Early history of *in vitro* fertilization

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Although *in vitro* fertilization (IVF) is used widely for a variety of purposes, it is often not appreciated how this technology was developed. A large number of experiments beginning in 1878 contributed to the first successful reports of IVF over 75 years later. The discovery of sperm capacitation in 1951 was central to the development of IVF technology, and it was rapidly followed by the first convincing reports of IVF in several species. The ability to fertilize oocytes *in vitro* has allowed major advances to be made into understanding the mechanisms involved in fertilization and early development, and IVF now supports reproductive biotechnology in animals and in humans. This article is a historical review of key experiments that helped to provide the basis for present day IVF procedures, placed into context with current practice.

In vitro fertilization (IVF) is a well-established technology with a variety of applications in basic and applied sciences. The technology supports the production of embryos used for research investigations, for treating human infertility, for enhancing the productivity of food animals, and for conservation of endangered mammals. For most species, IVF protocols are available that routinely provide high or at least acceptable proportions of fertilized ova, leading to the production of viable embryos that can develop into normal offspring after transfer to recipient females. In view of the extraordinary advances in IVF technology during the past few decades, and its current role in many areas of basic and applied science, it is easy to ignore the origins of IVF in basic research studies conducted as long ago as 1878, and to overlook the enormous significance of the discovery of sperm capacitation. For example, it is not well recognized that the basis for the first documented human IVF success in 1969 can be traced back to two groundbreaking articles with hamster gametes (Yanagimachi and Chang, 1963, 1964); these studies were the first to report mammalian IVF using spermatozoa capacitated *in vitro*. An earlier study by Chang (1959) showing that rabbit eggs fertilized *in vitro* could develop into normal young was also crucial to the acceptance starting 20 years later of human IVF as a clinical infertility treatment. Moreover, many of the key studies underlying the development of IVF were published before electronic databases such as Medline began cataloguing articles, and some reports appeared in sources not represented in these databases.

The present review attempts to describe the contributions to IVF made by numerous pioneering scientists up to 1971 and to illustrate some of the difficulties encountered in this

research. This cutoff date is used because most of the review was written as the Introduction of the author's unpublished PhD dissertation (Bavister, 1971). That Introduction has now been expanded and updated to recognize the current status and practice of IVF. The 1950s and 1960s were the 'golden age' of IVF, when, after the discovery of sperm capacitation, the first convincing successes were reported for several important species including the rabbit, mouse, hamster, rat and human. It seems timely to present the earliest work to remind present-day scientists how much is owed to the IVF pioneers, and how much the routine, protocol-driven IVF technology relies heavily on the information they provided.

The focus of this review is on spermatozoa and the changes they need to undergo before becoming able to fertilize an oocyte. It does not address the complexity of changes undergone by oocytes as a consequence of sperm penetration, in part because the author's early research focus was on sperm capacitation, and in part because so little was known before 1971 about parallel events occurring in mammalian oocytes during fertilization. Since the advent of IVF technology, many detailed reviews and articles have been published on the responses of oocytes to activation by spermatozoa (for example, Yanagimachi, 1994; Fissore *et al.*, 1998; Hewitson *et al.*, 2000). The present review does not address embryonic development subsequent to IVF, about which a very large number of articles have also been published (see Bavister, 1995; Niemann and Wrenzycki, 2000; Khosla *et al.*, 2001). The cited publication list is not exhaustive but includes those articles that were considered by the author in 1971 to be most relevant to sperm capacitation and IVF up to that time, as well as some representative contemporary references to illustrate how the field has progressed in theory and in practice since then.

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Early *in vitro* fertilization studies

In spite of intensive research efforts conducted since the latter half of the 19th Century, by 1971, knowledge of the physiology, biochemistry and morphology of mammalian fertilization and embryonic development was still sparse and inadequate, compared with the wealth of knowledge about that of amphibians and sea-urchins. The progress of research was hindered by the internal site of mammalian fertilization, which means that events involved in fertilization of mammalian eggs and early embryonic development cannot be investigated readily in their natural environment. Therefore, information concerning the relationship between the egg or the developing embryo and the immediate maternal environment is difficult to obtain. A potentially useful technique is to recover fertilized eggs or early embryos from the female reproductive tract, and to study their subsequent development *in vitro*. This approach has been used widely, but provides little information about the preceding events *in vivo*. Moreover, the timing of ovulation and of fertilization *in vivo* cannot be predicted accurately, which particularly restricts the investigation of rapidly occurring events, such as the penetration of spermatozoa through the egg investments and the changes undergone by the gametes during the early stages of fertilization.

Information can be derived much more readily from the study of eggs that are fertilized and then develop *in vitro*. Not only can the process of fertilization be closely observed, but also factors contributing to normal and abnormal fertilization and development can be examined. The progress of fertilization or embryogenesis can be frequently, if not continuously, observed and the conditions of culture can be varied to examine their effects on development. Thus, a wealth of information is available from studies *in vitro*, given the technical ability to accomplish them.

Early attempts to fertilize mammalian eggs *in vitro*

Between 1878 and 1953, numerous attempts were made to fertilize mammalian eggs *in vitro*. Although many reports were published claiming successful results, in the light of modern knowledge, most of these claims seem unjustified. Fertilization is a progressive, continuous process, beginning with the penetration of an egg by a spermatozoon, which results in the extrusion of the second polar body from the egg. This extrusion is followed by the formation, growth and juxtaposition of the pronuclei, and the process of fertilization culminates in the union of maternal and paternal chromosomes (syngamy), restoring the diploid state. Some authors still refer to the timing of specific events as occurring 'post-fertilization' when they actually mean 'post-insemination' of the oocytes. For example, erroneous and contradictory statements such as 'pronuclear formation begins [several] hours post-fertilisation' have been published.

Several early investigators attempted IVF using ovarian oocytes that were probably not mature enough to undergo

fertilization (Schenk, 1878; Pincus and Enzmann, 1935; Pincus, 1939). Other workers were aware of this problem. Rock and Menkin (1944) and Menkin and Rock (1948) cultured human ovarian oocytes for a time before insemination but, in retrospect, the time allowed for maturation of oocytes *in vitro*, which was based on the data of Pincus and Saunders (1939), was probably inadequate, judging from the work of Edwards (1965). Although it is possible that maturation of some oocytes could have been completed within the period during which the spermatozoa and eggs were incubated together, this still does not provide evidence that fertilization was accomplished *in vitro*.

Most of the criteria used to judge the results of IVF experiments can be misleading: for example, extrusion of the second polar body and development of eggs through the early cleavage stages may also be evoked by parthenogenetic activation, or mimicked by fragmentation. Eggs of the rabbit, the most commonly used species for the earliest IVF experiments, can be activated readily in culture by a variety of stimuli other than sperm penetration (Pincus, 1936, 1939; Thibault, 1949; Chang, 1954). The results of a study by Dauter and Thibault (1956) indicated that the incidence of parthenogenetic activation of rabbit eggs in culture was increased by the presence of spermatozoa. However, cleavage of unfertilized rabbit eggs in culture was reported to be much less common if the eggs were incubated with scrapings of Fallopian tube mucosa (Smith, 1951). Before the study of Smith (1951), only Schenk (1878) had used reproductive tract mucosa in the culture system. Parthenogenetic activation may be induced by cooling the eggs of some species; for example, the rat eggs used by Long (1912) are particularly susceptible in this respect (Austin, 1956a,b). With the exception of Smith (1951), none of the investigators in this field from Schenk (1878) to Shettles (1953) maintained the eggs at 37°C to preclude the possibility of cooling-induced activation. It is also possible that incubating eggs for prolonged periods at less than normal body temperature limited or even destroyed their ability to undergo normal fertilization and development.

As the eggs of some mammalian species may undergo parthenogenetic development in culture, the occurrence of apparently normal activation and cleavage of eggs *in vitro* is insufficient evidence for fertilization. The demonstration of sperm penetration into eggs *in vitro*, together with the formation of pronuclei and the second polar body, provides more convincing evidence for fertilization. No studies conducted before 1954 seem to have achieved this.

In some of the early experiments in rabbits, sperm heads that were apparently within the egg cytoplasm may have been superimposed during histological preparation (Moricard and Bossu, 1949; Moricard, 1950). Shettles (1953) reported that perivitelline spermatozoa were found in five human eggs that were inseminated *in vitro*, but the photographic evidence supporting this claim is by no means convincing and, in some cases, there appear to be breaks in the continuity of the zona pellucida through which spermatozoa might have gained entry. Smith (1951) claimed that 10 of 35

tubal rabbit eggs were penetrated by spermatozoa after finding sperm nuclei or developing male pronuclei in the egg cytoplasm; however, photographic evidence was not submitted to support this claim. One additional egg cleaved, but no sperm penetration was observed, and so parthenogenetic activation cannot be excluded.

The evidence for achievement of fertilization *in vitro* presented by Pincus and Enzmann (1934) appears more convincing. These workers obtained offspring after incubating rabbit eggs and spermatozoa *in vitro* and transferring the eggs, after washing them, to a recipient doe. However, these eggs may have been fertilized after transfer to the oviduct of the recipient animal. The removal of all adherent spermatozoa from freshly ovulated, cumulus-enclosed eggs is difficult (Chang, 1968), so it is likely that some spermatozoa escape the washing procedure and can be transferred into the oviducts along with the eggs. In the study by Pincus and Enzmann (1934), the interval between the start of gamete co-incubation *in vitro* and transfer of the eggs was only 20 min, so spermatozoa attached to oocytes were probably still capable of effecting fertilization *in vivo*. This criticism also applies to the experiments of Venge (1953), in which two litters of rabbits were obtained. In these experiments, the time interval between insemination of eggs *in vitro* and their transfer to recipients (3.5 h) was still insufficient to exclude the possibility of *in vivo* fertilization. This inherent problem is highlighted by the later adoption of this procedure as a treatment for infertile women, in which spermatozoa and eggs are mixed together *in vitro* and then transferred rapidly into the oviduct (Asch *et al.*, 1986). The high pregnancy success rates achieved with this 'gamete intra-Fallopian transfer' procedure indicate that the early claims for IVF success using this approach in animals were unwarranted.

In retrospect, it seems that most of the claims to have fertilized mammalian eggs *in vitro* made before 1954 were inadequately substantiated or were probably based on misinterpreted results. This conclusion is not surprising in view of the many experimental pitfalls discussed above; however, the possibility that IVF was obtained in some cases cannot be ruled out.

Discovery of capacitation

The probable cause of the lack of success of most of the early IVF experiments emerged with a major discovery by Austin (1951) and Chang (1951). These workers found that if spermatozoa were introduced into the oviducts of rabbits shortly after the predicted time of ovulation, very few eggs were fertilized, despite rabbit eggs remaining fertilizable for up to 8 h after ovulation (Chang, 1952; Adams and Chang, 1962a). In contrast, a high proportion of eggs was fertilized if oviductal insemination was performed several hours before ovulation. Observations carried out on rats showed that when spermatozoa were deposited directly into the ovarian capsule after ovulation, no penetrated eggs were recovered until about 4 h later, after which the number of

eggs undergoing fertilization increased rapidly (Austin, 1951). This significant delay before egg penetration was not an artifact of experimental interference, as in oestrous rats permitted to mate naturally but only after ovulation had occurred, penetration of eggs still did not start for about 2 h, even though spermatozoa rapidly reached the oviducts (Austin, 1952).

Both Austin and Chang postulated that the spermatozoa of some mammalian species need to reside for some time within the female reproductive tract before acquiring the capacity to penetrate eggs, and Austin (1951) coined the term 'capacitation' to refer to the change that spermatozoa undergo during this time. The need for rabbit spermatozoa to undergo capacitation almost certainly contributed to the failure of most attempts made before 1954 to achieve IVF in this species. In these experiments, spermatozoa were invariably recovered directly from the male reproductive tract. Today, it is known that spermatozoa from many species can be capacitated *in vitro*, but in several key animals, including rabbits, the spermatozoa must be treated in special ways before they can take part in fertilization (see section on 'Mechanisms of capacitation and the acrosome reaction').

