D. G. (1965). Methylation of nucleic acids during sea urchin embryo development. *J. Mol. Biol.* **11**, 851.

- Comb, D. G., and Brown, R. (1964). Preliminary studies on the degradation and synthesis of RNA components during sea urchin development. *Exptl. Cell Res.* **34**, 360.
- Costello, D. P. (1940). The fertilization of nucleated and nonnucleated fragments of centrifuged *Nereis* eggs. *J. Morphol.* **66**, 99.
- Cowden, R. R., and Lehman, H. E. (1963). A cytochemical study of differentiation in early echinoid development. *Growth* **27**, 185.
- Dalcq, A. (1928). "Les Bases Physiologiques de la Fécondation et de la Parthénogénèse." Presses Universitaires, Paris.
- D'Amelio, V. (1955). Trypsin sensitivity of some proteins of the sea urchin egg before and after fertilization. An electrophoretic analysis. *Experientia* **11**, 443.
- Davidson, E., Allfrey, V. G., and Mirsky, A. E. (1964). On the RNA synthesized during the lampbrush phase of amphibian oogenesis. *Proc. Natl. Acad. Sci. U. S.* **52**, 501.
- Decroly, M., Cape, M., and Brachet, J. (1964). Studies on the synthesis of ribonucleic acids in embryonic stages of *Xenopus laevis*. *Biochim. Biophys. Acta* **87**, 34.
- Delage, Y. (1901). Etudes expérimentales chez les Echinodermes. *Arch. Zool. Exptl. Gen.* **9**, 285.
- Denny, P. C., and Tyler, A. (1964). Activation of protein biosynthesis in non-nucleate fragments of sea urchin eggs. *Biochim. Biophys. Res. Commun.* **14**, 245.
- Dettlaff, T. A., Nikitina, L. A., and Stroeve, O. G. (1964). The role of the germinal vesicle in oocyte maturation in anurans as revealed by the removal and transplantation of nuclei. *J. Embryol. Exptl. Morphol.* **12**, 851.
- Ecker, R. E., and Brookbank, J. W. (1963). A ribosome fraction from sand-dollar (*Mellita quinquiesperforata*) ova. *Biochim. Biophys. Acta* **72**, 488.
- Edström, J. E., Grampp, W., and Schar, N. (1961). The intracellular distribution and heterogeneity of ribonucleic acid in starfish oocytes. *J. Biophys. Biochem. Cytol.* **11**, 549.
- Epel, D. (1964a). A primary metabolic change of fertilization: interconversion of pyridine nucleotides. *Biochim. Biophys. Res. Commun.* **17**, 62.
- Epel, D. (1964b). Simultaneous measurements of TPNH formation and respiration following fertilization of the sea urchin egg. *Biochim. Biophys. Res. Commun.* **17**, 69.
- Esper, H. (1965). Studies on the nucleolar vacuole in the oogenesis of *Arbacia punctulata*. *Exptl. Cell Res.* **38**, 85.
- Fauré-Fremiet, E. (1922). Echanges respiratoires des oeufs de *Sabellaria alveolata* L. au cours de la segmentation et de la cytolyse. *Compt. Rend. Soc. Biol.* **86**, 20.
- Ficq, A. (1955). Etude autoradiographique du métabolisme de l'oocyte d'*Asterias rubens* au cours de la croissance. *Arch. Biol. (Liege)* **66**, 509.
- Ficq, A. (1964). Effects de l'actinomycine D et de la puromycine sur le métabolisme de l'oocyte en croissance. *Exptl. Cell Res.* **34**, 581.
- Geuskens, M. (1965). A study of the ultrastructure of nucleate and anucleate fragments of unfertilized sea urchin eggs. *Exptl. Cell Res.* **39**, 413.
- Giardina, G., and Monroy, A. (1955). Changes in the proteins of the sea urchin egg at fertilization. *Exptl. Cell Res.* **8**, 466.
- Glisin, V. R., and Glisin, M. V. (1964). Ribonucleic acid metabolism following fertilization in sea urchin eggs. *Proc. Natl. Acad. Sci. U. S.* **52**, 1548.
- Gross, P. R. (1964). The immediacy of genomic control during early development. *J. Exptl. Zool.* **157**, 21.

- Gross, P. R., and Cousineau, G. H. (1964). Macromolecule synthesis and the influence of actinomycin on early development. *Exptl. Cell Res.* **33**, 368.
- Gross, P. R., and Cousineau, G. H. (1963). Effects of actinomycin D on macromolecule synthesis and early development in sea urchin eggs. *Biochem. Biophys. Res. Commun.* **10**, 321.
- Gross, P. R., Kraemer, K., and Malkin, L. I. (1965). Base composition of RNA synthesized during cleavage of the sea urchin embryo. *Biochem. Biophys. Res. Commun.* **18**, 569.
- Gross, P. R., Malkin, L. I., and Moyer, W. A. (1964). Templates for the first proteins of embryonic development. *Proc. Natl. Acad. Sci. U. S.* **51**, 407.
- Haggis, A. J. (1964). Quantitative determination of dextrinonucleic acid in embryos and unfertilized eggs of *Rana pipiens*. *Develop. Biol.* **10**, 358.
- Hagström, B., and Hagström, B. E. (1954a). Refertilization of the sea urchin egg. *Exptl. Cell Res.* **6**, 491.
- Hagström, B., and Hagström, B. E. (1954b). The action of trypsin and chymotrypsin on the sea urchin egg. *Exptl. Cell Res.* **6**, 532.
- Hamano, S. (1957). Physico-chemical studies on the activation and fertilization of fish eggs. *Mem. Fac. Fisheries, Hokkaido Univ.* **5**, 91.
- Harvey, E. B. (1956). "The American *Arbacia* and Other Sea Urchins." Princeton Univ. Press, Princeton, New Jersey.
- Hinegardner, R. T. (1961). The DNA content of isolated sea urchin egg nuclei. *Exptl. Cell Res.* **25**, 341.
- Hiramoto, Y. (1959a). Electric properties of echinoderm eggs. *Embryologia* **4**, 219.
- Hiramoto, Y. (1959b). Changes in electric properties upon fertilization in the sea urchin egg. *Exptl. Cell Res.* **16**, 421.
- Hoberman, H. D., Metz, C. B., and Graff, J. (1952). Uptake of deuterium into proteins of fertilized and unfertilized *Arbacia* eggs suspended in heavy water. *J. Gen. Physiol.* **35**, 639.
- Holter, H., and Zeuthen, E. (1944). The respiration of the egg and embryo of the ascidian *Ciona intestinalis* L. *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.* **25**, 33.
- Horwitz, B. A. (1965). Rates of oxygen consumption of fertilized and unfertilized *Asterias*, *Arbacia* and *Spisula* eggs. *Exptl. Cell Res.* **38**, 620.
- Hultin, T. (1950). The protein metabolism of sea urchin eggs during early development studied by means of N¹⁵-labelled ammonia. *Exptl. Cell Res.* **1**, 599.
- Hultin, T. (1952). Incorporation of N¹⁵-labelled glycine and alanine into the proteins of developing sea urchin eggs. *Exptl. Cell Res.* **3**, 494.
- Hultin, T. (1961). Activation of ribosomes in sea urchin eggs in response to fertilization. *Exptl. Cell Res.* **25**, 405.
- Hultin, T., and Bergstrand, Å. (1960). Incorporation of C¹⁴-L-leucine into protein by cell-free system from sea urchin embryos at different stages of development. *Develop. Biol.* **2**, 61.
- Ishida, J., and Nakano, E. (1950). Fertilization of activated sea urchin eggs deprived of fertilization membrane by washing with Ca-Mg-free media. *Annotiones Zool. Japon.* **23**, 43.
- Ishihara, K. (1957). Release and activation of aldolase at fertilization in sea urchin egg. *J. Fac. Sci. Univ. Tokyo, Sect. IV* **8**, 71.
- Ishihara, K. (1958a). Effect of butyric acid on aldolase complex in sea urchin eggs. *Sci. Rep. Saitama Univ., Ser. B* **3**, 11.

- Ishihara, K. (1958b). Enhanced respiration of sea urchin eggs induced by mechanical stimulation. *Sci. Rep. Saitama Univ., Ser. B.* **3**, 21.
- Ishihara, K. (1963). Isolation of aldolase complex in sea urchin eggs. *Sci. Rep. Saitama Univ., Ser. B* **6**, 173.
- Ishikawa, M. (1954). Relation between the breakdown of the cortical granules and permeability to water in the sea urchin egg. *Embryologia* **2**, 57.
- Isono, N. (1963). Carbohydrate metabolism in sea urchin eggs. IV. Intracellular localization of enzymes of the pentose phosphate cycle in unfertilized and fertilized eggs. *J. Fac. Sci. Univ. Tokyo, Sect. 4* **10**, 37.
- Isono, N., Isusaka, A., and Nakano, E. (1963). Studies on glucose-6-phosphate dehydrogenase in sea urchin eggs. *J. Fac. Sci. Univ. Tokyo, Sect. 4* **10**, 55.
- Izawa, M., Allfrey, V. G., and Mirsky, A. E. (1963). Composition of the nucleus and chromosomes in the lampbrush stage of the newt oocyte. *Proc. Natl. Acad. Sci. U. S.* **50**, 811.
- Kay, E. R. M. (1964). Direct isolation of deoxyribonucleic acid from cells. *Nature* **202**, 390.
- Klotz, M. (1958). Protein hydration and behaviour. *Science* **128**, 815.
- Krane, S. M., and Crane, R. K. (1960). Changes in levels of triphosphopyridine nucleotide in marine eggs subsequent to fertilization. *Biochim. Biophys. Acta* **43**, 369.
- Krane, S. M., and Crane, R. K. (1963). Alterations in pyridine nucleotides of dividing marine eggs. In "Control Mechanisms in Respiration and Fermentation" (B. Wright, ed.), pp. 157-166. Ronald Press, New York.
- Lardy, H. A., and Wellman, H. (1952). Oxydative phosphorylations: role of inorganic phosphate and acceptor systems in control of metabolic rates. *J. Biol. Chem.* **195**, 215.
- Laser, H., and Rothschild, L. (1939). The metabolism of the eggs of *Psammechinus miliaris* during the fertilization reaction. *Proc. Roy. Soc. (London), Ser. B* **126**, 539.
- Lentini, R. (1961). The oxygen uptake of *Ciona intestinalis* eggs during development in normal and in experimental conditions. *Acta Embryol. Morphol. Exptl.* **4**, 209.
- Lillie, R. S. (1916). Increase of permeability of water following normal and artificial activation in the sea urchin eggs. *Am. J. Physiol.* **40**, 249.
- Lindahl, P. E., and Holter, H. (1941). Ueber die atmung der ovozyten erster ordnung von *Paracentrotus lividus* und ihre Veränderung während der Reifung. *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.* **24**, 49.
- Lindberg, O., and Ernster, L. (1948). On carbohydrate metabolism in homogenized sea urchin eggs. *Biochem. Biophys. Acta* **2**, 471.
- Linderström-Lang, K. U. (1952). Proteins and enzymes. In "Lane Medical Lectures," Vol. VI. Stanford University Publications, Medical Sciences, Palo Alto, California.
- Loeb, J. (1913). "Artificial Parthenogenesis and Fertilization." Univ. of Chicago Press, Chicago, Illinois.
- Lundblad, G. (1949). Proteolytic activity in eggs and sperms from sea urchins. *Nature* **163**, 643.
- Lundblad, G. (1950). Proteolytic activity in sea urchin gametes. *Exptl. Cell Res.* **1**, 264.
- Maggio, R., and Catalano, C. (1963). Activation of amino acids during sea urchin development. *Arch. Biochem. Biophys.* **103**, 164.

- Maggio, R., and Ghiretti-Magaldi, A. (1958). The cytochrome system in mitochondria of unfertilized sea urchin eggs. *Exptl. Cell Res.* **15**, 95.
- Maggio, R., Monroy, A., Rinaldi, A. M., and Vittorelli, M. L. (1965). Activation *in vitro* des oeufs d'oursin. *Compt. Rend.* **260**, 1293.
- Maggio, R., Vittorelli, M. L., Rinaldi, A. M., and Monroy, A. (1964). *In vitro* incorporation of amino acids into protein stimulated by RNA from unfertilized sea urchin eggs. *Biochem. Biophys. Res. Commun.* **15**, 436.
- Metz, C. B. (1961). Use of inhibiting agents in studies on fertilization mechanisms. *Intern. Rev. Cytol.* **11**, 219.
- Metz, C. B., Schuel, H., and Bischoff, E. R. (1964). Inhibition of the fertilizing capacity of sea urchin sperm by papain digested, non-agglutinating antibody. *J. Exptl. Zool.* **155**, 261.
- Millonig, G., and Bosco, M. (1964). The cortical reaction in *Asterias* eggs. *Proc. 3rd Eur. Regional Conf. on Electron Microscopy, Praga*, Appendix to Vol. A and B, p. 40.
- Minganti, A. (1957). Experiments on the respiration of *Phallusia* eggs and embryos (ascidians). *Acta Embryol. Morphol. Exptl.* **1**, 150.
- Mirsky, A. E. (1936). Protein coagulation as a result of fertilization. *Science* **84**, 333.
- Mitchison, J. M., and Cummins, J. E. (1966). The uptake of valine and cytidine by sea urchin embryos and its relation to the cell surface. *J. Cell. Sci.* **1**, 35.
- Molinaro, M., and Hultin, T. (1965). The anomalous potassium requirement of amino acid incorporating systems from sea urchin eggs. *Exptl. Cell Res.* **38**, 398.
- Monroy, A. (1950). A preliminary electrophoretic analysis of proteins and protein fractions in sea urchin eggs and their changes on fertilization. *Exptl. Cell Res.* **1**, 92.
- Monroy, A. (1957). Studies of proteins of sea urchin eggs and of their changes following fertilization. In "The Beginning of Embryonic Development" (A. Tyler, R. C. von Borstel, and C. B. Metz, eds.), pp. 169-174. Am. Assoc. Advanc. Sci., Washington, D.C.
- Monroy, A. (1960). Incorporation of S³⁵-methionine in the microsomes and soluble proteins during the early development of the sea urchin egg. *Experientia* **16**, 114.
- Monroy, A. (1965). "Chemistry and Physiology of Fertilization." Holt, New York.
- Monroy, A., and Maggio, R. (1964). Biochemical studies on the early development of sea urchin. *Advan. Morphogenesis* **3**, 95.
- Monroy, A., Maggio, R., and Rinaldi, A. M. (1965). Experimentally induced activation of the ribosomes of the unfertilized sea urchin egg. *Proc. Natl. Acad. Sci. U. S.* **54**, 107.
- Monroy, A., and Monroy-Oddo, A. (1951). Solubility changes of proteins in sea urchin eggs upon fertilization. *J. Gen. Physiol.* **35**, 245.
- Monroy, A., and Tolis, H. (1964). Uptake of radioactive glucose and amino acids and their utilization for incorporation into proteins during maturation and fertilization of the eggs of *Asterias forbesii* and *Spisula solidissima*. *Biol. Bull.* **126**, 456.
- Monroy, A., and Tyler, A. (1963). Formation of active ribosomal aggregates (polysomes) upon fertilization and development of sea urchin eggs. *Arch. Biochem. Biophys.* **103**, 431.
- Monroy, A., and Vittorelli, M. L. (1960). On a glycoprotein of the sea urchin eggs and its changes following fertilization. *Experientia* **16**, 56.

- Monroy, A., and Vittorelli, M. L. (1962). Utilization of ^{14}C -glucose for amino acids and protein synthesis by the sea urchin embryo. *J. Cellular Comp. Physiol.* **60**, 285.
- Morgan, T. H. (1927). "Experimental embryology" Columbia Univ. Press, New York.
- Naora, H., Naora, H., Izawa, M., Allfrey, V. G., and Mirsky, A. E. (1962). Some observations on differences in composition between the nucleus and cytoplasm of the frog oocyte. *Proc. Natl. Acad. Sci. U. S.* **48**, 853.
- Nakano, E. (1953). Respiration during maturation and at fertilization of fish eggs. *Embryologia* **2**, 21.
- Nakano, E. (1954). Further studies on the fertilization of activated sea urchin eggs. *Japan. J. Zool.* **11**, 245.
- Nakano, E., Giudice, G., and Monroy, A. (1958). On the incorporation of S^{35} -methionine in artificially activated sea urchin eggs. *Experientia* **14**, 11.
- Nakano, E., and Monroy, A. (1957). A method for the incorporation of radioactive isotopes in sea urchin eggs. *Experientia* **13**, 416.
- Nakano, E., and Monroy, A. (1958). Incorporation of S^{35} -methionine in the cell fractions of sea urchin eggs and embryos. *Exptl. Cell Res.* **14**, 236.
- Nemer, M. (1962). Interrelation of messenger polyribonucleotides and ribosomes in the sea urchin egg during embryonic development. *Biochem. Biophys. Res. Commun.* **8**, 511.
- Nemer, M. (1963). Old and new RNA in the embryogenesis of the purple sea urchin. *Proc. Natl. Acad. Sci. U. S.* **50**, 230.
- Nemer, M., and Bard, S. G. (1963). Polypeptide synthesis in sea urchin embryogenesis: an examination with synthetic polyribonucleotides. *Science* **140**, 664.
- Nirenberg, M. W., and Matthaei, H. J. (1961). The dependence of cell-free protein synthesis in *E. coli* upon naturally occurring or synthetic polyribonucleotides. *Proc. Natl. Acad. Sci. U. S.* **47**, 1588.
- Ohnishi, T., and Sugiyama, M. (1963). Polarographic studies of oxygen uptake of sea urchin eggs. *Embryologia* **8**, 79.
- Okamoto, T., and Takanami, M. (1963). Interaction of ribosomes and natural polyribonucleotides. *Biochem. Biophys. Acta* **76**, 266.
- Örström, A. (1941). Ueber die Stickstoff-fractionen im Ei von *Paracentrotus lividus* vor und nach der Entwicklungserregung und ueber ihre Bedeutung für den osmotischen Druck und den Stoffwechsel. *Arkiv Kemi* **15A**, No. 1.
- Örstrom, A., and Lindberg, O. (1940). Ueber den Kohlenhydratstoffwechsel bei der Befruchtung des Seeigels. *Enzymologia* **8**, 367.
- Perlmann, P. (1959). Immunochemical analysis of the surface of the sea urchin egg—an approach to the study of fertilization. *Experientia* **15**, 41.
- Philips, F. S. (1940). Oxygen consumption and its inhibition in the development of *Fundulus* and various pelagic eggs. *Biol. Bull.* **78**, 256.
- Piatigorsky, J., Ozaki, H., and Tyler, A. (1966). RNA- and protein-synthesizing capacity of isolated oocytes of the sea urchin *Lytechinus pictus*. *Develop. Biol.* **13**, (in press).
- Piatigorsky, J., and Whiteley, A. H. (1965). A change in permeability and uptake of ^{14}C -uridine in response to fertilization in *Strongylocentrotus purpuratus* eggs. *Biochim. Biophys. Acta* **108**, 404.
- Piko, L., and Tyler, A. (1965). Deoxyribonucleic acid content of unfertilized sea urchin eggs. *Am. Zoologist* **5**, 636.

- Rebhun, L. I. (1962). Electron microscope studies on the vitelline membrane of the surf clam, *Spisula solidissima*. *J. Ultrastruct. Res.* **6**, 107.
- Reich, E. (1964). Binding of actinomycin as a model for the complex-forming capacity of DNA. In "The Role of the Chromosomes in Development" (M. Locke, ed.), pp. 73-81. Academic Press, New York.
- Reich, E., Franklin, R. M., Shatkin, A. J., and Tatum, E. L. (1962). Action of actinomycin D on animal cells and viruses. *Proc. Natl. Acad. Sci. U. S.* **48**, 1238.
- Rich, A., Warner, J. R., and Goodman, H. M. (1963). The structure and function of polyribosomes. Cold Spring Harbor Symposia. *Quant. Biol.* **28**, 269.
- Ricotta, C. M. (1956). Increase of the non-protein amino-nitrogen in sea urchin eggs upon fertilization. *Naturwissenschaften* **43**, 258.
- Robbie, W. A. (1946). The effect of cyanide on the oxygen consumption and cleavage of the sea urchin egg. *J. Cellular Comp. Physiol.* **28**, 305.
- Rossi, M., and Scarano, E., Personal communication.
- Rothschild, Lord (1949). The metabolism of fertilized and unfertilized sea urchin eggs. The action of light and carbon monoxide. *J. Exptl. Biol.* **26**, 100.
- Rothschild, Lord (1956). "Fertilization." Methuen, London.
- Rothschild, Lord, and Tyler, A. (1958). The oxidative metabolism of eggs of *Urechis caupo*. *Biol. Bull.* **115**, 136.
- Runnström, J. (1930). Atmungsmechanismen und Entwicklungserregung bei dem Seeigellei. *Protoplasma* **10**, 106.
- Salzen, E. A. (1957). The density of sea urchin eggs, embryos and larvae. *Exptl. Cell Res.* **12**, 615.
- Scarano, E., and Maggio, R. (1957). An exchange between P³²-labelled pyrophosphate and ATP catalyzed by amino acids in unfertilized sea urchin eggs. *Exptl. Cell Res.* **12**, 403.
- Schjeide, O. A., and Urist, M. R. (1956). Proteins and calcium in serums of estrogen-treated roosters. *Science* **124**, 1242.
- Shaver, J. R. (1956). Mitochondrial populations during development of the sea urchin. *Exptl. Cell Res.* **11**, 548.
- Shaver, J. R., and Barch, S. H. (1960). Experimental studies on the role of jelly coat material in fertilization in the frog. *Acta Embryol. Morphol. Exptl.* **3**, 180.
- Shivers, C. A., and Metz, C. B. (1962). Inhibition of fertilization in frog eggs by univalent fragments of rabbit antibody. *Proc. Soc. Exptl. Biol. Med.* **110**, 385.
- Shmerling, J. G. (1965). Isolation and properties of DNA of sturgeon oocytes. *Biokhimiya* **30**, 113 (in Russian).
- Spiegel, M., Ozaki, H., and Tyler, A. (1965). Electrophoretic examination of soluble proteins synthesized in early sea urchin development. *Biochem. Biophys. Res. Commun.* **21**, 135.
- Spirin, A. S., Belitsina, N. V., and Ajtkhozhin, M. A. (1964). Messenger RNA in early embryogenesis. *Dokl. Akad. Nauk SSSR* **25**, 321 (in Russian).
- Stafford, D. W., Sofer, W. H., and Iverson, R. M. (1964). Demonstration of polyribosomes after fertilization of the sea urchin egg. *Proc. Natl. Acad. Sci. U. S.* **52**, 313.
- Stefanelli, A. (1938). Il metabolismo dell'uovo e dell'embrione studiato negli anfibi anuri. II. L'assunzione de ossigeno. *Arch. Sci. Biol. (Naples)* **24**, 411.
- Sugiyama, M. (1951). Re-fertilization of the fertilized eggs of the sea urchin. *Biol. Bull.* **101**, 335.
- Tchou-Su and Wang Yu-lan. (1959). La parthénogénèse traumatique sur l'oeuf

- mature *in vitro* chez le *Bufo*, et la relation entre les degrés de maturité et le développement parthénogénétique. *Acta Exptl. Biol. Sinica* **6**, 276.
- Terman, S. A., and Gross, P. R. (1966). Translation-level control of protein synthesis during early development. *Biochem. Biophys. Res. Commun.* **21**, 595.
- Timourian, H., and Denny, P. C. (1964). Activation of protein synthesis upon fertilization in relation to magnesium and potassium ions. *J. Exptl. Zool.* **155**, 57.
- Tsusaka, A., and Nakano, E. (1965). The metabolic pattern during oogenesis in the fish, *Oryzias latipes*. *Acta Embryol. Morphol. Exptl.* **8**, 1.
- Tyler, A. (1941). Artificial parthenogenesis. *Biol. Rev. Cambridge Phil. Soc.* **16**, 291.
- Tyler, A. (1955). Gametogenesis, fertilization and parthenogenesis. In "Analysis of Development" (B. H. Williers, P. A. Weiss, and V. Hamburger, eds.), pp. 170-212. Saunders, Philadelphia, Pennsylvania.
- Tyler, A. (1959). Some immunobiological experiments on fertilization and early development in sea urchins. *Exptl. Cell Res. Suppl.* **7**, 183.
- Tyler, A. (1962). Introductory remarks. In *Proc. Conf. Immuno-Reprod.*, pp. 13-15. Population Council, New York.
- Tyler, A. (1963). The manipulation of macromolecular substances during fertilization and early development of animal eggs. *Am. Zoologist* **3**, 109.
- Tyler, A. (1964). Survey of some investigations of problems of reproduction by immunologic and protein-biosynthetic methods. *Proc. VIIth Intern. Conf. on Planned Parenthood; Excerpta Medica. Intern. Congr.*, Ser. No. 72, pp. 527-538.
- Tyler, A. (1965). The biology and chemistry of fertilization. *Am. Naturalist* **99**, 309.
- Tyler, A. (1966). Incorporation of amino acids into protein by artificially activated non-nucleate fragments of sea urchin eggs. *Biol. Bull.* **130**, 450.
- Tyler, A., and Brookbank, J. W. (1956). Antisera that block cell division in developing eggs of sea urchins. *Proc. Natl. Acad. Sci. U. S.* **42**, 304.
- Tyler, A., and Humason, W. D. (1937). On the energetics of differentiation. VI. Comparison of the temperature coefficients of the respiratory rates of unfertilized and fertilized eggs. *Biol. Bull.* **73**, 261.
- Tyler, A., and Monroy, A. (1959). Changes in rate of transfer of potassium across the membrane upon fertilization of eggs of *Arbacia punctulata*. *J. Exptl. Zool.* **142**, 675.
- Tyler, A., Monroy, A., Kao, C. Y., and Grundfest, H. (1956). Membrane potential and resistance of the starfish egg before and after fertilization. *Biol. Bull.* **111**, 153.
- Tyler, A., Monroy, A., and Metz, C. B. (1956). Fertilization of fertilized sea-urchin eggs. *Biol. Bull.* **110**, 184.
- Tyler, A., Piatigorsky, J., and Ozaki, H. (1966). Influence of individual amino acids on uptake and incorporation of valine, glutamic acid and arginine by unfertilized and fertilized sea urchin eggs. *Biol. Bull.* **131**, 204.
- Tyler, A., Ricci, N., and Horowitz, N. H. (1938). The respiration and fertilizable life of *Arbacia* eggs under sterile and non-sterile conditions. *J. Exptl. Zool.* **79**, 129.
- Tyler, A., and Schultz, J. (1932). Inhibition and reversal of fertilization in the eggs of the echiuroid worm, *Urechis caupo*. *J. Exptl. Zool.* **63**, 509.
- Tyler, A., Seaton, A., and Signoret, J. (1961). Further analysis of anti-developmental action of antisera against fertilizins and against other egg extracts of sea-urchins. *Am. Zoologist* **1**, 394.

- Tyler, A., and Tyler, B. S. (1966a). The gametes: some procedures and properties. In "Physiology of Echinodermata" (R. A. Boolootian, ed.), Chap. 27. Wiley, New York.
- Tyler, A., and Tyler, B. S. (1966b). Physiology of fertilization and early development. In "Physiology of Echinodermata" (R. A. Boolootian, ed.), Chap. 28. Wiley, New York.
- Vincent, W. S. (1963). Functions of the nucleolus. *Proc. 11th Intern. Congr. on Genetics*, pp. 343.
- Warburg, O. (1910). Ueber die Oxydationen in lebenden Zellen nach Versuchen am Seeigellei. *Z. Physiol. Chem.* **66**, 305.
- Whitaker, D. M. (1931a). On the rate of oxygen consumption by fertilized and unfertilized eggs. I. *Fucus vesiculosus*. *J. Gen. Physiol.* **15**, 167.
- Whitaker, D. M. (1931b). On the rate of oxygen consumption by fertilized and unfertilized eggs. II. *Cumingia tellinoides*. *J. Gen. Physiol.* **15**, 183.
- Whitaker, D. M. (1931c). On the rate of oxygen consumption by fertilized and unfertilized eggs. III. *Nereis limbata*. *J. Gen. Physiol.* **15**, 191.
- Whitaker, D. M. (1933a). On the rate of oxygen consumption by fertilized and unfertilized eggs. IV. *Chaetopterus* and *Arbacia punctulata*. *J. Gen. Physiol.* **16**, 475.
- Whitaker, D. M. (1933b). On the rate of oxygen consumption by fertilized and unfertilized eggs. V. Comparison and interpretation. *J. Gen. Physiol.* **16**, 497.
- Whiteley, A. H., and Chambers, E. L. (1960). The differentiation of a phosphate transport mechanism in the fertilized egg of the sea urchin. In "Symposium on Germ Cells and Development" (Pallanza S. Ranzi, ed.), pp. 387-401. Inst. Intern. d'Embryologie and Fondazione Baselli, Milan.
- Whiteley, A. H., McCarthy, B. J., and Whiteley, H. R. (1966). Changing populations of messenger RNA during sea urchin development. *Proc. Nat. Acad. Sci. USA* **55**, 519.
- Wilson, E. B. (1903). Experiments on cleavage and localization in the nemertine egg. *Arch. Entwicklungsmech. Organ.* **16**, 411.
- Wilt, F. H. (1964). Ribonucleic acid synthesis during sea urchin embryogenesis. *Develop. Biol.* **9**, 299.
- Wilt, F. H., and Hultin, T. (1962). Stimulation of phenylalanine incorporation by polyuridylic acid in homogenates of sea urchin eggs. *Biochem. Biophys. Res. Commun.* **9**, 313.
- Yasumasu, I., and Koshihara, H. (1963). Amino acyl RNA and transfer enzyme in sea urchin eggs. *Zool. Mag. (Tokyo)* **72**, 259 (in Japanese).
- Yasumasu, I., and Nakano, E. (1963). Respiratory level of sea urchin eggs before and after fertilization. *Biol. Bull.* **125**, 182.
- Yatsu, N. (1905). The formation of centrosomes in enucleated egg-fragments. *J. Exptl. Zool.* **2**, 287.
- Ycas, M. (1954). The respiratory and glycolytic enzymes of sea urchin eggs. *J. Exptl. Biol.* **31**, 208.
- Zeuthen, E. (1944). Oxygen uptake during mitosis. Experiments on the eggs of the frog (*Rana platyrrhina*). *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.* **25**, 191.

CHAPTER 9

Parthenogenesis in Vertebrates

Richard A. Beatty

AGRICULTURAL RESEARCH COUNCIL UNIT OF ANIMAL GENETICS,
AND UNIVERSITY OF EDINBURGH, EDINBURGH, SCOTLAND

I. Introduction	413
II. Normal Cytology of Female Meiosis	414
III. Normal Sex Chromosomes	415
IV. Cytogenetics of Parthenogenesis	418
V. Experimental Induction of Parthenogenesis	419
VI. Viability of Parthenogenones	420
VII. Fishes	421
VIII. Amphibians	423
A. <i>Rana japonica</i>	425
B. <i>Rana nigromaculata</i>	426
C. <i>Rana pipiens</i>	427
IX. Reptiles	427
X. Birds	428
XI. Mammals	430
A. Mouse	430
B. Rat	431
C. Hamster	431
D. Guinea Pig	431
E. Rabbit	432
F. Sheep	433
G. Ferret	433
H. Man	433
I. Discussion, Adult Parthenogenesis in Mammals	434
References	435

I. Introduction

Parthenogenesis is the production of an embryo from a female gamete without any genetic contribution from a male gamete, and with or without eventual development into an adult. This deliberately wide definition includes the special case of gynogenesis, in which a spermatozoon does enter the egg and activate it into development, but does not exercise its second role of contributing genetic material. A parthenogenetic organism is a parthenogenone, with a gynogenone as a special

type. Amphimixis means a fusion of reproductive cells of opposite sex. Apomixis means development without such fusion, and in vertebrates is synonymous with parthenogenesis (excluding gynogenesis, except in a special sense). Automixis in animals is a type of apomixis in which there is fusion of cells of the same sex, as when a polar body fuses with the oocyte. Androgenesis is the development of an egg with paternal chromosomes only.