Fertilization of eggs *in vitro* after the discovery of capacitation

Renewed interest in the fertilization of mammalian eggs *in vitro* followed the discovery of capacitation. Within a few years, rabbit eggs were fertilized *in vitro* for the first time, using spermatozoa recovered from the uterus (Dauzier *et al.*, 1954; Thibault *et al.*, 1954; Dauzier and Thibault, 1956). Many other workers confirmed these results, at first using spermatozoa capacitated *in vivo* (Brackett and Williams, 1965, 1968; Brackett, 1968). However, almost 20 years elapsed after the first rabbit IVF before this was repeated using spermatozoa capacitated *in vitro* (Ogawa *et al.*, 1972; Brackett and Oliphant, 1975; Brackett *et al.*, 1978; Hosoi *et al.*, 1981).

In a landmark study, Chang (1959) showed that *in vitro*-fertilized eggs could develop normally. After transfer of IVF rabbit eggs to foster mothers, living young were born that resembled their parents in coat colour. The observation that eggs showed morphological signs of fertilization (including cleavage) before transfer (Chang, 1959) precludes the possibility that eggs were simply fertilized *in vivo* by attached spermatozoa, which was not excluded in earlier studies. Numerous other studies duplicated these results (for example, Thibault and Dauzier, 1961; Brackett, 1969; Brackett and Oliphant, 1975).

During the 'golden age' of IVF, good morphological evidence was provided for success in several other important mammals. Technical aspects of IVF in a wide variety of species are thoroughly described and evaluated by Rogers (1978). Sperm penetration, emission of the second polar body and formation of pronuclei were all seen in golden (Syrian) and Chinese hamster eggs inseminated *in*

vitro (Yanagimachi and Chang, 1963, 1964; Barros and Austin, 1967; Pickworth and Chang, 1969). In one study, 45% of golden hamster eggs inseminated *in vitro* were fertilized and cleaved to the two-cell stage in culture (Yanagimachi and Chang, 1964). However, in spite of intensive efforts, none of these eggs developed further *in vitro* as a result of the 'two-cell block', which held up progress with cultured hamster embryos for 25 years after the landmark articles of Yanagimachi and Chang (1963, 1964) (Schini and Bavister, 1988), and it was not until 1992 that the first IVF hamster pups were born (Barnett and Bavister, 1992).

Whittingham (1968), Iwamatsu and Chang (1969, 1970) and Toyoda *et al.* (1971a,b) reported IVF of mouse eggs using spermatozoa capacitated *in vitro*. Whittingham (1968) transferred *in vitro*-fertilized mouse eggs at the two-cell stage to recipient mice, and subsequently recovered normal 17-day-old fetuses that genetically resembled their parents. Cross and Brinster (1970) and Mukherjee and Cohen (1970) confirmed this result with mouse eggs that were both matured and fertilized *in vitro*.

Toyoda and Chang (1968) were able to fertilize rat eggs *in vitro*, but only after removal of the zona pellucida. Miyamoto and Chang (1973) reported IVF of intact eggs with rat spermatozoa recovered from the uterus of mated females. Subsequently, *in vitro* capacitation and fertilization in the rat were accomplished by Toyoda and Chang (1974a,b).

Thibault and Dauzier (1961) reported the fertilization of a few sheep eggs and one pig egg *in vitro*, but many years elapsed before protocols were devised that supported consistent IVF results in these species and offspring were produced (Bondioli and Wright, 1980; Crozet *et al.*, 1987; Slavik and Fulka, 1991; Abeydeera, 2002).

Cat eggs that were inseminated *in vitro* with spermatozoa incubated *in utero* cleaved as far as the 16-cell stage (Hamner *et al.*, 1970). However, the first IVF using spermatozoa capacitated *in vitro* was not reported until 1977 (Bowen, 1977). IVF using spermatozoa capacitated *in vitro* has been used more recently by several laboratories to produce embryos for conservation of endangered species (Roth *et al.*, 1994a,b; Pope *et al.*, 2000, 2001).

Guinea-pig eggs were fertilized *in vitro* using epididymal spermatozoa (Yanagimachi, 1972), although it took up to 18 h before penetration began. It was later shown that guinea-pig sperm capacitation is retarded by glucose and by magnesium ions; in their absence, the time required for sperm capacitation was shortened to only 2–3 h (Rogers and Yanagimachi, 1975, 1976; Rogers *et al.*, 1979).

Fertilization of dog eggs *in vitro* was reported by Mahi and Yanagimachi (1976) and the time needed for capacitation of canine spermatozoa *in vitro* was estimated at 7 h. It was reported by Rogers (1978) that dog spermatozoa could become capacitated and undergo acrosome reactions in the absence of any egg or cumulus factors.

Fertilization of human eggs *in vitro* was first convincingly

achieved in 1969, as evidenced by sperm penetration, polar body emission and formation of pronuclei (Bavister *et al.*, 1969; Edwards *et al.*, 1969). This advance was based on modifications of the culture medium protocol used by Yanagimachi and Chang (1963, 1964), so it may be claimed that hamster IVF paved the way for human IVF (Bavister, 2002). In a classic understatement, Edwards commented that 'Human oocytes have been matured and fertilized by spermatozoa *in vitro*. There may be certain clinical and scientific uses for human eggs fertilized by this procedure.' (Edwards *et al.*, 1969). Some IVF human eggs were capable of undergoing apparently normal cleavage and differentiation in culture (Edwards *et al.*, 1970; Steptoe *et al.*, 1971). However, the first human birth from an IVF embryo was not reported until 1978 (Steptoe and Edwards, 1978), apparently because the ovarian stimulation regimens may have interfered with establishment of pregnancy or produced incompetent eggs. Since 1978, the clinical applications of human IVF have become widespread, so that probably more than a million IVF babies have been born worldwide. Originally proposed by Edwards and Steptoe as a treatment for infertility in women, human IVF and its derivative intracytoplasmic sperm injection (ICSI) are now used to treat male infertility, and even azoospermia.

Progress with IVF in non-human primates was slower than it was in humans. Successful IVF in the squirrel monkey was first reported by Gould *et al.* (1973) and by Kuehl and Dukelow (1975). However, in these studies, the presence of fertilizing sperm tails in the eggs was not shown, and the developmental ability of the IVF eggs was very low. Unequivocal evidence for IVF in rhesus macaques was reported by Bavister *et al.* (1983), who showed the presence of sperm tails in eggs, and considerable post-fertilization development. Stimulation of spermatozoa with caffeine and cyclic AMP was essential for supporting sperm fertilizing ability (Boatman and Bavister, 1984). Non-human primate IVF, mostly using macaque species, is now used by several laboratories world-wide to investigate primate fertilization and early embryonic development. In all cases, spermatozoa capacitated *in vitro* have been used. There appears to be only one report of IVF in chimpanzees (Gould, 1983). Oocytes were inseminated *in vitro* using Ham's F10 medium supplemented with serum and caffeine–cyclic AMP but it is not known whether these chemical stimulators are essential for sperm capacitation in chimpanzees. Of 18 oocytes that were mature at the time of collection, 11 were fertilized *in vitro* and five cleaved to about the three-cell stage.

Relationship between capacitation and the acrosome reaction

In most of the IVF studies carried out between 1954 and 1971, spermatozoa were either capacitated *in vivo* before insemination of the eggs, or it could be demonstrated or inferred from the timing of egg penetration that capacitation was required and had occurred *in vitro*. The spermatozoa of

mammals must undergo an acrosomal change called the acrosome reaction before penetration (Yanagimachi, 1994). The acrosome reaction was first demonstrated by Austin and Bishop (1958a,b), who used the novel technology of phase-contrast microscopy to discover that the spermatozoa of guinea-pigs, hamsters and Libyan jirds lost their acrosomes as they penetrated the zona pellucida. Later studies using the electron microscope, first with hamster spermatozoa, showed that this loss is restricted to the outer acrosomal membrane and acrosomal contents (Barros *et al.*, 1967; Franklin *et al.*, 1970). The acrosome reaction was found also to be prerequisite for fertilization in rabbits (Bedford, 1967, 1968), rats (Pikó and Tyler, 1964) and pigs (Dickmann and Dziuk, 1964), and bull spermatozoa were also found to undergo an acrosome reaction (Blom, 1963; Saacke and Almquist, 1964; see also Colwin and Colwin, 1967). In light of all this evidence, it was assumed that the acrosome reaction is a common characteristic of mammalian spermatozoa (Yanagimachi, 1994).

At first, there was considerable confusion over nomenclature concerning the changes that spermatozoa undergo before penetrating eggs. Austin and Bishop (1958b) suggested that the acrosome reaction constituted a part of the process of capacitation, whereas Yanagimachi (1969a) considered that the acrosome reaction '... must represent the final phase of sperm capacitation'. However, an acrosome reaction that is basically similar to that undergone by mammalian spermatozoa is exhibited by the spermatozoa of many marine invertebrates, including annelids, molluscs and echinoderms, and by spermatozoa of two other vertebrate groups, lampreys and sturgeons (Dan, 1956, 1967; Colwin and Colwin, 1965, 1967). However, capacitation seems peculiar to mammals. In agreement with the views of Bedford (1968) and Pikó (1969), it seems most appropriate to consider that capacitation is a discrete physiological change that enables mature spermatozoa to respond to appropriate stimuli, and thereby to undergo the acrosome reaction. This is the most logical way to view the changes undergone by spermatozoa within the female reproductive tract. Although the acrosome reaction is a fundamental change in structure undergone by the spermatozoa of animals from widely diverse groups, capacitation may be regarded as a process that has evolved to suit some peculiar feature of mammalian reproduction (for a review, see Pikó, 1969). Most likely, capacitation represents a conditioning process that prepares spermatozoa to undergo acrosome reactions very rapidly once they are exposed to mature oocytes within the oviduct. However, in the absence of appropriate stimuli (that is, zona pellucida or cumulus oophorus components), most capacitated spermatozoa would not automatically undergo acrosome reactions *in vivo*, thus maintaining them in a stable configuration for several hours, even days, before the arrival of ovulated oocytes. Thus, capacitation may be viewed as a mechanism for coordinating acrosome reactions with the availability of unfertilized oocytes, which has evolved together with internal fertilization. This view is

consistent with the apparent lack of capacitation in animals exhibiting external fertilization, in which spermatozoa are deposited in direct proximity to the oocytes, undergo acrosome reactions almost immediately, and have very short functional lifespans, usually encompassing only a few minutes. In contrast, it appears that mammalian spermatozoa may be sustained within the female reproductive tract without losing their fertility for many hours or even days.

Capacitation in different species

The evidence derived from early studies of a need for capacitation in mammals was necessarily circumstantial, being largely derived from the timing of penetration of eggs *in vivo* (Austin, 1951; Chang, 1951, 1969; Bedford, 1970a) or *in vitro* (Yanagimachi and Chang, 1963, 1964). Nevertheless, the sheer volume of this evidence in a wide variety of mammals (Austin, 1969; Yanagimachi, 1994) provided strong support for the concept of capacitation, even though very little was known about its mechanism.

In rabbits, a large mass of information was gathered concerning the need for spermatozoa to undergo capacitation before penetrating eggs (for a review, see Chang, 1969); however, much of these data were conflicting and confusing with regard to the conditions needed for capacitation. Most of the information related to capacitation in the rabbit uterus, but the oviduct was shown to be the major site of capacitation (Bedford, 1970a,b), although capacitation was accomplished in the shortest time (5–6 h) when spermatozoa passed from the uterus to the oviduct (Chang, 1955; Adams and Chang, 1962b). Bedford (1970a) provides the best discussion on the state of knowledge regarding capacitation in the rabbit and in other animals during the 'golden age' of IVF.

Further studies on the time relationships of fertilization *in vivo* in rats (Austin, 1952; Noyes, 1953; Austin and Braden, 1954) confirmed the original observation (Austin, 1951) that capacitation was required in this species. In golden hamsters, the need for capacitation was firmly established from the timing of fertilization *in vivo* (Strauss, 1956; Chang and Sheaffer, 1957; Yanagimachi, 1966; Hunter, 1969), together with IVF data (Yanagimachi and Chang, 1963, 1964; Yanagimachi, 1966, 1969a; Barros and Austin, 1967; Barros, 1968). In early IVF studies, high percentages of golden hamster eggs were fertilized *in vitro* with epididymal spermatozoa that had not been exposed to the female reproductive tract (Yanagimachi and Chang, 1963, 1964; Barros and Austin, 1967; Barros, 1968). Barros (1968) reported a delay of about 3 h before egg penetration took place *in vitro*. This delay was reduced to less than 30 min by preincubating spermatozoa with oviductal fluid containing ova in cumulus. These results were interpreted as indicating that golden hamster spermatozoa can be capacitated *in vitro* by incubation with cumulus oophorus fluids. Barros and Austin (1967) showed that hamster follicular eggs could be fertilized *in vitro*, and that follicular contents could induce the acrosome reaction in hamster spermatozoa.