Typically, a gamete is haploid, with one set of chromosomes. The zygote (fertilized egg, embryo, and adult) is diploid, with two sets. Higher multiples of the haploid number are polyploid: triploid, tetraploid, etc. All abnormal chromosome numbers are heteroploid, i.e., diploid gametes, haploid zygotes, and all polyploids and aneuploids. (In an evolutionary context, heteroploidy is not necessarily "abnormal," as in a triploid species, for example.) In aneuploids, one or more haploid sets of chromosomes is incomplete or else contains supernumerary chromosomes. (The term "aneuploid" as used by some biologists is synonymous with the word heteroploid, defined above.) The basic haploid number of chromosomes is usually symbolized as $1n$, diploids being $2n$, triploids $3n$, etc. An embryo developing with the genetic material of only one parent is a zygoid.

The total array of chromosomes in a cell is the chromosome complement. The centriole is a body close to the nucleus that plays an organizing role in cell division. The centromere is a body on each chromosome to which spindle fibers are attached during division. In germ cells, all the chromosomes of the haploid complement are different, or non-homologous. In a diploid cell, each chromosome is represented by two homologous partners. Crossing-over can occur only between homologous chromosomes. In sex chromosomes, a complication to be considered later is that part is homologous and part is not.

Some reviews of vertebrate parthenogenesis or of the wider field of chromosomal deviations are: general (Tyler, 1941; Bungenberg de Jong, 1957; Gallien, 1962; Peacock and Weidmann, 1964); amphibians and general (Fankhauser, 1945, 1955; Gallien, 1953); experimental mammals (Russell, 1962); mammals and general (Beatty, 1957); sex and general (Beatty, 1964a); gynogenesis (Beatty, 1964b.) Many studies of early parthenogenesis are more particularly relevant to the subject of activation, and are considered in Chapter 8.

II. Normal Cytology of Female Meiosis

In the division of a diploid body cell, the chromosomes divide into two strands called chromatids, still joined by the centromere of the chromosome. A pair of homologous chromosomes is therefore represented by four chromatids, and each set of chromatids is potentially a

set of chromosomes in a daughter cell. In ordinary cell division (mitosis), the four chromatids are distributed between two daughter cells, thus giving diploidy in each daughter cell. In meiosis they are actually or presumptively distributed among the four cells arising from two cell divisions, thus giving the reduced or haploid number in each gamete. There is usually no resting phase between the two divisions of meiosis. The second meiotic division resembles an ordinary mitosis. Some detailed events of female meiosis are as follows.

At the beginning of meiosis in the female the nucleus of the diploid oogonium resolves itself into two sets of chromosomes. The two chromosomes of a pair associate side by side as a bivalent, each chromosome splitting longitudinally into two chromatids still joined by the original centromere. The resulting group of four chromatids at a particular stage is called a tetrad. The paired chromosomes are held together by chiasmata, where interchange has occurred between a chromatid of one member and a chromatid of the other (crossing-over). At the first maturation division a dyad (one centromere with its two chromatids), together with a little cytoplasm, is cast out as a first polar body, symbolized as 1PB. The remaining dyad stays in the egg, the latter being now called a secondary oocyte. In most species, ovulation now intervenes. It will be realized that a dyad represents one original chromosome of the oogonium, except in regions where crossing-over has altered it.

Early in the second meiotic division, each dyad divides into its two chromatids after division of the centromere joining them. Completion of the second maturation division is stimulated by the entry of the spermatozoon; one chromatid of each dyad goes into a second polar body (2PB), while the other chromatid remains in what is now called the matured gamete, or ootid. The chromatids in the ootid are again now called chromosomes and constitute one haploid complement. The maternal and paternal sets of chromosomes each form into a pronucleus. The two pronuclei become apposed to one another and resolve into chromosomes that intermingle to give the diploid complement of the zygote. Ordinary mitosis gives the two diploid daughter cells at the first cleavage (1CL) of the zygote. It will be noted that the four sets of chromosomes produced by female meiosis are still in association at the end of meiosis. One set is in the egg, two in the first polar body, and one in the second polar body. We shall refer later to abnormalities in this distribution.

III. Normal Sex Chromosomes

In any normal diploid animal there are two haploid sets of chromosomes, one from each of its parents. Each chromosome of the haploid set is therefore represented in the diploid by a pair of homo-

logous chromosomes. They are homologous in the sense that they have an identical appearance under the microscope, contain identical genetic loci (though not necessarily the same alleles at each locus), associate during meiosis, and because crossing-over can occur between them at meiosis.

These remarks have to be modified for the special pair of sex chromosomes known in most vertebrates. (The other chromosomes are autosomes, one haploid set being symbolized as 1A). In mammals, for instance, the XX female has a pair of X chromosomes that are homologous in the full sense given above. In the male, however, the partner of the single X chromosome is a Y chromosome only partially homologous with the X. The parts of the X and Y chromosomes that are homologous are the homologous segments. The nonhomologous parts, generally at one end of the chromosomes, are the differential segments. These latter usually differ in appearance, do not contain identical loci, and chiasmata do not form between them. It will be realized that the concept of nonhomology of differential segments has meaning only when the X and Y of the male are being compared; in the female, the differential segments of the two X chromosomes *are* homologous with one another.

Essentially, the sex of offspring is determined by the inheritance of the differential segments. The homogametic sex is that with a fully homologous pair of sex chromosomes. The heterogametic sex is that with an unequal and only partially homologous pair. In mammals, some fishes, and some amphibians, the female is homogametic (XX) and the male heterogametic (XY), this being the XX-XY mechanism used as an example in the previous paragraph. In reptiles, some fishes, and some amphibians, the female is heterogametic and the male homogametic, a convenient terminology being ZW for the female and ZZ for the male (ZW-ZZ mechanism). In birds also the female is heterogametic, and the mechanism is often referred to as ZW-ZZ. However, there is no real evidence in birds that the W chromosome exists, and the mechanism is sometimes symbolized as ZO-ZZ, the female being supposed to have one sex chromosome less than the male. The utility of X and Y symbols versus Z and W is that the identity of the heterozygous sex is implicit in the symbolism.

The mechanics of normal meiosis produces equal numbers of X- and Y-bearing spermatozoa (or of Z- and W-bearing eggs) and hence allows for a sex ratio of unity at conception. With the XX-XY mechanism, the only sex chromosomes in an egg are X. The ZW-ZZ mechanism involves more complex possibilities, since the mode of inheritance of the differential segments in the female depends on the position of the centromere relative to the differential segments, and on the balance of pre- and post-

reduction. Let us consider, then, meiosis in a ZW female. "Z" and "W" will be used to symbolize the differential segments, "." to denote a centromere, and () to enclose the chromosome constitution of a cell.

First, in a ZW female, suppose the centromere to lie in the differential segments. At the tetrad stage of meiosis the original diploid oogonial pair of chromosomes ($Z \cdot + \cdot W$) will have become ($Z \cdot Z + W \cdot W$), i.e., a Z chromosome split into two sister chromatids still joined by a centromere, and a correspondingly split W chromosome. At the first meiotic division the centromeres will drag both chromatids of one differential segment into the first polar body, and both chromatids of the other differential segment are left in the oocyte, so that the content of the secondary oocyte is either ($Z \cdot Z$) or ($W \cdot W$). This is a reductional division of the segments, meaning that the products of division are unlike in the two daughter cells. At the second meiotic division, division is equational, since the centromere divides to give ($Z \cdot \cdot Z$) or else ($W \cdot \cdot W$) and the identical sister chromatids of the original chromosome are partitioned among two daughter cells, leaving either $Z \cdot$ or $\cdot W$ in the egg. The whole process is called prereduction, since unlike segments separated at the first meiotic division. Under the present assumption of the centromere lying in the differential segments, these various processes are unaffected by crossing-over that may have occurred between the homologous segments.

Now consider what happens when the centromere is assumed to lie in the homologous segments in the ZW female. If there is no crossing-over in the homologous region between the centromere and the differential segments, the chromosomes and chromatids will be partitioned as described in the previous paragraph, and prereduction will occur. But if a single chiasma exists between the centromere and the differential segment, a new situation arises. The switching of material now gives ($Z \cdot W + Z \cdot W$) for the tetrad constitution. At the first meiotic division there is therefore an equational division, ($Z \cdot W$) being the constitution of both the oocyte and the first polar body. At the second meiotic division the centromeres divide to give ($Z \cdot \cdot W$), and a reductional division leaves either $Z \cdot$ or $\cdot W$ in the matured gamete. The whole process is called postreduction. The type of reduction may be obligatory to the species or may vary within or among animals of a species (facultative pre- and postreduction). The occurrence of more than one chiasma in a cell brings complications. Clearly, one chiasma gives a ($Z \cdot W + Z \cdot W$) constitution for the tetrad, as just described, but a second chiasma might undo the switching of material and restore the differential segments to their original centromeres, to give ($Z \cdot Z + W \cdot W$) once again. This point will not be pursued, except to note the following: if all the

chiasmata in a given cell are fully known, then pre- or postreduction are necessary and predictable consequences; but if all we know is a cytologically observable pre- or postreduction, we cannot infer the nature of the chiasmatic processes.

The meiotic process as a whole reduces the number of chromosomes from diploid to haploid. Further, if we count chromosomes by counting the number of centromeres, then the chromosome number is always reduced at the first meiotic division. However, the word reductional is defined not in this sense but as in the preceding paragraphs.

In short, preréduction in sex chromosomes means a reductional division of the differential segments at the first meiotic division, and postreduction means a reductional division at the second meiotic division. In mammals, the only vertebrate group in which we can generalize, preréduction is typical. In normal meiosis, the end result, presumptive equality of the numbers of the sexes at conception, is unaffected by the type of reduction. But pre- and postreduction are highly relevant to the abnormal events of parthenogenesis, as we shall see in Section IV; for example, according to the type of reduction, a ZW mother might give parthenogenetic offspring all of her own sex or, alternatively, of both sexes.

IV. Cytogenetics of Parthenogenesis

Attention will be confined to the commoner types, haploid and diploid parthenogenesis, both arising by apomixis. Haploid parthenogenesis arises when the whole cytological background is normal except for the absence of a spermatozoon (or, in gynogenesis, the absence of a genetically effective spermatozoon). Diploid parthenogenesis can arise as a result of certain specific disturbances of female meiosis or of the first cleavage division, either of spontaneous origin or else experimentally imposed. Various types of parthenogenone may now be considered; the question marks indicate that there is *a priori* doubt about the viability or the sex of potential parthenogenones or both.

First, consider the case of the ZW mother. Haploid parthenogenones would be in the ratio of 1 Z (♂?) : 1 W (♀?). There are three main kinds of diploid. Diploid parthenogenesis Type 1PB arises by automixis when the first polar body is suppressed and all the strands of the tetrad remain in the egg. In the absence of crossing-over, the strands will still be in the oogonial form ($Z \cdot Z + W \cdot W$), reduced to ($Z \cdot + \cdot W$) at the second meiotic division, so that all parthenogenones will be ZW (♀). In the presence of one chiasma the strands will be in the altered form ($Z \cdot W + Z \cdot W$) and, with independent segregation of sister chromatids at the second meiotic division, will give rise to offspring in the ratio

1 ZZ (♂):2 ZW (♀):1 WW (♀?). Diploid parthenogenesis Type 2PB arises by automixis when the second polar body is suppressed and two sets of chromatids remain in the egg instead of one set. If prereduction had occurred at the first meiotic division, the constitution of the secondary oocyte would have been either (Z·Z) or (W·W) and, in the absence of a second polar body, the parthenogenones are in the ratio 1 ZZ (♂):1 WW (♀?). With postreduction, the oocytes after emission of the first polar body would contain only (Z·W), and the parthenogenones would all be ZW (♀). Diploid parthenogenesis Type 1CL occurs when a suppression of the first cleavage of a developing haploid egg leaves two identical sets of chromosomes in the egg, the sex chromosomes being either ZZ (♂) or WW (♀?) with equal expectation. Since the products of chromosomal replication at the first mitosis have been left together in one cell, this kind of parthenogenone must be homozygous at every genetic locus.

Second, consider the XX mother. Pre- and postreduction are no longer of interest, and each parthenogenone will contain, simply, a number of X chromosomes equal to its ploidy. All haploid parthenogenones will be X (♀?), all diploid parthenogenones XX (♀).

V. Experimental Induction of Parthenogenesis

There are two main experimental requirements in the induction of parthenogenesis. First, the egg must be activated to begin development as a potentially haploid parthenogenone. If diploid parthenogenesis is required, it is necessary also to double the chromosome number. General references are Tyler (1941) and Fankhauser (1945), and laboratory procedures are given by Rugh (1962).

Some classical means of activation are hypo- and hypertonic solutions, acid or alkali, pricking the egg in the presence of blood, heat treatment, and cold treatment. In induced gynogenesis (see Beatty, 1964b), a genetically inactive spermatozoon is used to activate the egg, the principal modes of inactivation of the spermatozoon being by X-rays, radium irradiation, ultraviolet rays, photodynamic chemicals in the presence of light, and by use of nitrogen mustard; all except the last involve radiation of some kind. Induced gynogenesis has now become a routine method for the production of haploid amphibian cells (Selman, 1958).

The main methods of doubling chromosome number by suppression of a polar body or of the first cleavage are the application of either heat or cold treatment, with best results when the change in temperature is made abruptly. Temperature treatments may also activate the egg, so that Type 2PB parthenogenesis can be produced by one experimental

operation. The optimum temperature treatment lies within a narrow range. The egg is not readily available for experimental suppression of the first polar body, but is more accessible at the time of the second polar body or the first cleavage. Chromosome doubling sometimes occurs spontaneously in an activated egg, so that, once again, a single operation may produce a few diploid parthenogenones.

A good example of experimental activation followed by experimental doubling of chromosome number is found in Fischberg (1947).

VI. Viability of Parthenogenones

Deleterious recessive genes in heterozygous form are surprisingly common in animal populations. For instance, only about 1 in 20,000 human beings are albinos (homozygotes), yet about 1 in 70 carry the gene in heterozygous form; in cattle, a gene causing dropsy in calves is manifest in about 1 in 300 births, yet roughly 1 out of 10 bulls is a heterozygote (after Falconer, 1960). Deleterious genes affecting viability come to notice especially when inbreeding is practiced and there is a decrease in heterozygosity; an accumulation of such genes causes a rise in the incidence of abnormalities, death of individuals, and extinction of inbred lines in the first stages of inbreeding. In diploid animals a decrease in heterozygosity simply means an increase in homozygosity, and any deleterious gene will express itself as soon as it becomes homozygous and is no longer "covered" by a normal allele. A phenomenon analogous to homozygosity is the hemizyosity of haploid animals, where every gene is present in single dose and there is necessarily no normal allele to "cover" it. Parthenogenesis involves decreased heterozygosity and therefore decreased viability. Another factor is heteroploidy per se; it is a general rule in heteroploid animals that cell size is positively correlated and cell number negatively correlated with the number of chromosome sets (Fankhauser, 1945), and these unusual circumstances can lead to physiological disturbances. The effects of decreased heterozygosity and of heteroploidy per se may now be considered in the various classes of parthenogenones, most of the information coming from the amphibians.

In haploid parthenogenesis, as also in haploid androgenesis, amphibians develop a typical haploidy syndrome. This syndrome (after Hamilton, 1963) begins with a delay in gastrulation and the existence of unusually small cells at the animal pole. After closure of the neural folds of the abnormally short neural plates, the embryos show microcephaly, lordosis, and ventral oedema. After hatching, the animals are sluggish, inactive, and with abnormal muscle tissue. The death rate is high, and nearly all animals perish at an early stage. A few become

tadpoles, but metamorphosis (reported by Miyada, 1960) is very rare. An important paper by Subtelny (1958) permits one to disentangle the effects of heteroploidy *per se* and absence of heterozygosity in causing the syndrome, though he was not working with parthenogenones. He made haploids by transplanting nuclei from androgenetic haploid blastulas into enucleated eggs. The resulting haploids, necessarily nonheterozygous, exhibited the typical haploidy syndrome. Some of the eggs underwent a spontaneous chromosome doubling by suppression of the first cleavage division. As explained in Section IV, such eggs are also wholly nonheterozygous—but they did *not* show the haploidy syndrome. Hence, the haploidy syndrome must be due to heteroploidy (haploidy) *per se*.

In diploid parthenogenesis there is no heteroploidy, and only the degree of homozygosity is relevant to viability. Types 1PB and 2PB will be homozygous except in some cross-over regions, and a deleterious effect on viability is expected. Type 1CL, however, is equivalent to the theoretical extreme of inbreeding, since the animals contain two identical chromosome complements and are homozygous at every genetic locus, and low viability is expected. In Subtelny's (1958) work the (androgenetic) homozygous diploids were normal up to the late tail-bud stage, but subsequent development varied with the individual and was often retarded and abnormal. Part of the variability might have been due to segregation of deleterious genes among the androgenones and part to the general genetic phenomenon that highly homozygous animals (and therefore, presumably, hemizygous animals) are not genetically "buffered" against adversity, and so their development is easily influenced by local environmental conditions.

In polyploid parthenogenesis, which is rarely encountered, the polyploidy itself would not necessarily give poor viability, since nonparthenogenetic triploids and tetraploids in amphibians often have good viability, and pentaploids are known as adults. Further, viable nonparthenogenetic triploids are known in fishes, reptiles, and birds.

In experimental studies of parthenogenesis there would be every reason to employ whenever possible long-established inbred strains. The very existence of such a strain implies that major deleterious genes have been reduced in number or even eliminated during the process of inbreeding.

VII. Fishes

As in any vertebrate, the production of fatherless young in fishes is not necessarily equivalent to parthenogenesis, since self-fertilizing hermaphroditism is a known phenomenon in fish. For instance, Spurway

(1957) demonstrated functional hermaphroditism in *Lebistes reticulatus*, thereby correcting an earlier report of parthenogenesis. All specimens of *Rivulus marmoratus* are hermaphrodites, and the normal mode of reproduction is by self-fertilization (Kallman and Harrington, 1964).

Lestage (1934) reviewed the work of Pirola, who had claimed parthenogenetic development of eggs of *Paralosa lacustris lariana* after treatment with a special solution and the passage of a weak electric current. Lieder (1955) recorded an all-female race of *Carassius auratus gibelio*. Artificial insemination with spermatozoa of other races gave "hybrid" offspring with a purely maternal phenotype. He noted (1959) that the egg is ovulated after emission of the first polar body and is at metaphase of the second meiotic division at the time of "fertilization." When "fertilized" by *Carassius carassius*, the egg is found to contain the foreign spermatozoon only in the form of a small pycnotic mass at the time of the first cleavage division. Since both polar bodies had been emitted, the regulation of the gynogenetically developing egg to diploidy probably occurred by a failure of cytoplasmic division at the time of the first nuclear division of the zygoid. In *Esox lucius*, Kasansky (1934) reported early parthenogenetic development after experimental treatments of unfertilized eggs or after exposure to foreign spermatozoa. In 1935, he recorded parthenogenetic development up to 70 days in eggs of *Cyprinus carpio* after treatment with human saliva. Of these various claims that of Lieder seems to be the best established.

Svärdson (1945) inseminated eggs of *Salmo salar* ($1n = 30$) with spermatozoa of *Salmo trutta* ($1n = 40$) and subjected the eggs to a cold treatment. Three haploid embryos with a chromosome number of 30 were evidently parthenogenetic. Haploid embryos of *Gasterosteus aculeatus* at an advanced embryonic stage were recorded by Swarup (1956) as a by-product of successful experiments for the induction of triploidy by temperature treatments of fertilized eggs. These haploids were probably androgenetic, but the same treatment of eggs in the absence of spermatozoa yielded parthenogenetic haploids that could reach the gastrula stage (Swarup, 1957, 1959).

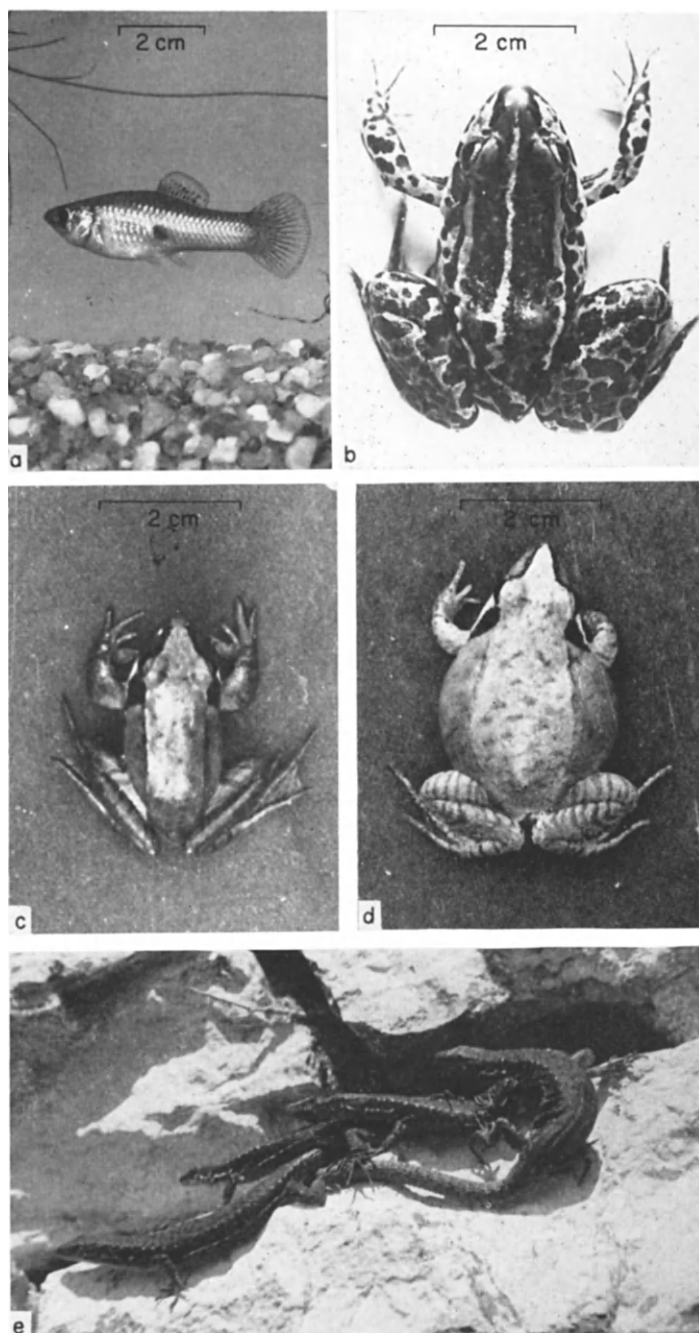
It has been known since the pioneer paper of Hubbs and Hubbs (1932) that gynogenesis occurs in the teleost *Mollienesia formosa* (see Fig. 1, a). Males are virtually unknown, and females kept in isolation do not reproduce. Young are produced after mating to males of *M. sphenops*, *M. latipinna*, and other species, and this is thought to be the natural mode of reproduction of *M. formosa*. The young are gynogenetic "false hybrids" to which the spermatozoa contribute no hereditary material. The young resemble only the maternal species, *M. formosa*, and this holds over many generations of "back-crossing" to males of

other species. In such a mode of reproduction, one would expect clones to develop, in which there might be genetic differences in histocompatibility between clones, but there should be a virtual genetic identity of individuals within clones. This point was tested by Kallman (1962), who transplanted tissues from offspring to parents, between members of the same sib, between members of different sibs from the same clone, and between members of different clones. In accordance with expectation, grafts within a clone were successful, grafts between clones unsuccessful.

VIII. Amphibians

In amphibians, very many young parthenogenones have been reported in many species. Attention will be restricted to some general points and to selected examples of survival to the age when sex can be determined or to the adult stage.

The study of parthenogenesis and heteroploidy in amphibians has been hampered by lack of knowledge about a highly important factor, the identity of the heterogametic sex. Much of the earlier literature is pervaded with the idea that the heterogametic sex tends to be the male in Anurans and the female in Urodeles. This idea must be allowed to die. It is necessary merely to mention that the only modern cytological proofs in any amphibians show female heterogamety in two Anurans, *Xenopus laevis* (Weiler and Ohno, 1962; Morescalchi, 1963) and *Discoglossus pictus* (Morescalchi, 1964), and male heterogamety in five Urodeles, *Oedipina* species (Kezer, unpublished). From this and other evidence we now know that, according to the species, both types of heterogamety can be found in Urodeles and perhaps also in Anurans. Direct evidence comes from consideration of cytologically visible sex chromosomes, from breeding of sex-reversed or hermaphrodite animals, and (though not yet in amphibians) from sex-linkage studies. Indirect and less crucial evidence comes from the sex of triploids, the sex of androgenones and parthenogenones, and from the sex of offspring after species hybridization (Haldane, 1922: "When in the F₁ offspring of two animal races one sex is absent, rare, or sterile, that sex is the heterozygous sex"). The author, in surveying these forms of evidence, has attempted an appraisal of the identity of the heterogametic sex (Beatty, 1964a). The following is a summary. The heterogametic sex is marked by an asterisk when it is particularly well established and is in brackets when it is doubtful: *Hynobius* spp., ♂; *Triturus* spp., ♂; *Oedipina* spp., ♂*; *Pleurodeles* ♀*; *Ambystoma* (*Siredon*) ♀* (the absence of an asterisk in the original survey is regretted); among the Anurans, *Bufo vulgaris* ♀; *Xenopus* ♀*; *Rana arvalis* ♀; *R. japonica*, *nigromaculata*, and



temporaria, ♂; *Discoglossus* ♀*, may now be added to the Anurans, as mentioned above.

A. *Rana japonica*

Diploid parthenogenesis after pricking unfertilized eggs in the presence of blood and then suppressing the second polar body by cold treatment has been reported in a series of papers by Moriwaki (1957, 1959, 1960a,b). Examples are shown in Fig. 1, c, d. The parthenogenones were diploid, Type 2PB. Mature animals comprised seven males and nine females. The female of the species was considered to be the homogametic sex, and the presence of both sexes among parthenogenones was therefore surprising. It was assumed that the male parthenogenones were genetic females that had undergone a sex reversal. One such male when crossed to a normal female (the latter presumed XX) did, indeed, produce all-female offspring, as if it were itself XX, i.e., a "false male" that was genetically female. In the later papers, the assumption of an XX constitution for parthenogenones of both sexes was supported by further breeding tests, except that one male produced from a bisexual cross of two parthenogenetic animals and with only X chromosomes in its ancestry behaved, surprisingly, as if it were YY. In an extension of the work, Moriwaki (1963) reported that in a strain of frogs originating by ordinary bisexual inbreeding from parthenogenones, males appeared more frequently as the number of generations increased, and in the F₃ the sex ratio soon after metamorphosis was about unity. This was believed to be a consequence of genetic changes accumulated during the inbreeding.

Due weight must be given in the first place to the argument of the worker concerned, that the male is the heterogametic sex in this species. There is independent and direct evidence in support: Kawamura and Yokota (1959) showed that artificially sex-reversed females when mated

FIG. 1. (a) The gynogenetic fish *Poecilia (Mollienesia) formosa*. The dark central patch is a transplant (see text). (From a photograph supplied by Dr. K. D. Kallman.) (b) Diploid parthenogenetic female of *Rana nigromaculata*, aged 6 months. The diploidy is thought to have arisen by suppression of the first cytoplasmic cleavage (Type 1CL parthenogenesis). (After Kawamura, 1939b, by permission of the author and of the *J. Sci. Hiroshima Univ.*) (c, d) Diploid parthenogenetic male (c) and female (d) of *Rana japonica*, aged 1 year. The diploidy is presumed to have arisen by suppression of the second polar body (Type 2PB parthenogenesis). (Male reproduced from an unpublished photograph supplied by Dr. T. Moriwaki; female reproduced from Moriwaki, 1957, by permission of the author and the *J. Sci. Hiroshima Univ.*) (e) The parthenogenetic lizard *Lacerta saxicola armeniaca* in natural surroundings. (From a photograph supplied by Dr. I. S. Darevskii.)

to normal females gave virtually all-female offspring. There is, however, a conflict with Haldane's rule, since species hybrids involving *R. japonica* gave all-male offspring (Kawamura and Kobayashi, 1960; Kawamura, 1950), thus indicating female heterogamety (see Section VIII, second paragraph). The direct evidence must be preferred to the indirect, yet a certain discrepancy remains, and there are other difficulties discussed by Beatty (1964a).

B. *Rana nigromaculata*

Heteroploidy and parthenogenesis have been studied intensively in the species by Japanese workers, who feel that the male is the heterogametic sex.

Miyada (1960) obtained gynogenetic haploids after insemination with spermatozoa treated with toluidine blue. Parthenogenones near or after metamorphosis comprised 14 females, 3 males, and 1 hermaphrodite. Evidently, the production of both sexes in a species with supposed female homogamety presents a problem, and Miyada considered that all parthenogenones were chromosomally female (X), but that the abnormal physiology of the haploid condition could have caused some of the genetic females to undergo sex reversal and become males.

Diploid parthenogenesis is well known from the work of Kawamura (1939a,b, 1949) (see Fig. 1, b). In his earlier work in that series of publications, 86 parthenogenetic diploids were identified after pricking unfertilized eggs in the presence of blood. Twenty sexed parthenogenones comprised 14 with normal ovaries, 3 with abnormal ovaries, 2 nearly transformed into males, and 1 with a fairly normal male gonad. In his 1949 paper, Kawamura reported that 17 out of 18 metamorphosed frogs had female gonads, while the remaining one had a male gonad with female tendencies. As with the haploids mentioned above, the main trend once again is that parthenogenones are female, and the rare males could be explained by sex reversal of genetic females.

Tetraploid and hexaploid parthenogenones have been reported by Kawamura (1939a) after pricking unfertilized eggs in the presence of blood.

Again, one should consider in the first place the argument of the workers concerned, that the male is the heterogametic sex in this species, and the hypothesis receives independent support from Haldane's rule, since racial and subspecific crosses yield fertile females and almost sterile males (Moriya, 1960). Nevertheless, the sexes among parthenogenetic and heteroploid offspring in the species can almost equally well be accounted for in terms of female heterogamety, and a direct identification of the heterogametic sex would be welcome.

C. Rana pipiens

In reviewing the classical experiments of J. Loeb, Parmenter (1925, 1926) pointed out that all parthenogenetic metamorphosed tadpoles were diploid, and parthenogenetic adults comprised seven males and two females. Parmenter (1933) recorded development up to an age of 7 months after pricking unfertilized eggs; development was *not* of the ICL type, since there was no delay in the first cleavage, and was thought to be Type IPB. The existence of both sexes among parthenogenones suggests female heterogamety in the species, but no independent confirmation from other lines of evidence seems to be available.

IX. Reptiles

A remarkable fact discovered by Lantz and Cyrén (1936) is that males are totally absent in the *armeniaca* subspecies of the lizard *Lacerta saxicola* Eversmann (see Fig. 1, e), although they are present in other subspecies. Darevskii (1958) found that males are also absent in the subspecies *rostombekowi* and *dahli*. No males were found over the entire period of activity of the lizards between hibernation periods, nor among newly hatched young in field material or in the laboratory. No spermatozoa were ever seen in the sex ducts and cloacae of females. Thus, the data indicated a complete absence of males. The possibility of gynogenesis after copulation with males of bisexually reproducing forms could be rejected, because such species did not come into contact with the all-female subspecies. The existence of a natural nongynogenetic parthenogenesis was proved as follows. Thirty young females of *L. saxicola armeniaca*, including 15 immature females, were overwintered in the absence of males of any description. The 56 young born the next summer, some from the originally immature specimens, were all female. Darevskii and Kulikova (1961) again reported that the subspecies *dahli* and *rostombekowi* were parthenogenetic, while there were both parthenogenetic and bisexual races of the subspecies *defilippii*. In the parthenogenetic forms, which are diploid, the first and second chromosomal meiotic divisions take place normally, and it is assumed that diploidy arises by second polar body suppression or by first cleavage suppression. Malformed embryos, some male, are often found in the early stages of development of eggs from parthenogenetic females.