These workers concluded that hamster epididymal spermatozoa could be capacitated *in vitro*, in the absence of any contribution from the female reproductive tract, as reported by Yanagimachi and Chang (1963, 1964). Later work by Yanagimachi (1969a) amply supported this conclusion. It became accepted that capacitation of hamster spermatozoa is a change undergone normally within the female reproductive tract, but which may occur *in vitro* under special conditions. Follicular fluid is able to provide these conditions (Yanagimachi, 1969a,b), and the presence of this fluid was essential in these early studies to allow hamster spermatozoa to undergo the acrosome reaction and penetrate eggs *in vitro*. Alternatively, follicular fluid can be replaced by serum albumin (Bavister, 1969) or by the fluid component of the cumulus oophorus (Bavister, 1982). Although the need for capacitation was firmly established by 1971 in rabbits, rats and golden hamsters, the demonstration of capacitation in other mammalian species was less well documented. After their introduction into the tubal ampulla via the ostium, ferret spermatozoa did not penetrate eggs within the oviduct until 3.5 h had elapsed (Chang and Yanagimachi, 1963). This delay presumably represents the time needed for capacitation of ferret spermatozoa in the oviduct. The time relationships of hybrid fertilization of rabbit eggs by snowshoe hare spermatozoa indicated that capacitation of these spermatozoa was necessary and took 6–10 h in the rabbit oviduct; this timing is very similar to that for oviductal capacitation of rabbit spermatozoa (Chang *et al.*, 1971). In a study of the time relationships of fertilization in the Mongolian gerbil and in the deer mouse, animals were artificially inseminated after the time of induced ovulation (Marston and Chang, 1966). The time interval between insemination and the earliest recovery of penetrated eggs was about 4 h for the gerbil and 3 h for the deer mouse. These data were consistent with the idea that capacitation occurs in both of these species. The time needed for IVF of a few Chinese hamster eggs by epididymal spermatozoa was approximately the same as that required for golden hamster eggs (Pickworth and Chang, 1969).

In mice, the time relationships for fertilization *in vivo* indicated that either capacitation occurred within a very short time (about 1 h) or else capacitation was not required in this species (Braden and Austin, 1954). However, Iwamatsu and Chang (1970) subsequently reported that although 1 h or more was needed for untreated epididymal spermatozoa to penetrate eggs *in vitro*, spermatozoa that had been preincubated in heterologous follicular fluid were able to effect penetration in only 20 min. This result was interpreted as indicating that capacitation of mouse spermatozoa was required, and occurred within about 1 h. This timing is consistent with later estimates made using a quantitative fluorescence assay of between 30 and 90 min for mouse sperm capacitation (Ward and Storey, 1984).

The IVF experiments of Hamner *et al.* (1970) indicated that cat spermatozoa require about 0.5–2.0 h of incubation *in utero* for capacitation to occur. This supposition has been

amply confirmed using IVF with spermatozoa capacitated *in vitro* in domestic and endangered felids (Andrews *et al.*, 1992; Roth *et al.*, 1994a,b; Pope *et al.*, 2000, 2001). The results of several early studies indicated a need for capacitation of ram spermatozoa. When ejaculated spermatozoa were introduced into the oviducts of ewes shortly after ovulation, no penetrated eggs were recovered until 3–5 h later. After incubation for several hours in the ligated uterus, spermatozoa were able to penetrate eggs 1.5–2.0 h after deposition into the oviduct (R. Moore, C. Polge and L. Rowson, unpublished). However, Mattner (1963) found that tubally inseminated spermatozoa needed only 1.5 h to penetrate eggs even without prior incubation *in utero*. Dauter and Thibault (1959) were able to fertilize sheep eggs *in vitro* with spermatozoa recovered from the uterus of a mated ewe, but not with ejaculated spermatozoa. In retrospect, this failure was probably due to unsuitable culture conditions for supporting capacitation *in vitro*, because several authors have since reported successful IVF in sheep using spermatozoa taken directly from the male reproductive tract (Bondioli and Wright, 1980; Crozet *et al.*, 1987; Slavik and Fulka, 1991) and the need for ovine sperm capacitation is now well established.

The few critical observations available on the timing of fertilization in the pig up until 1971 were consistent with a need for capacitation in this species. Boar spermatozoa introduced into the oviducts soon after ovulation did not penetrate eggs until 4 or 5 h later (Polge, 1969). Hunter and Dziuk (1968) found that only 2 h elapsed between the time of artificial insemination and the beginning of egg penetration. This delay presumably represents the time needed for capacitation and the acrosome reaction, as the time needed for sperm transport to the oviducts is only 15 min in this species (First *et al.*, 1968). In view of the observation made by Polge (1969), capacitation does not appear to proceed optimally in the oviduct alone. Although production of viable pig embryos by IVF is now quite routine using *in vitro*-matured (IVM) oocytes, the problem of polyspermic fertilization remains and the aetiology of this defect is not understood (Abeydeera, 2002). Moreover, embryos produced by IVM and IVF have low average developmental competence *in vitro*, which may also be the result of inadequate maturation of the egg cytoplasm (Niemann, 2001).

The early observations of Ericsson (1967a) and Lauderdale and Ericsson (1970) indicated that capacitation was necessary for bull spermatozoa, and an unpublished result of Edwards (quoted by Austin, 1969), concerning the time needed for the penetration of cow eggs *in vitro*, supported this suggestion. However, Mahajan and Menge (1966) were unable to demonstrate any advantage of uterine-incubated bull spermatozoa over control spermatozoa in terms of ability to fertilize eggs *in vivo*. Thus, the need for capacitation of bull spermatozoa was not convincingly demonstrated by these early studies. However, it has since been shown unequivocally that bull spermatozoa, like those of all other mammals studied, do require capacitation,

which can be achieved *in vitro* under special conditions (see 'Mechanisms of capacitation and acrosome reaction'). The technique of IVF in cattle (*Bos taurus*) is among the most successful of all species; 40% or more of inseminated IVM oocytes can develop into blastocysts, and many calves have been born that were derived from these processes (Brackett *et al.*, 1978; Hasler, 1998).

It was uncertain from the early studies whether primate spermatozoa require capacitation. In humans, the IVF experiments of Edwards *et al.* (1969) showed a delay of about 7 h between insemination of the eggs and the beginning of penetration; it seemed likely that this delay reflected a need for sperm capacitation. Unfortunately, insufficient material was available at that time to investigate this possibility more thoroughly. The observations of Marston and Kelly (1968) on the time relationships of fertilization in rhesus monkeys did not allow any conclusions to be drawn regarding the need for capacitation in this species. Now, it is clear that primate spermatozoa do need a period of capacitation. Macaque monkey spermatozoa require treatment with chemical mediators to acquire the capacity to undergo acrosome reactions and to bind and then penetrate the zona pellucida (Boatman and Bavister, 1984). This finding is curious because human spermatozoa require only washing and incubation for a few hours in a culture medium, without any specific chemical treatments, to become capable of fertilizing oocytes *in vitro* (Mortimer, 1994, 2000).

It has also been claimed that some amphibian spermatozoa undergo capacitation. Shivers and James (1970) showed that untreated frog spermatozoa penetrated very few eggs from which the jelly-coats had been removed; however, spermatozoa that had been preincubated with uterine eggs (that is, with intact jelly-coats) were subsequently able to penetrate a high proportion of de-jellied eggs. These workers inferred a need for capacitation of frog spermatozoa. In addition, Wolf and Hedrick (1971) found that *Xenopus laevis* spermatozoa needed to react with jelly-coat materials before penetrating eggs, and concluded that capacitation was required. However, these studies do not indicate whether the short time-interval between insemination and penetration of eggs (5 min in frogs; 20–30 min in *Xenopus*) includes a period of capacitation, similar to that in mammals, or whether it represents only the time needed for an acrosome reaction to take place. If capacitation does not occur, the prolonged time needed for acrosome reactions in amphibian spermatozoa may represent an interesting evolutionary intermediate between the spermatozoa of some invertebrates in which the acrosome reaction occurs immediately on contact with the egg capsule (Dan, 1956; Colwin and Colwin, 1967), and the spermatozoa of mammals, which need a period of capacitation usually requiring several hours.

It can be concluded that capacitation of spermatozoa is peculiar to mammalian reproduction and is a prerequisite for fertilization. Capacitation has not been found to be unnecessary in any mammal so far investigated, although it

may occur very rapidly in some species (for example, mice), so it may be assumed that capacitation is a universal phenomenon in mammals.

Hyperactivated motility of spermatozoa

Concomitant with the completion of capacitation *in vitro*, golden hamster spermatozoa exhibit 'whiplash' flagellar activity that can easily be observed under a phase-contrast microscope (Yanagimachi, 1970). After 3–4 h incubation under capacitating culture conditions, most motile spermatozoa show this vigorous 'hyperactivated' pattern of activity characterized by high amplitude flagellar bending, whereas in the early IVF studies, spermatozoa incubated under non-capacitating conditions never showed activation. Hyperactivated sperm motility was also observed through the wall of the oviductal ampulla in mated hamsters and mice, and hyperactivated spermatozoa have been flushed from ampullae of several other species near the time of fertilization (Katz and Yanagimachi, 1980, 1994; Demott and Suarez, 1992). These observations indicate that this change in flagellar activity is a natural event in capacitation that is involved in sperm penetration through the egg investments (Yanagimachi, 1970; Katz *et al.*, 1989; Suarez *et al.*, 1991) and is perhaps also necessary for spermatozoa to reach the site of fertilization (Shalgi *et al.*, 1992). Hyperactivated motility of spermatozoa incubated under capacitating conditions also occurs in many other species (Yanagimachi, 1994). Although the precise functional significance of hyperactivation is still not clear, this marked change in motility could be a useful indicator of capacitation in individual spermatozoa. However, hyperactivation can also occur under non-capacitating conditions. When golden hamster spermatozoa are incubated in the absence of serum albumin and cumulus oophorus factors, after 3–4 h they begin to exhibit hyperactivated motility that appears identical to that seen under capacitating conditions. However, these spermatozoa incubated in the absence of serum albumin and cumulus oophorus factors are not capacitated, do not undergo spontaneous acrosome reactions and cannot fertilize oocytes (Bavister, 1981, 1982).

Mechanisms of capacitation and the acrosome reaction

It seems surprising that research into the nature of capacitation proceeded very slowly for 20 years after its discovery (1951–1971). Some of the early developments in this research were reviewed by Austin (1961, 1967) and later developments were reviewed by Yanagimachi (1994). A major obstacle to progress was, and remains, the absence of a suitable means of determining whether individual spermatozoa have been capacitated, other than by observing their ability to penetrate eggs. In a sperm suspension incubated *in vitro*, there will be a heterogeneous population of living and dead, motile and non-motile,

capacitated and non-capacitated spermatozoa, and the relative proportions of all these will change over time. This fluctuating heterogeneity of sperm suspensions *in vitro* makes biochemical studies into the mechanism of capacitation difficult, and it may be unwise to ascribe particular chemical or physical properties found in the population as a whole to those few spermatozoa capable of fertilizing eggs. Bavister (1986) suggested that '...characteristics evaluated in a large sperm population may be more representative of nonfertile than of capacitated spermatozoa'.

In spite of this concern, a large amount of information has been gathered on the changes undergone by spermatozoa during capacitation *in vivo* and *in vitro*. The results of several early studies indicated that, at least in rabbit spermatozoa, no readily detectable morphological change occurs during capacitation (Adams and Chang, 1962b; Austin, 1963; Bedford, 1963, 1964). A report of progressive elevation or loosening of the rabbit sperm plasma membrane during capacitation (Bernstein, 1966) was unsubstantiated by other workers, and the observations probably resulted from fixation artifacts (Bedford, 1969a,b). Nevertheless, some subtle changes do occur in the surface of spermatozoa during capacitation. Spermatozoa within the uterus of the oestrous rabbit were readily engulfed by leucocytes, but in the pseudopregnant uterus, where little or no capacitation occurs, leucocytes failed to ingest intact motile spermatozoa (Bedford, 1965). In addition, a potent substance found in the seminal plasma of a fertile rabbit merely agglutinated ejaculated or epididymal spermatozoa, but immediately immobilized spermatozoa recovered from the uterus (Bedford, 1970c). Furthermore, the net negative charge was reduced on the surface of rabbit spermatozoa after incubation in the oestrous uterus (Vaidya *et al.*, 1971).