Mertens (1960) recalled that peculiar sex ratios have been found in gekkos. Maslin (1962) found that six species of the lizard genus *Cnemidophorus* are wholly or virtually all female. He had included a careful search for alternative ecological niches that males, if they existed, might have occupied.

X. Birds

Early parthenogenesis in avian eggs was first reported nearly a century ago. In the chicken (e.g., Olsen, 1956), a limited growth of membranes was observed in some unfertilized eggs. The incidence of parthenogenesis varied between individuals and between breeds, and the extent of the development seemed to be responsive to selective breeding. Fowl pox virus increased the incidence of parthenogenesis, perhaps as a direct cause or by means of some associated contaminant. Poole and Olsen (1958) found the Dark Cornish breed to be outstanding as a producer of eggs showing early parthenogenesis.

In turkeys also, early spontaneous parthenogenesis and the role of fowl pox were demonstrated. The special interest of turkeys, however, is that parthenogenones have survived to the adult stage. Olsen (1960) summarized this fascinating work. A selection for high incidence of parthenogenetic eggs was carried out. In the initial stock of Beltsville Small White turkeys in 1952, the incidence of parthenogenones was as follows: those consisting of membranes only, 16.7%; those with blood as well as membranes (not recorded, but 0.8% in 1953); embryos, 0.2%; all categories, 16.9% (14.1% in 1953). As selection progressed there was a steady rise in the incidence of parthenogenesis, the corresponding figures in 1960 being 25.6%, 4.4%, 9.8%, and 38.8%. The percent hatching also increased. Sixty-seven hatched from 1956 onward, many of these living for a time, with some reaching maturity. All were male. Motile spermatozoa were observed in several. One parthenogenetic cock (see Fig. 2) produced semen that was used to inseminate seven virgin turkeys and another seven whose last mating had been 8 months before. The sex ratio among the offspring was about unity. Hence, the sex chromosomes of the cock were presumably those of a normal male (ZZ).

In arguing the mode of origin of these parthenogenetic turkeys, we start with the knowledge that they are all diploid (Yao and Olsen, 1955; Poole and Olsen, 1957; Poole, 1959). Further, they are all male, and presumed to have the normal sex chromosome constitution ZZ; the results of the breeding test just mentioned are in agreement. As Olsen (1962) pointed out, the most likely mode of origin is through suppression of the second polar body of the egg (Type 2PB parthenogenesis). This gives diploidy, and gives an expectation of male ZZ offspring, as found, together with OO offspring, presumed to be inviable. Type 2PB parthenogenesis is consistent with the immunological evidence from tissue transplantation of Poole *et al.* (1963), in that the parthenogenones were not fully homozygous. Of other possible modes of origin, Type 1CL would have given complete homozygosity and an

expectation of low viability. Type IPB parthenogenesis should have produced at least some females (ZO), but none were encountered. Thus the preferred explanation, suppression of the second polar body, invokes a well-known mechanism for the production of parthenogenesis in un-

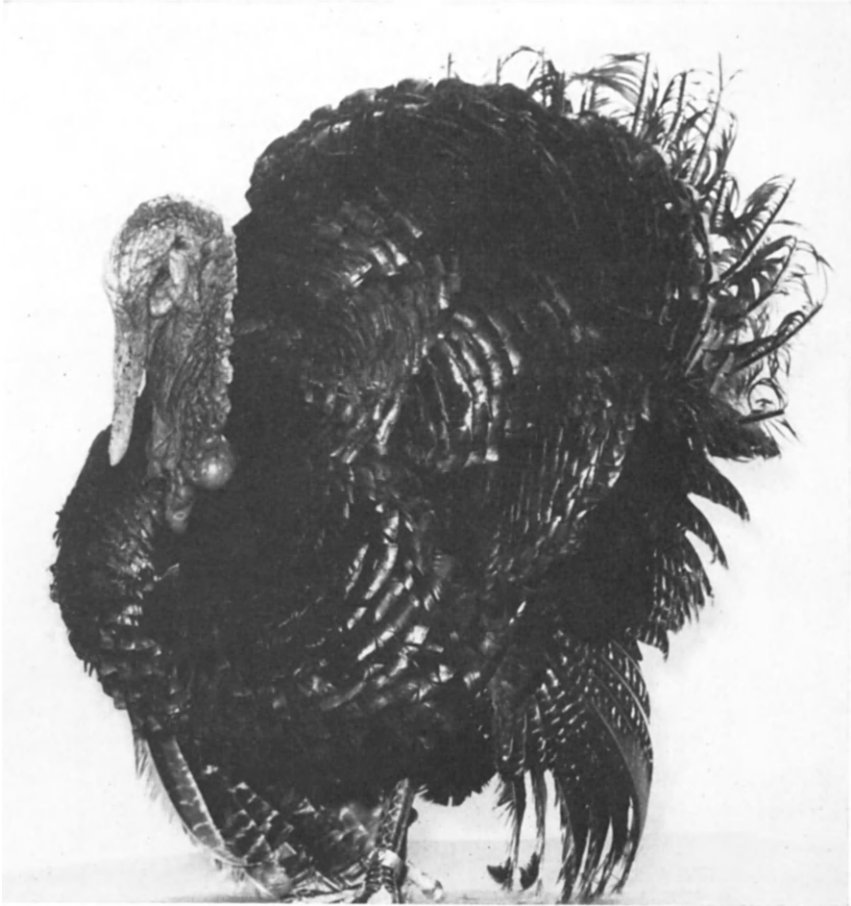


FIG. 2. Mature parthenogenetic male turkey. (By permission of Dr. Marlow W. Olsen, U. S. Department of Agriculture, A.R.S., Animal Husbandry Research Division, Beltsville, Maryland, and the *J. of the British Turkey Federation*.)

fertilized eggs in amphibians and of triploidy in fertilized eggs of fishes, amphibians, and mammals.

The parthenogenetic status of these turkeys is further supported by histocompatibility studies (Healey *et al.*, 1962). In this work, the sex chromosome mechanism is stated as XX in the male and XY in the female (or, in our alternative terminology, a ZW-ZZ mechanism rather

than ZO-ZZ). YY is assumed inviable, the parthenogenones all being XX. In normal nonparthenogenetic animals, sons contain paternal genes not common to the mother; and the mother, with her Y chromosome and because meiotic segregation does not pass all her genes to her son, contains maternal genes not common to the son. It was found as expected that homografts from dam to normal son or normal son to dam were rejected. There is a different situation in parthenogenesis, since all the genes in the parthenogenone also occur in the mother, but not every gene in the mother is shared with her parthenogenetic son. Again as a result of meiotic segregation, brother parthenogenones would not necessarily be genetically alike. These genetic expectations were reflected faithfully in the results from tissue transplantation; grafts from parthenogenone to dam were accepted and those from dam to parthenogenone or between brother parthenogenones were rejected.

In short, all the evidence supports the existence of spontaneous Type 2PB parthenogenesis in the turkey. It will be noted that parthenogenesis in the turkey gives only males, while in the lizard described in Section IX there were fully viable females and some males dying as embryos. Presumably there is a difference in the cytological origin of parthenogenones in the two groups, or else there is a difference in viability between male and female parthenogenones.

XI. Mammals

A. MOUSE

The first maturation division, normally peripheral in the ovarian oocyte, sometimes lay deep in the oocyte, no first polar body was produced, and all chromosomal products of division remained in the egg at that stage (Pesonen, 1946a,b). In one egg, the four sets of chromatids were subsequently arrayed on the metaphase plate of the second meiotic division; if this division could have been completed in an unfertilized egg, with extrusion of a second polar body, we should have had an example of Type 1PB parthenogenesis, or of triploidy if the egg had been fertilized.

Braden and Austin (1954) subjected unfertilized eggs of the mouse to a heat treatment at ca. 45°C with the intention of suppressing the second polar body and causing Type 2PB parthenogenesis. In many such eggs, however, polar bodies were observed, and it was considered that the treatment had resulted in haploid parthenogenesis. Development to the four-cell stage was recorded. They also noted that anaesthesia alone caused some unfertilized eggs to develop a pronucleus and a second polar body, and these again were presumably haploids.

Gynogenesis has been studied intensively in the mouse. Edwards (1954, 1957a) inseminated mice with X-ray-treated spermatozoa and examined the resulting embryos at the blastocyst stage. The higher the dosage of X-rays, the smaller was the number of chromosomes in the blastocysts. In certain mitoses at the higher dosages he observed normal chromosomes in haploid number, presumably the maternal ones, and abnormal or fragmented chromosomes, presumably the paternal ones. Development of eggs was retarded in correspondence with the degree of dosage of the spermatozoa with X-rays. In short, X-rays are an effective gynogenetic agent in the mouse, but the embryos soon come to grief. Ultraviolet light applied to spermatozoa could also act as a gynogenetic agent, about one-third of the embryos being haploid, but development was again retarded (Edwards, 1954, 1957b). Neither nitrogen mustard (Edwards, 1958) nor the radiomimetic substance tryptaflavine (Edwards, 1954, 1958) gave certainly gynogenetic eggs after insemination of treated spermatozoa.

B. RAT

Parthenogenetic development of rat eggs after treatment with 2,4-dichlorophenoxyacetic acid has been claimed by Pesonen and Marjanen (1949). Anaesthesia, with or without chilling of eggs, always caused extrusion of the second polar body, i.e., the beginning of parthenogenetic development (Thibault, 1949). The effect of anaesthesia combined with chilling was confirmed by Austin and Braden (1954b), with development to two-cell stages of normal appearance. Early spontaneous parthenogenesis was reported by Austin (1951) and Austin and Braden (1954a).

C. HAMSTER

The hamster is unusual among mammals in that some 80% of unfertilized eggs become spontaneously activated and extrude a second polar body within a day of ovulation (Austin, 1956). A pronucleus often formed, and a haploid first cleavage spindle was observed. The latest stage seen was a two-cell egg of normal appearance. Usually, however, activated eggs degenerated.

D. GUINEA PIG

Parthenogenesis up to the blastocyst stage, presumably haploid, was reported in a few ovarian eggs by Courrier (1923), Courrier and Oberling (1923), and Lelièvre *et al.* (1927). The beginning of a first cleavage, or possibly an unusual meiotic division, was observed in the ovarian egg by Bacsich and Wyburn (1945).

E. RABBIT

An eight-cell embryo resulted after culturing *in vitro* an unfertilized egg that had initially been subjected to hypertonic treatment (Pincus, 1936, 1939b). Pincus and Enzmann (1936), using ultraviolet irradiation of spermatozoa, noted a probably gynogenetic embryo at the two-cell stage. Cleaved eggs were found after tonicity treatments of unfertilized eggs (Pincus and Shapiro, 1940a). Eggs developing gynogenetically after insemination of spermatozoa treated with X-rays reached the metaphase of the first cleavage (Amoroso and Parkes, 1947). After cold treatment of unfertilized eggs, Thibault (1949) noted a few that had developed a haploid pronucleus. In all these various reports, the developing eggs were presumably haploid.

Diploid parthenogenesis Type 2PB, after chilling of unfertilized eggs and consequent suppression of the second polar body, is a well-established phenomenon in the rabbit. Cleaved eggs, morulae, and a blastocyst originating in this way have been reported (Pincus and Shapiro, 1940a,b; Shapiro, 1942), but the existence of two polar bodies in some eggs was inconsistent with Type 2PB. Thibault (1949) confirmed that Type 2PB eggs develop, and explained the two polar bodies by showing that they are the products of a division of the first polar body, neither being the true second polar body. His latest stage was the 6½-day blastocyst. Ample further confirmation came from Chang (1952, 1954), who noted that about 18% of chilled unfertilized ova developed into diploid blastocysts after suppression of the second polar body.

Diploid parthenogenones at term have been reported in the rabbit. Two live females reported by Pincus (1939b, with additional details in 1939a) may not have been parthenogenetic (see Beatty, 1957). More critical experiments, in which male rabbits were wholly excluded during the experiment, yielded one dead female offspring after treating virgin eggs with hypertonic solution and transferring them to host females (Pincus, 1939b). In another experiment described by Pincus (1939a), virgin eggs from a cc^{ch} dam (i.e., heterozygous for albino and chinchilla) were treated with hypotonic salts, followed by serum and then hypertonic salts, and were finally transferred into host females. Two female young were born, both chinchilla in appearance, and therefore either cc^{ch} or else $c^{ch}c^{ch}$. One of these was fertile and shown by breeding tests to be cc^{ch} . This excluded Type 1CL parthenogenesis, which can give only homozygous animals, but is compatible with Type 2PB parthenogenesis after postreduction at the albino locus.

The rabbit is the mammalian species *par excellence* in which experimental treatments can produce large numbers of well-developed Type

2PB blastocysts. Whether they can really survive to birth must now be regarded as an open question, since no confirmation has come during the quarter of a century that has elapsed since the first and only reports of such survival.

F. SHEEP

Thibault (1949) found that 2 out of 19 unfertilized sheep eggs subjected to cold treatment emitted the second polar body and that 1 had a haploid pronucleus. (In studying experimental gynogenesis in mammals, he found a low incidence of early activation when sheep eggs were exposed to spermatozoa that had been treated with the gynogenetic substance trypanflavine.) The other egg had started diploid parthenogenetic development, with a central diploid pronucleus (see also Thibault and Ortavant, 1949), and another egg with an unknown number of chromosomes was segmenting.

G. FERRET

Chang (1950) reported 12 tubal and uterine ova resembling normal two-six-cell stages in uninseminated immature ferrets, one egg being proved haploid. Altogether, 43% of the eggs showed evidence of parthenogenetic development. In experiments with cold treatment, Chang (1957) noted development up to the morula stage.

H. MAN

An ovarian ovum, cultured *in vitro* in serum with a little ethyl ester of acetic acid, and then pricked, developed to an apparent cleavage stage (Reimann and Miller, 1939). Shettles (1957) reported a two-cell, four-cell, and blastula stage within the intact ovarian follicle, with no apparent access for spermatozoa. However, in 12 cases of ovarian pregnancy examined by Ashley (1959), nuclear sex was of the male type in 7 cases and of the female type in 5. If these embryos were diploid, then parthenogenesis would seem unlikely, and one would think rather of fertilization following entry of spermatozoa into the follicle. The apparent males might, alternatively, have been haploid parthenogenones, or else XO + 2A, but neither possibility was thought likely.

A lecture by Dr. H. Spurway (see "Editorial," 1955), in raising the matter of parthenogenesis in man and in outlining the criteria for recognizing it, set in motion a train of events that led to scientific examination of some possible cases. A newspaper asked mothers to come forward who believed that they had a parthenogenetic child. It would have been interesting at this first enquiry to see if more female than male children were claimed as parthenogenones, since the expectation

is that all parthenogenones should be female, but unfortunately the newspaper mentioned that parthenogenetic children would be female. However, this press activity did bring forward 19 women whose claims were examined carefully by Balfour-Lynn (1956). Eleven claims were immediately eliminated. In the eight remaining cases the mothers and daughters were blood typed. Six of these were eliminated because of antigens possessed by the child but not by the mother, and another was eliminated because of an eye-color factor not shared with the mother. In the remaining case, "Mrs. Alpha and daughter," there was an identity of blood groups between mother and daughter, as also in ability to taste phenyl thiocarbamide, in secretor activity, and in the pattern of direct filter-paper serum electrophoresis. The probability of so good an agreement between mother and daughter after bisexual reproduction was stated to be below 0.01. As a final check, skin grafts were carried out. A graft from daughter to mother was shed in about 4 weeks, while one from mother to daughter appeared to remain healthy for 6 weeks and was then removed. The author found the significance of the results from skin grafting "obscure," but a straightforward interpretation must surely be that the rejection of a graft from daughter to mother means that the daughter possesses antigens not possessed by the mother, and is therefore not parthenogenetic. Perhaps the first lesson of this work is that even a small number of cases can produce one instance in which there is a very substantial similarity between the genotype of mother and daughter, even though a final test suggests normal fertilization rather than parthenogenesis. However, Balfour-Lynn's approach is certainly the right one in investigating problems of this kind.

I. DISCUSSION, ADULT PARTHENOGENESIS IN MAMMALS

Numerous examples of embryonic parthenogenesis in mammals have been quoted in the previous sections, but there was thought to be no confirmed evidence that parthenogenones have come to term. The special importance of mammals warrants some discussion of the possibility of birth, the following being based primarily on "Editorial" (1955, 1956) and Beatty (1957).

An important first point is that we do not expect to find parthenogenesis in mammals, and scientist and layman alike are inhibited from bringing possible cases to notice. In laboratory mammals or in pedigree stocks of domestic animals the breeder might prefer not to draw attention to an inexplicable birth that could signify error or a misalliance rather than parthenogenesis. In mammals having access to males, there would usually be nothing to mark an occasional parthenogenone born in a series of normally conceived offspring. No claim of parthenogenesis

seems ever to have been made from prisons or other establishments where women are segregated from men.

There are also special biological reasons against the conception or continued development of a mammalian parthenogenone. Homozygosity in a diploid parthenogenone is likely to be high or even complete, and the resulting equivalent of inbreeding depression would lower viability. In vertebrates other than mammals haploids have poor viability. There is plenty of evidence in mammals themselves that parthenogenones die before implantation. In mammals such as the rabbit and cat the act of coitus itself is normally necessary for ovulation to occur. In polytocous animals, a litter of one offspring is notoriously inviable, and this is precisely the litter size expected from the development of a rare parthenogenone in an uninseminated dam.

Familiar biological processes can sometimes offer an alternative explanation for apparent cases of parthenogenesis. Even in a genetics laboratory, some unusual modes of inheritance are likely to be ascribed to mutation, though compatible with parthenogenesis; indeed, this point might repay examination. Hermaphroditism is a further possibility; in the end, we can probably never disprove the logical *reductio ad absurdum* that a single spermatozoon might somehow develop in the female, fertilize an egg, and thus give an offspring that has no apparent father but is, nevertheless, not parthenogenetic. As against this, the internal body temperature of mammals is inimical to spermatozoon development, this being the teleological reason why testes are external.

Comparative grounds, however, give some support for the possibility of born parthenogenones in mammals. It may first be noted that heteroploidy and parthenogenesis are allied phenomena, since one and the same cytological abnormality may give rise to parthenogenesis in the absence of a spermatozoon or to heteroploidy in the presence of a spermatozoon. Adult heteroploidy is known in fishes, amphibians, reptiles (Darevskii and Kulikova, 1964), birds and mammals, the discovery in mammals being fairly recent. Adult parthenogenesis is known in fishes, amphibians, reptiles, and birds—and parthenogenetic embryos certainly occur in mammals. We would not be altogether surprised if a finding of adult mammalian parthenogenesis were to provide a last link in this already almost complete parallel. In the meantime, the comparative argument can, at least, be regarded as a strong incentive for further research.

REFERENCES

- Amoroso, E. C., and Parkes, A. S. (1947). Effects on embryonic development of X-irradiation of rabbit spermatozoa *in vitro*. *Proc. Roy. Soc. (London)*, Ser. B 134, 57.

- Ashley, D. J. B. (1959). Are ovarian pregnancies parthenogenetic? *Am. J. Human Genetics* **11**, 305.
- Austin, C. R. (1951). Activation and the correlation between male and female elements in fertilization. *Nature (London)* **168**, 558.
- Austin, C. R. (1956). Activation of eggs by hypothermia in rats and hamsters. *J. Exptl. Biol.* **33**, 338.
- Austin, C. R., and Braden, A. W. H. (1954a). Induction and inhibition of the second polar division in the rat egg and subsequent fertilization. *Australian J. Biol. Sci.* **7**, 195.
- Austin, C. R., and Braden, A. W. H. (1954b). Anomalies in rat, mouse and rabbit eggs. *Australian J. Biol. Sci.* **7**, 537.
- Bacsich, P., and Wyburn, G. M. (1945). Parthenogenesis of atretic ova in the rodent ovary. *J. Anat.* **79**, 177.
- Balfour-Lynn, S. (1956). Parthenogenesis in human beings. *Lancet* **2**, 1071.
- Beatty, R. A. (1957). "Parthenogenesis and Polyploidy in Mammalian Development." Cambridge Univ. Press, London and New York.
- Beatty, R. A. (1964a). Chromosome deviations and sex in vertebrates. In "Intersexuality in Vertebrates Including Man" (C. N. Armstrong and A. J. Marshall, eds.), Chap. 2, pp. 17-143. Academic Press, New York.
- Beatty, R. A. (1964b). Gynogenesis in vertebrates: fertilization by genetically inactivated spermatozoa. *Proc. Intern. Symp. on Effects of Ionizing Radiation on the Reproductive System, Fort Collins, Colorado, 1962* (W. D. Carlson and F. X. Gassner, eds.), p. 229.
- Braden, A. W. H., and Austin, C. R. (1954). Reactions of unfertilized mouse eggs to some experimental stimuli. *Exptl. Cell Res.* **7**, 277.
- Bungenberg de Jong, C. M. (1957). Polyploidy in animals. *Bibliog. Genetica* **17**, 111.
- Chang, M. C. (1950). Cleavage of unfertilized ova in immature ferrets. *Anat. Record* **108**, 31.
- Chang, M. C. (1952). Fertilizability of rabbit ova and the effects of temperature *in vitro* on their subsequent fertilization and activation *in vivo*. *J. Exptl. Zool.* **121**, 351.
- Chang, M. C. (1954). Development of parthenogenetic rabbit blastocysts induced by low temperature storage of unfertilized ova. *J. Exptl. Zool.* **125**, 127.
- Chang, M. C. (1957). Natural occurrence and artificial induction of parthenogenetic cleavage of ferret ova. *Anat. Record* **128**, 187.
- Courrier, R. (1923). Vésicule blastodermique parthénogénétique dans un ovaire de cobaye impubère. *Arch. Anat. Histol. Embryol.* **2**, 455.
- Courrier, R., and Oberling, C. (1923). Parthénogénèse spontanée dans l'ovaire du cobaye. *Bull. Soc. Anat. Paris* **93**, 724.
- Darevskii, I. S. (1958). Natural parthenogenesis in certain subspecies of rocky lizard, *Lacerta saxicola* Eversmann. *Dokl. Akad. Nauk SSSR* **122**, (in Russian). (Translation by Am. Inst. Biol. Sci., Washington, D.C., *Biol. Sci. Sect.* 877.)
- Darevskii, I. S., and Kulikova, W. N. (1961). Natürliche Parthenogenese in der polymorphen Gruppe der kaukasischen Felseidechse (*Lacerta saxicola* Eversmann). *Zool. Jahrb. Systematik* **89**, 119.
- Darevskii, I. S., and Kulikova, V. N. (1964). Natural triploidy within a polymorphic group of *Lacerta saxicola* Eversmann, resulting from hybridization between bisexual and parthenogenetic subspecies of this species. *Dokl. Akad. Nauk SSSR* **158**, 202 (in Russian).

- "Editorial." (1955). Parthenogenesis in mammals? *Lancet* **2**, 967.
- "Editorial." (1956). The virgin births newspaper feature. *S. African Med. J.* **30**, 804.
- Edwards, R. G. (1954). The experimental induction of pseudogamy in early mouse embryos. *Experientia* **10**, 499.
- Edwards, R. G. (1957a). The experimental induction of gynogenesis in the mouse. I. Irradiation of the sperm by X-rays. *Proc. Roy. Soc. (London)*, *Ser. B* **146**, 469.
- Edwards, R. G. (1957b). The experimental induction of gynogenesis in the mouse. II. Ultra-violet irradiation of the sperm. *Proc. Roy. Soc. (London)*, *Ser. B* **146**, 488.
- Edwards, R. G. (1958). The experimental induction of gynogenesis in the mouse. III. Treatment of sperm with trypanflavine, toluidine blue, or nitrogen mustard. *Proc. Roy. Soc. (London)*, *Ser. B* **149**, 117.
- Falconer, D. S. (1960). "Introduction to Quantitative Genetics." Oliver & Boyd, Edinburgh and London.
- Fankhauser, G. 1945. The effects of changes in chromosome number on amphibian development. *Quart. Rev. Biol.* **20**, 20.
- Fankhauser, G. (1955). The nucleus and cytoplasm in development. In "Analysis of Development" (B. H. Willier, P. A. Weiss, and V. Hamburger, eds.), Chap. 1, pp. 126-150. Saunders, Philadelphia, Pennsylvania.
- Fischberg, M. (1947). Experimentelle Auslösung von haploider und diploider parthenogenese bei den Urodelen *Triton palmatus* und *Triton alpestris*. *Arch. Julius Klaus-Stift. Vererbungsforsch. Sozialanthropol. Rassenhyg.* **22**, 331.
- Gallien, L. (1953). L'hétéropléidie expérimentale chez les amphibiens. *Année Biologique* **29**, 5.
- Gallien, L. (1962). Hétéropléidie et sexualité chez les vertébrés. *Année Biologique* **1**, 53.
- Haldane, J. B. S. (1922). Sex ratio and unisexual sterility in hybrid animals. *J. Genet.* **12**, 101.
- Hamilton, L. (1963). An experimental analysis of the development of the haploid syndrome in embryos of *Xenopus laevis*. *J. Embryol. Exptl. Morphol.* **11**, 267.
- Healey, W. V., Russell, P. S., Poole, H. K., and Olsen, M. W. (1962). A skin-grafting analysis of fowl parthenogens: evidence for a new type of genetic histocompatibility. *Ann. N. Y. Acad. Sci.* **99**, 698.
- Hubbs, C. L., and Hubbs, L. C. (1932). Apparent parthenogenesis in nature, in a form of fish of hybrid origin. *Science* **76**, 628.
- Kallman, K. D. (1962). Gynogenesis in the teleost, *Mollienesia formosa* (Girard), with a discussion of the detection of parthenogenesis in vertebrates by tissue transplantation. *J. Genet.* **58**, 7.
- Kallman, K. D., and Harrington, R. W. (1964). Evidence for the existence of homozygous clones in the self-fertilizing hermaphroditic teleost *Rivulus marmoratus* (Poey). *Biol. Bull.* **126**, 101.
- Kasansky, W. J. (1934). Die parthenogenetische Entwicklung der Hechteier (*Esox lucius*, L.). *Zool. Anz.* **106**, 161.
- Kasansky, W. J. (1935). Parthenogenetisch entwickelte junge Karpfen (*Cyprinus carpio*, L.). *Zool. Anz.* **110**, 191.
- Kawamura, T. (1939a). Artificial parthenogenesis in the frog. I. Chromosome numbers and their relation to cleavage histories. *J. Sci. Hiroshima Univ.*, *Ser. B.* **1** **6**, 115.
- Kawamura, T. (1939b). Artificial parthenogenesis in the frog. II. The sex of parthenogenetic frogs. *J. Sci. Hiroshima Univ.*, *Ser. B* **1** **7**, 39.

- Kawamura, T. (1949). Further observations on diploid and triploid parthenogenetic frogs of *Rana nigromaculata*. *J. Sci. Hiroshima Univ., Ser. B* 1 2, 1.
- Kawamura, T. (1950). Studies on hybridization in amphibians. II. Interspecific hybrids in red-colored frogs. *J. Sci. Hiroshima Univ., Ser. B* 1 11, 61.
- Kawamura, T., and Kobayashi, M. (1960). Studies on hybridization in amphibians. VII. Hybrids between Japanese and European brown frogs. *J. Sci. Hiroshima Univ., Ser. B* 1 18, 221.
- Kawamura, T., and Yokota, R. (1959). The offspring of sex-reversed females of *Rana japonica* Guenther. *J. Sci. Hiroshima Univ., Ser. B* 1 18, 31.
- Lantz, L. A., and Cyrén, O. (1936). Contribution à la connaissance de *Lacerta saxicola* Eversmann. *Bull. Soc. Zool. France* 61, 159.
- Lelièvre, Peyron, A., and Corsy, F. (1927). La parthénogénèse dans l'ovaire des mammifères et le problème de l'origine des embryomies. *Bull. Etude Cancer* 16, 711.
- Lestage, J. A. (1934). Notes de limnobiologie. V. L'industrialization de la parthénogénèse artificielle chez l'agone (Clupeidae: *Paralosa lacustris lariana* Pir.). *Ann. Soc. Zool. Belg.* 64, 69.
- Lieder, U. (1955). Männchenmangel und natürliche Parthenogenese bei der Silberkarausche *Carassius auratus gibelio* (Vertebrata, Pisces). *Naturwissenschaften* 21, 590.
- Lieder, U. (1959). Über die Eientwicklung bei männchenlosen Stämmen der Silberkarausche *Carassius auratus gibelio* (Bloch) (Vertebrata, Pisces). *Biol. Zentr.* 78, 284.
- Maslin, T. P. (1962). All-female species of the lizard genus *Cnemidophorus*, Teiidae. *Science* 135, 212.
- Mertens, R. (1960). "The World of Amphibians and Reptiles" (H. W. Parker, transl.). Harrap, London.
- Miyada, S. (1960). Studies on haploid frogs. *J. Sci. Hiroshima Univ., Ser. B* 1 19, 1.
- Morescalchi, A. (1963). Conferma della presenza di eterocromosomi in *Xenopus laevis* Daudin. *Rend. Accad. Sci. Fis. Mat. (Soc. Nazl. Sci., Napoli)*, Ser. 4 30, 310.
- Morescalchi, A. (1964). Il corredo cromosomico di *Discoglossus pictus* Otth: cromosomi sessuali, spiralizzazione cromosomica e zone eterocromatiche. *Caryologia* 17, 327.
- Moriwaki, T. (1957). Studies on matured parthenogenetic frogs. I. The development and the reproductive ability. *J. Sci. Hiroshima Univ., Ser. B* 1 17, 13.
- Moriwaki, T. (1959). Studies on matured parthenogenetic frogs. II. The offspring of a male parthenogenetic frog. *J. Sci. Hiroshima Univ., Ser. B* 1 18, 45.
- Moriwaki, T. (1960a). Studies on matured parthenogenetic frogs. III. Offspring of female parthenogenetic frogs. *J. Sci. Hiroshima Univ., Ser. B* 1 18, 157.
- Moriwaki, T. (1960b). Studies on matured parthenogenetic frogs. IV. On second-generation offspring of parthenogenetic frogs. *J. Sci. Hiroshima Univ., Ser. B* 1 18, 183.
- Moriwaki, T. (1963). Studies on matured parthenogenetic frogs. V. On the F₁ offspring of parthenogenetic frogs. *Bull. Suzugamine Women's Coll. (Nat. Sci.)* 10, 11.
- Moriya, K. (1960). Studies on the five races of the Japanese pond frog, *Rana nigromaculata* Hallowell. III. Sterility in interracial hybrids. *J. Sci. Hiroshima Univ., Ser. B* 1 18, 125.