In the early studies, a considerable volume of information was accumulated on the metabolic changes that occur in spermatozoa after incubation in the female reproductive tract, or during incubation *in vitro* with secretions from the tract (for a review, see Iritani *et al.*, 1969). It is not known to what extent these metabolic changes are involved with capacitation; they may reflect changes in membrane permeability resulting from capacitation, facilitating the exchange of ions and substrates between the spermatozoon and its environment, or they may be merely contemporaneous with capacitation.

It does seem paradoxical that, whereas capacitation of spermatozoa is accepted as being universally required in mammals, methods for supporting this process *in vitro* differ markedly among species. The mechanism of capacitation, and hence the *in vitro* conditions for capacitating spermatozoa, might be expected to be very similar across species, but this is clearly not so. Mouse spermatozoa can be capacitated simply by diluting epididymal contents into a suitable culture solution and incubating them for as little as 30 min; guinea-pig spermatozoa can be capacitated in the same way by incubation for 2–3 h (Rogers, 1978). This simple approach also works for human and dog

spermatozoa, after removal of seminal plasma by washing or gradient centrifugation (Mahi and Yanagimachi, 1976, 1978; Mortimer, 1994, 2000). Rabbit spermatozoa can also be capacitated *in vitro* by simply washing and incubation, but there must be a second wash step after an initial short period of incubation, probably to eliminate soluble seminal plasma factors from the culture environment (Brackett and Oliphant, 1975; Hosoi *et al.*, 1982). In contrast to human spermatozoa, in another primate genus, the macaque monkeys, spermatozoa require both washing and treatment with caffeine and cyclic AMP to support acquisition of fertilizing ability *in vitro* (Boatman and Bavister, 1984). Golden hamster epididymal spermatozoa under *in vitro* conditions also require treatment with chemical mediators, both to sustain motility (hypotaurine) and to stimulate acrosome reactions (catecholamines such as adrenaline; Leibfried and Bavister, 1982; Bavister, 1989; Boatman *et al.*, 1990). Although hamster, mouse, dog and human spermatozoa thrive on or require glucose in the culture medium (Bavister and Yanagimachi, 1977; Mahi and Yanagimachi, 1978; Fraser and Herod, 1990; Rogers and Perreault, 1990; Travis *et al.*, 2001), glucose inhibits or greatly delays the completion of capacitation in guinea-pig and bull spermatozoa (Rogers and Yanagimachi, 1975; Parrish *et al.*, 1989a; Vredenburg-Wilberg and Parrish, 1995). In bull spermatozoa, this inhibition is the result of glycolysis blocking the normal increase in intracellular pH that accompanies capacitation with heparin (Parrish *et al.*, 1989a). An increase in intracellular pH also appears to accompany the acrosome reaction of mouse spermatozoa (Rockwell and Storey, 2000). Even if they are artifacts of the *in vitro* culture of spermatozoa, these apparently contradictory capacitation requirements need to be resolved if a common mechanism is to be established and the events involved elucidated.

One consistent feature of early culture systems for supporting *in vitro* sperm capacitation and acrosome reactions was the use of biological fluids (usually follicular or cumulus oophorus fluids; Iwamatsu and Chang, 1969; Yanagimachi, 1969a,b; Bavister, 1982). These fluids were later replaced by serum albumin (Bavister, 1969, 1981, 1982, 1989; Miyamoto and Chang, 1973a,b,c; Hoppe and Whitten, 1974; Davis, 1976). In modern IVF protocols, either bovine or human serum albumin is almost always used, although cumulus oophorus fluids may also be present. The way in which serum albumin supports capacitation and acrosome reactions is still not fully understood. At least two mechanisms have been proposed: removal of either lipids or zinc ions from sperm membranes by albumin, leaving them more unstable and thus able to undergo membrane fusion leading to acrosome reactions. Some of the earliest work on this topic was done by Davis *et al.* (1979, 1980) who showed that in a capacitating culture medium, serum albumin depleted cholesterol from rat spermatozoa while transferring phosphatidylcholine to them. Further evidence for involvement of cholesterol depletion in sperm capacitation was provided by Langlais

and Roberts (1985) and Langlais *et al.* (1988). Alternatively or additionally, serum albumin depletes zinc from spermatozoa by chelation (zinc stabilizes membranes and is a counter-ion to calcium, inhibiting many of its functions). Hamster spermatozoa can be capacitated *in vitro* without albumin, using a chemical chelator that depletes zinc concentrations in spermatozoa by 40% or more (Andrews *et al.*, 1994), but these spermatozoa could not undergo acrosome reactions without addition of serum albumin, even in the presence of zona pellucida (Andrews and Bavister, 1989). When albumin was separated from spermatozoa by a semi-permeable membrane, acrosome reactions did not occur (Dow and Bavister, 1989). In spite of much attention, the way in which albumin supports acquisition of sperm fertilizing ability is unknown, in part because of the problem of discriminating which spermatozoa in a suspension are actually capacitated, but also because of fluctuating concentrations of contaminants in commercial serum albumin preparations, such as fatty acids (Chen, 1967) and even a factor that activates the phosphatidylinositol system in a number of cells (Tigyi *et al.*, 1991), which may counteract the biological activities of serum albumin preparations, or which conversely may even be responsible for them. Certainly, the biological activity of these preparations is batch variable, but not particularly species-specific. Alternative methods for capacitating spermatozoa would be particularly useful as there is potential for disease transmission by using bovine or human serum albumin preparations. The induction of acrosome reactions by lysophosphatidylcholine showed that sulphated glycoconjugates, including heparin, could capacitate bovine spermatozoa (Parrish *et al.*, 1989b). Further research into non-serum-derived capacitating compounds is needed in view of the potential for transmitting bovine spongiform encephalopathy or Creutzfeldt-Jakob disease from infected animal or human donors, as well as for elucidating the mechanism of capacitation.

Assessment of sperm fertilizing ability

Egg penetration or fertilization as tests for capacitation and the acrosome reaction

In early studies, in the absence of more suitable methods, most information relating to capacitation was derived from experiments involving penetration or fertilization of eggs. Rabbits were the most widely used animal in such experiments, not only because the need for capacitation had been clearly established in this species, but the relatively short and predictable fertilizable life of rabbit eggs was particularly useful in the capacitation test system (as described by Soupart, 1967). The major disadvantage of this method was that the onset and duration of ovulation in rabbits are highly variable (Walton and Hammond, 1928; Harper, 1961, 1963). Although this objection was largely overcome by the technical refinement of adding recently ovulated eggs to the preincubated spermatozoa instead of

vice versa (Harper, 1970), the method as a whole lacks precision. It is not easy to examine the biochemical conditions prevailing in the female reproductive tract during capacitation of spermatozoa, and it is not possible to control these conditions, except crudely by making drastic hormonal or surgical changes to investigate the effect upon capacitation (for example, see Soupart and Orgebin-Crist, 1966; Bedford, 1970b). In addition, once capacitated spermatozoa are incubated with eggs in the female reproductive tract, little further information can be gained concerning the acrosome reaction and other events associated with egg penetration.

It was realized that more precise and perhaps less conflicting information could be gained by capacitating rabbit spermatozoa *in vitro*, but the validity of early claims to have achieved this (Kirton and Hafs, 1965; Ericsson, 1969; Johnson and Hunter, 1971) is in some doubt because of the lack of confidence in the rabbit capacitation test system (see above). This situation would be improved by fertilizing rabbit eggs consistently *in vitro*. However, the necessary degree of reproducibility was initially difficult to achieve (for example, see Fraser *et al.*, 1971) and reproducible protocols were devised only after methods were developed for capacitating spermatozoa *in vitro* (Brackett and Oliphant, 1975; Hosoi *et al.*, 1982), by which time, IVF in rodents had become the predominant approach for assessment of sperm capacitation and the acrosome reaction.

In vitro fertilization in the hamster

Golden hamsters are highly suitable for the study of sperm capacitation, and a well-defined protocol for achieving IVF in this species has been described by Bavister (1989). There were numerous advantages when hamsters were used for IVF studies: not only were capacitation of spermatozoa and penetration of eggs accomplished easily *in vitro*, but the hamster acrosome reaction (like that in rabbits, guinea-pigs and dogs) is pronounced enough to be discernable under the phase-contrast microscope in living, motile spermatozoa (Yanagimachi and Chang, 1964; Yanagimachi, 1966, 1972, 1994; Mahi and Yanagimachi, 1978), so that the percentage of reacted spermatozoa can be readily assessed at any time.

However, a problem encountered with the early attempts to fertilize hamster eggs *in vitro* was the highly variable outcome of the experiments, ranging from near 0 to 80–90% fertilization of eggs. This variability was the result of differences in the pH of the culture medium used for IVF, which was uncontrolled and altered by the numbers of live spermatozoa present. After pH was controlled using a bicarbonate–CO₂ buffer system, high, reproducible frequencies of IVF were obtained (Bavister, 1969). These early experiments with hamster spermatozoa were the first to show that occurrence of the acrosome reaction, sperm binding to the zona pellucida and fertilization are pH-dependent events. This culture system was then used successfully for the first documented human IVF (Bavister

et al., 1969; Edwards *et al.*, 1969). The sequence of experiments that connected hamster IVF to the first human IVF is described by Bavister (2002). Studies by Miyamoto and Chang (1974) showed that pH was also important for IVF in mice and rats.

In virtually all IVF experiments with hamster gametes, spermatozoa have been obtained from the cauda epididymidis. Not only is it easy to obtain viable spermatozoa in this way, but the time-course of capacitation and penetration of eggs *in vitro* with epididymal spermatozoa (Barros, 1968; Yanagimachi, 1969b) is similar to that observed *in vivo* after normal mating (Strauss, 1956; Chang and Sheaffer, 1957; Yanagimachi, 1966). Moreover, methods for routinely collecting ejaculated hamster spermatozoa have not been developed. Although it is more practical to investigate capacitation using hamster epididymal spermatozoa and the IVF approach, it must be kept in mind that capacitation of epididymal spermatozoa may not be identical to capacitation of ejaculated spermatozoa, that events occurring in culture conditions may not be the same as events after normal mating, and that the process of capacitation in hamster spermatozoa may not be fully representative of capacitation in other mammals. One obvious difference between conditions *in vitro* and *in vivo* is that under most IVF conditions, a very large population of spermatozoa is present but only a small proportion seems capable of penetrating zonae pellucidae in a timely manner, whereas *in vivo*, a small number of spermatozoa is present at the site and time of fertilization but most of the spermatozoa appear to be capable of fertilizing oocytes (Austin, 1951; Zamboni, 1970; Yanagimachi and Mahi, 1976). The total number of spermatozoa in a typical IVF culture drop often exceeds the number of eggs present by three or more orders of magnitude. However, when the culture conditions for hamster sperm survival and capacitation *in vitro* were optimized, very small numbers of spermatozoa (≤ 5) could be used for gamete co-incubation and were capable of fertilizing eggs (Bavister, 1979).

Alternative sperm fertility assays

The ability of spermatozoa to penetrate the zona pellucida and to fertilize eggs is the only unequivocal test for completion of capacitation and the ensuing physiological acrosome reaction. However, IVF is far from satisfactory as a means to evaluate sperm fertilizing ability in a large sperm population, as it introduces large experimental variations, such as differences in the fertilizability of eggs, and yields no information on the frequency of capacitation. One obvious reason for this problem is the rapid operation of the block to polyspermy (Austin and Braden, 1954; Stewart-Savage and Bavister, 1988), such that only one spermatozoon, or at most several, can gain access to the oocyte before the properties of the zona pellucida are altered, preventing further sperm entry. One way to overcome this limitation to testing sperm fertilizing ability is to inhibit the block to polyspermy, for example, by storing oocytes for a time in

concentrated salt solutions (Yanagimachi *et al.*, 1979; Boatman *et al.*, 1988; Yoshimatsu *et al.*, 1988). When the block to polyspermy is destroyed in this way, many, perhaps hundreds of, spermatozoa can penetrate each zona pellucida, thus increasing the efficiency of this 'capacitation detection' system by two or more orders of magnitude (Boatman *et al.*, 1988). This method has also been used to assess the fertility of spermatozoa from endangered species using oocytes from related common animals (Andrews *et al.*, 1992). A different approach is to count the proportion of spermatozoa able to respond to a challenge that induces acrosome loss, such as lysophosphatidylcholine (Parrish *et al.*, 1988, 1989b), after capacitation treatments, although how accurately this procedure mimics the natural acrosome reaction is not clear.

An early attempt to devise a chemical test for capacitation was reported by Ericsson (1967a,b). This test involved the fluorescent labelling of spermatozoa with tetracycline hydrochloride and the subsequent removal of fluorescence during capacitation. Unfortunately, the time courses, sites and conditions of fluorescence removal and of capacitation were not strictly correlated (Vaidya *et al.*, 1969), so the test was of dubious value for the study of capacitation. Nevertheless, this approach was a valuable stimulus to further attempts to develop chemical tests for capacitation. A refinement of this approach uses chlor-tetracycline (CTC) fluorescence to quantitate mouse sperm capacitation (Saling and Storey, 1979); distinct patterns of fluorescence change as capacitation progresses (Ward and Storey, 1984). This assay has also been used with human spermatozoa (Lee *et al.*, 1987).

Johnson and Hunter (1970) used a fluorescent antibody technique to identify rabbit sperm-specific and sperm-coating antigens; the sperm-coating antigens were removed after prolonged incubation in rabbit uteri, re-exposing acrosomal sperm-specific sites. This approach seemed promising, not only as a potential test for capacitation, but also as a method of investigating the nature of this process. Brackett and Oliphant (1975) measured the removal of rabbit sperm surface antigens as a progressive indicator of capacitation taking place *in vitro*.

The occurrence of the sperm acrosome reaction, in the absence of fertilization, has often been used to indicate that capacitation has taken place (Barros and Garavagno, 1970). However, structural changes in the acrosome are not necessarily the result of capacitation of spermatozoa, but can also occur under other circumstances, as degenerative changes (Blom, 1945; Hancock, 1952; Austin and Bishop, 1958c) or 'false' acrosome reactions that are not dependent on previous capacitation (Bedford, 1969b). Thus, the occurrence of an acrosome reaction is an insufficient indication that capacitation has occurred, except in species in which the acrosomes are very large (for example, golden hamsters and guinea-pigs), so that the acrosome reaction (detachment or loss) can be seen clearly in freely motile spermatozoa that do not appear to be moribund (Yanagimachi, 1994).

Two remarkable procedures derived from IVF have been devised for evaluating sperm fertilizing ability: one procedure uses oocytes without zonae pellucidae, and the other uses zonae pellucidae without oocytes. The hamster egg can be fertilized by spermatozoa of other species if the zona pellucida is removed (Yanagimachi, 1994). This 'zona-free penetration assay' is very useful for evaluating human sperm chromosomes (Rudak *et al.*, 1978; Martin, 1985) and has been used as a surrogate test for human sperm fertility (Yanagimachi *et al.*, 1976; Rogers *et al.*, 1979); however, its predictive value is dubious, and most clinical IVF programmes no longer use it. A major problem with this assay is that, in the absence of the zona pellucida, which is a natural stimulator of acrosome reactions, spermatozoa may fail to exhibit acrosome reactions or to fertilize hamster eggs, yet remain capable of effecting fertilization of homologous zona-intact (human) eggs (giving a false negative result). Conversely, spermatozoa from an infertile male may be artificially induced to undergo acrosome reactions by the conditions of the test, such as overnight incubation of the sperm sample at 4°C, and thus become able to fuse with hamster egg plasma membranes and form pronuclei (giving a false positive result). The 'hemi-zona assay' avoids the problems with the hamster egg zona-free penetration assay by using human zonae pellucidae to gauge sperm-binding capability, which should correlate with fertilizing ability. Each zona pellucida is bisected and one half is inseminated with a sperm sample of unknown fertility. The resulting sperm binding frequency is compared directly with sperm binding to the matched half-zona pellucida using a control sample of known fertility (Oehninger *et al.*, 1992; Franken *et al.*, 1993; Coddington *et al.*, 1994). This assay does not measure ability of the spermatozoa to form pronuclei because no egg is present.

A variant of IVF that has gained great popularity in recent years is ICSI, in which a single spermatozoon is selected and injected into the egg cytoplasm with a fine pipette. The first successful human ICSI was reported by Palermo *et al.* (1992) but this approach was first demonstrated with animal gametes many years earlier (Uehara and Yanagimachi, 1977). Uehara and Yanagimachi (1977) showed that hamster spermatozoa from the cauda epididymidis could undergo decondensation and form pronuclei after injection into eggs. This demonstration that testicular spermatozoa could form pronuclei after ICSI pre-dated by many years the use of this technique for azoospermic men (Palermo *et al.*, 1999). ICSI in humans is very effective for addressing 'male factor' infertility (van Steirteghem *et al.*, 1993; Oehninger *et al.*, 1995) but some clinics routinely use ICSI in place of IVF for convenience. Although numerous offspring have been born after ICSI in humans (Palermo *et al.*, 1992, 1999; van Steirteghem *et al.*, 1993; Oehninger *et al.*, 1995; Bonduelle *et al.*, 1999, 2002) and in animals, there are concerns about potential abnormalities during fertilization and even in the offspring themselves as a result of this technique (Kent-First *et al.*, 1996; Wakayama *et al.*, 1998; Hewitson *et al.*, 2000;

Terada *et al.*, 2000) indicating a need for more animal testing with ICSI to provide a more complete understanding of the long-term consequences (Yanagimachi, 1995). Although one major study showed no differences in defects at birth (Bonduelle *et al.*, 2002), it is possible that problems resulting from ICSI will be revealed later in life.

Conclusions (2002)

During the last 30 years, it has become clear that IVF can be achieved in many mammals and has important practical applications in human clinical medicine and in animal breeding. It is apparent from evaluating both the early and the contemporary literature that successful fertilization of mammalian eggs *in vitro* has depended upon the use of spermatozoa that have undergone capacitation, either *in vivo* or *in vitro*. However, precise information concerning the nature of capacitation has been gained by studying the requirements for accomplishing IVF. Therefore, the studies of IVF and of capacitation are complementary, so it is not surprising that knowledge about these processes has advanced in parallel.

The preceding account of early IVF studies illustrates the rather primitive state of knowledge during the first 20 years after the discovery of capacitation about the mechanisms of this process and of the ensuing acrosome reaction, and about the functional significance of these events for fertilization in mammals. In the ensuing 30 years, much has been learned on these topics, and IVF technology is currently being applied routinely in a wide variety of species. However, several key questions remain completely or partially unanswered. For example, what is the stimulus for the initiation of capacitation within the female reproductive tract? What molecular events are involved in capacitation? How exactly does the completion of capacitation lead to the acrosome reaction? How does serum albumin induce capacitation and the acrosome reaction? Considering that it takes only one or a few spermatozoa to fertilize oocytes within the oviduct, why are so many spermatozoa needed to effect fertilization *in vitro*? How is capacitation stimulated within the female reproductive tract in species such as the hamster or rhesus monkey that require chemical stimulators for supporting sperm capacitation *in vitro*? Does the application of ICSI make IVF obsolete or redundant, or does IVF, by preserving the natural mechanisms for selecting against the entry of abnormal spermatozoa into the oocyte, safeguard against defects that may be introduced using ICSI? Full answers to these perplexing questions concerning fertilization should be provided during the next 30 years of research into this fundamental aspect of mammalian reproduction.

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References

Key references are identified by asterisks.

- Abeydeera LR** (2002) *In vitro* production of embryos in swine *Theriogenology* **57** 256–273
- Adams CE and Chang MC** (1962a) The effect of delayed mating on fertilisation in the rabbit *Journal of Experimental Zoology* **151** 155–158
- Adams CE and Chang MC** (1962b) Capacitation of rabbit spermatozoa in the Fallopian tube and in the uterus *Journal of Experimental Zoology* **151** 159–165
- Andrews JC and Bavister BD** (1989) Hamster zonae pellucidae cannot induce physiological acrosome reactions in chemically capacitated hamster spermatozoa in the absence of albumin *Biology of Reproduction* **40** 117–122
- Andrews JC, Howard JG, Bavister BD and Wildt DE** (1992) Sperm capacitation in the domestic cat (*Felis catus*) and leopard cat (*Felis bengalensis*) as studied with a salt-stored zona pellucida penetration assay *Molecular Reproduction and Development* **31** 200–207
- Andrews JC, Nolan JP, Hammerstedt RH and Bavister BD** (1994) Role of zinc during hamster sperm capacitation *Biology of Reproduction* **51** 1238–1247
- Asch RH, Balmaceda JP, Ellsworth LR and Wong PC** (1986) Preliminary experiences with gamete intrafallopian transfer (GIFT) *Fertility and Sterility* **45** 366–371
- *Austin CR** (1951) Observations on the penetration of the sperm into the mammalian egg *Australian Journal of Scientific Research Series B* **4** 581–589
- Austin CR** (1952) The ‘capacitation’ of the mammalian sperm *Nature (London)* **170** 326
- Austin CR** (1956a) Activation of eggs by hypothermia in rats and hamsters *Journal of Experimental Biology* **33** 338–347
- Austin CR** (1956b) Effects of hypothermia and hyperthermia on fertilisation in rat eggs *Journal of Experimental Biology* **33** 348–357
- Austin CR** (1961) Fertilisation of mammalian eggs *in vitro*. *International Review of Cytology* **12** 337–359
- Austin CR** (1963) Acrosome loss from the rabbit spermatozoa in relation to entry into the egg *Journal of Reproduction and Fertility* **6** 313–314
- Austin CR** (1967) Capacitation of spermatozoa *International Journal of Fertility* **12** 25–31
- Austin CR** (1969) Sperm capacitation – biological significance in various species. In *Mechanisms Involved in Conception: Advances in the Biosciences* **4** pp 5–10 Ed. G Raspe. Pergamon Press, Oxford
- Austin CR and Bishop MWH** (1958a) Role of the rodent acrosome and perforatorium in fertilisation *Proceedings of the Royal Society, Series B* **149** 241–248
- Austin CR and Bishop MWH** (1958b) Capacitation of mammalian spermatozoa *Nature (London)* **181** 851
- Austin CR and Bishop MWH** (1958c) Some features of the acrosome and perforatorium in mammalian spermatozoa *Proceedings of the Royal Society, Series B* **149** 234–240
- Austin CR and Braden AWH** (1954) Time relations and their significance in the ovulation and penetration of eggs in rats and rabbits *Australian Journal of Biological Science* **7** 179–194
- Austin CR and Braden AWH** (1956) Early reaction of the rodent egg to spermatozoa penetration *Journal of Experimental Biology* **33** 358–365
- Barnett DK and Bavister BD** (1992) Hypotaourine requirement for *in vitro* development of golden hamster one-cell embryos into morulae and blastocysts, and production of term offspring from *in vitro* fertilized ova *Biology of Reproduction* **47** 297–304
- Barros C** (1968) *In vitro* capacitation of golden hamster spermatozoa *Anatomical Record* **160** 310 (Abstract)
- Barros C and Austin CR** (1967) *In vitro* fertilisation and the sperm acrosome reaction in the hamster *Journal of Experimental Zoology* **166** 317–323
- Barros C and Garavagno A** (1970) Capacitation of hamster spermatozoa with blood sera *Journal of Reproduction and Fertility* **22** 381–384
- Barros C, Bedford JM, Franklin LE and Austin CR** (1967) Membrane vesiculation as a feature of the mammalian acrosome reaction *Journal of Cell Biology* **34** C1–C5
- Bavister BD** (1969) Environmental factors important for *in vitro* fertilisation in the hamster *Journal of Reproduction and Fertility* **18** 544–545
- Bavister BD** (1971) *A Study of In Vitro Fertilisation and Capacitation in the Hamster* PhD Dissertation, University of Cambridge
- Bavister BD** (1979) Fertilization of hamster eggs *in vitro* at sperm egg ratios close to unity *Journal of Experimental Zoology* **210** 259–264
- Bavister BD** (1981) Substitution of a synthetic polymer for protein in a mammalian gamete culture system *Journal of Experimental Zoology* **217** 45–51
- Bavister BD** (1982) Evidence for a role of post-ovulatory cumulus components in supporting fertilizing ability of hamster spermatozoa *Journal of Andrology* **3** 365–372
- Bavister BD** (1986) Animal *in vitro* fertilization and embryo development. In *Manipulation of Mammalian Development* pp 81–148 Ed. RBL Gwatkin. Plenum Press, New York
- Bavister BD** (1989) A consistently successful procedure for *in vitro* fertilization of golden hamster eggs *Gamete Research* **23** 139–158
- Bavister BD** (1995) Culture of preimplantation embryos: facts and artifacts *Human Reproduction Update* **1** 91–148
- Bavister BD** (2002) How animal research led to the first documented human IVF *Reproductive Biomedicine Online* **4** Supplement 1 24–29
- Bavister BD and Yanagimachi R** (1977) The effects of sperm extracts and energy sources on the motility and acrosome reaction of hamster spermatozoa *in vitro*. *Biology of Reproduction* **16** 228–237
- Bavister BD, Edwards RG and Steptoe PC** (1969) Identification of the midpiece and tail of the spermatozoon during fertilisation of human eggs *in vitro*. *Journal of Reproduction and Fertility* **20** 159–160
- Bavister BD, Boatman DE, Leibfried ML, Loose M and Vernon MW** (1983) Fertilization and cleavage of rhesus monkey oocytes *in vitro*. *Biology of Reproduction* **28** 983–999
- Bedford JM** (1963) Morphological reaction of spermatozoa in the female reproductive tract of the rabbit *Journal of Reproduction and Fertility* **6** 245–255
- Bedford JM** (1964) Fine structure of the sperm head in ejaculate and uterine spermatozoa of the rabbit *Journal of Reproduction and Fertility* **7** 221–228
- Bedford JM** (1965) Effect of environment on phagocytosis of rabbit spermatozoa *Journal of Reproduction and Fertility* **9** 249–256
- Bedford JM** (1967) Experimental requirement for capacitation and observations on ultrastructural changes in rabbit spermatozoa during fertilisation *Journal of Reproduction and Fertility Supplement* **2** 35–48
- Bedford JM** (1968) Ultrastructural changes in the sperm head during fertilisation in the rabbit *American Journal of Anatomy* **123** 329–357
- Bedford JM** (1969a) Limitations of the uterus in the development of the fertilising ability (capacitation) of spermatozoa *Journal of Reproduction and Fertility Supplement* **8** 19–26
- Bedford JM** (1969b) Morphological aspects of sperm capacitation in mammals. In *Mechanisms Involved in Conception: Advances in the Biosciences* **4** pp 35–50 Ed. G Raspe. Pergamon Press, Oxford
- Bedford JM** (1970a) Sperm capacitation and fertilisation in mammals *Biology of Reproduction Supplement* **2** 128–158
- Bedford JM** (1970b) Influence of oestrogen and progesterone on sperm capacitation in the reproductive tract of the female rabbit *Journal of Endocrinology* **46** 191–200
- Bedford JM** (1970c) Observations on the properties of a potent sperm head agglutinin in the semen of a fertile rabbit *Journal of Reproduction and Fertility* **22** 193–196
- Bernstein MH** (1966) Modification of sperm structure in capacitation *Journal of Cell Biology* **31** 12A (Abstract)
- Blom E** (1945) Spontaneous detachment of the galea capitis in spermia of bull and stallion *Scandinavisk Veterinaertidsskrift* **35** 779–783
- Blom E** (1963) The galea capitis and apical body in bull sperm and the fertilisation process *International Journal of Fertility* **8** 447–452
- Boatman DE and Bavister BD** (1984) Stimulation of rhesus monkey sperm capacitation by cyclic nucleotide mediators *Journal of Reproduction and Fertility* **77** 357–366
- Boatman DE, Andrews JC and Bavister BD** (1988) A quantitative assay for capacitation: evaluation of multiple sperm penetration through the zona pellucida of salt-stored hamster eggs *Gamete Research* **19** 19–29