- Olsen, M. W. (1956). Fowl pox vaccine associated with parthenogenesis in chicken and turkey eggs. *Science* **124**, 1078.
- Olsen, M. W. (1960). Nine-year summary of parthenogenesis in turkeys. *Proc. Soc. Exptl. Biol. Med.* **105**, 279.
- Olsen, M. W. (1962). The occurrence and possible significance of parthenogenesis in eggs of mated turkeys. *J. Genet.* **58**, 1.
- Parmenter, C. L. (1925). The chromosomes of parthenogenetic frogs and tadpoles. *J. Gen. Physiol.* **8**, 1.
- Parmenter, C. L. (1926). The chromosomes of parthenogenetically developed young tadpoles and early cleavages of *Rana pipiens*. *Anat. Record* **34**, 150 (Abstr.).
- Parmenter, C. L. (1933). Haploid, diploid, triploid and tetraploid chromosome numbers and their origin in parthenogenetically developed larvae and frogs of *Rana pipiens* and *Rana palustris*. *J. Exptl. Zool.* **66**, 409.
- Peacock, A. D., and Weidmann, U. (1964). A review of recent researches on the cytology of animal parthenogenesis. *Przegląd. Zool.* **8**, 16 (in Polish; English summary).
- Pesonen, S. (1946a). Abortive egg cells in the mouse. *Hereditas* **32**, 93.
- Pesonen, S. (1946b). Über Abortiveier. I. *Acta Obstet. Gynecol. Scand., Suppl. II* **25**, 152.
- Pesonen, S., and Marjanen, P. (1949). Artificial parthenogenesis of ova of rats. *Ann. Chir. Gynaecol. Fenniae, Suppl. 3* **38**, 353.
- Pincus, G. (1936). The parthenogenetic activation of rabbit eggs. *Anat. Record, Suppl.* **1**, 67.
- Pincus, G. (1939a). The breeding of some rabbits produced by recipients of artificially activated ova. *Proc. Natl. Acad. Sci. U. S.* **25**, 557.
- Pincus, G. (1939b). The comparative behavior of mammalian eggs *in vivo* and *in vitro*. IV. The development of fertilized and artificially activated rabbit eggs. *J. Exptl. Zool.* **82**, 85.
- Pincus, G., and Enzmann, E. V. (1936). The comparative behavior of mammalian eggs *in vivo* and *in vitro*. II. The activation of tubal eggs of the rabbit. *J. Exptl. Zool.* **73**, 195.
- Pincus, G., and Shapiro, H. (1940a). The comparative behavior of mammalian eggs *in vivo* and *in vitro*. VII. Further studies on the activation of rabbit eggs. *Proc. Am. Phil. Soc.* **83**, 631.
- Pincus, G., and Shapiro, H. (1940b). Further studies on the parthenogenetic activation of rabbit eggs. *Proc. Natl. Acad. Sci. U. S.* **26**, 163.
- Poole, H. K. (1959). The mitotic chromosomes of parthenogenetic and normal turkeys. *J. Heredity* **50**, 151.
- Poole, H. K., Healey, W. V., Russell, P. S., and Olsen, M. W. (1963). Evidence of heterozygosity in parthenogenetic turkeys from homograft responses. *Proc. Soc. Exptl. Biol. Med.* **113**, 503.
- Poole, H. K., and Olsen, M. W. (1957). The sex of parthenogenetic turkey embryos. *J. Heredity* **48**, 217.
- Poole, H. K., and Olsen, M. W. (1958). Incidence of parthenogenetic development in eggs laid by three strains of Dark Cornish chickens. *Proc. Soc. Exptl. Biol. Med.* **97**, 477.
- Reimann, S. P., and Miller, B. J. (1939). Studies of human ova. I. Description of artificially induced parthenogenetic activities in one. *Arch. Pathol.* **27**, 412 (reprinted with additions).
- Rugh, R. (1962). "Experimental Embryology: Techniques and Procedures," 3rd. ed. Burgess, Minneapolis, Minnesota.

- Russell, L. B. (1962). Chromosome aberrations in experimental mammals. In "Progress in Medical Genetics" (A. G. Steinberg and A. G. Bearn, eds.), Vol. 2, pp. 230-294. Grune & Stratton, New York.
- Selman, G. G. (1958). An ultra-violet light method for producing haploid amphibian embryos. *J. Embryol. Exptl. Morphol.* **6**, 634.
- Shapiro, H. (1942). Parthenogenetic activation of rabbit eggs. *Nature (London)* **149**, 304 (editorial summary of lecture).
- Shettles, L. B. (1957). Parthenogenetic cleavage of the human ovum. *Bull. Sloane Hospital for Women* **3**, 59.
- Spurway, H. (1957). Hermaphroditism with self-fertilization, and the monthly extrusion of unfertilized eggs, in the viviparous fish *Lebistes reticulatus*. *Nature (London)* **180**, 1248.
- Subtelny, S. (1958). The development of haploid and homozygous diploid frog embryos obtained from transplantations of haploid nuclei. *J. Exptl. Zool.* **139**, 263.
- Svårdson, G. (1945). Chromosome studies on Salmonidae. *Medd. Statens Undersökn.-O. Försöksanst. F. Sötvattensfisket*, No. 23. 151 pp.
- Swarup, H. (1956). Production of heteroploidy in the three-spined stickleback, *Gasterosteus aculeatus* (L.). *Nature (London)* **178**, 1124.
- Swarup, H. (1957). Production of haploidy in experimentally treated eggs of *Gasterosteus aculeatus* (L.). *Proc. Natl. Acad. Sci., India* **27**, 274.
- Swarup, H. (1959). Production of triploidy in *Gasterosteus aculeatus* (L.). *J. Genet.* **56**, 129.
- Thibault, C. (1949). L'oeuf des mammifères. Son développement parthénogénétique. *Ann. Sci. Nat. Zool., Ser. II* **11**, 136.
- Thibault, C., and Ortavant, R. (1949). Parthénogénèse expérimentale chez la brebis. *Compt. Rend.* **228**, 510.
- Tyler, A. (1941). Artificial parthenogenesis. *Biol. Rev. Cambridge Phil. Soc.* **16**, 291.
- Weiler, C., and Ohno, S. (1962). Cytological confirmation of female heterogamety in the African water frog (*Xenopus laevis*). *Cytogenetics* **1**, 217.
- Yao, T. S., and Olsen, M. W. (1955). Microscopic observations of parthenogenetic embryonic tissues from virgin turkey eggs. *J. Heredity* **46**, 133.

Author Index

Numbers in *italic* show the page on which the complete reference is listed.

A

Aalbers, J. G., 110, *114*
 Abe, S., 75, 85
 Abraham, K. A., 28, 85, 86
 Adams, C. E., 102, *116*, 284, 292
 Afzelius, B. A., 32, 33, 34, 40, 86, 204,
 205, 207, 209, 210, 225, 246, 257,
 258, 259, 288, 321, 323, 351, 356,
 359, 360, 379, 403
 Agin, D. P., 353, *366*
 Ajtkhozhin, M. A., 397, 401, *410*
 Åkerman, Å., 139, 158
 Aketa, K., 387, 403
 Allen, W. S., 26
 Allfrey, V. G., 371, 376, 405, 407, 409
 Almquist, J. O., 38, 39, 95, 335, 337,
 339, 340, 350, 355, 365
 Alonso, A., 173, 230
 Alvarez, B., 173, 230
 Amoroso, E. C., 432, *435*
 Ånberg, Å., 335, 360
 Andersen, H., 69, 94
 Anderson, W. A., 86
 André, J., 35, 40, 86, 245, 265, 267, 268,
 277, 279, 288, 345, 359, 360
 Angleton, M., 58, 97
 Annison, E. F., 110, *115*
 Applegate, A., 59, 86
 Ardao, M. I., 75, 86
 Arstila, A. U., 359, 364
 Ashley, D. J. B., 433, 436
 Austin, C. R., 38, 39, 41, 86, 107, *114*,
 118, 158, 164, 166, 171, 172, 177,
 220, 224, 232, 243, 264, 275, 280,
 284, 287, 288, 290, 292, 331, 341,
 343, 350, 360, 363, 430, 431, 436
 Axelrod, R., 387, 403

B

Backus, M. P., 153, *158*
 Bacsich, P., 431, *436*
 Bäckström, S., 388, 403
 Baggett, B., 45, 72, 95
 Bahr, G. F., 35, 86

Bajpai, P. K., 79, 86
 Balfour-Lynn, S., 434, *436*
 Balitsina, N. V., 397, 401, *410*
 Ballentine, R., 384, 403
 Ballowitz, E., 343, 360
 Balogh, K., Jr., 49, 86
 Baltus, E., 378, 395, 400, 403
 Banbury, G. H., 146, *158*
 Bane, A., 38, 93, 335, 350, 359, 360,
 364, 365
 Bangham, A. D., 79, 86
 Barany, K., 76, 86
 Barany, M., 76, 86
 Barch, S. H., 212, 213, 215, 224, 233,
 380, *410*
 Bard, S. G., 390, 409
 Barksdale, A. W., 128, 151, *158*
 Barnes, H., 46, 86, 107, *114*
 Barnett, B. D., 51, 89
 Barquet, J., 173, 230
 Barron, E. S. G., 75, 86, 384, 403
 Barsky, G., 357, 360
 Barth, R. F., 70, 86, 177, 225
 Barthélémy, M. H., 211, 225
 Bawa, S. R., 32, 34, 86, 264, 288
 Baxandall, J., 199, 204, 205, 207, 209,
 210, 225, 379, 403
 Bayreuther, K. E., 21, 26
 Beale, G. H., 70, 86
 Beatty, R. A., 414, 419, 423, 426, 432,
 434, 436
 Bedford, J. M., 166, 171, 220, 225, 335,
 341, 349, 350, 360
 Benda, C., 237, 288
 Bennett, H. S., 358, 360
 Berelson, B., 5, 26
 Berg, W. E., 164, 225, 281, 288
 Bergstrand, Å., 376, 390, 406
 Bernstein, M. H., 68, 87, 246, 257, 258,
 288, 319, 321, 360
 Berthold, G., 120, 125, *158*
 Bhargava, P. M., 28, 85, 86
 Bianchetti, R., 387, 403
 Binford, R., 335, 360

- Birch-Andersen, A., 270, 288, 359, 360
 Bischoff, E. R., 186, 198, 200, 201, 202, 203, 225, 230, 380, 408
 Bishop, D. W., 27, 28, 41, 42, 43, 47, 52, 56, 57, 66, 69, 73, 82, 87, 96, 131, 158, 168, 170, 171, 172, 225, 229, 234
 Bishop, H., 150, 158
 Bishop, M. W. H., 28, 87, 224, 280, 287, 288, 341, 350, 360
 Bistis, G., 153, 154, 158
 Black, D. L., 29, 30, 83, 90
 Black, R. E., 383, 385, 403, 404
 Blackshaw, A. W., 87
 Blakeslee, A. F., 143, 144, 158
 Blandau, R. J., 29, 30, 42, 87
 Bloch, F., 335, 360
 Blom, E., 270, 288, 340, 350, 355, 359, 360
 Bobr, L. W., 83, 87
 Boettcher, B., 173, 225
 Boone, M. A., 51, 89
 Bordet, J., 128, 158
 Borei, H., 381, 384, 385, 404
 Bosco, M., 382, 408
 Bourcart, C., 358, 360
 Bourne, G. H., 179, 228
 Bowen, R. H., 238, 239, 288, 352, 353, 361
 Boyd, M., 384, 404
 Brachet, J., 215, 226, 375, 377, 378, 384, 389, 393, 395, 400, 401, 403, 404, 405
 Brackett, B. G., 46, 87
 Braden, A. W. H., 430, 431, 436
 Bradfield, J. R. G., 59, 87
 Bradt, O., 211, 212, 234
 Brandt, P. W., 353, 361
 Branham, J. M., 61, 78, 87, 189, 225
 Braunitzer, G., 146, 161
 Brefeld, O., 122, 158
 Briggs, R., 347, 361, 373, 404
 Bril-Petersen, E., 166, 225
 Brock, N., 384, 404
 Brock, T. D., 182, 225
 Brokaw, C. J., 42, 43, 53, 75, 76, 81, 87, 141, 142, 143, 158
 Brookbank, J. W., 164, 187, 194, 203, 204, 205, 225, 234, 379, 380, 398, 404, 405, 411
 Brown, D. D., 376, 401, 404
 Brown, G. G., 166, 225, 245, 265, 275, 277, 278, 288, 359, 361
 Brown, Harrison, 5, 26
 Brown, P. C., 172, 173, 226, 228
 Brown, R., 400, 405
 Bruchmann, H., 140, 159
 Bulbring, E., 59, 87
 Buller, A. H. R., 146, 159
 Buller, R., 139, 159
 Bungenberg de Jong, C. M., 414, 436
 Burge, R. E., 89
 Burgeff, H., 144, 145, 159
 Burgos, M. H., 239, 244, 249, 267, 269, 288, 289, 335, 343, 350, 351, 361
 Burn, J. H., 59, 87
 Burnasheva, S. A., 28, 44, 45, 54, 87, 88
 Buruiana, L. M., 57, 88
 Byers, H. L., 193, 226
- C**
- Caldeyro-Barcia, R., 7, 26
 Campbell, D. H., 168, 226
 Cape, M., 401, 405
 Capers, C. R., 357, 361
 Caplan, S. R., 62, 84, 92
 Carlile, M. J., 127, 128, 158, 159
 Carlsen, E. N., 375, 404
 Carlson, F. D., 81, 88
 Casida, L. E., 177, 230
 Catalano, C., 390, 407
 Ceas, M. P., 402, 404
 Celano, M. J., 173, 176, 229
 Chambers, E. L., 209, 235, 382, 412
 Chambers, L. S., 168, 195, 228
 Chang, M. C., 69, 70, 83, 88, 97, 171, 220, 226, 281, 287, 293, 341, 343, 361, 367, 432, 433, 436
 Chang, M. S., 80, 92
 Child, F. M., 41, 94
 Choong, C. H., 77, 80, 88, 97
 Claybrook, J. R., 55, 88
 Cleland, K. W., 384, 385, 386, 404
 Clermont, Y., 39, 40, 41, 88, 90, 247, 269, 289, 350, 361
 Coale, A., 5, 26
 Cohen, R. B., 49, 86
 Cohen, S., 168, 226
 Cohn, F., 121, 159
 Cole, F. J., 10, 11, 18, 26
 Coleman, A. W., 118, 123, 159

- Collier, J. R., 164, 226, 288, 243, 246,
282, 284, 293
- Colwin, A. L., 179, 181, 226, 242, 243,
245, 246, 258, 260, 264, 283, 285,
286, 287, 288, 296, 297, 298, 299,
301, 303, 305, 307, 309, 311, 312,
313, 315, 317, 319, 321, 323, 325,
326, 327, 328, 329, 331, 332, 334,
340, 341, 344, 348, 349, 351, 352,
353, 355, 357, 361, 362
- Colwin, L. H., 179, 181, 226, 242, 243,
245, 246, 258, 260, 264, 283, 285,
286, 287, 288, 296, 297, 298, 299,
301, 303, 305, 307, 309, 311, 312,
313, 315, 317, 319, 321, 323, 325,
326, 327, 328, 329, 331, 332, 334,
340, 341, 344, 348, 349, 351, 352,
353, 355, 357, 361, 362
- Comb, D. G., 400, 402, 404, 405
- Conklin, E. G., 179, 226
- Conti, A. M., 72, 88
- Cook, A. H., 129, 130, 159
- Coombs, R. R. A., 173, 175, 176, 226,
228
- Cooper, W. G., 357, 362
- Cori, O., 47, 88
- Cornefert, F., 357, 360
- Corsy, F., 431, 438
- Costello, D. P., 177, 183, 226, 371, 405
- Couch, J. N., 150, 159
- Courrier, R., 431, 436
- Cousineau, G. H., 393, 400, 406
- Cowden, R. R., 402, 405
- Cragle, R. G., 95
- Crane, R. K., 388, 407
- Cresseri, A., 72, 88
- Cummins, J. E., 390, 408
- Cushing, J. E., 168, 226
- Cyrén, O., 427, 438
- Dabich, D., 82, 91
- Daems, W. T., 88
- Dalcq, A., 215, 226, 379, 405
- D'Amelio, V., 402, 405
- Dan, J. C., 164, 178, 182, 226, 227,
242, 243, 244, 245, 246, 257, 258,
260, 270, 272, 274, 282, 283, 284,
290, 292, 293, 296, 297, 321, 323,
325, 329, 331, 350, 351, 362, 365
- Daniel, J. F., 212, 227
- Danielli, J. F., 353, 362
- D'Anna, T., 219, 231
- Darevskii, I. S., 427, 435, 436
- Davidson, E., 376, 405
- Davison, J. A., 78, 88
- Davson, H., 353, 356, 362
- Dawid, W., 146, 161
- de Bary, A., 119, 120, 122, 150, 159
- Decroly, M., 401, 405
- de Harven, E., 179, 232, 258, 260, 263,
292, 329, 350, 355, 365
- Delage, Y., 371, 372, 405
- Denny, P. C., 393, 395, 398, 405, 411
- De Silva, P. L. G., 77, 88
- Dettlaff, T. A., 243, 246, 290, 343, 362,
372, 405
- Dickmann, Z., 118, 159, 246, 287, 290,
341, 350, 359, 362
- Docton, F. L., 176, 226
- Dodel, A., 120, 159
- Dodge, B. O., 153, 159
- Dorfman, R. I., 7, 26
- Dorn, H., 5, 26
- Dott, H. M., 63, 88, 101, 115
- Drevius, L. O., 37, 66, 67, 88, 90
- Druckrey, H., 384, 404
- Dunham, P. R., 65, 95, 96
- Dziuk, P. J., 287, 290, 341, 350, 362
- E**
- Ecker, R. E., 398, 405
- Edström, J. E., 376, 405
- Edwards, R. G., 49, 69, 73, 88, 97, 173,
175, 176, 226, 431, 437
- Eichwald, E. J., 177, 226
- Einberg, E., 269, 289, 350, 361
- Elliott, A. M., 345, 363
- Elvidge, J. A., 129, 130, 159
- Ely, F., 176, 226
- Emerson, R., 126, 159
- Endo, Y., 356, 363
- Engelhardt, V. A., 44, 88
- Enzmann, E. V., 432, 439
- Epel, D., 388, 405
- Ernster, L., 49, 92, 387, 407
- Errera, M., 128, 159
- Esper, H., 375, 405
- Esping, U., 189, 226
- Ezell, S. D., 166, 226

F

- Falconer, D. S., 420, 437
 Fallon, J. F., 264, 275, 290, 331, 363
 Fankhauser, G., 414, 419, 420, 437
 Farmer, J. B., 128, 159
 Farquhar, M. G., 357, 363
 Fasten, N., 335, 363
 Fauré-Fremiet, E., 28, 88, 384, 405
 Fawcett, D., 28, 37, 88, 166, 227, 239, 244, 249, 267, 269, 288, 289, 335, 343, 350, 351, 353, 359, 361, 363
 Fedorova, L. G., 28, 88
 Ferguson, L. C., 173, 175, 176, 226
 Ferin, J., 7, 26
 Fernández-Morán, H., 353, 363
 Ficq, A., 375, 376, 378, 393, 395, 400, 403, 404, 405
 Finean, J. B., 353, 363
 Finkler, A. E., 170, 235
 Fischberg, M., 420, 437
 Fischer, A., 122, 159
 Fischer, F. G., 122, 151, 152, 159
 Flake, G. P., 168, 198, 200, 227
 Flaks, J. G., 164, 203, 227
 Flipse, R. J., 51, 89, 110, 111, 115
 Fol, H., 327, 363
 Folkers, K., 73, 93
 Folkes, B. P., 145, 159, 219, 227
 Follenius, E., 240, 292, 344, 359, 363, 365
 Fox, Sidney W., 21, 26, 193, 235
 Franklin, L. E., 165, 181, 205, 227, 319, 321, 323, 337, 351, 355, 363
 Franklin, R. M., 402, 410
 Freund, J., 171, 172, 227
 Friedmann, I., 339, 344, 347, 364
 Fritsch, F. E., 132, 159
 Fujita, H., 357, 363
 Fulton, H. R., 122, 123, 159
 Gibbons, I. R., 34, 52, 56, 89
 Gibbons, R. A., 62, 96
 Ginsburg, A. S., 83, 89, 166, 227, 241, 243, 246, 290, 343, 344, 345, 362, 363
 Giudice, G., 389, 409
 Glick, R. N., 214, 219, 227
 Glisin, M. V., 400, 405
 Glisin, V. R., 400, 405
 Glover, T. D., 72, 89
 Glynn, L. E., 172, 173, 226, 228
 Goldberg, B., 357, 363
 Goldberg, E., 80, 89, 93
 Gonsse, P. H., 45, 73, 74, 75, 89, 107, 115
 Good, G. M., 212, 227
 Goodman, H. M., 391, 410
 Goroschankin, J., 131, 159
 Grabar, P., 169, 227
 Graff, J., 376, 389, 406
 Grampp, W., 376, 405
 Grant, R. A., 219, 227
 Graves, A. H., 122, 159
 Graves, C. N., 63, 71, 91, 95, 101, 115, 169, 231
 Gray, J., 29, 81, 89, 288, 290
 Gray, R. S., 169, 229
 Graziano, K. D., 227
 Green, H., 357, 363
 Green, W. W., 166, 227
 Greep, R. V., 7, 26
 Gregg, K. W., 193, 194, 197, 227
 Gresham, G. A., 173, 228
 Griffiths, P. D., 47, 90
 Gross, P. R., 393, 395, 400, 405, 406, 411
 Grove, D., 61, 90
 Grundfest, H., 381, 411
 Gullbring, B., 174, 176, 227
 Guraya, S. S., 29, 89

G

- Gaddum, P., 72, 89
 Gale, P. H., 73, 93
 Gallien, L., 414, 437
 Galtsoff, P. S., 258, 260, 290, 329, 350, 363
 Gass, G. H., 72, 96
 Geitler, L., 144, 159
 Geuskens, M., 396, 405
 Chiretti-Magaldi, A., 386, 408
 Giardina, G., 402, 405

H

- Hadarag, E. I., 57, 88
 Hadek, R., 258, 269, 270, 280, 287, 290, 335, 341, 349, 350, 363
 Hafs, H. D., 169, 228
 Hagen-Seyfferth, M., 124, 159
 Haggis, A. J., 378, 406
 Hagiwara, Y., 258, 274, 290
 Hagström, B., 380, 406
 Hagström, B. E., 118, 123, 131, 161, 181, 184, 193, 194, 195, 209, 227, 232, 233, 380, 406

- Haino, K., 164, 189, 227, 282, 290, 291
Haldane, J. B. S., 423, 437
Hamano, S., 402, 406
Hamilton, L., 420, 437
Hammond, M., 51, 89
Hamner, C. E., 101, 116, 171, 236
Hanstein, J., 121, 136, 159
Hara, S., 46, 90
Harde, S., 194, 231
Harding, C. B., 189, 227
Harrington, R. W., 422, 437
Harris, G. W., 7, 26
Harris, J. E., 89
Hartman, C. G., 89
Hartmann, M., 125, 126, 159, 238, 291
Hartree, E. F., 47, 48, 91
Hartree, E. F., 102, 108, 115, 116, 171, 173, 227, 248, 284, 291, 292
Harvey, C., 76, 77, 89
Harvey, E. B., 393, 406
Hathaway, R. R., 68, 89, 164, 171, 181, 190, 191, 203, 227, 248, 291
Hauschka, S. D., 164, 228, 281, 291
Hawes, M. D., 173, 228
Hawker, L. E., 145, 159
Hayashi, T., 28, 89
Healey, W. V., 428, 429, 437, 439
Heilbron, Sir I., 129, 159
Hellinga, G., 170, 195, 233
Henle, G., 168, 195, 228
Henle, W., 168, 195, 228
Hepden, P. M., 145, 159
Herken, H., 384, 404
Hibbard, H., 238, 281, 291
Hinegardner, R. T., 378, 406
Hiramoto, Y., 347, 363, 382, 406
Hirosawa, K., 46, 90
Hoberman, H. D., 376, 389, 406
Hoffman, L. R., 131, 134, 135, 159
Hoffmann-Berling, H., 52, 56, 57, 66, 87, 89
Holborow, E. J., 172, 173, 226, 228
Holter, H., 373, 384, 406, 407
Holwill, M. E. J., 89, 112, 115
Hope, J., 179, 228
Hopkins, J. M., 33, 93, 97
Hopsu, V. K., 359, 364
Horiuchi, K., 108, 115
Horne, H. W., Jr., 83, 90
Horowitz, N. H., 381, 411
Horstmann, E., 249, 291, 335, 350, 364
Horwitz, B. A., 381, 382, 383, 406
Howe, G. R., 29, 30, 83, 90
Hraba, T., 176, 230
Hrudka, F., 49, 90
Hubbs, C. L., 422, 437
Hubbs, L. C., 422, 437
Huggett, A. St. G., 7, 26
Hughes, W. N., 213, 228
Hultin, T., 165, 191, 228, 376, 389, 390, 398, 406, 408, 412
Humason, W. D., 384, 385, 411
Humphreys, W. J., 178, 228, 283, 291
Humphries, A. A., Jr., 179, 211, 213, 228
Hunter, A. G., 169, 228
Hutner, S. H., 124, 159
- I
- Iiuzuka, R., 46, 90
Impellizzeri, M. A., 402, 404
Inman, F. P., 168, 231
Ishida, J., 380, 406
Ishihara, K., 193, 194, 228, 388, 406, 407
Ishikawa, M., 382, 407
Isono, N., 388, 389, 407
Isusaka, A., 388, 389, 407
Ito, S., 359, 363
Iversen, S., 90
Iverson, R. M., 393, 398, 410
Izawa, M., 371, 407, 409
- J
- Jaisle, F., 76, 86
Jensen, R. G., 45, 90
Jensen, N. A., 356, 364
Jones, Alan W., 47, 90
Jones, J. K. N., 219, 227
- K
- Kabat, E. C., 168, 228
Kakabayashi, N., 170, 235
Kallman, K. D., 422, 423, 437
Kambara, S., 212, 214, 219, 220, 228
Kamboj, V. P., 90
Kaminer, B., 84, 96
Kao, C. Y., 381, 411
Kar, A. B., 7, 26, 90
Kasansky, W. J., 422, 437
Katagiri, C., 213, 214, 228
Katsh, G. F., 70, 90, 169, 170, 172, 177, 229
Katsh, S., 70, 90, 97, 164, 169, 170, 171, 172, 177, 228, 229, 236

- Kavanau, J. L., 353, 364
 Kawamura, T., 425, 426, 437, 438
 Kay, E. R. M., 378, 407
 Kaye, G. I., 252, 293, 335, 367
 Kaye, J. S., 244, 250, 251, 256, 258, 264,
 291, 335, 351, 364
 Kehl, H., 145, 159
 Kelly, P. G., 52, 90
 Kemp, N. E., 179, 229
 Kessel, R. G., 179, 229
 Kigawa, N., 282, 291
 Kihlman, 122, 159
 Kille, R. A., 243, 246, 291, 343, 364
 King, T. J., 347, 361, 373, 404
 Kinoshita, S., 52, 56, 66, 90
 Kirkpatrick, C. H., 172, 229
 Kloetzel, J. A., 198, 229
 Klotz, M., 403, 407
 Kobayashi, M., 426, 438
 Kobayashi, T., 7, 26
 Koefoed-Johnson, H. H., 69, 94
 Köhler, E., 125, 159
 Köhler, F., 145, 146, 159
 Köhler, K., 168, 186, 191, 195, 197, 198,
 204, 229, 230
 König, E., 122, 159
 Koltzoff, N. K., 335, 364
 Komarek, R. J., 45, 90
 Kominz, D. R., 75, 90
 Königsberg, I. R., 357, 362
 Korsh, G., 110, 116
 Koshihara, H., 390, 412
 Kotsevalov, O., 169, 170, 235
 Kotte, W., 129, 159
 Koyama, J. K., 61, 96
 Kraemer, K., 400, 406
 Krafczyk, H., 145, 146, 159
 Krane, S. M., 388, 407
 Krauss, M., 164, 229, 281, 291
 Kulikova, V. N., 427, 435, 436
 Kunieda, H., 126, 160
 Kuramoto, C., 46, 90
 Kusano, S., 125, 160
 Kushida, H., 257, 258, 272, 290, 321,
 323, 325, 351, 362

L

- Lagarrique, M., 47, 88
 Landsteiner, K., 168, 173, 229
 Lantz, L. A., 427, 438
 Lanz, R. N., 45, 90
 Lardy, H. A., 44, 90, 387, 407

- Larson, B. L., 169, 229
 Laser, H., 382, 407
 Lavallard, P., 358, 360
 Lavin, L. H., 220, 221, 222, 229, 231
 Lazear, E. J., 176, 226
 Leblond, C. P., 39, 88, 90, 247, 269,
 288, 350, 361
 Lederberg, J., 6, 26
 Leech, J. H., 357, 364
 Lehman, H. E., 402, 405
 Lehmann, H., 47, 90
 Lelievre, Peyron A., 431, 438
 Lenhossek, M. von, 238, 291
 Lentini, R., 384, 407
 Lestage, J. A., 422, 438
 Leuchtenberger, C., 247, 291
 Levi, J. D., 144, 160
 Levine, J., 82, 91
 Levine, P., 173, 176, 229
 Levring, T., 130, 131, 160
 Lewin, R., 118, 120, 123, 124, 160
 Lidforss, B., 139, 160
 Lieder, U., 422, 438
 Liess, J., 61, 90
 Lillie, F. R., 68, 90, 181, 183, 187, 193,
 229, 230, 238, 291, 331, 348, 349,
 364
 Lillie, R. S., 382, 407
 Lindahl, P. E., 37, 67, 74, 90, 91, 101,
 115, 373, 407
 Lindberg, O., 387, 407, 409
 Linderström-Lang, K. U., 403, 407
 Linskens, H., 118, 160
 Lipton, M. M., 171, 172, 227
 Littna, E., 376, 401, 404
 Lodge, J. R., 28, 95, 100, 101, 111, 115
 Loeb, J., 165, 182, 193, 230, 238, 239,
 242, 291, 346, 364, 379, 407
 Lönning, S., 323, 364
 Loewenstein, W. R., 357, 367
 Lorenz, F. W., 83, 87
 Lubet, P., 358, 360
 Lundblad, G., 398, 403, 407
 Lutwak-Mann, C., 74, 77, 91, 105, 107,
 114, 115
 Lybing, S., 385, 404
 Lyubimova, M. N., 28, 88

M

- McCarthy, B. J., 400, 401, 412
 McCartney, J. L., 172, 230

- McElroy, W. D., 5, 26
Macey, R. I., 64, 93
Machin, K. E., 53, 60, 91
Machlis, L., 118, 123, 126, 127, 128,
132, 133, 134, 135, 155, 157, 158,
159, 160, 161
McKeen, W. E., 44, 91
MacLeod, J., 75, 91
MacLeod, R. A., 64, 91
MacLeod, T. M., 82, 83, 95
McVittie, A., 33, 93
Maggio, R., 376, 386, 390, 396, 397,
407, 408, 410
Mainx, F., 125, 133, 160
Majsky, A., 176, 230
Malkin, L. I., 400, 406
Mancini, R. E., 173, 230
Mancuso, V., 178, 179, 232, 233
Mann, H., 77, 91
Mann, T., 28, 44, 47, 51, 65, 68, 74, 77,
80, 91, 100, 105, 107, 108, 110,
111, 114, 115, 164, 230
Manton, I., 339, 344, 347, 364
Marcus, F., 47, 88
Marcus, M., 66, 91
Mardachev, S. R., 7, 26
Marjanen, P., 431, 439
Markert, C. L., 5, 26
Marré, E., 387, 403
Maruyama, K., 64, 97
Masaki, J., 47, 48, 91
Mascarenhas, J. P., 118, 123, 155, 157,
160
Maslin, T. P., 427, 438
Masouredis, S. P., 176, 232
Mather, R. E., 92
Mathews, H. P., 73, 87
Matousek, J., 169, 171, 177, 230
Matthaei, H. J., 390, 409
Mattner, P. E., 83, 91
Mayer, A. M., 66, 91
Mayer, M. M., 168, 228
Mazia, D., 203, 235
Meisenheimer, J., 24, 26
Melander, S. E. J., 72, 93
Mellen, W. J., 172, 235
Menge, A. C., 177, 230
Mertens, R., 427, 438
Metz, C. B., 118, 160, 164, 165, 168,
181, 182, 183, 184, 185, 186, 187,
188, 189, 190, 191, 193, 194, 195,
197, 198, 200, 201, 202, 203, 204,
209, 212, 213, 215, 219, 225, 227,
229, 230, 233, 235, 236, 245, 246,
291, 331, 364, 376, 380, 382, 389,
406, 408, 410, 411
Meves, F., 241, 291, 349, 364
Meyer, A. W., 26
Miller, B. J., 433, 439
Miller, R., 68, 91
Miller, R. L., 3, 26, 118, 160
Millonig, G., 382, 408
Minganti, A., 166, 219, 231, 384, 408
Minotakis, C. S., 110, 115
Mirsky, A. E., 371, 376, 402, 403, 405,
407, 408, 409
Mitchison, J. M., 31, 91, 390, 408
Mittal, K. K., 71, 91, 169, 231
Mixner, J. P., 92
Miyada, S., 421, 426, 438
Moewus, F., 124, 160
Moghissi, K. S., 82, 91
Mohri, H., 45, 48, 49, 52, 55, 61, 91,
92, 96, 101, 108, 111, 115, 116
Mohri, T., 49, 92
Molinaro, M., 398, 408
Molisch, H., 155, 160
Mollenhauer, H. H., 357, 364
Monesi, V., 174, 175, 231
Monné, L., 194, 231
Monroy, A., 68, 92, 181, 189, 190, 193,
231, 351, 353, 364, 374, 376, 377,
378, 381, 382, 384, 387, 388, 389,
390, 391, 392, 396, 397, 398, 399,
400, 401, 402, 403, 404, 405, 408,
409, 411
Monroy-Oddo, A., 402, 408
Moore, R. D., 357, 366
Morescalchi, A., 423, 438
Morgan, T. H., 8, 26, 117, 160, 165,
231, 379, 409
Moriber, L. C., 244, 291, 359, 366
Moricard, R., 246, 280, 287, 291, 341,
364
Moriwaki, T., 425, 438
Moriya, K., 426, 438
Morowitz, H. J., 353, 365
Morrill, J. B., 245, 246, 291, 331, 364
Moser, F., 243, 291
Moses, M. J., 166, 231, 252, 253, 255,
258, 285, 292, 335, 364
Motamura, I., 193, 194, 231

Moule, G. R., 77, 92
 Mounib, M. S., 72, 80, 92
 Moyer, W. A., 406
 Mumaw, V. R., 357, 366
 Murray, A., 246, 288, 321, 360

N

Nace, G. W., 213, 220, 221, 222, 231
 Näf, U., 157, 160
 Nagano, T., 34, 49, 92, 249, 250, 269,
 292, 343, 350, 364
 Nakano, E., 373, 374, 376, 380, 381,
 384, 388, 389, 390, 406, 407, 409,
 411, 412
 Naora, H., 371, 409
 Naora, H., 371, 409
 Nashed, N., 92
 Needham, J., 10, 26
 Neilands, J. B., 92
 Nelson, L., 35, 44, 48, 49, 51, 52, 53,
 54, 55, 56, 57, 59, 60, 64, 75, 78,
 81, 84, 86, 92, 93, 118, 160
 Nelson, W., 5, 26
 Nemer, M., 390, 400, 409
 Nemirovsky, M., 173, 230
 Nevo, A., 45, 61, 62, 84, 92, 95, 101,
 116
 Newhaus, O. W., 82, 91
 Newton, A. A., 46, 47, 92, 111, 115
 Nicander, L., 38, 41, 93, 335, 350, 359,
 360, 364, 365
 Nickerson, W. J., 144, 160
 Nijijima, L., 258, 260, 270, 283, 292,
 331, 365
 Nikitina, L. A., 372, 405
 Nirenberg, M. W., 390, 409
 Nishikawa, Y., 247, 255, 292
 Nisonoff, A., 168, 231
 Norman, C., 80, 89, 93
 Noyes, R. W., 82, 93
 Nutting, W. H., 127, 160

O

Oberling, C., 431, 436
 O'Brien, T. F., 173, 228
 Örström, A., 387, 403, 409
 Ogasawara, F. X., 83, 87
 Ohnishi, T., 381, 382, 409
 Ohono, S., 423, 440
 Ohori, Y., 257, 258, 272, 290, 321, 323,
 325, 351, 362

Okamoto, T., 396, 409
 Oketa, K., 194, 231
 Okubo, F., 46, 90
 Olsen, M. W., 428, 429, 437, 439, 440
 Omachi, A., 64, 93
 O'Melveny, K., 185, 195, 199, 235
 Onofrey, E., 64, 91
 Onuma, H., 247, 255, 256, 292
 Ortavant, R., 433, 440
 Osanai, K., 194, 231
 O'Shea, T., 63, 64, 93
 Oshima, H., 187, 231
 Ouchterlony, O., 169, 231
 Overton, C. E., 144, 160
 Ozaki, H., 375, 390, 395, 409, 410, 411

P

Page, A. C., Jr., 73, 93
 Palade, G. E., 357, 363, 365
 Pappas, G. D., 252, 293, 335, 367
 Parkes, A. S., 7, 26, 432, 435
 Parmenter, C. L., 427, 439
 Pascher, A., 125, 131, 160
 Pasteels, J. J., 179, 215, 226, 231, 232,
 258, 260, 263, 292, 321, 329, 331,
 337, 350, 355, 359, 365
 Pautard, F. G. E., 52, 55, 90, 93
 Pawlowski, R., 353, 366
 Peacock, A. D., 414, 439
 Pease, D. C., 35, 93
 Pence, V. W., 89
 Pequengnat, W., 187, 232
 Perez y Perez, F., 72, 93
 Perlmann, H., 205, 209, 232
 Perlmann, P., 118, 123, 131, 161, 181,
 186, 189, 193, 195, 203, 204, 205,
 207, 209, 210, 225, 232, 233, 379,
 403, 409
 Perloff, W. H., 82, 93
 Pernot, E., 69, 93, 169, 171, 232
 Persijn, J. P., 88
 Persson, B. H., 72, 93
 Pesonen, S., 430, 431, 439
 Pfeffer, W., 119, 133, 137, 142, 160
 Phillips, F. S., 384, 409
 Phillips, P. H., 44, 90
 Philpott, D. E., 245, 258, 260, 283, 289,
 290, 297, 301, 329, 350, 362, 363
 Piatigorsky, J., 164, 232, 243, 292, 375,
 390, 399, 409, 411
 Pickett, B. W., 45, 90

- Pikó, L., 93, 171, 232, 246, 258, 269,
280, 281, 287, 292, 341, 342, 347,
365, 378, 409
Pincus, G., 432, 439
Plempel, M., 145, 146, 160, 161
Plowman, K., 54, 92, 93
Pochon-Masson, J., 359, 365
Polge, C., 110, 114, 115
Polin, D., 73, 93
Pollister, A. W., 244, 292
Poole, H. K., 428, 429, 437, 439
Popivanov, R., 174, 232
Porte, A., 240, 292, 344, 365
Porter, R. R., 168, 186, 190, 200, 226,
232
Porterfield, I. D., 80, 93
Posalaky, Z., 51, 93
Preer, J. R., Jr., 70, 93
Pringsheim, N., 120, 121, 133, 161
Proskauer, J., 136, 161
Provasoli, L., 124, 159

Q

- Quertier, J., 378, 395, 400, 403
Quinlivan, W. L. G., 176, 232

R

- Randall, J., 33, 93
Rao, S. S., 170, 232
Raper, J. R., 118, 150, 151, 153, 157,
161
Rapoport, H., 127, 160
Rasmusen, B. A., 71, 91, 169, 231
Rawitscher-Kunkel, E., 132, 133, 134,
135, 161
Razin, S., 353, 365
Rebhun, L. I., 178, 179, 232, 331, 365,
382, 410
Reger, J. F., 166, 232, 252, 265, 267,
292, 335, 343, 365
Reich, E., 402, 410
Reimann, S. P., 433, 439
Renaud, F. L., 40, 94
Reverberi, G., 178, 179, 232, 233
Reynolds, A. J., 60, 78, 94
Ricci, N., 381, 411
Rice, E. W., 66, 94
Rich, A., 391, 410
Richards, A. G., 77, 78, 88, 94
Rickert, D. A., 95
Ricotta, C. M., 403, 410

- Rikmenspoel, R., 29, 37, 39, 42, 43, 44,
53, 80, 82, 94
Rinaldi, A. M., 396, 397, 408
Ris, H., 338, 342, 347, 366
Rivera, J. A., 28, 94
Robbie, W. A., 410
Robbins, W. J., 129, 161
Robertson, J. D., 296, 353, 357, 365
Rodenburg, J. M., 170, 235
Romig, W. R., 21, 26
Ronsdorf, L., 145, 146, 161
Rosenbaum, J. L., 41, 94
Rossi, 387, 410
Roth, L. E., 94
Rothschild, Lord, 24, 26, 31, 33, 39, 46,
47, 60, 61, 78, 81, 92, 94, 111, 112,
115, 117, 118, 138, 140, 141, 161,
181, 233, 244, 280, 292, 382, 383,
384, 385, 386, 404, 407, 410
Rottenstein, K., 69, 94
Rowe, A. J., 56, 89
Rozin, R., 69, 95
Rozin, S., 69, 95
Ruegg, J. C., 76, 95
Rümke, P. H., 170, 195, 233
Rugh, R., 211, 233, 419, 439
Rumery, R. E., 29, 30, 42, 87
Runnström, J., 118, 123, 131, 161, 181,
189, 193, 195, 231, 233, 353, 365,
383, 410
Runnström, T., 191, 233
Russell, L. B., 414, 440
Russell, P. S., 70, 86, 177, 225, 428, 429,
437, 439

S

- Saacke, R. G., 38, 39, 63, 95, 335, 337,
339, 340, 350, 355, 365
Sachs, J. von, 161
Sadri, K. K., 170, 232
Saito, A., 258, 290
Sakai, Y. T., 241, 292
Salisbury, G. W., 28, 63, 65, 71, 91, 95,
100, 101, 111, 115, 169, 229, 231
Salzen, E. A., 403, 410
Sanger, S. W., 53, 95
Sapsford, C. S., 39, 41, 86
Sargeant, T., 58, 97
Satir, P., 53, 95
Sawada, Y., 46, 90
Scarano, E., 390, 410

- Schabtach, E., 166, 233, 235
 Schar, N., 376, 405
 Schartau, O., 238, 291
 Schenkman, J. B., 95
 Schindler, H. J., 45, 61, 62, 84, 92, 95
 Schirren, C., 73, 95
 Schjeide, O. A., 375, 404, 410
 Schmid, O. J., 77, 91
 Schmidt, R. P., 101, 115
 Schneider, L., 345, 356, 365, 366
 Schoenberg, M. D., 357, 366
 Schrader, F., 247, 291
 Schreiber, E., 125, 161
 Schuel, H., 186, 200, 201, 202, 203, 230, 380, 408
 Schultz, J., 380, 411
 Scott, T. W., 45, 72, 95, 110, 115
 Seaton, A., 379, 411
 Sekine, T., 59, 95
 Selman, G. G., 419, 440
 Shahani, S., 174, 175, 233
 Shapiro, H., 432, 439, 440
 Shatkin, A. J., 402, 410
 Shaver, J. R., 212, 213, 214, 215, 219, 220, 224, 227, 233, 378, 380, 410
 Shelley, H. J., 59, 87
 Shettles, L. B., 433, 440
 Shibata, K., 139, 140, 142, 161
 Shivers, C. A., 198, 209, 212, 213, 215, 216, 218, 219, 233, 380, 410
 Shmerling, J. G., 378, 410
 Signoret, J., 379, 411
 Silvester, N. L., 112, 115
 Singer, S. J., 168, 233
 Sjöstrand, F. S., 353, 366
 Slautterback, D. B., 194, 231
 Sleigh, M. A., 28, 95
 Smith, A. U., 67, 95
 Smith, A. V., 168, 169, 233
 Smith, G., 120, 161
 Smith, H. W., 353, 366
 Smith, M. C., 73, 93
 Smith, N., 213, 222, 231
 Snell, G. D., 173, 177, 233
 Sobrero, A. J., 82, 83, 95
 Sofer, W. H., 393, 398, 410
 Southam, A. L., 174, 175, 233
 Spallanzani, Lazzaro, 26
 Spiegel, M., 395, 410
 Spirin, A. S., 397, 401, 410
 Spiro, D., 357, 367
 Spurway, H., 421, 422, 440
 Srivastava, P. N., 102, 115, 116, 173, 227, 248, 284, 291, 292
 Stadler, D. R., 122, 161
 Stafford, D. W., 393, 398, 410
 Stefanelli, A., 384, 410
 Steinbach, H. B., 64, 65, 95, 96
 Steinberger, E., 82, 93
 Stephens, R. E., 53, 96
 Stern, S., 190, 191, 192, 234
 Stoeckenius, W., 353, 366
 Stone, G. M., 109, 116
 Stone, W. H., 177, 230
 Strasburger, E., 120, 121, 136, 159, 161
 Straub, R. W., 76, 95
 Stroeva, O. G., 372, 405
 Stuckey, R. E., 76, 77, 89
 Stumpf, P. K., 92
 Subtelny, S., 211, 212, 234, 421, 440
 Sugiyama, M., 381, 382, 409, 410
 Suto, S. H., 126, 160
 Suyama, T., 213, 222, 231
 Suzuki, R., 68, 96
 Svårdson, G., 422, 440
 Swann, M. M., 280, 292
 Swarup, H., 422, 440
 Swift, H., 40, 94
 Szent-Gyorgyi, A. G., 53, 84, 95, 96
 Szollosi, D., 338, 342, 347, 366
 Szumowski, P., 169, 232
- T
- Taguchi, S., 45, 96, 111, 116
 Takanami, M., 396, 409
 Takashima, R., 245, 258, 264, 275, 292, 321, 366
 Takashima, Y., 245, 258, 264, 275, 292, 321, 366
 Talmage, D. W., 177, 229
 Tampion, D., 62, 96
 Tandler, B., 359, 366
 Tates, A. D., 88
 Tatum, E. L., 402, 410
 Tchou-Su, 211, 212, 234, 373, 410
 Tencer, R., 393, 404
 Terman, S. A., 395, 411
 Ternner, C., 45, 96, 110, 111, 116
 Terry, T. M., 353, 365

Thibault, C., 93, 431, 432, 433, 440
 Thibault, J. P., 82, 83, 90
 Thimann, K. V., 144, 160
 Thompson, G. E., 171, 172, 227, 230
 Thunquist, L. O., 101, 115
 Thuret, G., 120, 128, 161
 Tibbs, J., 48, 52, 59, 63, 74, 96
 Tiffany, L. H., 133, 161
 Timourian, H., 398, 411
 Tiselius, A., 191, 233
 Tobias, J. M., 353, 366
 Tolis, H., 377, 399, 400, 401, 408
 Tolmach, L. J., 348, 349, 366
 Tormey, J. McD., 353, 366
 Trautwein, W., 76, 86
 Traverso-Cori, A., 47, 88
 Trelle, G. J., 375, 404
 Tremor, J. W., 345, 363
 Tsubo, I., 252, 293, 335, 367
 Tsubo, Y., 123, 124, 161
 Tsuchiya, S., 45, 96
 Tsusaka, A., 374, 411
 Twarog, B. M., 76, 95
 Tyler, A., 5, 6, 7, 20, 26, 69, 93, 96,
 117, 161, 164, 165, 166, 168, 170,
 171, 172, 178, 179, 181, 182, 183,
 184, 185, 186, 187, 189, 190, 191,
 193, 195, 199, 200, 203, 204, 205,
 232, 234, 235, 238, 243, 244, 246,
 258, 269, 281, 285, 287, 292, 293,
 331, 341, 342, 346, 347, 348, 351,
 359, 365, 366, 375, 378, 379, 380,
 381, 382, 383, 384, 385, 390, 391,
 392, 393, 395, 398, 402, 403, 404,
 405, 408, 409, 410, 411, 412, 414,
 419, 440
 Tyler, B. S., 20, 26, 378, 382, 412
 Tyler, P., 166, 235
 Tyler, W. J., 177, 230

U

Umberger, E. J., 72, 96
 Urist, M. R., 375, 410
 Ursprung, H., 166, 233, 235

V

Valentine, R. C., 49, 73, 88
 Vandemark, N. L., 61, 95, 96
 Van Duijn, C., 96

Van Wagtendonk, W. J., 70, 96
 Vasseur, E., 182, 189, 190, 191, 233,
 235
 Verkaik, C., 145, 161
 Vincent, W. S., 398, 412
 Vittorelli, M. L., 387, 396, 397, 408, 409
 Vivier, E., 345, 360
 Vloedman, D. A., Jr., 70, 96
 Vulchanov, V. H., 174, 232

W

Wada, S. K., 242, 243, 244, 245, 282,
 284, 290, 293
 Wagner, S., 269, 289, 350, 361
 Waites, G. M. H., 77, 92
 Waldeck, J. G., 64, 93
 Waldeyer, W., 237, 293
 Wales, R. G., 45, 62, 63, 64, 72, 73, 77,
 80, 88, 93, 95, 97, 101, 116
 Wallace, J. C., 45, 72, 95, 101, 109, 116
 Wallenfels, K., 238, 291
 Walton, H., 28, 87
 Wang, Yu-lan, 211, 212, 234, 373, 410
 Warburg, O., 381, 412
 Ward, H. M., 119, 123, 161
 Ward, M., 135, 136, 161
 Warner, J. R., 391, 410
 Warr, J. R., 33, 93
 Warren, L., 164, 203, 227
 Watanabe, S., 58, 64, 97
 Watson, M. R., 97
 Wedin, K., 74, 91
 Weidmann, U., 414, 439
 Weil, A. J., 69, 97, 166, 169, 170, 171,
 235
 Weiler, C., 423, 440
 Weiner, A. S., 173, 235
 Weinman, D. E., 46, 87, 171, 236
 Weisberger, A. S., 357, 366
 Wellman, H., 387, 407
 Went, H. A., 203, 235
 Wentworth, B. C., 172, 235
 Werner, G., 122, 151, 152, 159
 Westenbrink, H. G. H., 166, 225
 Westerfeld, W. W., 95
 Whaley, W. G., 357, 364
 Whitaker, D. M., 382, 384, 412
 White, I. G., 45, 62, 63, 72, 73, 88, 95,
 97, 101, 109, 110, 115, 116

Whiteley, A. H., 209, 235, 382, 399,
400, 401, 409, 412
Whiteley, H. R., 400, 401, 412
Wiener, J., 357, 367
Wiese, L., 118, 161, 165, 182, 235
Wigglesworth, V. B., 265, 293
Wilkie, D., 141, 161
Williams, C. A., Jr., 169, 227
Williams, D. E., 201, 236
Williams, J. L., 128, 159
Williams, L., 129, 161
Williams, M. W., 127, 160
Williams, W. L., 46, 87, 101, 116, 171,
236
Willson, J. T., 70, 97, 172, 236
Wilson, C. M., 126, 159
Wilson, E. B., 166, 236, 371, 412
Wilson, L., 169, 170, 235
Wilt, F. H., 390, 400, 412
Winge, Ö., 144, 161
Wintrebert, P., 238, 293
Wright, L., 42, 87
Wyburn, G. M., 431, 436

Y

Yamakami, K., 173, 236
Yamamoto, H., 335, 367
Yamamoto, Y., 252, 293
Yanagimachi, R., 246, 281, 287, 293, 341,
359, 367
Yanagisawa, T., 111, 116
Yanigamachi, R., 83, 97
Yao, T. S., 428, 440
Yasumasu, I., 45, 61, 92, 96, 101, 111,
115, 116, 381, 390, 412
Yasuzumi, G., 166, 236, 252, 265, 285,
293, 335, 367
Yatsu, N., 371, 412
Ycas, M., 386, 412
Yokota, R., 425, 438
Yonge, J. G., 380, 404
Young, M. R., 49, 84, 97

Z

Zeitler, E., 35, 86
Zeuthen, E., 384, 406, 412
Zickler, H., 153, 161

Index to Genus and Species Names

A

Acetabularia, 125
A. mediterranea, 120
Acheta domestica, 258
Achlya, 44, 122, 128, 150, 151, 157
A. ambisexualis, 150
A. bisexualis, 150
Acipenser güldenstädti, 246(T)
A. stellatus, 246(T)
Adiantum capillus-veneris, 137, 139
A. cuneatum, 137, 139
Allomyces, 44, 126, 127
A. arbuscula, 40
Amblyomma, 265
A. dissimili, 252
Ambystoma (*Siredon*), 423
Anthocidaris crassipina, 246(T), 381
Arbacia, 55, 61, 64, 65, 68, 78, 185, 186,
 187, 189, 190, 197, 201, 202, 203,
 204, 319, 321, 323, 380, 383
A. lixula, 193, 204, 205, 387(T)
A. punctulata, 164, 165, 193, 194, 195,
 198, 199, 200, 210, 211, 246(T),
 258, 336, 337, 381, 384(T)
Ascidia, 166
A. nigra, 335
Ascobolus, 157
A. carbonarius, 153
A. stercorarius, 153, 154
Ascophyllum nodosum, 128
Asellus militaris, 267
Astasia longa, 41
Asterias, 55, 270, 323, 371, 383
A. amurensis, 246(T), 257, 258, 260,
 261, 262, 274, 275, 276
A. forbesi, 246(T), 400
A. glacialis, 384
A. vulgaris, 246(T)
Asterina pectinifera, 246(T), 258
Astriclypeus manni, 246(T)
Astropecten scoparius, 246(T)

B

Bacillus megatherium, 349
B. subtilis, 21
Balanus balanus, 46, 107

Barnea, 179, 264, 329, 331, 354
B. candida, 258, 260, 263, 264, 337
Blechnum fraxinum, 137, 139
Bombardia lunata, 153
Brachythecium rivulare, 137, 139
Brissopsis, 323
Bufo, 267, 269
B. bufo, 384(T)
B. bufo asiaticus, 212, 214, 219, 372
B. viridis, 372
B. vulgaris, 211, 423

C

Calcar haematragum, 245(T)
Callinectes sapidus, 245(T), 265, 270,
 275, 277, 278
Cambaroides, 252
Campanularia, 118
C. calceolifera, 68
C. flexuosa, 68
Carassius auratus gibelio, 422
C. carassius, 422
Ceratium, 42
Ceratopteris thalictroides, 136, 137, 138,
 139
Cerebratulus, 371
Chaetopterus, 75, 370
C. variopedatus, 384(T)
Chama retroversa, 245(T)
Chara, 122
Cladostephus spongiosus, 125, 126
Chlamydomonas, 125, 158, 182
C. braunii, 23
C. coccifera, 23, 131
C. eugametes, 124
C. moewusii, 124
C. moewusii var. *reinhardii*, 124
C. moewusii var. *rotunda*, 123, 124, 125
C. paupera, 125
C. pulvisculus, 120
C. reinhardii, 33
C. snowiae, 66
Chlorogonium oogonium, 131
Ciona, 75, 165, 166, 179, 370
C. intestinalis, 23, 384(T)
Clypeaster, 242

C. japonicus, 246(T)
Clypidina, 245(T)
Cnemidophorus, 427
Colpoda, 71
Colpomenia sinuosa, 126
Comanthus japonicus, 245(T), 258
Crassostrea, 329
C. echinata, 245(T)
C. gigas, 245(T)
C. nippona, 245(T)
C. virginica, 258, 260
Cumingia tellinoides, 384
Cutleria multifida, 125, 126
Cyprinus carpio, 422

D

Dasycladus, 125
D. clavaeformis, 120
Dasypus novemcinctus, 22
Dendraster excentricus, 384(T)
Diadema setosum, 246(T), 258
Dictyota dichotoma, 129
Dictyuchus monosporus, 150
Discoglossus, 281, 425
D. pictus, 423

E

Echinarachnius, 197, 200, 201, 202
Echinocardium cordatum, 246(T), 258
Echinus esculentus, 107
Ectocarpus siliculosus, 120, 125, 126
Enteromorpha, 125
Equisetum arvense, 139, 140, 141
E. palustre, 139
Eriocheir japonicus, 265
Eschscholus lucius, 422

F

Fucus, 120, 125, 128, 129, 158, 187, 189
F. platycarpus, 128
F. serratus, 128, 130
F. spiralis, 130
F. vesiculosus, 128, 130, 384(T)
Funaria hygrometrica, 137, 139
Fundulus heteroclitus, 384

G

Gasterosteus aculeatus, 422
Gerris, 359
Gorgonocephalus eucnemis, 258
Gymnogramme martensii, 139

H

Haliotus, 164
Hemicentrotus pulcherrimus, 111, 246(T), 381, 388
Himanthalia lorea, 128
Holothuria atra, 246(T), 329, 344
Homosira banksii, 130
Hydrodictyon reticulatum, 125
Hydroides, 179, 181, 243, 264, 270, 283, 284, 285, 296, 297, 298, 301, 302, 303, 305, 307, 309, 310, 311, 312, 315, 317, 319, 337, 341, 343, 346, 347, 348, 352, 354, 357, 358
H. hexagonus, 245(T), 258, 264
Hyla arborea japonica, 213, 214
Hynobius, 423

I

Isaria farinosa, 122
Isoetes japonica, 139, 140, 141

L

Lacerta saxicola armeniaca, 425, 427
L. saxicola dahli, 427
L. saxicola defilippi, 427
L. saxicola rostombekowi, 427
Lampetra, 343
L. fluviatilis, 246(T)
L. planeri, 246(T), 359
Lebistes reticulatus, 240, 422
Leptobryum pyriforme, 137, 139
Limulus, 359
Lymantria, 24
Linckia, 22
Lithophaga curta, 245(T)
Loligo pealii, 107
Lunella coronata coreensis, 245(T)
Lycopodium clavatum, 140
Lytechinus, 75, 80, 197, 200, 201, 319, 321, 323, 378, 380
L. pictus, 200, 378, 381, 391, 394, 395
L. variegatus, 190, 191, 192, 194, 195, 210, 211, 246(T), 381

M

Mactra lateralia, 384(T)
M. sulcataria, 245(T)
M. veniformis, 245(T)
Marchantia, 139
M. polymorpha, 122, 136, 137, 139

Marsilea, 121, 122, 136, 158

M. drummondi, 137, 139

Marthasterias glacialis, 384(T)

Megathura, 164, 281

M. crenulata, 245(T)

Melanospora parasitica, 122

Mellita, 197

M. quinquiesperforata, 164, 165

Menippe mercenaria, 245(T), 265, 266, 270, 275

Mespilia globulus, 246(T)

Miaster, 24

Misgurnus fossilis, 397

Mollienesis formosa, 422, 425

M. latipinna, 422

M. sphenops, 422

Monodonta labio, 245(T)

Mucor, 157

M. hiemalis, 145

M. mucedo, 125, 143, 144, 145, 146, 147, 149, 158

Mytilus, 51, 57, 59, 64, 65, 75, 78, 164, 178, 244, 263, 270, 271, 281, 282, 283, 284, 285, 329, 331, 354

M. edulis, 59, 76, 178, 241, 243, 244, 245(T), 258, 260, 270, 271, 272

M. perna, 358

N

Nereis, 7, 177, 183, 270, 331, 371

N. japonica, 245(T), 258, 264, 265

N. limbata, 245(T), 264, 275, 370, 384(T)

N. mirabilis, 245(T)

N. succinea, 384(T)

Neurospora sitophila, 153

O

Ochromonas danica, 41

Octopus vulgaris, 105

Oedipina, 423

Oedogonium, 121, 131, 132, 133, 134, 135, 157, 158

O. cardiacum, 131, 134, 135

O. geniculatum, 135

Ophiarachnella gorgonia, 246(T)

Ophioplocus japonicus, 246(T)

Oryzias, 370

O. latipes, 241, 374, 384(T)

Oscillatoria, 75

Osmunda javanica, 141

P

Pandorina morum, 75, 120

Palaemonetes paludosus, 167

Paracentrotus, 321

P. lividus, 193, 203, 204, 205, 206, 337, 384(T), 387(T), 391, 397

Paralosa lacustris lariana, 422

Paramecium, 70, 182, 356

Parastychopus californicus, 246(T), 258

Pecten irradians, 53

Penicillium, 123

Perinereis cultrifera, 245(T)

Periplaneta americana, 77

Petricola japonica, 245(T)

Phallusia, 370

P. mammillata, 384(T)

Phascolosoma, 65

Phlebodium aureum, 135

Phycomyces, 146

P. blakesleeianus, 145, 147, 158

P. nitens, 145

Phytophthora, 44

Pilobolus crystallinus, 145

Pityrogramma sulphurea, 141

Plagiobrissus, 197

Platynereis dumerilii, 245(T)

Pleurodeles, 423

Poecilia formosa, see also *Mollienesis formosa*, 425

Polytoma, 23, 76, 81

P. uvella, 42

Pomatoleios crosslandi, 245(T)

Prasiola, 344

P. stipitata, 238

Procambarus, 252, 253

P. clarkii, 258

Psammecinus microtuberculatus, 193

P. miliaris, 193, 384(T)

Pseudocentrotus, 242, 270, 321

P. depressus, 111, 246(T), 257, 258, 259, 272, 273, 381

Pteridium aquilinum, 141, 142

Pteris serrulata, 121, 136, 137, 139

Pycnopodia helioanthoides, 246(T)

R

Radula complanata, 137, 139

Rana, 216, 370

R. arvalis, 423, 425, 426

R. catesbiana, 216

R. clamitans, 215, 216
R. japonica, 423, 425, 426
R. nigromaculata, 423, 425, 426
R. pipiens, 211, 212, 213, 214, 215, 216,
 217, 219, 371, 427
R. platyrrhina, 384(T)
R. sylvatica, 215, 216, 218
R. temporaria, 214, 425
Rattus norvegicus, 258
R. rattus, 338
Rhizopus nigricans, 144, 145
Rivulus marmoratus, 422

S

Sabellaria, 179, 242, 331, 337, 350
S. alveolata, 384(T)
S. vulgaris, 180, 245(T)
Saccharomyces cerevisiae, 144
Saccoglossus, 270, 284, 296, 297, 305,
 307, 309, 313, 314, 315, 316, 317,
 318, 319, 320, 321, 322, 323, 324,
 325, 326, 327, 328, 330, 331, 332,
 333, 334, 343, 346, 347, 348, 349,
 352, 353, 354, 357
S. kowalevskii, 246(T), 258
Salmo salar, 422
S. trutta, 422
Salvinia natans, 139, 140, 141
Saprolegnia, 44, 122, 123, 150
S. ferax, 151
S. mixta, 151
Sapromyces reinschii, 150
Saxostrea commercialis, 384(T)
Scalpellum, 33
Scutus scapha, 245(T)
Scythosiphon lomentarium, 120
Selaginella erythropus, 137, 139
Sphaerechinus granularis, 205, 386
Sphaeroplea, 121, 131, 132
Spirogyra, 123, 143, 144
Spisula, 74, 178, 331, 382, 383, 399
S. solidissima, 370, 401
Spondylus cruentus, 245(T)
Squalus acanthias, 105
Strongylocentrotus, 184, 321

S. droebachiensis, 246(T), 258, 260
S. franciscanus, 246(T)
S. purpuratus, 194, 195, 246(T), 378,
 384(T), 385, 393
Styella, 179
Sycon raphanus, 24
Synchitrium endobioticum, 125
S. fulgens, 125

T

Tegula, 282
T. pfeifferi, 245(T), 282
Temnopleurus toreumaticus, 246(T)
Tetrahymena, 34, 52, 56, 71, 345
Thermobia, 264
Thraustotheca, 151
Thyone briareus, 246(T)
Trapezium sublaevigatum, 245(T)
Triturus, 179, 423
T. torosus, 212
T. viridescens, 211, 213, 371
Turbo cornutus, 245(T)
T. stenogyrus, 245(T)

U

Ulothrix zonata, 120
Ulva, 125
Umbonium, 245(T)
Urechis, 179, 182, 200, 331, 334, 359,
 381, 383
Urechis caupo, 200, 370, 380, 384(T),
 385

V

Vaucheria, 121

X

Xenopus, 423
X. laevis, 376, 423
Xiphosura polyphemus, 245(T), 265,
 267, 268, 270, 277, 279

Z

Zirfaea subconstricta, 245(T)