- Boatman DE, Bavister BD and Cruz E** (1990) Addition of hypotaurine can reactivate immotile golden hamster spermatozoa *Journal of Andrology* **11** 66–72
- Bondioli KR and Wright RW, Jr** (1980) Influence of culture media on *in vitro* fertilization of ovine tubal oocytes *Journal of Animal Science* **51** 660–667
- Bonduelle M, Camus M, De Vos A et al.** (1999) Seven years of intracytoplasmic sperm injection and follow-up of 1987 subsequent children *Human Reproduction* **14** Supplement **1** 243–264
- Bonduelle M, Liebaers I, Deketelaere V, Derde MP, Camus M, Devroey P and van Steirteghem A** (2002) Neonatal data on a cohort of 2889 infants born after ICSI (1991–1999) and of 2995 infants born after IVF (1983–1999) *Human Reproduction* **17** 671–694
- Bowen RA** (1977) Fertilization *in vitro* of feline ova by spermatozoa from the ductus deferens *Biology of Reproduction* **17** 144–147
- Brackett BG** (1969) Effects of washing the gametes on fertilisation *in vitro* *Fertility and Sterility* **20** 127–142
- Brackett BG and Oliphant G** (1975) Capacitation of rabbit spermatozoa *in vitro*. *Biology of Reproduction* **12** 260–274
- Brackett BG, Hall JL and Oh YK** (1978) *In vitro* fertilizing ability of testicular, epididymal, and ejaculated rabbit spermatozoa *Fertility and Sterility* **29** 571–582
- Brackett BG, Oh YK, Evans JF and Donawick WJ** (1978) *In vitro* fertilization of cow ova *Theriogenology* **9** 89 (Abstract)
- Braden AWH and Austin CR** (1954) Fertilisation of the mouse eggs and the effect of delayed coitus and of hot-shock treatment *Australian Journal of Biological Science* **7** 552–565
- *Chang MC** (1951) Fertilising capacity of spermatozoa deposited into the Fallopian tubes *Nature (London)* **168** 697
- Chang MC** (1952) Fertilisability of rabbit ova and the effects of temperature *in vitro* on their subsequent fertilisation and activation *in vivo*. *Journal of Experimental Zoology* **121** 351–381
- Chang MC** (1954) Development of parthenogenetic rabbit blastocysts induced by low temperature storage of unfertilised ova *Journal of Experimental Zoology* **125** 127–150
- Chang MC** (1955) Development of fertilising capacity of rabbit spermatozoa in the uterus *Nature (London)* **175** 1036–1037
- *Chang MC** (1959) Fertilisation of rabbit ova *in vitro*. *Nature (London)* **179** 466–467
- Chang MC** (1968) *In vitro* fertilisation of mammalian eggs *Journal of Animal Science* **27** Supplement **1** 15–22
- Chang MC** (1969) Hormonal regulation of sperm capacitation. In *Mechanisms Involved in Conception: Advances in the Biosciences* **4** pp 13–24 Ed. G Raspe. Pergamon Press, Oxford
- Chang MC and Sheaffer D** (1957) Number of spermatozoa ejaculated at copulation transported into the female tract and present in the male tract of the golden hamster *Journal of Heredity* **48** 107–109
- Chang MC and Yanagimachi R** (1963) Fertilisation of ferret ova by deposition of epididymal sperm into the ovarian capsule with special reference to the fertilisable life of ova and the capacitation of sperm *Journal of Experimental Zoology* **154** 175–188
- Chang MC, Hunt DM and Marston JH** (1971) The capacitation time and fertilising life of snowshoe hare spermatozoa in the female tract of rabbit *Journal of Reproduction and Fertility* **25** 287–289
- Chen RF** (1967) Removal of fatty acids from serum albumin by charcoal treatment *Journal of Biological Chemistry* **242** 173–181
- Coddington CC, Oehninger SC, Olive DL, Franken DR, Kruger TF and Hodgen GD** (1994) Hemizona index (HZI) demonstrates excellent predictability when evaluating sperm fertilizing capacity in *in vitro* fertilization patients *Journal of Andrology* **15** 250–254
- Colwin AL and Colwin LH** (1965) Suggested similarity between early events of fertilisation in certain mammals and vertebrates *Anatomical Record* **151** 447
- Colwin LH and Colwin AL** (1967) Membrane fusion in relation to sperm–egg association. In *Fertilisation* Vol. I pp 295–367 Eds CB Metz and A Monroy. Academic Press, New York
- Cross PC and Brinster RL** (1970) *In vitro* development of mouse oocytes *Biology of Reproduction* **3** 298–307
- Crozet N, Huneau D, Desmedt V, Theron MC, Szollosi D, Torres S and Sevellec C** (1987) *In vitro* fertilization with normal development in the sheep *Gamete Research* **16** 159–170
- Dan JC** (1956) The acrosome reaction *International Review of Cytology* **5** 365–393
- Dan JC** (1967) Acrosome reaction and lysins. In *Fertilisation* Vol. I pp 237–293 Eds CB Metz and A Monroy. Academic Press, New York
- Dauzier L and Thibault C** (1956) Recherche experimentale sur la maturation des gametes males chez les mammiferes par l'etude de la fecondation *in vitro* de l'oeuf de lapine *Proceedings of the 3rd International Congress on Animal Reproduction (Cambridge)* pp 58–61
- Dauzier L and Thibault C** (1959) Donnees nouvelles sur la fecondation *in vitro* de la lapine et de la brebis *Comptes Rendus de l'Academie de Science (Paris)* **248** 2655–2656
- Dauzier L, Thibault C and Wintenberger S** (1954) La fecondation *in vitro* de l'oeuf de lapine *Comptes Rendus de l'Academie de Science (Paris)* **238** 844–845
- Davis BK** (1976) Influence of serum albumin on the fertilizing ability *in vitro* of rat spermatozoa *Proceedings of the Society for Experimental Biology and Medicine* **151** 240–243
- Davis BK, Byrne R and Hungund B** (1979) Studies on the mechanism of capacitation. II. Evidence for lipid transfer between plasma membrane of rat sperm and serum albumin during capacitation *in vitro*. *Biochimica et Biophysica Acta* **558** 257–266
- Davis BK, Byrne R and Bedigian K** (1980) Studies on the mechanism of capacitation: albumin-mediated changes in plasma membrane lipids during *in vitro* incubation of rat sperm cells *Proceedings National Academy of Sciences USA* **77** 1546–1550
- Demott RP and Suarez SS** (1992) Hyperactivated sperm progress in the mouse oviduct *Biology of Reproduction* **46** 779–785
- Dickmann Z and Dziuk PJ** (1964) Sperm penetration of the zona pellucida of the pig egg *Journal of Experimental Biology* **41** 603–608
- Dow MPD and Bavister BD** (1989) Direct contact is required between serum albumin and hamster spermatozoa for capacitation *in vitro*. *Gamete Research* **23** 171–180
- Edwards RG** (1965) Maturation *in vitro* of human ovarian oocytes *Lancet* **ii** 926–929
- Edwards RG, Bavister BD and Steptoe PC** (1969) Early stages of fertilisation *in vitro* of human oocytes matured *in vitro*. *Nature (London)* **221** 632–635
- Edwards RG, Steptoe PC and Purdy JM** (1970) Fertilisation and cleavage *in vitro* of preovulatory human oocytes *Nature (London)* **227** 1307–1309
- Ericsson RJ** (1967a) Technology, physiology and morphology of sperm capacitation *Journal of Reproduction and Fertility Supplement* **2** 65–74
- Ericsson RJ** (1967b) Fluorimetric method for the measurement of sperm capacitation *Proceedings of the Society for Experimental Biology and Medicine* **125** 1115–1118
- Ericsson RJ** (1969) Capacitation *in vitro* of rabbit sperm with mule eosinophils *Nature (London)* **221** 568–569
- First NL, Short RE, Peters JB and Stratman FW** (1968) Transport of boar spermatozoa in estrual and luteal sows *Journal of Animal Science* **27** 1032–1036
- Fissore RA, Gordo AC and Wu H** (1998) Activation of development in mammals: is there a role for a sperm cytosolic factor? *Theriogenology* **49** 43–52
- Franken DR, Acosta AA, Kruger TF, Lombard CJ, Oehninger S and Hodgen GD** (1993) The hemizona assay: its role in identifying male factor infertility in assisted reproduction *Fertility and Sterility* **59** 1075–1080
- Franklin LE, Barros C and Fussell EN** (1970) The acrosomal region and the acrosome reaction in sperm of the golden hamster *Biology of Reproduction* **3** 180–200
- Fraser LR and Herod JE** (1990) Expression of capacitation-dependent changes in chlortetracycline fluorescence patterns in mouse spermatozoa requires a suitable glycolysable substrate *Journal of Reproduction and Fertility* **88** 611–621
- Fraser LR, Dandekar PV and Vaidya RA** (1971) *In vitro* fertilisation of tubal rabbit ova partially or totally denuded of follicular cells *Biology of Reproduction* **4** 229–233
- Gould KG** (1983) Ovum recovery and *in vitro* fertilization in the chimpanzee *Fertility and Sterility* **40** 378–383