Subject Index

A

- A (activating) antigen, 205, 209, 210
 - location of, 205
- Acetyl phosphate, 46
- Acetylcholine,
 - effect on ciliary movement in gill plate of *Mytilus edulis*, 59
- Acetylcholinesterase,
 - in bull semen, 48
 - and conductile mechanisms, 60
 - in fish sperm, 48, 59
 - flagellar, distribution of, 60
- Acroblast,
 - Golgi membranes of, 251
 - migration of acrosomal granule from, 249
- Acrosomal cap, 249, 250
 - of birds, 269
 - enzyme released on disruption of, 287
 - formation of, 249
 - of rabbit spermatozoon, 269
- Acrosomal filament, 329
 - of *Lampreta*, 343
 - of starfish, 325
 - of sturgeon, 343
- Acrosomal granule, 249, 250
 - of annelids, 331
 - aspermatic antigen as component of, 171-173
 - of echinoderms, fate in egg sperm association, 321
 - role in passage of spermatozoon through egg jelly, 323
 - structure of, 321
 - fate in chicken spermiogenesis, 249
 - in humans, synthesis by Golgi complex, 249
 - of *Hydroides*, 299, 306, 307, 315
 - as source of capsule lysins, 301
 - contact with egg capsule, 307
 - fate during zygote formation, 301, 306, 307, 315
 - lack in crayfish, 335
 - lipids in, 255
 - of mammals, 335
 - of polychaet annelids, 264, *see also* of *Hydroides*
 - of *Saccoglossus*, 307
 - fate during zygote formation, 307, 315
 - of sea urchin, 272
 - fine structure of, 258, 259
 - of starfish, 323
 - of toad, 343
 - of *Urechis*, 334
 - vitelline coat lysin as homologue of, 283
- Acrosomal lumen, of sea urchin, 272
- Acrosomal membrane,
 - of annelids, fusion with sperm and egg plasma membrane, 331
 - of *Barnea*, 264
 - of echinoderms, structure of, 321
 - eversion of, 303
 - in starfish, 325
 - fusion with egg plasma membrane, 342
 - of *Hydroides*, 298, 299, 315
 - insertion into sperm plasma membrane, 301, 302, 303, 304, 305, 306, 307, 315, 341
 - structure of, 315
 - loss in dehiscence, 340
 - of mollusks, conversion to acrosomal tubule, 329
 - of rabbit, 269
 - of *Sabellaria*, perforatorium-like structure in tubula, invagination of, 350
 - of *Saccoglossus*, 307, 315
 - conversion to acrosomal tubule, 307, 309, 317
 - structure of, 315
 - site of contact with sperm plasma membrane, 358
 - of vertebrates, 337
- Acrosomal process,
 - formation of, 285-286
 - of *Mytilus edulis*, 271, 272
 - of *Pseudocentrotus depressus*, 272

- formation of, 272
 - function of, 273
- of starfish, 274, 275
 - membrane of, 286
 - fusion with egg plasma membrane, 286
 - plasma membrane and, 286
- Acrosomal region,
 - of arthropods, development of, 335
 - egg activation and, 352-353
 - of house cricket, 335
 - of *Hydroides*, 298-301, 315
 - changes during progression of sperm head through egg envelope, 307, 314-315
 - dehiscence of, 299, 301, 307, 315, 319
 - membrane loss in, 337, 340
 - structure of, 298-299, 300, 301, 315
 - of unactivated, 298, 299, 300, 301
 - of *Sabellaria*, 336, 337
 - of *Saccoglossus*, 305, 313, 319, 320
 - dehiscence of, 307, 315, 319
 - structure of, 305, 307, 314-315
 - of unactivated, 313
 - of sea urchin, 336, 337
 - during zygote formation, 337, 338
 - of starfish, changes preceding egg-sperm association in, 323
 - structure of, 323
- Acrosomal tubule, 342
 - of annelids, 306, 307, 330, 331
 - contact with egg plasma membrane, 317, 348
 - in echinoderms, 323
 - in *Hydroides*, 302, 303, 308, 309
 - formation of, 315, 317
 - in echinoderms, 321
 - of holothurians, 329
 - of invertebrates, perforatorium-like
 - structure in, 351
 - membrane, fusion with egg plasma membrane, 353
 - of mollusks, 329
 - of *Saccoglossus*, 319
 - conversion of acrosomal membrane to, 307, 309
 - core of, 313
 - fusion with egg plasma membrane, 309, 317
 - of starfish, formation of, 325
 - fusion with egg plasma membrane, 325
 - of *Urechis*, 334, 335
 - of vertebrates, perforatorium in lumen of, 350
 - Acrosomal vesicle, 249, 250, 357
 - of amphibians, 269
 - of annelids, 331
 - of arthropods, 335
 - as source of material within acrosomal invagination, 257
 - of *Barnea*, 263-264
 - of birds, 269
 - of bull, 270
 - of crab, 277, 278, 279
 - membrane of, fusion with plasma membrane, 277, 279
 - structure of, 265
 - of crayfish, formation and fate of, 253, 254
 - membrane of, 256
 - staining of, 254
 - of decapods, trigger layer of, 285
 - eversion of, 317
 - fate in spermiogenesis, 249, 250, 251
 - of toad, 249-250
 - formation of, 252
 - of house cricket, perforatorium-like
 - structure in, 351
 - of *Hydroides*, material with lytic activity in, 283
 - separation of acrosomal granule from, 320, 321
 - material contained in, segregation of, 255, 256
 - of mollusks, as source of capsule lysins, 329
 - structure of, 329
 - of *Mytilus*, 262-263, 270, 271
 - membrane of fusion with plasma membrane, 270
 - vitelline coat lysin in, 283
 - of *Nereis*, 264, 275
 - nuclear envelope and, 256
 - of polychaet annelids, 264, *see also* of *Hydroides*
 - of rat, structure of, 269
 - release of lysins from, 284
 - of *Saccoglossus*, 320, 321

- size of, 255
- of starfish, 323
- terminology, 260
- of tick, formation and fine structure of, 252
- of toad, 343
- Acrosome, 238
 - aspermatogetic antigen of, 171-173
 - association with perforatorium, 349, 350
 - changes during early stages of fertilization in, 239-240, 352-353, *see also* Acrosome reaction
 - cap of, terminology, 249
 - of decapods, formation of, 252
 - decrease in volume during spermiogenesis, 256
 - of echinoderms, reaction with egg jelly coat, 285
 - structure of, 321
 - egg-sperm association in species with, 319-343
 - without, 343-346
 - enzymes in, 3
 - function of, 19
 - fine structure of, 259-270
 - formation, during spermiogenesis, 244, 247-257
 - fine structure studies of, 249-254
 - role of Golgi complex in, 238
 - function of, 19, 238, 352
 - hyaluronidase in, 164, 173
 - of *Hydroides*, 299
 - structure of, 315
 - invagination cavities, material accumulated in, 256
 - nature of, 256
 - sources of, 256
 - isolation of, 248
 - lack of, 166
 - in algae, 343, 344
 - in ciliates, 345-346
 - in teleosts, 240, 241, 343, 344-345
 - lipids in, 255
 - lipoglycoprotein complex in, 101
 - of mammals, terminology of, 335
 - perforatoria in, role of, 239
 - of *Saccoglossus*, 305, 315
 - of sea urchin, 321
 - Pseudocentrotus depressus*, 257, 259
 - of starfish, fine structure of, 260, 261
 - surface of, antigenic structure, 199
 - of tick, formation of, 252
- Acrosome reaction, 163, 194, 240-241, 296, *see also* Acrosome, Sperm, activation of
 - in annelids, 275
 - components of, 239-240
 - condition conducive to, 241-244
 - in crabs, 275-279
 - effect of antibody treatment on, 203
 - fertilizin-antifertilizin system as possible initiator of, 164, 224
 - formation of acrosomal process membrane during, 286
 - general aspects of, 239-244
 - in *Hydroides*, 243
 - initiation of, 164, 222, 224
 - induction by egg jelly, 240
 - inhibition of, 242-243
 - of invertebrates, 220
 - fine structure studies, 270-279
 - of marine, 270
 - experimental induction, 244
 - pH of seawater and, 242
 - in *Mytilus edulis*, experimental induction, 243, 244
 - incomplete, 244
 - phase-contrast microscopic studies on, 239
 - in sea urchin, 257ff, 272-273
 - elements of, 257ff
 - fine structure of, 257, 259
 - substances participating in, 257
 - species showing, 245(T), 246(T)
 - in starfish, 274-275, 276
 - surface structure studies of, 223
 - in vertebrates, 280-281
- Acromosin,
 - composition of, 101
 - of ram sperm, properties of, 101-102
- Actin,
 - sperm, isolation and purification, 55
- Actinomycin,
 - binding of DNA by, 378, 402
 - effect on RNA synthesis, 402
- Activation,
 - egg, *see* Egg, activation
 - parthenogenetic, effect on protein synthesis, 389

- sperm, *see* Acrosome reaction, Sperm, activation
- "Activation antigen," 379
- Actomyosin, ATP and, 54
- Actospermosin, ATP and, 54
- Adenosine diphosphate, *see* ADP
- Adenosine triphosphate, *see* ATP
- Adenosinetriphosphatase, *see* ATPase
- Adenylic acid, in bull sperm extracts, 44
- Adenylpyrophosphatase, in bull semen, 48
- ADP,
 - in boar epididymal sperm, 45
 - in bull semen, 46
 - in bull sperm extracts, 44
 - in sea urchin sperm, 45
 - in spermatozoa, 111
- Adrenaline, effect on ciliary movement
 - in gill plate of *Mytilus edulis*, 59
- Agranular vesicles, of crayfish, 253
- Algae,
 - anisogamy in, 125
 - attraction of sperm to eggs in, 128ff
 - brown, attraction of motile swimmers to nonmotile, 125-126
 - extrusion of nonmotile eggs by, 128
 - flagella of, 126
 - reaction of isogametes in, 120
 - sperm attractants in, 128-131
 - conjugating, directional growth of sexual elements in, 143
 - copulation processes in, 120
 - fertilization, role of conjugation tubes, 118
 - fusion of sperm and egg plasma membrane in, 347
 - sites of, 358
 - green, formation of zygote membrane in, 344-345
 - sperm attractants in, 131, 132
 - lack of acrosome in, 343, 344
 - sperm attractants in, 128-132
- Amino acids, of seminal plasma, 104
- AMP,
 - in boar epididymal sperm, 45
 - in bull sperm extracts, 44
 - in bull semen, 46
 - in sea urchin sperm, 45
- Amphibians,
 - acrosomes of, spermiogenesis in, 267, 269
 - adult heteroploidy in, 435
 - cessation of protein synthesis in maturing egg of, 377
 - egg of, onset of RNA synthesis in, 401
 - egg jelly, antigens of, 213-220
 - origin of, 177
 - fertilization in, 211-222
 - role of egg cortex antigens, 220, 222
 - of egg jelly antigens, 213-220
 - germinal vesicle of, 371
 - heterogametic sex in, 423, 425
 - parthenogenesis in, 423-427
 - adult, 435
 - types of, 420-421
 - sex chromosomes in, 416
- Amphimixis, 414
- Ampulla, 101
- Anastomosis, arterio-venous, 10
- Androgenesis, 414
- Androspores,
 - attachment to oogonium, species specificity for site of, 121
 - chemotactic attraction to oogonium in *Oedogonium*, 133-135
- Animals, *see also* individual animals
 - generation of, historical aspects, 14-16
- Anisogametes, 123
 - physiological and morphological, 125-128
- Anisogamy, 123
 - in algae and fungi, 125ff
- Annelids,
 - acrosomal changes during early stage of fertilization, 239
 - acrosome reaction in, 245(T)
 - egg membranes of, 179
 - fragmentation in, 21
 - growth of oocytes in, 370
 - polychaet, fine structure of acrosome in, 264, *see also* *Hydroides*
 - sperm-egg association in, 331
- Antheridial hyphae, 149-152
 - chemotropic response to nutrients, 152
- Antheridial hyphae,
 - directed growth toward oogonia, 119, 121, 143, 149, 150
 - hormonal regulation of, 150ff

- induction of, 119, 122
 - of sperm by, in *Sphaeroplea*, 131-132
- role in fertilization of fungi, 118
- Antherozoids, 129
 - chemotactic reactions of, 120
- Antibodies, 3
 - acrosome-specific, from mouse sera, 70
 - against fertilizin, 379
 - against material from sea urchin eggs, 37
 - anti-egg jelly, effect on cleavage of sea urchin egg, 211
 - effect on sperm motility, 68-71, 72, 73
 - specific, use in control of development, 6
 - univalent, 209
 - effect on sea urchin eggs, 210
 - fertilization-inhibiting action, 209
- Anti-bull sperm serum, sperm-agglutinating action, 168
- Antiegg jelly serum,
 - effect of fertilizability of amphibian eggs, 212-213
 - mechanism of, 212
 - univalent, effect on cleavage of amphibian eggs, 212
- Antifertilizin(s), 3, 19, 20
 - antisera to, 380
 - blocking of, by combination with fertilizin, 185
 - chemistry of, 191, 193
 - egg, relation to sperm antifertilizin, 193
 - extracts, antigens in, 191
 - combination with fertilization products, 195
 - effect on fertilizin, 182
 - inactivation by *Fucus* extracts, 189
 - isolation from sperm, 182, 185
 - location of, 178, 223
 - physicochemical properties, 191
 - reaction with egg, 185
 - role in fertilization, 183-189
 - specificity of, 182, 183
- Antigen F, of amphibian egg, location of, 222
- Antigens, 3
 - of amphibians
 - of egg cortex, 220-222
 - of egg jelly, 213-220
 - distribution in oviduct, 214-215
 - interrelationships of, 216
 - localization of, 215-216, 217
 - number of, 213, 215
 - of essential, 216, 217
 - specificity of, 216
 - sites essential for fertilization in, 219
 - use of univalent antibody in study of, 219
 - in antifertilizin, 191
 - aspermato-genic, 171-173
 - blood group, *see* Blood group antigens
 - erythrocyte, *see* Erythrocyte antigens
 - in fertilization product, 194
 - of sea urchin eggs, 224
 - chemical properties, 204(T)
 - distribution of, 199
 - egg jelly and, 210
 - heat lability of, 210
 - location of, 198-199, 205, 210
 - physiological effects, 204ff
 - role in cleavage and development, 203
 - of sea urchin sperm, 195ff, 224
 - fertilizin action of, 198
 - number of, 195, 198
 - role in fertilization, 199-203, 224
 - soluble, 195, 197, 199, 201
 - of seminal plasma, 169
 - adsorption to sperm, 170, 171
 - sperm-surface (combining sites), 168
 - number of soluble, on sperm tail, 169
- Antisera,
 - to sea urchin egg jelly, 187
 - effect on eggs, 187
- Antiserum,
 - to sperm, *see* Antisperm sera
- Antisperm sera,
 - effect on fertilizing capacity of sperm, 186
 - inhibition of antifertilizin by, 186
 - of fertilizin by, 186
 - rabbit, 185
- Aphids,
 - sexual reproduction in, 24
 - "winter eggs" of, 24
- Apical body, 349-350
 - of bull spermatozoon, 270

- perforatorium and, 350
- of rabbit spermatozoon, 287
- Apical cap, of crab acrosome, 265, 275
- Apical vesicle,
 - of *Hydroides*, 298, 299, 315, 340
 - of *Lytechinus*, 321
- Apomixis, 414
- Archegonium(a), 123
 - chemotactic reaction of plant sperm to, 119, 120
 - response to water, 135
 - sperm and, 135-143
 - structure of, 135, 136
 - of water fern, sperm entrance into, 121
- Arthropods,
 - acrosomal region, fine structure of, 335-359
 - acrosomal vesicle of, 335
 - acrosome, fine structure of, 264-267
 - acrosome reaction in, 245(T)
 - sperm-egg association in, 25, 335
- Articulation plates, 38
 - composition, 39
 - γ -fibers in, 38
 - formation, 38
 - proximal centriole and, 39
- Artificial insemination, 10
 - and disproof of *aura seminalis*, 16-17
 - dog, 16
 - frog, 17
 - media used in storing and diluting of semen for, 99
 - preparation of semen for, 67
- Ascidians,
 - barrier to cross-fertilization in, site of, 166
 - differences in sperm surface of, 167
 - hermaphroditic, self-sterility of, 165, 222
 - zygote formation by membrane fusion, 335
- Ascogonium, 153
 - development of trichogynes from, 153
- Ascospores, of parasite, growth towards hyphae of host, 122
- Aspermatogenic antigen,
 - acrosomal glycoproteins and, 173
 - as component of acrosomal granule, 172
 - fertilization and, 172
 - hyaluronidase and, 172
 - physicochemical properties of, 172
- Aspermatogenesis, antigen responsible for, 171-173
- Asteroids,
 - acrosome reaction in, 246(T)
 - calcium and, 243
- ATP,
 - activation of flagella models by, 47
 - as main energy-rich sperm phosphagen, 111
 - as source of contractile energy, 44
 - as substrate in mechanochemical coupling process, 50
 - in boar epididymal sperm, 45
 - in bull semen, 46
 - in bull sperm, 44
 - effect on precipitation of *Mytilus* sperm tails, 51
 - of starfish sperm flagella, 52
 - on sperm cell motility, 44, 45
 - on turbidity of suspension of *Tetrahymena* cilia, 52
 - energy made available by splitting of, 112-113
 - enzyme splitting terminal phosphate from, 48
 - in flagellar contraction-relaxation cycle, 112
 - in flagellar movement, 42, 43, 44, 46, 50, 51, 52, 66, 75, 76, 81, 84
 - plasticizing effect, 43
 - half-life of human spermatozoa and, 46
 - hydrolysis of, 113
 - by flagella, 44
 - in mammalian sperm, 51
 - diffusion through principal piece, 50
 - down sperm flagella, rate of, 41
 - in mammalian spermatozoa, 46
 - motile flagella and, 48
 - motility of ejaculated spermatozoa and, 51
 - motility of human spermatozoa and, 46
 - production in sperm, effect of carbohydrate on, 45
 - regeneration, 47
 - in γ -fibers, 51

- resynthesis, 45, 47, 113
 reversible complex formation between
 Mg and, 58
 in sea urchin sperm, 45
 sperm cell constituents and, 45
 synthesis, 49
 during spermatogenesis, 51
 in sperm, effect of cations on, 63
 utilization in mammalian sperm, 50
 transport to sites of, 47
 ATPase,
 activity of, 54, 55
 of flagella, 55
 potentiation of, 74
 in spermosin, 54
 from *Arabacia* flagella, 55
 in fish sperm, 52
 in flagellum, 104
 isolation from, 52
 sperm, activation by Mg^{++} , 57
 in spermatozoa, 111
 Atropine, effect on ciliary movement in
 gill plate of *Mytilus edulis*, 59
 Autoagglutinins, animal, effect on sperm
 motility, 69
 Autogamy, 22
 in ciliate protozoa, 23
 Autoimmunization, aspermatogenic
 phenomenon and, 172
 Automixis, 314
 Axial filament complex, 103
 composition, 34
 "contractile units" of, 112
 role in flagellar activity, 112
 β -fibers of, 36
 γ -fibers and, 34
 location of, 35
 movements, 39
 Axolotl, neoteny in, 23
- B**
- Bacillus subtilis*,
 complete virus formation in, following
 infection with free virus DNA, 21
 Bacteria, "mating behavior," 8
 Barnacle, ATP in spermatozoa of, 46
 Bedbugs, meeting of egg and sperm in,
 25
- Birds, *see also* individual birds
 parthenogenesis in, 428-430
 adult, 435
 sex chromosomes in, 416
 spermatozoa, γ -fibers in, 32
 Blastocyst stage, polyembryony at late,
 22
 Blood group antigens, 173-177
 of nonsecretors, 173, 174
 of secretors, 173, 174
 on sperm, origin of, 173
 other than the ABO system, 175-
 176
 Boar,
 acrosome, structure of, 335
 perforatorium of, 350
 sperm, AT level of epididymal, 45
 reversible inactivation with CO_2 , 61
 spermatids, effect of fixative compon-
 ents on stability of PAS-positive
 acrosomal material of, 248(T)
 tests for glycogen and acid muco-
 polysaccharides in developing
 acrosome of, 247(T)
 spermatozoa, cholinesterase activity of,
 59
 Bracken fern, chemotaxis of, 141
 Bryozoans,
 budding in, 22
 internal buds (statoblasts) of, 22
 permanent association of buds in, 22
 Budding, in bryozoans and freshwater
 sponges, 22
 Buds, strobilization of, 22
 Buffalo, semen, number of antigens in,
 170
 Bulbourethral gland, 101
 Bull,
 acrosome, structure of, 335
 perforatorium of, 350
 semen, anacrobic incubation and heat
 production, 46
 antisera for, metabolic effects of, 71
 composition, 46
 decapitated spermatozoa in, 47
 effect of γ -rays on, 80
 motile flagella in, 47
 number of precipitin bands in, 169
 respiratory activity, 48

- sperm, absorption spectra of, 80
 - acetylcholinesterase in, 60
 - antigens of, 171
 - blood group, 176-177
 - subsurface, 168
 - surface, 168, 195
 - biplanar waves in, 43
 - cells, substances from, effect on
 - seminal plasma, 69
 - in egg yolk citrate buffer, 42
 - rotating, 42
 - nonrotating, 42
 - forward velocity of, 42
 - decapitated, *in vitro* assay of heads
 - from, 60
 - effect of visible radiation on, 80
 - extracts, composition of, 44
 - flagella, enzymic activity, 59
 - movement of, 37
 - stiffness of, 53
 - synchronic action of, 60
 - lipids in, 45
 - loss of cytochromes in, 80
 - mitochondria of, 49
 - succinic dehydrogenase in, 49
 - motility of, 44, 45
 - effect of ATP on, 44
 - of CO₂ on, 61
 - of dienestrol on, 72
 - of temperature on, 77
 - of ultraviolet radiation on, 80
 - of viscosity of medium on, 81
 - of visible radiation on, 79
 - energy expenditure, 82
 - resumption of, on aeration, 61
 - reversible inhibition by fluoride, 74
 - myokinase in, 52
 - osmotic effects on, 66
 - oxygen consumption of, effect of
 - steroid hormones on, 72
 - penetration of antibodies into, 168
 - spontaneous dehiscence in, 340
 - spermosin from, 44, 52
 - stiffness of tail, 44
 - structure of head, 339
 - vesiculation, 337
 - spermatozoa of, "apical body" in, 270
 - ATP:ADP ratio of, 47
 - flagellar activity of, 287
 - lytic agent in, 284
 - motility inhibition of, 74
 - tissue specificity in, 169
- C**
- C (cortical) antigens, 210
 - Calcium,
 - effect on fertilizin agglutination of
 - sperm, 182
 - role in fertilization, 242, 243
 - of sperm, 7
 - changes following, in mammals, 172, 220
 - role in fertilization, 343
 - SCA and, 171
 - Capillary method, Pfeffer's, 137, 141, 142
 - Carbohydrates,
 - as major component of fertilizin, 181
 - breakdown, ATP production and, 45
 - synthesis of lipids in sperm and, 45
 - Carbon dioxide tension, effect on sperm
 - metabolism, 101
 - Cat,
 - acrosome of, structure, 335
 - ovulation, role of coitus in, 435
 - perforatorium-like structure in, 350
 - Cations, role in sperm motility, 62-66
 - Cells,
 - permeability, in sperm senescence, 109
 - phagocytic, destruction of spermatozoa by, 25
 - transformations of, by administration
 - of DNA to fertilized egg, 6
 - Central tubule, of activated crab acrosome, 275
 - Centriole(s),
 - absence of, 166
 - distal, and animal spermatid, 41
 - mitochondrial sheath and, 35
 - spermiogenesis and, 35
 - occurrence in pairs, 40
 - proximal, articulation plates and, 39
 - orientation, 38
 - in reptilian spermatozoa, 38
 - of rat spermatid, 39
 - ring, termination of mitochondrial
 - sheath at, 36
 - "Cercaria," resembling spermatid worms
 - of ram, 13

- Chemotactic agents, 124-125, *see also*
 Chemotaxis
 in *Allomyces*, 126
 in *Chlamydomas*, 123-125
 clustering as response to, 126
 in *Fucus*, 125, 128, 129, 130
 in *Sphaeroplea*, 132
 stability of, 124-125
- Chemotaxis,
 between gametes, 3
 mechanism of, 118
 role in attachment of sperm to egg,
 128, 129
 role in sexual reproduction of algae,
 128ff
 of animals, 117-118
 of plants, 123-143
 historical background, 118-123
- Chemotropism,
 between hyphal fungi, 122
 mechanism of, 118
 of pollen tubes, 123
 role in sexual reproduction of plants,
 122-123, 143-155
 historical background, 118-123
- Chicken,
 acrosome formation in, 255
 domestic, fate of acrosomal granule
 during spermiogenesis, 249
 of acrosomal vesicle, 249
 invagination cavities of sperm nucleus
 in, 256
 parthenogenesis in, 428
 perforatorium of, 350
- Choanocytes, 24, 25
- Choline, in seminal plasma, 104
- Cholinesterase, in bull semen, 48
- p*-Chloromercuribenzoate (PCMB),
 effect on flagellar movement, 75
- Chorion, 223
 of ascidian egg, role of, 179
 follicle cells and, 179
 role in self-sterility, 165
- Chromosomes,
 deviations, 414
 sex, *see* Sex chromosomes
- Cilia,
 abnormal, centriolar induction in, 40
 activity of, 27
 effect of viscosity on performance of,
 81
 β -fibers in conductile system of, 59
 fine structure of, 28
 inactive regions of, 53
 morphology of, 31ff
 movement, in gill plates of *Mytilus*
 edulis, 59
 effect of chemical agents on, 59
 sliding filament model of, 53
 protozoan, protein with metal-activated
 ATPase activity from, 56
 of *Tetrahymena*, contractile elements
 in, 34
 effect of ATP on turbidity of sus-
 pensions of, 52
 protein from, 56
- Ciliates,
 membrane fusion in, 345-346
 site of, 358
- Citrate,
 attraction of club moss sperm to, 140
 in seminal plasma, 104
- Cleavage,
 of sea urchin egg, inhibition by antiegg
 serum, 204
- Clubmosses,
 sperm of, chemotactic reactions, 119
 chemotropic reactions, 140
- Cock, Z_{02} value of semen, 107
- Cockroach, 78
 sperm motility of, 77
- Coelenterates,
 alternations of generation (metagene-
 sis) in, 22
 external budding in, 22
 permanent association of buds in, 22
- Coenzyme Q vitamins, effect on sperm
 motility, 73
- Cold shock,
 effect on spermatozoa, 109
 proteins as protective agents against,
 77
- Conductile system, in cilia, β -fibers and,
 59
- Conidium, attraction between trichogyne
 and, 153
- Conjugation, 22, 23
 in ciliate protozoa, 22

- Contractile elements,
 in flagellum, 53
 of mammalian sperm, γ -fibers and, 53
 Contraction-relaxation cycle, 51-60
 in γ -fiber of mammalian sperm flagellum, 58
 Copulatory tube, 144
 Cortical granule,
 breakdown, failure in antiegg serum treated eggs, 209
 in parthenogenetically activated sea urchin eggs, 205, 200
 release of fertilization product on, 194
 contact with egg plasma membrane, site of, 358
 membranes of, insertion into egg plasma membrane, 353, 356
 polysaccharide content, 194
 of *Sabellaria* egg, 180, 181
 of sea urchin egg, location of, 179
 Cow, cervical mucus, protection against dilution of sperm, 62
 Crab,
 acrosome of, fine structure of, 252, 265, 266
 acrosome reaction in, 275-279
 horseshoe, contraction of striated muscles in, 53
 spermatozoa, fine structure, 267
 nature of trigger layer of, 285
 Crayfish,
 acrosome, fine structure of, 252ff
 development of acrosomal region in, 335
 lack of acrosomal granule in, 335
 spermatid, acrosomal complex of, 255
 spermatozoa, staining properties of, 247
 trigger layer of, 285
 Creatine,
 enzymic phosphorylation, 46, 47
 in seminal plasma, 104
 Creatine phosphate, 46
 Creatine phosphokinase, in mammalian seminal plasma, 47
 Cricket,
 acrosome of, differentiation, 250, 251, 252
 fine structure of, 264
 invagination cavities in, 256
 development of acrosomal region in, 335
 perforatorium-like structure in, 351
 spermiogenesis in, 251
 Crinoids, acrosome, fine structure of, 257
 Crustaceans,
 acrosome reaction in, 245(T)
 parthenogenesis in, 24
 Curare, effect on ciliary movement in gill plate of *Mytilus edulis*, 59
 Cyclostomata, acrosome reaction in, 246(T)
 Cytochrome system,
 in sea urchin eggs, 386
 of spermatozoa, location of, 103
 role in sperm respiration, 107, 111
 Cytochromes, 36
 loss in bull sperm, 80
 Cytofertilizin, 193-195
 isolation of, 193
 release at fertilization, 193
 from jellyless eggs, 193
 Cytoplasm, sperm, location of, 35
- D**
- Decapitation, SCA and, 171
 acrosomal, in *Hydroides*, 299, 301, 302, 303, 304, 305, 307, 341
 spontaneous, 337
 in mammals, 342
 membrane loss in, 337
 in mollusks, 329
 release of zona lysin at, 342
 sperm activation and, 301, 315, 342
 Dehydrogenases,
 in flagellum, 104
 in mammalian sperm, 49
 Deoxyribonucleic acid, *see* DNA
 Dermal secretion,
 of *Arbacia*, fertilizin and, 187, 188(T)
 as fertilization inhibitor, 187, 188
 mode of action, 187
 nature of, 187
 Dictyosomal vesicles, 252
 Dictyosomes, acroblast formation from, 244
 Dienestrol, effect on bull sperm motility, 72

- Dilution effect,
 role in sperm respiration, 108-109
 on sea urchin sperm, 61
- DNA,
 binding by actinomycin, 378, 402
 cell transformation by administration
 of, to fertilized egg, 6
 cytoplasmic, 377-378
 in oocyte, 378
 RNA synthesis by transcription
 from, 395
 significance of, 378
 information coded in, expression dur-
 ing development of new individ-
 ual, 21
 transfer to RNA, 21
 RNA-primed *in vitro* synthesis of, 21
 in sperm head, 101
 conjugation with protamines and
 histones, 101
 in sperm nucleus, composition of, 101
- Dog,
 artificial insemination, 16
 perforatorium of, 350
 spermatozoa, motility of, inhibition, 74
 by antibiotics, 73
 sperm, viability, 62
 spermatc worms in, 11
- Drones, nature of, 24
- Dynein, from *Tetrahymena* cilia, 56

E

- Echinoderms,
 acrosome reaction in, 239, 245(T)
 aging and, 240-241
 egg-sperm association in, 319, 321,
 323, 325, 327-329
 lack of acrosomal vesicle in, 286
 sperm, metabolic processes in, 100
 spermatozoa, fine structure of, 257
- Echinoids,
 acrosome reaction in, 246(T)
 calcium and, 243
- Echiuroids, egg-sperm association in,
 331, 334, 335
- Egg(s), 177-179
 activation of, 4, 369-412
 acrosomal region and, 352-353
 breakdown of germinal vesicle and,
 371-372, 373
 effect on protein structure, 402-403
 fertilization and, 379-381
 periacrosomal material and, 353
 sites of membranes meeting in, 358
 in species with cortical granules,
 353, 356
 agglutination, by sperm extract, 184
 amphibian, antigens of cortex, 220-222
 coelomic, effect of treatment with
 antisera on, 220
 fertilizability of, 211
 parthenogenetic activation, 211
 fertilizability
 of antiegg jelly serum treated,
 212-213
 of coelomic, 211
 role of egg jelly in, 212
 of uterine, 211
 maturation of, 211
 ovulation in germinal vesicle stage,
 211
 parthenogenetic activation, of coe-
 lomic, 211
 inhibition by antibody treat-
 ment, 222
 ascidian, chorion of, as barrier to self-
 and cross-fertilization, 179
 association with sperm by membrane
 fusion, 295-362
 comparative aspects, 319-346
 mechanism of, 353, 356-358
 attachment of sperm to, 128, 129
 in *Fucus*, 129
 chorion, *see* Chorion
 cortex, antigens of, *see* Antigens
 cytolysis, 238
 development, inhibitors of, 379
 extracellular layers of, 223
 penetration by sperm, 3, 4
 mechanism of, 3, 4
 fertilized, fertilization of, 380-381
 respiratory metabolism of, 381-389
 fertilizability, effect of antifertilizin on,
 185
 gelatinous coat, passage of semen
 through, 16
 of *Hydroides*, association with sperm,
 mechanism of, 296-305

- capsule of, 297-298
 - contact with plasma membrane, 301
- cytoplasm, penetration into sperm cell, 305
- plasma membrane, 297-298
 - as part of zygote membrane, 303
 - contact with sperm plasma membrane, 303, 304, 305
- structure of, 179
 - of capsule and plasma membrane, 297-298
- inertness of mature unfertilized, 376-377
- in vitro* culture of, 7
- jellyless, release of cytofertilizin from, 193
- maturation of, 370
- membrane(s), composition in *Tegula*, 282
 - molecular differences between, 165
- meeting of sperm and, mode of, 24-25
- micropyle, in teleosts, 241
- microvilli, role in fertilization, 347-348
- of nonechinoid species, protein synthesis in, 399, 400
- plasma membrane, joining with sperm plasma membrane, 4, 20
 - penetration by sperm, possible mechanisms of, 4
- polyspermic, 297
- rabbit, zona penetration by perforatorium in, 350
- respiration, effect of antiegg serum on, 204, 205, 209
- of *Sabellaria vulgaris*, structure of, 181
- of *Sacroglossus*, association with sperm, mechanism of, 305-313
- capsule and plasma membrane of, 305
- sea urchin, antigens of, 203-211
 - effect of antiegg serum on
 - on cortex, 209-211
 - inhibition of fertilization, 204, 209
 - morphological effects, 204, 206, 207
 - parthenogenetic activation, 204-205, 208, 209
 - specificity of, 204
 - shedding of, in germinal vesicle stage, 370, 373, 377
 - specific adherence of sperm to, 3
 - surface, effect of vitelline coat lysins on, 283
 - in *Hydroides*, 283
 - in *Mytilus*, 283
 - structural changes at fertilization, 382
 - trout, after insemination, 345
- unfertilized, capacitation of ribosomes of, 396-398
 - inhibition of protein synthesis in, 389-390
 - basis for, 390-396
 - location of active RNA fraction in, 397
 - "masked" mRNA in, 396
 - oxygen consumption of, ADP level and, 387
 - of *Urechis*, structure of, 335
- Egg jelly, 223
 - action on sperm, comparative aspects, 219-220
 - amphibian, antigens of, *see* Antigens, of amphibian egg jelly
 - chemistry of, 219
 - layers of, 213
 - origin of, 177, 224
 - oviduct as source of, 224
 - role in fertilization, 211-220
 - as barrier to fertilization and polyspermy, 184
 - fertilization product and, 194-195, 196
 - function of, 224
 - induction of acrosome reaction by, 240, 243
 - in microvilli, 178
 - precipitation by antiegg serum, 204
 - by antifertilizin, 185
 - precursor granules, 183
 - release of lysin from, antibody treatment and, 203
 - of *Sabellaria* egg, 179
 - sea urchin, antisera to, 186-187
 - fertilizin as major component, 181, 182, 223
 - origin of, 177, 183

precipitation by antifertilizin, 182
 Egg water, activation of spermatozoon by, 321
 Ejaculation, activation of mammalian sperm by, 3
 Enteropneusts, acrosome reaction in, 239, 246(T)
 Enzymes, *see also* individual compounds
 amino-acid activating, of unfertilized egg, 390
 egg, activation by fertilization, 403
 inhibition, by compartmentalization, 389
 in seminal plasma, 104, 105
 sperm, activity, site of, 51
 effect of sulphhydryl reagents on, 75
 flagellar components and, 41
 inhibition of, 75
 by sodium fluoride, 74
 proteolytic, effect on sperm penetration of cervical mucus, 82
 sperm, mammalian, 49
 motility and, 73
 of *Mytilus*, inhibition of, 75
 Epididymis, 101
 ram, spermatozoa in, 45
 Epithelium, seminiferous, cycle of, in rat, 39
 Ergothioneine, in seminal plasma, 104
 Erythrocyte antigens, 171, 223
 absence from sperm in nonsecretors, 223
 in seminal plasma of bull, 176-177
 on bull sperm, 176-177
 Eserine,
 effect on ciliary movement in gill plate of *Mytilus edulis*, 59
 on rate of flagellation in *Mytilus* sperm, 59
 Estrogens,
 effect on cervical mucus, 82
 on formation of yolk proteins in fowl, 375
 on liver transfer RNA's for serine, 375
 Eugenic methods, of selective breeding, 5
 Euphenics,
 methods of, 6
 tools of, 6

F

F antigens, *see* Fertilization antigens
 Ferns, *see also* individual ferns
 archegonia of, 135ff
 chemotactic response of sperm to, 135, 136, 137
 interspecific attraction in, 139
 sperm of, attraction to chemical agents, 139, 140
 configuration and, 142
 receptor sites for, 140
 chemotactic reactions, 119, 121
 chemotaxis between gametes in, 3
 trapping of sperm in, 121
 Ferret, parthenogenesis in, 433
 Fertility,
 control of, 5-8
 aspermatogenic phenomenon and, 172
 comparative approach to, 7-8
 by eugenic methods, 5-6
 experimental approach to, 8-9
 qualitative, 5-6
 reasons for, 5
 Fertilization, *see also* Syngamy
 in amphibians, 211-222
 antisera inhibitory to, 220, 221(T)
 role of egg jelly in, 211-220
 in animals, definition of, 23
 blocking of, 380
 effect on enzymes, 389
 egg activation and, 379-381
 of fertilized eggs, 380-381
 inhibitors of, 185-189
 in vitro, in mammals, 7
 role of fertilizin and antifertilizin in, 183-189, 224
 role of gamete surface components in, 163-236
 sea urchin, egg, inhibition by antiegg serum, 204, 209
 role of sperm antigens in, 199-203
 specificity of, 164-166, 177
 fertilizin-antifertilizin reaction and, 189, 224
 membrane fusion and, 165
 species, 3
 studies on, historical survey
 animality of spermatric worms, 11-14

- artificial insemination and disproof of *aura seminalis*, 16-17
- demonstration of external insemination, 16
- ovist's theory of preformation, 17, 18
- role of spermatozoon, 17-19
- Spallanzani's role in, 9-14
- views of Harvey and Leeuwenhoek, 14-19
- Fertilization (F) antigens, 209, 210
 - of sea urchin sperm, 200, 201-203
 - in egg cortex, 220, 222
 - reaction with fertilizin, 201
- Fertilization cone, 297, 298
 - of *Hydroides*, 303, 308, 309, 310
 - fate in zygote formation, 305, 313
 - role of, 351
 - of *Saccoglossus*, 297, 309, 333, 334
 - formation, 303
- Fertilization membrane, 205
 - formation in antiegg serum treated eggs, 209
 - of *Saccoglossus* egg, 305
- Fertilization product,
 - egg jelly and, 194-195, 196
 - fertilizin and, 195
 - of sea urchin egg, 193-195
 - antigens in, 194
 - combination with antifertilizin, 193, 195
 - effect on sperm motility, 194
 - sperm-agglutinating action, 193ff
 - reversal of, 193
 - specificity of, 193
- Fertilizin, 3, 17, 20
 - antibodies against, 379
 - antiserum to, 183, 380
 - of *Arbacia*, physicochemical properties of, 190
 - as major component of sea urchin egg jelly, 223
 - chemistry of, 189-191
 - concept, 68
 - effect of activation by, on sperm tip in *Nereis*, 331
 - electrophoretic behavior, 190-191, 192
 - historical aspects, 181
 - inert, release from sperm, 190, 191
 - inhibitor of fertilizing capacity of sperm by, 185
 - location of, 178
 - nature of, 179
 - reactive sites, 190
 - release on breakdown of cortical granule, 194
 - removal from eggs, 183-185
 - role in fertilization, 183-189
 - secretion of, fertilization and, 193
 - sources of, 183
 - specificity of, 182
 - sperm-agglutinating action of, 181
 - neutralization by antifertilizin, 182
 - reversal of, in sea urchin, 181-182, 185
 - species specificity of, 182
 - in starfish, 182
 - in *Urechis*, 182
 - sperm-combining sites of, 191
 - structure of, 190
 - univalent, 190-191
 - comparison with multivalent, 190, 191(T)
 - conversion of multivalent to, 190
- Fertilizin-antifertilizin system, 164, 181, 223
 - as initiator of acrosomal reaction, 164, 189, 224
 - distribution of, 182
 - mature of, 181
 - role in fertilization, 224
 - in gametic contact during fertilization, 348
- Fertilizin haptens,
 - from unfertilized sea urchin eggs, 194
 - relation to cytofertilizin or fertilization product, 194
- Fertilizin theory, 238
- Fibers,
 - flagellar, conformational change of, 56
 - identity of, 53
 - supernumerary,
 - formation of new shell of filaments and, 40
- α -Fibers,
 - of *Chlamydomonas reinhardtii*, subunits of, 34
 - fate of experimental cryptorchidism, 34

- physiological, in spermatozoa, 84
- fine structure of, 34, 35
- of mammalian sperm, 31, 40
- in sperm of organisms lacking γ -fibers, 40
- β -Fibers,
 - absence in *Scalpellum*, 33
 - of axial filament complex, 30
 - bending of bull sperm flagellum toward, 67
 - of *Chlamydomonas reinhardtii* mutants, 33
 - ATPase in, 34
 - in conductile system of cilia, 59
 - cytochemical reactivity of bull sperm and, 60
 - fate in experimental cryptorchidism, 34
 - of mammalian sperm, 31, 32
 - fine structure, 35
 - role of, 33, 34
 - in spermatozoa, 84
- γ -Fibers,
 - anchoring in articulation plates, 38
 - appearance, 39
 - axial filament and, 34
 - coarse, axial filament complex as complement of, 35
 - lack in primitive sperm, 36
 - composition, 54
 - conductile function in mammalian sperm flagella and, 59
 - contractile elements in, 44, 53
 - flagellar, contraction-relaxation cycle in, 58
 - enzymes in, 57
 - lack of, 85
 - of rat, 54
 - of mammalian sperm, 31, 32, 36
 - function of, 33, 85
 - in midpiece, 36
 - in mitochondrial sheath, 35
 - motility of sperm and, 81, 85
 - movement of bull sperm flagellum and, 38
 - organisms lacking, 85, 401
 - origin and formation, 40
 - outer, 34
 - of rat epididymal sperm, enzymes in, 49
 - regeneration of ATP in, 51
 - relation between proximal centriole and origin of, 38
 - rudimentary, 85
 - in spermatozoa, α - and β -fibers and, 84
 - of reptiles, 38
 - stability of, 35
- Fibrous sheath,
 - incorporation of fibers into, 37
 - sperm, formation, 41
 - stiffness of bull sperm flagellum and, 53
- Fish(es),
 - adult heteroploidy in, 435
 - egg, effect of fertilization on protein structure in, 403
 - onset of RNA synthesis in, 401
 - oxygen consumption of oocytes, during maturation, 373
 - at time of fertilization, 373, 374
 - parthenogenesis in, 421-423
 - adult, 435
 - self-fertilizing hermaphroditism in, 421-422
 - sex chromosomes in, 416
 - sperm, acetylcholinesterase in, 59
 - ATPase in, 52
 - teelost, lack of acrosome in, 240, 343, 344, 345
- Fission,
 - binary, 21
 - multiple, 21
- Flactin, isolation and properties, 55, 56
- Flactospermosin, isolation and properties, 56
- Flagella,
 - abnormal, centriolar induction in, 40
 - absence of, 166
 - activity of, 27
 - contractile units of axial filament and, 112
 - ATPase from, 52
 - from *Arbacia*, 55
 - ATPase activity of glycerinated, 55
 - attachment to eggs in *Fucus* by, 128, 129
 - bending waves, 60
 - of *Ceratium*, 42
 - bull, enzymic activity, 59
 - movement of, 37
 - chemistry, energy sources, 44-50

- conduction and contraction of, 29
 - contractile elements in, 53
 - contractile protein of, 30, 52
 - diffusion of ATP down, 41
 - entry into egg cytoplasm, 305, 312, 313, 345
 - enzymes and coenzymes in, 104
 - fine structure of, 28, 33
 - fish, actinlike component of, 54, 55
 - inactive regions, 53
 - internal viscosity, 43
 - lack of α -fibers in, 85
 - models, activation by ATP, 47
 - morphology of, 31ff
 - motile, activity of semen and, 48
 - ATP and, 48
 - in bull semen, 47
 - hyaluronidase of semen and, 48
 - plasmalogen of semen and, 48
 - propagation of undulatory wave by, 57
 - rat, composition of γ -fibers of, 54
 - role in sperm metabolism, 100
 - similarity in behavior of various kinds of, 44
 - starfish, ATP sensitivity of, 55
 - effect of ATP on precipitation of, 52
 - structure of, 102
 - transport within, 51
 - water uptake by sperm tails and, 52
- Flagellar action, synchrony of, 60
- Flagellar anlage, motility as intrinsic or myogenic property of, 41
- Flagellar movement,
 - ATP in, 42, 43, 50, 56, 66, 81, 84
 - effect of methyl cellulose on, 42, 43
 - of *Chlamydomonas snowiae*, 66
 - generation of, 44
- Flagellar wave, 54
 - coordination of, 59-60
 - head of spermatozoon and, 47
 - initiation and production, 39
 - nature of, 41-51, 56
 - physical aspects, 42
 - propagated, relaxation of contractile cycle, 56
 - propagation of contractile, 30
- Flagellation,
 - chemistry, energy sources, 44-50
 - cycle, shuttling of metals between contraction and relaxation systems during, 57
- Flatworms, external budding in, 22
- Follicle cell layer (corona radiata), 223
 - of mammalian egg, 177
 - role in fertilization, 177
- Follicle cells,
 - of ascidian egg, 179
 - of rabbit ova, lysis of, 284
- Fowl,
 - domestic, fertilization in, 83
 - effect of estrogen on formation of yolk proteins in, 375
 - semen, effect of visible radiation on, 79
 - storage of, 67
 - sperm, acid production *in vitro*, 62
 - agglutination on inactivation, 61
 - dispersion on reactivation, 61
 - motility of, 45
 - effect of temperature on, 77
- Fragmentation, in metazoans, 21
- Frog,
 - artificial insemination, 17
 - eggs, activation of, 347
 - sperm, motility of, 72
 - spermatic worms of, 17
 - synthesis of high molecular RNA during oogenesis, 375
 - type of, 375-376
- Fructolysis,
 - in whole semen, 105-106
 - index of, 105
 - inhibitors of, 106
 - rate of, factors affecting, 105-106
- Fructose,
 - metabolism, and ATP content of mammalian sperm, 51
 - in seminal plasma, 104
 - transport in bull spermatozoa, 51
 - utilization by spermatozoa, 105
 - in washed sperm suspensions, 106
- Fucose, in fertilizin, 189
- Fucus* extract, inhibition of fertilization by, 189
- Fungi,
 - anisogamy in, 125

- ascomycetous, reproduction of, 152-154
 - copulation processes, 12
 - fertilization in, 118
 - higher, directional growth of sexual elements in, 143
 - hyphal fusions in, 122
 - hyphal tube of, direction to oogonium, 131, 150
 - transport of sperm in, 118
- G**
- Galactose, transport in bull spermatozoa, 51
 - Galea capitis, 249
 - of mammals, 335
 - possible role in fertilization, 340
 - shape in bull semen, 340
 - Gametangia,
 - of *Allomyces*, 126, 127
 - grouping of male gametes near female, 126
 - female, growth of trichogyne by, 143
 - Gametes,
 - animal, kinds of, 23
 - attraction between nonmotile and motile, 125
 - male, attraction to sirenin, 127
 - inactivation of sirenin by, 127
 - plasma membranes of
 - receptor substances on, 19, *see also* Fertilizins and Antifertilizins
 - of *Polyoma*, 23
 - sea urchin, antigens of, 195-211
 - specific adherence to each other, 19
 - specificity of, 23
 - sperm and enclosed nonmotile female, 131-135
 - and free, nonmotile female, 128-131
 - Gametogenesis, 7, 8
 - hypothalamic-pituitary-gonadal feedback in, 3
 - problems of, 2ff
 - Gametology, comparative, problems and procedures of syngamy and, 26
 - Gamones, 147
 - Gastropods,
 - acrosome reaction in, 245(T)
 - calcium and, 243
 - pH of sea water and, 242
 - GDP, in bull sperm, extracts, 44
 - Gemmules, *see* Buds, internal
 - Genes, specific activity, protein synthesis and, 4
 - Germinal vesicle, 371
 - amphibian, 371
 - breakdown of, 371-375
 - egg activation and, 371-372, 373
 - protein synthesis in *Spisula* eggs and, 399
 - time of, 373
 - DNA in, 378
 - eggs shed as, 211, 370, 373, 377
 - Glucose,
 - resynthesis of ATP and, 45
 - utilization by spermatozoa, 105
 - in washed sperm suspensions, 106
 - Glucose-6-phosphate dehydrogenase (G-6-P hydrogenase)
 - egg, activation of, 388-389
 - α -Glycerophosphate, site of activity, 51
 - Glycerolphosphorylcholine, in seminal plasma, 104
 - Glycolysis, *see also* Fructolysis,
 - energy, in flagellar movement, 45
 - in semen, 99
 - enzymes and coenzymes for, location of, 104
 - rate of, factors affecting, 100
 - in sperm, 105-107
 - intermediary stages of, 106-107
 - in washed suspensions of, 106-107
 - Glycolytic enzyme system, matrix as substratum of, 35
 - Glycoproteins, acrosomal, aspermatogenic hormone and, 173
 - Glycosidases, in seminal plasma, 104
 - Goat antirabbit sperm serum,
 - sperm-agglutinating action of, 169(T)
 - Golgi apparatus, 238
 - acrosomes originating in, 238, 244, 247-248
 - structures resembling, in decapods, 252, 254
 - Conadotropins, mare serum, effect on amphibian sperm motility, 72
 - Gonads, ripening of, 3
 - GTP, in bull sperm extracts, 44
 - Graafian follicle, 14

- Growth, mechanism of, 2
- Guinea pig,
 effect of aspermatogenic antigen in,
 171-172
 parthenogenesis in, 431
 perforatorium of, 350
 sperm, antigenicity of tails, 69
 antigens of, 169, 171
 on surface, 168
 spermatozoa of, precipitin bands in
 rabbit antisera to, 70
 tissues specificity in, 169
- Cuppy, lack of acrosome in, 240
- Gynogenesis, 413, 418
 induced, 418, 419
 in *Mollinisia* sp., 422
 in mouse, 431
 in reptiles, 427
- ## H
- Hamster,
 ascent of sperm through oviduct of, 83
 golden, acrosome reaction in, 246
 perforatorium of, 281
 spermatozoon, penetration of zona
 pellucida, 280
 parthenogenesis in, 431
 sperm antigens of Chinese, 171
 spermatozoa, membrane arrangement
 at tip of zona penetrating, 341
- Haploid males, drones as, 24
- Haploidy syndrome, 420
- Hawk moth, 105
- Head cap, 249
- Hectocotyl, of cephalopod mollusks, 25
- Helical sheath, in mammalian spermatids,
 40
- Hermaphrodite, 23
- Hermaphroditism, self-fertilizing, in
 fishes, 421-422
- Heteroagglutinins, animal, effect on sperm
 motility, 69
- n*-Hexane, as chemotactic agent for plant
 sexual cells, 125
- Hexokinase, role in sperm glycolysis, 106
- Hexose phosphates, egg, changes in fer-
 tilization, 387
- Histocompatibility, aspermatogenic phe-
 nomenon and, 172
- Histocompatibility antigens, on sperm,
 177
- Histones, conjugation with sperm, DNA,
 101
- Holothurians, sperm-egg association in,
 325, 327, 328-329
- Holothurioids, acrosome reaction in,
 246(T)
- Homunculi, 14
- Honeybee, control of reproductive
 process in, 24
- Hormone(s),
 antheridial-inducing (hormone A of
 Raper), 151
 induction of antheridial initials in
 Achlya by, 128
 sirenin and, 128
 chemotactic, 123
 chemotropic, 123, 145
 (hormone C of Raper), 150-151,
 152
 effect on sperm metabolism, 71
 on sperm motility, 71
 plant, sperm-attracting, *see* Sirenin
 steroid, effect on bull sperm oxygen
 consumption, 72
 zygotropic, 145-149
 transmission of, 149
- Horse, intestinal worms of, 13
- Horsetails,
 sperm of, chemotactic reactions, 119
 chemotropic reactions, 140
- Humans, polyembryony in, 22
- Hyaline (ectoplasmic) layer
 of sea urchin egg
 precipitation by antiegg serum, 204,
 205, 206, 207
- Hyaluronic acid, 4, 164
 as intercellular cement, 280, 283
 lysis by zona lysin, 284
- Hyaluronidase,
 acrosomal, lytic action, 164
 as guinea pig sperm antigens, 169
 occurrence, in bacteria, 3
 in bull semen, 47, 48
 in mammalian sperm, 3
 immunological species differences, 164
 in rodent acrosome, 280
 role in fertilization, 284
 in sperm head, 101

- Hydroides-Saccoglossus* pattern,
 of sperm-egg association, 297-305,
 306, 307, 308, 309, 310, 311, 312,
 313, 315, 317, 319, 331
 in arthropods, 335, 359
 changes in acrosomal region, 315
 in echinoderms, 323, 346
 in echinroids, 334-335, 359
 in mammals, 341, 342, 359
 in mollusks, 329, 346
 significance of, 317, 319
 in vertebrates, 346
- Hyphae,
 antheridial, *see* Antheridial hyphae
 chemotropic responses of, 119
 fungal, chemotropism between, 122
 vegetative, 152
- Hypothalamic-pituitary-gonadal feed-
 back, role in gametogenesis, 3
- I**
- Implantation plates, *see* Articulation
 plates
 "Informosome," 398
- Infusion animals, spontaneous generation
 in, 10
- Inner cone, spermatid, 251, 252
- Inosine, in bull semen, 46
- Inosinic acid, in bull sperm extracts, 44
- Inositol, in seminal plasma, 104
- Insects,
 γ -fibers in spermatozoa of, 32
 parthenogenesis in, 24
- Insemination,
 artificial, *see* Artificial insemination
 external, 16
in vitro, sperm survival in human
 cervical mucus and, 82
- Insulin,
 effect on carbohydrate transport in
 bull spermatozoa, 51
- Interstitial membrane, 250, 251
- Invertebrates,
 marine, acrosome reaction in, calcium
 and, 243, 244
 experimental induction of, 244
 perforatorium of, 350-351
- Ions, concentration of, effect on sperm
 metabolism, 101
- Isogametes, 23, 123-125
 copulation, historical aspects, 120
 flagella of, 120
 group formation, 120
 chemotaxis and, 123, 125
- Isogamy, 123
- J**
- J (jelly) antigen, 204, 205, 209
 on bull sperm, 176-177
- Japanese quail, aspermatogenic antigen
 effect in, 172
- Jelly layer, of sea urchin egg, 179
- K**
- Kinoplasmic droplet, 29, 72
- L**
- Lactate, in female reproductive tract, 45
- Lactic acid,
 formation, in anaerobically incubated
 semen, 105
 in washed sperm suspensions, 106
- Lamprey,
 acrosomal region of, 359
 acrosome reaction in, 243
- Large oocyte supernatant (LOS)
 antigen, 222
- Lecithin,
 in acrosomal material from ram
 spermatozoa, 248
 of sperm, 104
 species differences in content of, 104
- Leeches,
 destruction of spermatozoa by phago-
 cytic cells in, 25
 meeting of egg and sperm in, 25
 sarcotubules of, 34
 spermatophores of, 25
 enzymic material in, 25
- Light, effect on sperm metabolism, 101
- Lipids,
 in acrosomal structures, 255
 in bull sperm, 45
 synthesis in sperm, effect of carbo-
 hydrates on, 45
- Lipoglycoproteins,
 acrosomal, 101, *see also* Acrosomin
 function of, 101-102

- Lipovitellin, formation, estrogen and, 375
- Liverworts, sperm of, chemotactic reaction, 119, 136, 137
- Lizards, parthenogenetic, absence of males in, 427
diploidy in, 427
viability of, 430
- Lysins, acrosomal, role of, 164, 165
egg membrane, release of, 222
sperm, effect on jelly coat and egg membrane, 281
isolation and properties of, 281-284
localization in acrosome, 282-283, 284
of *Mytilus*, 281-282
of *Tegula*, 282
source in mollusks, 329
of vitelline membranes, 19
zona, release at dehiscence, 341, 342
source of, 342
- M**
- Macrogametes, *see* Ova
- Magnesium, role in relaxation and contraction phase of cycle, 58
- Malate, attraction of fern sperm to, 138-140, 141, 142
- Mammals, *see also* individual animals
acrosome, structure of, 335
acrosome reaction in, 246(T)
distal centriole and spermatid in, 41
parthenogenesis in, 430-435
adult, 434-435
ferret, 433
guinea pig, 431
hamster, 431
man, 433-434
mouse, 430-431
rabbit, 432-433
rat, 431
sex chromosomes in, 416
sperm-egg association in, 335-343, 359
spermatozoa, arrangement of membranes on tip of zona-penetrating, 341
- Man, acrosome, structure of, 335
parthenogenesis in, 433-434
perforatorium-like structure in, 350
sperm, regional surface antigens of, 195
Mannose, utilization by spermatozoa, 105
Marsh-Bendall relaxing factor, 76
sensitivity of sperm flagellar models to, 56
Mating behavior, studies on, 8
Mating type, of an isolate of *Chlamydomonas*, 123, 124
Mating-type substances, 182
Matrix, composition, function and nature, 35
Maturation, 370
egg, begin of, 371
metabolic aspects of oogenesis and, 371-378
Medusas, 22
Megagametophyte, of *Marsilea*, 137
structure of, 121
Meiosis, female, cytology of normal, 414-415
Membrane fusion, definition, 295
sperm-egg association by, 295-367
Membranes, sperm, instability of, 285
Metagenesis, 22
Metazoans, gametes of, 23
Methyl cellulose, in flagellar movement, 42, 43
Microgametes, *see* Spermatozoa
Micronuclei, 22
in autogamy, 23
in conjugation, 23
transfer of, 23
union of, 23
Microtubular fibrous sheath, sperm, 36
Microvilli, 178, 179
egg, of *Hydroides*, 298
role in fertilization, 303, 347-348
of *Sabellaria*, 180, 181
of *Saccoglossus*, 305
egg jelly in, 178
egg receptors on, 4
fertilizin-coated surface of, 183
penetration of vitelline membrane by, 178, 179
in *Hydroides*, 179
in *Triturus*, 179

- of sea urchin oocytes, 181
 - Micropyle, 119
 - of insect egg, contact with spermatozoa, 265
 - Middle piece,
 - sperm, γ -fibers and, 36
 - metabolic activities, 49-50
 - Mitochondria,
 - midpiece, structure, 35
 - sperm, absence of, 166
 - motility and, 68
 - spermiogenesis and, 40
 - Mitochondrial sheath,
 - of spermatozoa, 35, 36, 103
 - axial filament complex in, 35
 - of nonmotile flagella, 36
 - role of, 35
 - structure of, 35
 - Molecular biology, 8
 - Mollusks,
 - acrosome reaction in, 239, 245(T)
 - bivalve, fine structure of acrosome in, 260, 262-264
 - perforatorium-like structures in, 350
 - sperm-egg association in, 329, 331
 - spermatozoa, γ -fibers in, 32
 - Monkeys,
 - aspermatogetic antigen in testicular tissue of, 172
 - spermiogenesis of, 39
 - Mosses, *see also* Clubmosses
 - sperm of, chemotactic reactions, 119, 121, 137
 - Mouse,
 - parthenogenesis in, 430-431
 - sperm, histocompatibility antigens in, 177
 - surface antigens on, 168
 - Mucorales,
 - directional growth of sexual elements in, 143, 144
 - zygophores of, 143, 144-145
 - hormones controlling, 145-149
 - Mucus,
 - cervical, effect of estrogen on, 82
 - motility of human sperm in, 82
 - movement of sperm through, 33
 - penetration by semen, effect of proteolytic enzymes on, 82
 - sperm distribution in, 83
 - Muscles,
 - biochemistry and ultrastructure of, 28
 - striated, contraction in *Pecten irradians*, 53
 - Myokinase, in sperm, 52
 - Myxomyosin, protein from *Tetrahymena* cilia and, 56
- N**
- Necrospermia, 82
 - Nemerteans, fragmentation in, 21
 - Neoteny, 23
 - Nuclear envelope, 307
 - acrosomal, of crab, 267
 - of *Mytilus*, 262
 - of starfish, 260
 - Nuclear membrane,
 - of *Bufo*, perforatorium as outgrowth of, 269
 - of crab acrosome, 265
 - Nucleic acid, in guinea pig sperm antigens, 169
 - Nucleotides, in boar epididymal sperm, 45
 - Nuptial dances of spiders, 25
 - "Nurse cells," 25, 370
- O**
- Oidium, attraction of trichogynes to, 154
 - Ophiuroids, acrosome reaction in, 246(T)
 - Oocyte,
 - DNA in cytoplasm of, 378
 - localization in amphibians, 378
 - growth of, 375-376
 - oxygen consumption during maturation, 373
 - Oogamy, 123
 - Oogenesis,
 - cessation of protein synthesis during, 376-377
 - possible mechanism for, 377
 - information produced by, control of embryonic development and, 370
 - inhibition and activation of enzymes during, 389
 - metabolic aspects of maturation and, 371-378
 - synthesis of ribosomes during, 376
 - of RNA during, 375-376

- Oogonium(a),
 attachment of androspore and sperm
 to, in *Oedogonium*, 133-135
 chemotactic agent responsible for,
 133-134
 attraction of antheridial hyphae to, 119
 entrance of sperm into, 121, 131
 in fungi, 131
 extrusion of eggs by brown algae from,
 128
 induction of antheridial hyphae by,
 119, 122
- Ootids, 370
- Outer cone, sperm, 251, 252
- Ova, *see also* Egg
 of metazoans (macrogametes), 23
- Oviduct,
 amphibian, antigens in, 214-215
 as source of egg jelly, 224
- Ovist's theory of preformation, 17, 18
- Oxygen consumption,
 during maturation of oocytes, 373-374
 of unfertilized egg, 386
 effect of fertilization on, 381ff
- Oxygen tension, effect on sperm metabo-
 lism, 101
- P**
- Paedogenesis, in the gallfly, 24
- Palps of spiders, transfer of sperm by,
 25
- Parthenogenesis, 22, 24
 alternating with fertilization in aphids,
 24
 in amphibians, 211
 antisera and, 220
 in *Rana japonica*, 425-426
 in *Rana nigromaculata*, 426
 in *Rana pipiens*, 427
 in antiegg serum activated sea urchin
 eggs, 204-205, 209
 artificial, 238, 239, 379-380
 autogamy as analog of, 23
 in birds, 428-430
 cytogenetics of, 418-419
 definition, 413
 diploid, 418, 419
 in *Rana*, 425
 in rabbit, 432
 experimental induction of, 419-420
 in fishes, 421-423
 haploid, 418, 420
 in *Rana*, 426
 in mammals, 430-435
 mode of, 24
 natural, 24
 polyploid, 421
 pre- and postreduction of sex chromo-
 somes in, 418
 in reptiles, 427
 in vertebrates, 413-440
- Parthenogenones, 413, 430
- human, sex of, 433
- mammalian, 434
- types of, 418ff
- viability of, 420-421
- sex differences in birds, 430
- Pasteur effect, in spermatozoa, 107
- Pelecypods,
 acrosome reaction in, 245(T), 260,
 262-264
 calcium and, 243
 pH of sea water and, 242
- Perch,
 sperm, ATP and water uptake by tail,
 52
 effect of sodium fluoride on, 74
 flagellar ATPase of, 63
- Pertoratorium, 237, 238, 249, 250, 287-
 288, 341
 of amphibian spermatozoon, 269
 as part of periacrosomal material, 349
 association with acrosome, 349, 350
 of bird spermatozoon, 269
 chicken, 250
 of bull spermatozoon, 270
 formation during spermiogenesis, in
 chicken, 249, 250
 in toad, 249-250
 function of, 240, 287, 351-352
 or of equivalents, 351-352
 of golden hamster, 281
 lytic action, 287
 possible mechanism of, 288
 of invertebrates, 350-351
 formation following sperm activa-
 tion, 351
 location of, 349, 351
 of mammalian, 337
 structure of, 349