- Gould KG, Cline EM and Williams WL (1973) Observations on the induction of ovulation and fertilization *in vitro* in the squirrel monkey (*Saimiri sciureus*). *Fertility and Sterility* **24** 260–268
- Hamner CE, Jennings LL and Sojka NJ (1970) Cat (*Felis catus* L) spermatozoa require capacitation *Journal of Reproduction and Fertility* **23** 477–480
- Hancock JL (1952) The morphology of bull spermatozoa *Journal of Experimental Biology* **29** 445–453
- Harper MJK (1961) The time of ovulation in the rabbit following the injection of luteinizing hormone *Journal of Endocrinology* **22** 147–152
- Harper MJK (1963) Ovulation in the rabbit. The time of follicular rupture and expulsion of the eggs in relation to injection of luteinizing hormone *Journal of Endocrinology* **26** 307–316
- Harper MJK (1970) Factors influencing sperm penetration of rabbit eggs *in vivo*. *Journal of Experimental Zoology* **173** 47–62
- Hasler JF (1998) The current status of oocyte recovery, *in vitro* embryo production, and embryo transfer in domestic animals, with an emphasis on the bovine *Journal of Animal Science* **76** 52–74
- Hewitson L, Simerly C, Dominko T and Schatten G (2000) Cellular and molecular events after *in vitro* fertilization and intracytoplasmic sperm injection *Theriogenology* **53** 95–104
- Hoppe PC and Whitten WK (1974) An albumin requirement for fertilization of mouse eggs *in vitro*. *Journal of Reproduction and Fertility* **39** 433–436
- Hosoi Y, Niwa K, Hatanaka S and Iritani A (1981) Fertilization *in vitro* of rabbit eggs by epididymal spermatozoa capacitated in a chemically defined medium *Biology of Reproduction* **24** 637–642
- Hunter RHF (1969) Capacitation in the golden hamster with special reference to the influence of the uterine environment *Journal of Reproduction and Fertility* **20** 223–237
- Hunter RHF and Dziuk PJ (1968) Sperm penetration of pig eggs in relation to the timing of ovulation and insemination *Journal of Reproduction and Fertility* **15** 199–208
- Iritani A, Gomes WR and VanDemark NL (1969) The effect of whole, dialysed and heated female genital tract fluids on respiration of rabbit and ram spermatozoa *Biology of Reproduction* **1** 77–82
- Iwamatsu T and Chang MC (1969) *In vitro* fertilization of mouse eggs in the presence of bovine follicular fluid *Nature* **224** 919–920
- Iwamatsu T and Chang MC (1970) Further investigation of capacitation of sperm and fertilisation of mouse eggs *in vitro*. *Journal of Experimental Zoology* **175** 271–281
- Johnson WL and Hunter AG (1970) Immunofluorescent changes associated with maturation of rabbit sperm *Biology of Reproduction, Society for the Study of Reproduction 3rd annual meeting, Ohio Abstract* 15
- Johnson WL and Hunter AG (1971) Partial *in vitro* capacitation with β -amylase and β -glucuronidase of rabbit sperm and association of sperm and semen antigens *Journal of Dairy Science* **54** 786 (Abstract)
- Katz D and Yanagimachi R (1980) Movement characteristics of hamster spermatozoa within the oviduct *Biology of Reproduction* **22** 759–764
- Katz DF, Drobnis EZ and Overstreet JW (1989) Factors regulating mammalian sperm migration through the female reproductive tract and oocyte vestments *Gamete Research* **22** 443–469
- Kent-First MG, Kol S, Muallem A, Ofir R, Manor D, Blazer S, First N and Itskovitz-Eldor J (1996) The incidence and possible relevance of Y-linked microdeletions in babies born after intracytoplasmic sperm injection and their infertile fathers *Molecular Human Reproduction* **2** 943–950
- Khosla S, Dean W, Reik W and Feil R (2001) Culture of preimplantation embryos and its long-term effects on gene expression and phenotype *Human Reproduction Update* **7** 419–427
- Kirton KT and Hafs HD (1965) Sperm capacitation by uterine fluids or β -amylase *in vitro*. *Science (New York)* **150** 618–619
- Kuehl TJ and Dukelow WR (1975) Fertilization *in vitro* of *Saimiri sciureus* follicular oocytes *Journal of Medical Primatology* **4** 209–216
- Langlais J and Roberts KD (1985) A molecular membrane model of sperm capacitation and the acrosome reaction of mammalian spermatozoa *Gamete Research* **12** 183–224
- Langlais J, Kan FW, Granger L, Raymond L, Bleau G and Roberts KD (1988) Identification of sterol acceptors that stimulate cholesterol efflux from human spermatozoa during *in vitro* capacitation *Gamete Research* **20** 185–201
- Lauderdale JW and Ericsson RJ (1970) Physiological conditions affecting the ability of cattle uteri to influence the fertilising capacity of sperm *Biology of Reproduction* **2** 179–184
- Lee MA, Trucco GS, Bechtol KB, Wummer N, Kopf GS, Blasco L and Storey BT (1987) Capacitation and acrosome reactions in human spermatozoa monitored by a chlortetracycline fluorescence assay *Fertility and Sterility* **48** 649–658
- Leibfried ML and Bavister BD (1982) Effects of epinephrine and hypotaurine on *in vitro* fertilization in the golden hamster *Journal of Reproduction and Fertility* **66** 87–93
- Long JA (1912) The living eggs of rats and mice with a description of apparatus for obtaining and observing them *Publications in Zoology of the University of California (Berkeley)* **9** 105–136
- Mahajan SC and Menge AC (1966) Influence of uterine environment on the fertilising capacity of sperm in cattle *Journal of Animal Science* **25** 1083–1086
- Mahi CA and Yanagimachi R (1976) Maturation and sperm penetration of canine ovarian oocytes *in vitro*. *Journal of Experimental Zoology* **196** 189–196
- Mahi CA and Yanagimachi R (1978) Capacitation, acrosome reaction, and egg penetration by canine spermatozoa in a simple defined medium *Gamete Research* **1** 101–109
- Marston JH and Chang MC (1966) Morphology and timing of fertilisation and early cleavage in Mongolian gerbil and deer mouse *Journal of Embryology and Experimental Morphology* **15** 169–191
- Marston JH and Kelly WA (1968) Time relationship of spermatozoon penetration into the egg of the rhesus monkey *Nature (London)* **217** 1073–1074
- Martin RH (1985) Chromosomal abnormalities in human sperm *Basic Life Sciences* **36** 91–102
- Mattner PE (1963) Capacitation of ram spermatozoa and penetration of the ovine egg *Nature (London)* **199** 722–723
- Menkin MF and Rock J (1948) *In vitro* fertilisation and cleavage of human ovarian eggs *American Journal of Obstetrics and Gynecology* **55** 440–452
- Miyamoto H and Chang MC (1973a) *In vitro* fertilization of rat eggs *Nature* **241** 50–52
- Miyamoto H and Chang MC (1973b) Fertilization of rat eggs *in vitro*. *Biology of Reproduction* **9** 384–393
- Miyamoto H and Chang MC (1973c) The importance of serum albumin and metabolic intermediates for capacitation of spermatozoa and fertilization of mouse eggs *in vitro*. *Journal of Reproduction and Fertility* **32** 193–205
- Miyamoto H, Toyoda Y and Chang MC (1974) Effect of hydrogen-ion concentration on *in vitro* fertilization of mouse, golden hamster, and rat eggs *Biology of Reproduction* **10** 487–493
- Moricard R (1950) Penetration of the spermatozoon *in vitro* into the mammalian ovum oxydo potential level *Nature (London)* **165** 763
- Moricard R and Bossu J (1949) Premieres etudes du passage du spermatozoide au travers de la membrane pellucide d'ovocytes de lapine fécondes 'in vitro'. *Comptes Rendus de l'Academie de Medicine* **33** 659–665
- Mortimer D (1994) *Practical Laboratory Andrology* Oxford University Press, New York
- Mortimer D (2000) Sperm preparation methods *Journal of Andrology* **21** 357–366
- Mukherjee AB and Cohen MM (1970) Development of normal mice by *in vitro* fertilisation *Nature (London)* **228** 472–473
- Niemann H and Wrenzycki C (2000) Alterations of expression of developmentally important genes in preimplantation bovine embryos by *in vitro* culture conditions: implications for subsequent development *Theriogenology* **53** 21–34
- Noyes RW (1953) Fertilising capacity of spermatozoa *Western Journal of Surgery, Obstetrics and Gynecology* **61** 342–349
- Oehninger S, Franken D, Alexander N and Hodgen GD (1992) Hemizona assay and its impact on the identification and treatment of human sperm dysfunctions *Andrologia* **24** 307–321
- Oehninger S, Veeck L, Lanzendorf S, Maloney M, Toner J and Muasher S (1995) Intracytoplasmic sperm injection: achievement of high