- in vertebrates, 269, 343, 350
- of vertebrates, 269
- function of, 280
- structure of, 350
 - rat, 269
 - toad, 343, 350
- Periacrosomal material,
 - in annelids, 331
 - in arthropods, 335
 - egg activation and, 353
 - of *Hydroides*, location of, 298, 299, 300, 301, 307, 309, 315
 - in mollusks, 329
 - perforatorium as part of, 349
 - of *Saccoglossus*, 305
 - location of, 315
 - starfish, location of, 323
- Permeability, of flagellar surface, 36
- Phage, adhesion to bacterial wall, 348
- Phagocytosis, 20
- Phosphagens, in sperm metabolism, 46
- Phosphatases, movement of male gametes and, 58
- Phosphate, uptake by fertilized eggs, inhibition by antiegg serum, 209
- Phosphorus compounds,
 - energy-rich, *see also* ATP and other individual compounds
 - sperm motility and, 111
 - intracellular, utilization by spermatozoa, 108
 - sperm, breakdown of, 108
 - role in endogenous respiration, 108
- Phosphorylcholine, in seminal plasma, 104
- Phosvitin, formation, estrogen and, 375
- Physiology,
 - reproductive, studies on, comparative approach, 7-8
 - experimental methods, 8-9
- Fig.,
 - acrosome reaction in, 246(T)
 - egg of, sperm penetration filament of, 350
- Pinocytosis, 20
 - specific, as sperm-engulfing process, 346
- Pisces, *see also* Fishes
 - acrosome reaction in, 246(T)
- Planarians, reproduction by transverse fission, 21
- Plants,
 - chemotactic systems of, 123-143
 - chemotropic systems in, 143-155
 - mechanism of gametic approach in, 117-161
- Plasma, seminal, *see* Seminal plasma
- Plasma membrane, 178
 - acrosomal, of bivalve mollusks, 262
 - of starfish, 260
 - antidevelopmental antibodies and, 379
 - of antiegg serum-treated sea urchin eggs, 205
 - antigens located in outside layer of, 205, 210
 - association of fertilizin with, 183
 - egg, 223
 - acrosomal changes previous to contact with, 352-353
 - contact and fusion of acrosomal surface with, 222
 - fusion of acrosomal tubule membrane with, 353
 - of *Hydroides*, 290
 - microvilli as extension of, 178
 - penetration of, 4
 - structure of, 296
 - species differences in, 296
 - nomenclature, 296
 - fusion with acrosomal membrane, 341
 - in zygote formation, 353, 356
 - begin of, 357
 - partial, 357
 - sites of, 357-358
 - in species with vitelline envelopes, 358
- lack in mitochondrial sheath of non-motile flagella, 36
- model of, 356
- nature of, 353
 - of invertebrate spermatozoa, 286
- outer spermatozoon membrane corresponding to, 166
- rupture of, role in egg activation, 347
- sperm, 242
 - of *Barnea*, 264
 - contact with egg capsule, 348
 - dissolution of, 340
 - products of, 248

- effect of egg jelly substance on, 285
- fate in spermiogenesis in crayfish, 252
- friability, 35
- fusion with egg plasma membrane, 4, 20, 308, 309, 310, 340, 342, 348
- of *Hydroides*, 299, 315
 - as part of zygote membrane, 303
 - contact with acrosomal membrane, 301
 - contact with egg envelope, 301, 304, 305, 315
 - loss in dehiscence, 340
 - of *Saccoglossus*, 305, 315
 - contact with egg plasma membrane, 323
 - of sea urchin, 257
 - structure of, 222
 - of teleosts, fusion with egg plasma membrane, 344
 - of tick, 252
- vesiculation of, 357
- zygote, formation, 352, 353, 356, 357
 - in algae, 345
 - in *Hydroides*, 303, 317
 - in mammals, 342
 - by membrane fusion, 353, 356, 357
 - in *Saccoglossus*, 309, 313, 317
 - of *Hydroides*, 311
 - of mollusks, 331
 - of *Saccoglossus*, composition of, 309
- Plasma serum, antiseminal, 170
- Plasmalogen, *see also* Phospholipids
 - in acrosomal material from ram spermatozoa, 248
 - in bull flagella, 48
 - of sperm, 104
 - species differences in content of, 104
- Platyhelminths, fragmentation in, 21
- Pleurodeles, heterogametic sex in, 423
- Pollen tubes, 154-155
 - chemotropic response of, 154-155
 - conduction to egg, 118, 119, 120, 122
 - directional growth of, 143
 - role in fertilization, historical background, 119
- Polychaeta,
 - acrosome reaction in, 245(T)
 - pH of seawater and, 242
- Polyembryony,
 - in armadillos, 22
 - in humans, 22
 - of *Fucus*, as fertilization inhibitors, 189
 - properties of, 189
 - inhibition of fertilizin agglutination by, 189
 - mechanism of, 189
- Polysaccharide, as guinea pig sperm antigen, 169
- Polysomes,
 - fertilization and, 391-393
 - protein synthesis and, 393
- Polyspermy,
 - block to, 4, 381
 - opening of cortical vessels and, 4
 - unavailability of egg receptors to sperm and, 4
- Polyuridylic acid, 6
- Populations, growth of, control of fertility and, 5
- Postreduction, of sex chromosomes, 417, 418
- Potassium,
 - egg, compartmentalization at fertilization, 398
 - sperm motility and, 62-63
- Potassium gradient, in sperm, 65
- Prereduction, of sex chromosomes, 417, 418
- Principle piece of spermatozoa, γ -fibers and, 36
- Proacrosomal granule, 244, 247, 250
 - of crayfish, 253, 254
 - properties, 253
 - fate in cat spermiogenesis, 249
 - formation, 244
 - from acroblast, in house cricket, 250, 251
 - move towards nucleus, 254
- PAS positive reaction of, 244
- reduction in water content of, during spermiogenesis, 256
- role in acrosome reaction, 285
- staining properties, 255
- synthesis by Golgi apparatus, 254

- Progesterone, effect on bull sperm oxygen consumption, 72
- Proglottids, 22
- "Prostaglandins," 104
- Prostate, 101
- Protamines, conjugation with spermatic DNA, 101
- Proteases, as activators of protein synthesis in developing egg, 398
- Proteins,
 as guinea pig sperm antigen, 169
 attraction of sperm of liverworts to, 140
 conjugates with mRNA
 as "messenger protector," 398
 in unfertilized egg, 398
 release of mRNA from, 398
 contractile, in γ -fibers of rat sperm flagella, 54
 from starfish sperm flagella, 54
 synthesis of, acrosomal RNA metabolism and, 28-29
 cessation during oogenesis, 376-377
 in eggs of nonechinoid species, 399, 400
 initiation of, specific gene activity and, 4
 site of, 391
 stimulation by fertilization, 389-390
- Protista,
 flagellate, mating gametes of, 23
 "mating behavior," 8
 reproduction by binary fission, 21
- Protozoa, ciliate, forms of sexual reproduction in, 22, 23
- Pyrophosphatase, in sperm and seminal plasma, 57
- Pyrophosphate, as relaxing agent in glycerinated muscle, 57
- Q**
- Quillworts, sperm, attraction to chemical agents, 139, 140
- R**
- Rabbit,
 acrosome, fine structure of, 258, 335
 acrosome reaction in, 246(T)
 distal centriole and spermatid, 41
- egg, antigenic site on surface of, role in fertilization, 210
- fertilization *in vitro*, 7
- ovulation, role of coitus in, 435
- parthenogenesis in, 432-433
- perforatorium of, 340, 351
- sperm, surface antigens on, 168
- spermatic worms in, 11
- spermatozoa, chemotaxis of, 119
 fine structure of, 269-270
 lytic agents in, 284
 membrane arrangement at tip of zona-penetrating, 341
 motility reduction by nitro blue tetrazolium, 73
 unsheathing of "apical body" of, 287
- Radiation,
 effect on motility of sperm, 80
 bull, 80
- Ram,
 perforatorium of, 350
 semen, effect of sodium fluoride on, 74
 sperm, antigens of, 171
 motility of, 63
 effect of temperature on, 77
 effect of ultraviolet radiation on, 80
 spermatozoa, composition of acrosomal material, 248
 lytic agent in, 284
- Rat,
 acrosome reaction in, 246(T)
 acrosome, structure following penetration of zona, 341
 aspermatic antigen effect in, 172
 distal centriole and spermatid, 41
 effect of spermatic lysin on zona of, 341
 fusion of sperm and egg plasma membrane in, 347
 parthenogenesis in, 431
 perforatorium in, 350
 sperm, antimyosin reactive material from, 55
 frozen-dried, immunocytochemical reaction, 71
 spermatid, centrioles of, 39
 spermatozoa, fine structure of, 269
 testis extracts, precipitin bands in, 170

- zygote of, composition of plasma membrane in, 342
- Refertilization, 380-381
- Relaxation, reversible, 64
- Relaxation phenomena, 56-59
- Relaxation system, composition, 57
- Reproduction,
 - asexual, forms of, 21-22
 - origin of new living organisms in, 21
 - patterns of, 20-25
 - sexual, forms of, 22-24
 - in *Oedogonium*, 133-135
 - origin of new living organisms in, 21
 - syngamy as principal form of, 22
- Reproductive tract,
 - female, sperm motility in, 82
 - male, sperm motility in, 82
- Reptiles,
 - adult heteroploidy in, 435
 - parthenogenesis in, 427
 - adult, 435
 - sex chromosomes in, 416
- Respiration, *see also* Oxygen consumption
 - change at fertilization, 373-374, 382, 383
 - of sperm, 100, 107-111
 - endogenous, 107-109
 - enzymes and coenzymes for, location of, 104
 - exogeneous, 109-111
 - species differences in, 109-110
 - substrates for, 109
 - role of cytochrome system in, 107
- Reticulum, endoplasmic, fate in spermatogenesis, 29
- Rh antigens, on sperm, 176
- Ribaga's organ,
 - of bedbugs, 25
 - phagocytic cells in, 25
- Ribonucleic acid(s), *see* RNA, mRNA
- Ribosomes, fate in spermatogenesis, 29
- RNA,
 - as messenger between DNA and protein, 21
 - cytoplasmic from sturgeon oocytes, as primer for DNA and RNA synthesis, 378
 - extracts from unfertilized eggs, mRNA activity of, 396
 - incorporation of uridine into, by artificially activated nonnucleate sea urchin egg fragments, 378
 - metabolism, acrosomal protein synthesis associated with, 28-29
- RNA,
 - protein synthesis during egg development and, 395
 - replicative capacity, 21
 - synthesis, in chemically enucleated eggs, 393
 - during oogenesis, 375
 - rate of, 375
 - types of, 375-376
 - effect of actinomycin on, 402
 - in sea urchin egg, 399ff
 - fertilization and, 400
 - onset of, 400
 - type of, 401
 - in unfertilized, 399
 - synthetic, effect on incorporation of amino acids into proteins of cell-free systems, 390-391
- mRNA,
 - activation of ribosomes by, 397
 - conjugates with proteins, "as messenger protector," 398
 - release of, 398
 - synthetic, 6
 - as tool of euphenics, 6
 - masked, 396
 - embryonic determination and, 402
 - unmasking by fertilization or artificial activation, 396, 402
 - of unfertilized eggs, 391, 393
 - conjugates with protein, 397-398
- Ribosomes,
 - synthesis during oogenesis, 376
 - of unfertilized eggs, 390
 - activation by mRNA, 397
 - by trypsin, 397
 - capacitation of, 396-398
 - protein synthesis and, 390-391, 396ff
- Rodents,
 - spermatozoa, effect of hyaluronidase on, 280
 - penetration of egg envelope, 287
- Roosters,
 - aspermatic antigen in testicular tissue of, 172

- sperm, effect of vitamins on motility of, 73
- Roots, chemotropic responses of, and root hairs, 119
- Rotifers,
 - meeting of egg and sperm in, 25
 - parthenogenesis in, 24
- Rusts, connections between germinating sporidia in, 122

S

- Salamanders,
 - spermatophores of, 25
 - spermatozoa, perforation of, 269
- Salyrgan, flagellar detachment caused by, 66
- Saprolegniales, directional growth of sexual elements in, 143
- Schizogony, formation of new individuals in, 21
- Sea anemones, reproduction by longitudinal fission, 21
- Sea cucumber, acrosome reaction in, pH of sea water and, 242
- Sea robin, enzyme from sperm tail of, 48
- Sea urchin,
 - acrosomal process, formation of, 270
 - acrosome, fine structure of, 257
 - acrosome reaction in, 270-272
 - calcium and, 242
 - pH of medium and, 242
 - cessation of protein synthesis in mature unfertilized egg of, 376
 - egg, activation of, 347
 - antibodies to, 379
 - antigens of, 203-211
 - chemical properties of, 204(T)
 - cytochrome system in, 386
 - effect of actinomycin on, 393
 - of antisera on, 203ff
 - of fertilization on protein structure in, 402
 - fertilization product of, 193-195
 - relation to egg jelly fertilizin, 193
 - ions as activators of protein synthesis, 398
 - isolation of antifertilizin from, 193
 - oxygen consumption, fertilization and, 381ff, 383
 - RNA acting as template RNA in unfertilized, 396
 - RNA synthesis in, 399ff
 - fertilization and, 400
 - in unfertilized egg, 399
 - structure of, 179
- egg jelly, origin of, 177
- entrance of internal sperm organelles into egg of, 329
- maturation of female gamete in, 370
- oxygen consumption of oocytes during maturation, 373-374
 - at time of fertilization, 373-374
- perforatorium-like structures in, 351
- refertilization in, 380
- sperm, 68
 - antisera for, 71
 - association with egg, according to *Hydroides-Saccoglossus* pattern, 319, 321, 323
 - composition, 45
 - dilution effect in, 61
 - effect of cations on, 66
 - effect of viscosity of medium on, 81
 - fertilizing capacity of, 61
 - flagellar action, 61
 - motility, 64, 65
 - myokinase in, 52
 - oxygen uptake of, 61
 - rate of motility, 61
 - tail enzyme of, 48
- spermatozoa, breakdown of phospholipids in, 108
 - dilution effect in, 108
 - effect of egg water on, 108
 - of egg jelly solution on, 272, 285
 - oxygen consumption on addition of extracellular substrates in, 110, 111
 - time needed to reach egg surface, 280
 - trigger layer of, 285
 - Z_{O₂} value in, 107
- Selection, natural, 20
- Selective breeding, "eugenic" methods of, 5-6
- Self-fertilization,
 - barrier to, 165
 - localization of, 165
 - spermatic lysins and, 166
- Self-sterility, 165-166
- Semen, *see also* Sperm, Spermatozoa
 - animal, oxidation of testosterone to androstenedione by, 72

- chemical characteristics of, 101-105
 - of seminal plasma, 104-105
 - of spermatozoa, 101-104
- coagulation, enzymes participating in, 104
- fowl, lactate dehydrogenase and, 51
- fructose in, effect of oxygen deprivation on, 105
- glycolysis in, 100
 - species differences in, 100
- human, aspermatogenic antigen in, 172
 - blood group substances in, 173
- motility of, inhibitors of, 106
- nature of, historical aspects, 11
- penetration into cervical mucus, effect of proteolytic enzymes on, 82
- respiration of, 100
- spermatozoa as constituents of, 11
- Seminal fluid, fertilizing agents in, 10
- Seminal plasma, 104-105
 - activation of mammalian sperm by, 30
 - antigens in, 169, 173
 - adsorption on sperm, 170, 171
 - blood group antigens, 173
 - erythrocyte antigens, 223
 - of bull sperm, total lipids in, 45
 - chemical composition, 101, 103
 - complement-fixing action, 170
 - components of mammalian, 104-105
 - of nonmammalian, 105
 - effect of substances from bull sperm cells on, 69
 - human, number of precipitin bands in, 170
 - mammalian composition, 47, 104-105
 - effect of cations on, 65
 - movement of sperm through, 33
 - from oligospermic patients, 68
 - rabbit, number of precipitin bands in, 170
 - substances from, effect on sperm motility, 68
 - substrates for exogenous sperm respiration in, 109
- Seminal vesicle, 101
 - antigens of, 170
 - SCA, 171
- Serotonin, in sexual secretions of dogfish and octopus, 105
- Sertoli cells,
 - cytoplasm of, exchange of material between acrosome and, 249
- Sex,
 - heterogametic, in amphibians, 423, 426
 - homogametic, in *Rana*, 425
- Sex chromosomes,
 - cytologically visible, 423
 - normal, 415-418
 - pre- and postreduction in parthenogenesis, 418
 - of vertebrates, 416
- Sex reversal, 2
- Sexual somatic fusion, in higher fungi, 143
- Sirenin, 126
 - assay of, 127
 - attraction of male gametes to, 127
 - hormone A and, 128
 - inactivation by male gametes, 127
 - preparation, 127
 - structure, 127
- Sheep, parthenogenesis in, 433
- Snails, spermatozoa, γ -fibers in, 32
- Snakes, spermatozoa, γ -fibers in, 32
- Sodium, role in sperm motility, 65
- Sodium fluoride, as tool in studies on sperm metabolism and motility, 74
- Sodium malate, response of sperm to, 137-138, 140
- Sorbitol, in seminal plasma, 104
- Spactomyosin complex, ATPase activity of, 54
- Sperm, *see also* Semen and Spermatozoa
 - acrosome of, *see* Acrosome
 - activation of, 296, *see also* Acrosome reaction
 - acrosomal dehiscence and, 301
 - formation of acrosomal process following, 325
 - sites of membranes meeting in, 358
 - in species with acrosome, 353-356
 - agglutination of, 168
 - by fertilizin, 181, 182, 185
 - amphibian, motility, effect of mare serum gonadotropins on, 72
 - antigenic differentiation, 174-175
 - association with egg by membrane fusion, 295-367
 - comparative aspects, 319-346, 354, 355

- fertilizin-antifertilizin interaction in,
178
in *Holothuria atra*, 344
mechanism of, 353, 356-358
 in *Hydroides*, 296-305
 in *Saccoglossus*, 296-305
role of microvilli in, 17
specificity of, 222
stages of, 353-358
 in *Saccoglossus*, 314, 315, 316,
 317-331
attachment to eggs, chemotaxis and,
128, 129, 130
 to egg plasma membrane, 163
avian, changes in environment of, 82
"bathing cap" detachment of, 340
bull, *see* Bull, sperm
capacitation of, 7, 172
cells, binding of antigenic seminal
 plasma material, 69
 radiation and photosensitivity of, 79
chemotactic response to archegonia,
135-143
 in ferns, 135, 136, 137
 in liverworts, 136, 137
 in mosses, 137
complement-fixing action of epi-
 didymal and seminal, 170
conductile system in, role of, 59
density, effect on respiration and gly-
 colysis in, 100
enclosed nonmotile female gametes
 and, 131-135
epididymal, fertilizing of eggs by, 171
 metabolism, 45
 motion of, 29
erythrocyte antigens on, 223
 adsorption from seminal plasma in
 nonsecretors, 223
extracts, antigens in, nature of, 170
 effect of egg membranes on, 166
flagellum of, *see* Flagella
free, nonmotile female gametes and,
128-131
glycolysis in, 100
 species differences in, 100
head, 101
 chemical constituents of, 101
 proximal centriolar region of, 29
 structure of, 101
human, motility, in cervical mucus, 82
 effect of temperature on, 77
irradiated, properties of, 100
mammalian, activation of, 30
 ATP in, 51
 utilization of, 50
biologically important substances in,
50
changes in environment of, 82
coiling or spiralization in, 66
contractile elements of, and γ -fibers,
53
contraction-relaxation, mechanisms
 in, 67
cross sections through principal
 piece and end piece of tail, 37
enzymes in, 49
 of rat, 49
function of γ -fibers in, 85
motility, osmotic effects on, 66-67
 reduction by chemicals, 73
storage, 67
viability, 62
metabolism of, 99-116
 effect of hormones on, 71
 factors affecting, 101
 role in survival of sperm in female
 tract, 114
 studies on, 74
models, coordination of reactivated, 59
 effect of Ca^{++} on, in presence of
 relaxing factor, 57
 motility, 65
 reactivation in presence of ATP, 57
motility, 3, 28
 of cockroach, 77
 effect of extraneous physical factors
 on, 77
 of γ -fibers on, 85
 of hormones on, 72
 of natural biological fluids and
 extracts on, 68-71
 of pH on, 61
 on respiration and glycolysis, 100
 of temperature on, 77
energy sources for, 63
exogenous respiration and, 109-110
of human, effect of antibiotics on,
73
in male and female reproductive
 tracts, 82-83
of marine invertebrate, 67, 68

- of models, 68
- of *Mytilus*, 59, 64, 65
- of oligospermic, 69
- role of energy-rich phosphorus compounds in, 111ff
 - of potassium in, 62-63
 - of sodium in, 65
 - of zinc in, 66
- studies on, 74
- substances from seminal plasma and, 68
- succinic dehydrogenase and, 73
- motility-inducing factor from, 56
- Mytilus*, effect of eserine on rate of flagellation, 59
 - enzyme activity, 59
 - inhibition of, 75
 - pyrophosphatase of, 57
 - tails of, 51
- nucleus, fate in zygote formation, 313, 332, 333
 - penetration into egg cytoplasm, 313, 323
- origin, fertilizing power and, 29
- penetration into egg, mechanism of, 3-4
 - of extracellular egg membranes, 163
- plasma membrane, *see* Plasma membrane, sperm
- removal of antifertilizin from, 185
 - effect on fertilizing capacity, 185
- respiration of, exogenous, 109-111
- response to chemical agents, 137-138, 139-140
- rotating, wave action of, 37
- sea urchin, *see* Sea urchin, sperm
- senescence of, 108-109
 - stages of, 109
- specific adherence to egg, 3
- Spisula*, sperm, motility of, 74
- starfish, myokinase in, 52
- stimulating factor for, 68
- surface, antifertilizin of, *see* Antifertilizin
 - antigen of, in sea urchin, *see* Antigens, sea urchin, sperm
- tail, distribution of contractile elements along, 44
- transport, of energetic material in, 50, 51
 - rate of, 83
 - swarming around egg in *Homosira banksii*, 130-131
- Sperm agglutinins, 170
- Sperm serum, antiseminal, 170
- Spermateliosis, 40
- Spermatia, growth of trichogyne towards, 154
- Spermatid,
 - animal, and distal centriole, 41
 - mammalian, helical sheath in, 40
- Spermatogenesis, ATP formation during, 51
- Spermatophores, species forming, 25
- Spermatozoa, 10, 11, 166-167, *see also* Sperm, Semen
 - acrosomal region, *see* Acrosomal region
 - acrosome of, *see* Acrosome
 - activation by apical dehiscence, 242
 - antigenicity, 71
 - as constituent of semen, 11
 - boar, cholinesterase activity of, 59
 - bull, hexoses and, 51
 - different response of ejaculated and epididymal, to damaging agents, 109
 - of echinoderms, effect of activation on acrosomal region, 321
 - passage through egg jelly, 323
 - effect of antibiotics on motility of, 73
 - of sulfhydryl reagents on metabolism of, 75
 - of sulfhydryl reagents on motility of, 75
 - epididymal, motility, 69
 - extra and intracellular nutrients of, 99
 - fish, stimulation, 68
 - head, flagellar wave and, 47
 - human, effect of ATP on motility and half-life, 46
 - Hydroides*, dehiscence of, 337, 339
 - immobility, 12
 - mammalian, antigenic structure of, 168-171
 - ATP content, 46
 - attachment of neck to head of, 38
 - cross section of midpiece of, 32
 - glycolysis, 45

- hormonal interactions governing
 - metabolic shifts in, 72
 - of metazoans (microgametes), 23
- middle piece of, 36
- motility, effect of extraneous chemical factors on, 60
- of mollusks, change in, following activation, 329
- nature of, historical aspects, 11, 12
- naturally decapitated, in bull semen, 47
- neck, structure, 38
- nutrients of, extracellular, 99
 - intracellular, 99
- principle piece of, 36
- proximal centriole and γ -fibers, 38
- rabbit, chemotaxis of, 119
- reptilian, γ -fibers in, 38
- respiration, 68
- role in fertilization, 19
- of shrimp, 167
- vertebrate, marine, activation of, 31
 - reversible inactivation, 61
- Z_{O_2} values of, 107
- Spermatozoon-coating antigen (SCA), 170-171
 - occurrence in man and rabbit, 171
 - role in capacitation and decapacitation phenomena in rabbit, 171
 - seminal vesicle as source of, 171
- Spermidine, 104
- Spermine, 104
- Spermiogenesis, 39
 - cat, fate of proacrosomal granule in, 249
 - chicken, fate of acrosomal vesicle in, 249
 - formation of perforatorium during, 249, 250
 - crayfish, 252ff, 255
 - decrease in acrosome volume during, 256
 - fate of Golgi complex in, 254
 - flagellar motion during, 40
 - formation of acrosome during, 244, 247-257
 - in house cricket, 251
 - of perforatorium in toad during, 249
 - mitochondria and, 40
 - mitochondrial sheath and, 35
 - in monkey, stages in, 39
 - in rat, 39
 - in tick, 252
- Spermiokyst, 25
- Spermosin,
 - ATP and, 54
 - ATPase activity of, 44, 54
 - from bull sperm, 44, 54
 - formation of reactive dual protein complex by, 54
 - isolation and properties, 56
 - removal from *Asterias* flagella, 55
- Spiders,
 - meeting of egg and sperm in, 25
 - palps of, transfer of sperm by, 25
- Spiralization, in mammalian sperm, 66
- Sponges,
 - budding in, 22
 - meeting of egg and sperm in, 24
 - permanent association of buds in, 22
- Sporangiophores, conversion of zygophores to, 149
- Sporozoa, reproduction by multiple fission, 21
- Sporulation, formation of new individual in, 21
- Squid, spermatozoa, γ -fibers in, 32
- Starfish,
 - acrosome reaction in, 274-275
 - egg of, maturation, 370
 - oxygen consumption fertilization and, 382
 - protein synthesis in, 399
 - entrance of internal sperm organelles into egg of, 329
 - fertilizin action in, metal-binding agent required for, 182
 - flagella, actinlike component from, 54
 - ATPase activity of, 55
 - formation of acrosomal process in, 270
 - perforatorium-like structures in, 351
 - regeneration in, 22
 - sperm-egg association in, 323, 325, 329
 - according to *Hydroides-Saccoglossus* pattern, 325
 - sperm, effect of cations on, 66
 - myokinase in, 52
 - spermatozoa, trigger layer of, 285
- Sterility, relief of, 5
- Strobilization, 22

- Sturgeon, acrosome reaction in fresh-water, magnesium and, 243
- Succinic dehydrogenase,
activation in sperm senescence, 109
in middle piece of rabbit sperm, 49
- Sucrose, attraction of moss sperm to, 140
- Sulfhydryl reagents, effect on metabolism and motility of spermatozoa, 75
- Surface active agents, spermicidal, effect on sperm motility, 76
- Syngamy, 297
as principal form of sexual reproduction, 22
in lower plants and animals, 8
mechanism of, 19-20
edge-on joining of sperm and egg plasma membranes, 4, 20
problems and procedures of comparative gametology and, 1-26
- T**
- Teleosts,
lack of acrosome in, 240, 241, 343, 344-345
micropyles in chorion of, 344
function of, 344
- Temperature coefficients of eggs, 385
- Test cells, of ascidian egg, 179
- Testosterone, effect on sperm oxygen consumption, 72
- Thiourea,
effect on flagellar activity, 75
in studies of contractile systems, 76
- Tick,
aberrant spermatozoon of, 335
spermiogenesis in, 252
- Tissue respiration, 10
- Toad,
differentiation of acrosomal vesicle during spermiogenesis, 249-250
perforatorium of, 350, 351
spermatic worms of, 17
spermatogenesis in, 343
- TPNH, egg, change at fertilization, 388
- Trichogyne(s), 152-154
attraction to oidium, 154
chemotropic growth of, 153-154
function of, 152
of fungi, role in fertilization, 118
- Trigger layer,
sperm, 284-285
location of, 285
- Trap action, 3
- Trout,
sperm, motility of, 83
inhibition by detergents, 77
spermatozoa, exogenous respiration in, 110
- Trypsin,
agglutinability and fertility in eggs treated with, 184
as activation of ribosomes, 397
- Tunicates,
external budding in, 22
neoteny in, 23
permanent association of buds in, 22
- Turkeys,
parthenogenesis in, 24
parthenogenetic, 428-430
diploidy of, 428ff
fowl pox and, 428
origin of, 428-430
viability of, 429-430
- Twins, monocytotic, 22
- U**
- Uric acid,
in boar epididymal sperm, 45
- Urodeles, heterogametic sex in, 423
- Uterotubal junction, passage of sperm through, 30
- V**
- Vas deferens, 101
- Vertebrates, *see also* individual animals
acrosome, fine structure of, 267-270
acrosome reaction in, 246(T)
parthenogenesis in, 413-440
perforatorium of, 350
- Viruses, "mating behavior," 8
- Vitamins, coenzyme Q, effect on sperm motility, 73
- Vitelline capsule, 297
- Vitelline coat, of *Hydroides*, acrosome reaction at, 24
- Vitelline envelope, 296
of *Hydroides*, lysis of, 283
of *Saccoglossus* egg, 305

- of *Urechis*, 334
- Vitelline membrane, 165, 177, 178, 296, 298, *see also* Plasma membrane, egg
- action of *Arbacia* dermal secretion on, 187
- of plant phenols on, 189
- of *Hydroides* egg, 298
- location of, 223
- of parthenogenetically activated sea urchin eggs, 205
- penetration by microvilli, 178-179
- removal of, 165
- of *Sabellaria* egg, 179
- of *Triturus*, penetration by microvilli, 179
- Voltage gradient, effect on movement of fern sperm, 142-143

W

- Water ferns, *see also* Ferns
- archegonia of, 136
- structure of egg-containing apparatus of, 121
- Water mold,
 - attraction between motile and non-motile gametes in, 126
 - gamete-bearing apparatus of, 126, 127
 - growth of hyphae towards insect larvae, 122
 - reproduction in, 122
- Welfare, human, problems of reproductive physiology relating to, 2, 5
- Working hypothesis, as tool in experimental science, 9
- World Health Organization "Division of Maternal and Child Health" of, 7
- Worms, *see also* individual members
- meeting of egg and sperm in, 25
- sperm, 11

X

- X-rays, effect on sperm, 100

Y

- Yeast, copulation, hormonal control of, 144

Z

- Z₀₂,
 - definition, 167
 - of mammalian and nonmammalian spermatozoa, 107
- Zinc, role in sperm motility, 66
- "Zona lysis," 102, 284, *see also* Lysins
- in rodent acrosome, 280
- Zona pellucida, 296
 - effect of acrosomal lipoproteins on, 101
 - lysis of, 284
 - of mammalian eggs, 177
 - role in fertilization, 177
 - penetration by perforatorium in rodents, 280
 - by sperm, 164
 - role of hyaluronidase in contact of spermatozoa with, 284
- Zygophores, 144-149
 - conversion to sporangiophores, 149
 - interspecific chemotropic response of, 158
 - of mucorales, 143, 144-145
 - hormonal control of, 145-149
 - of algae, 338, 339
 - of ascidians, 338, 339
 - formation, 23
 - in echinoderms, 323
 - in *Hydroides*, stages of, 301-305, 315, 317
 - in mammals, 342
 - mechanism of, 346-348, 353-358
 - in mollusks, 336, 337
 - in *Saccoglossus*, stages of, 307, 309, 313, 314, 315, 317, 325, 326, 327, 328, 330, 331, 332
 - stages of, 356
 - in starfish, 325
 - of *Hydroides*, 312
 - of rat, 338
 - of *Saccoglossus*, 333
 - composition of plasma membrane, 309
- Zygotropic factor, 147-148
- Zygotropic hormones, *see* Hormones, zygotropic
- Zygotropism, 144, *see also* Zygophores and Hormones, zygotropic