- pregnancy rates in couples with severe male factor infertility is dependent primarily upon female and not male factors *Fertility and Sterility* **64** 977–981
- Ogawa S, Satoh K, Hamada M and Hashimoto H (1972) *In vitro* culture of rabbit ova fertilized by epididymal sperms in chemically defined media *Nature* **238** 270–271
- Palermo G, Joris H, Devroey P and van Steirteghem AC (1992) Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte *Lancet* **340** 17–18
- Palermo GD, Schlegel PN, Hariprasad JJ, Ergun B, Mielnik A, Zaninovic N, Veeck LL and Rosenwaks Z (1999) Fertilization and pregnancy outcome with intracytoplasmic sperm injection for azoospermic men *Human Reproduction* **14** 741–748
- Parrish JJ, Susko-Parrish J, Winer MA and First NL (1988) Capacitation of bovine sperm by heparin *Biology of Reproduction* **38** 1171–1180
- Parrish JJ, Susko-Parrish JL and First NL (1989) Capacitation of bovine sperm by heparin: inhibitory effect of glucose and role of intracellular pH *Biology of Reproduction* **41** 683–699
- Parrish JJ, Susko-Parrish JL, Handrow RR, Ax RL and First NL (1989) Effect of sulfated glycoconjugates on capacitation and the acrosome reaction of bovine and hamster spermatozoa *Gamete Research* **24** 403–413
- Pickworth S and Chang MC (1969) Fertilisation of Chinese hamster eggs *in vitro*. *Journal of Reproduction and Fertility* **19** 371–374
- Pikó L (1969) Gamete structure and sperm entry in mammals. In *Fertilisation* Vol. II pp 325–403 Eds CB Metz and A Monroy. Academic Press, New York
- Pikó L and Tyler A (1964) Fine structural studies of sperm penetration in the rat *Proceedings of the 5th International Congress on Animal Reproduction (Trento)* **2** 372–377
- Pincus G (1936) *The Eggs of Mammals* Macmillan, New York
- Pincus G (1939) The comparative behaviour of mammalian eggs *in vivo* and *in vitro*. IV. Development of fertilised and artificially activated rabbit eggs *Journal of Experimental Zoology* **82** 65–129
- Pincus G and Enzmann EV (1934) Can mammalian eggs undergo normal development *in vitro*? *Proceedings National Academy of Sciences USA* **20** 121–132
- Pincus G and Enzmann EV (1935) The comparative behaviour of mammalian eggs *in vivo* and *in vitro*. *Journal of Experimental Medicine* **62** 665–675
- Pincus G and Saunders B (1939) The comparative behaviour of mammalian eggs *in vivo* and *in vitro*. VI. The maturation of human ovarian ova *Anatomical Record* **75** 537–542
- Polge C (1969) Advances in reproductive physiology in pigs *Journal of the Australian Institute of Agricultural Science* **September** 147–153
- Pope CE (2000) Embryo technology in conservation efforts for endangered felids *Theriogenology* **53** 163–174
- Pope CE, Gomez MC, Mikota SK and Dresser BL (2000) Development of *in vitro* produced African wildcat (*Felis silvestris*) embryos after cryopreservation and transfer into domestic cat recipients *Biology of Reproduction* **62** Supplement **1** 321
- Pope CE, Gomez MC, Davis AM, Harris RF, Mikota SK, Boyd EH and Dresser BL (2001) Oocyte retrieval, *in vitro* fertilization and embryo transfer in the caracal (*Caracal caracal*). *Theriogenology* **55** 397 (Abstract)
- Rock J and Menkin MF (1944) *In vitro* fertilisation and cleavage of human ovarian eggs *Science (New York)* **100** 105–106
- Rockwell PL and Storey BT (2000) Kinetics of onset of mouse sperm acrosome reaction induced by solubilized zona pellucida: fluorimetric determination of loss of pH gradient between acrosomal lumen and medium monitored by dapoxyl (2-aminoethyl) sulfonamide and of intracellular Ca(2+) changes monitored by fluo-3 *Molecular Reproduction and Development* **55** 335–349
- Rogers BJ (1978) Mammalian sperm capacitation and fertilization *in vitro*: a critique of methodology *Gamete Research* **1** 165–223
- Rogers BJ and Perreault SD (1990) Importance of glycolysable substrates for *in vitro* capacitation of human spermatozoa *Biology of Reproduction* **43** 1064–1069
- Rogers BJ and Yanagimachi R (1975) Retardation of guinea pig sperm acrosome reaction by glucose: the possible importance of pyruvate and lactate metabolism in capacitation and the acrosome reaction *Biology of Reproduction* **13** 568–575
- Rogers BJ and Yanagimachi R (1976) Competitive effect of magnesium on the calcium-dependent acrosome reaction in guinea pig spermatozoa *Biology of Reproduction* **15** 614–619
- Rogers BJ, Chang L and Yanagimachi R (1979) Glucose effect on respiration: possible mechanism for capacitation in guinea pig spermatozoa *Journal of Experimental Zoology* **207** 107–112
- Rogers BJ, Van Campen H, Ueno M, Lambert H, Bronson R and Hale R (1979) Analysis of human spermatozoal fertilizing ability using zona-free ova *Fertility and Sterility* **32** 664–670
- Roth TL, Howard JG, Donoghue AM, Swanson WF and Wildt DE (1994a) Function and culture requirements of snow leopard (*Panthera uncia*) spermatozoa *in vitro*. *Journal of Reproduction and Fertility* **101** 563–569
- Roth TL, Swanson WF and Wildt DE (1994b) Developmental competence of domestic cat embryos fertilized *in vivo* versus *in vitro*. *Biology of Reproduction* **51** 441–451
- Rudak E, Jacobs PA and Yanagimachi R (1978) Direct analysis of the chromosome constitution of human spermatozoa *Nature* **274** 911–913
- Saacke RG and Almquist JO (1964) Ultrastructure of bovine spermatozoa. I. The head of normal ejaculated sperm *American Journal of Anatomy* **115** 143–162
- Saling PM and Storey BT (1979) Mouse gamete interactions during fertilization *in vitro*. Chlortetracycline as a fluorescent probe for the mouse sperm acrosome reaction *Journal of Cell Biology* **83** 544–555
- Schenk SL (1878) Das Säugetierei Künstlich befruchtet ausserhalb des Muttertieres *Mittheilungen aus dem Embryologischen Institute der Kaiserlich-Königlichen Universität in Wien* **1** 107
- Schini SA and Bavister BD (1988) Two-cell block to development of cultured hamster embryos is caused by phosphate and glucose *Biology of Reproduction* **39** 1183–1192
- Shalgi R, Smith TT and Yanagimachi R (1992) A quantitative comparison of the passage of capacitated and uncapacitated hamster spermatozoa through the uterotubal junction *Biology of Reproduction* **46** 419–424
- Shettles LB (1953) Observations on human follicular and tubal ova *American Journal of Obstetrics and Gynecology* **66** 235–247
- Shivers CA and James JM (1970) Capacitation of frog sperm *Nature (London)* **227** 183–184
- Slavik T and Fulka J (1991) Pregnancies after transfer of sheep embryos produced from oocytes matured and fertilized *in vitro*. *Folia Biologica (Praha)* **37** 94–100
- Smith AU (1951) Fertilisation *in vitro* of the mammalian egg *Biochemical Society Symposia (Cambridge)* **7** 3–10
- Soupart P (1967) Studies on the hormonal control of rabbit sperm capacitation *Journal of Reproduction and Fertility Supplement* **2** 49–62
- Soupart P and Orgebin-Crist MC (1966) Capacitation of rabbit spermatozoa delayed *in vivo* by double ligation of uterine horn *Journal of Experimental Zoology* **163** 311–318
- *Steptoe PC and Edwards RG (1978) Birth after the reimplantation of a human embryo *Lancet* **2** 366
- Steptoe PC, Edwards RG and Purdy JM (1971) Human blastocysts grown in culture *Nature (London)* **229** 132–133
- Stewart-Savage J and Bavister BD (1988) A cell surface block to polyspermy occurs in golden hamster eggs *Developmental Biology* **128** 150–157
- Strauss F (1956) Time and place of fertilisation of the golden hamster egg *Journal of Embryology and Experimental Morphology* **4** 42–56
- Suarez SS, Katz DF, Owen DH, Andrew JB and Powell RL (1991) Evidence for the function of hyperactivated motility in sperm *Biology of Reproduction* **44** 375–381
- Terada Y, Luetjens CM, Sutovsky P and Schatten G (2000) Atypical decondensation of the sperm nucleus, delayed replication of the male genome, and sex chromosome positioning following intracytoplasmic human sperm injection (ICSI) into golden hamster eggs: does ICSI itself introduce chromosomal anomalies? *Fertility and Sterility* **74** 454–460
- Thibault C (1949) L'oeuf des mammifères son développement parthenogénétique *Annales de la Science Naturelle et de Zoologie et Biologie Animale* **11** 136–219
- Thibault C and Dautier L (1961) Analyse des conditions de la fécondation *in vitro* de l'oeuf de la lapine *Annales de la Biologie Animale et de Biochimie et Biophysique* **1** 277–294

- Thibault C, Dauzier L and Wintenberger S** (1954) Etude cytologique de la fecondation *in vitro* de l'oeuf de la lapine *Comptes Rendue de la Societe de Biologie (Paris)* **148** 789–790
- Tigyi G, Henschen A and Miledi R** (1991) A factor that activates oscillatory chloride currents in *Xenopus* oocytes copurifies with a subfraction of serum albumin *Journal of Biological Chemistry* **266** 20 602–20 609
- Toyoda Y and Chang MC** (1968) Sperm penetration of rat eggs *in vitro* after dissolution of zona pellucida by chymotrypsin *Nature (London)* **220** 589–591
- Toyoda Y and Chang MC** (1974a) Fertilization of rat eggs *in vitro* by epididymal spermatozoa and the development of eggs following transfer *Journal of Reproduction and Fertility* **36** 9–22
- Toyoda Y and Chang MC** (1974b) Capacitation of epididymal spermatozoa in a medium with high K-Na ratio and cyclic AMP for the fertilization of rat eggs *in vitro*. *Journal of Reproduction and Fertility* **36** 125–134
- Toyoda YM, Yokoyama M and Hoshi T** (1971a) Studies on the fertilization of mouse eggs *in vitro*. I. *In vitro* fertilization of eggs by fresh epididymal sperm *Japanese Journal of Animal Reproduction* **16** 147–151
- Toyoda YM, Yokoyama M and Hoshi T** (1971b) Studies on the fertilization of mouse eggs *in vitro*. II. Effects of *in vitro* preincubation on time of sperm penetration of mouse eggs *in vitro*. *Japanese Journal of Animal Reproduction* **16** 152–157
- Travis AJ, Jorgez CJ, Merdiushev T, Jones BH, Dess DM, Diaz-Cueto L, Storey BT, Kopf GS and Moss SB** (2001) Functional relationships between capacitation-dependent cell signaling and compartmentalized metabolic pathways in murine spermatozoa *Journal of Biological Chemistry* **276** 7630–7636
- Uehara T and Yanagimachi R** (1977) Behavior of nuclei of testicular, caput and cauda epididymal spermatozoa injected into hamster eggs *Biology of Reproduction* **16** 315–321
- Vaidya RA, Bedford JM, Glass RH and Morris JM** (1969) Evaluation of the removal of tetracycline fluorescence from spermatozoa as a test for capacitation in the rabbit *Journal of Reproduction and Fertility* **19** 483–489
- Vaidya RA, Glass RH, Dandekar P and Johnson K** (1971) Decrease in the electrophoretic mobility of rabbit spermatozoa following intra-uterine incubation *Journal of Reproduction and Fertility* **24** 299–301
- van Steirteghem AC, Nagy Z, Joris H, Liu J, Staessen C, Smits J, Wisanto A and Devroey P** (1993) High fertilization and implantation rates after intracytoplasmic sperm injection *Human Reproduction* **8** 1061–1066
- Venge O** (1953) Experiments on fertilisation of rabbit ova *in vitro* with subsequent transfer to alien does. In *Mammalian Germ Cells* Ciba Foundation Symposium pp 243–252 Ed. GEW Wolstenholme. Churchill Press, London
- Vredenburg-Wilberg WL and Parrish JJ** (1995) Intracellular pH of bovine sperm increases during capacitation *Molecular Reproduction and Development* **40** 490–502
- Wakayama T, Whittingham DG and Yanagimachi R** (1998) Production of normal offspring from mouse oocytes injected with spermatozoa cryopreserved with or without cryoprotection *Journal of Reproduction and Fertility* **112** 11–17
- Walton A and Hammond J** (1928) Observations on ovulation in the rabbit *British Journal of Experimental Biology* **6** 190–204
- Ward CR and Storey BT** (1984) Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay *Developmental Biology* **104** 287–296
- Whittingham DG** (1968) Fertilisation of mouse eggs *in vitro*. *Nature (London)* **220** 592–593
- Wolf DP and Hedrick JL** (1971) A molecular approach to fertilisation. III. Development of a bioassay for sperm capacitation *Developmental Biology* **25** 360–376
- Yanagida K, Bedford JM and Yanagimachi R** (1991) Cleavage of rabbit eggs after microsurgical injection of testicular spermatozoa *Human Reproduction* **6** 277–279
- Yanagimachi R** (1966) Time and process of sperm penetration into hamster ova *in vivo* and *in vitro*. *Journal of Reproduction and Fertility* **11** 359–370
- Yanagimachi R** (1969a) *In vitro* capacitation of hamster spermatozoa by follicular fluid *Journal of Reproduction and Fertility* **18** 275–286
- Yanagimachi R** (1969b) *In vitro* acrosome reaction and capacitation of golden hamster spermatozoa by bovine follicular fluid and its fractions *Journal of Experimental Zoology* **170** 269–280
- Yanagimachi R** (1970) The movement of golden hamster spermatozoa before and after capacitation *Journal of Reproduction and Fertility* **23** 193–196
- Yanagimachi R** (1972) Fertilization of guinea pig eggs *in vitro*. *Anatomical Record* **174** 9–19
- Yanagimachi R** (1994) Mammalian fertilization. In *The Physiology of Reproduction* 2nd Edn pp 189–317 Eds E Knobil and JD Neill. Raven Press, New York
- Yanagimachi R** (1995) Is an animal model needed for intracytoplasmic sperm injection (ICSI) and other assisted reproduction technologies? *Human Reproduction* **10** 2525–2526
- Yanagimachi R and Chang MC** (1963) Fertilisation of hamster eggs *in vitro*. *Nature (London)* **200** 281–282
- *Yanagimachi R and Chang MC** (1964) *In vitro* fertilisation of golden hamster ova *Journal of Experimental Zoology* **156** 361–376
- Yanagimachi R and Mahi CA** (1976) The sperm acrosome reaction and fertilization in the guinea-pig: a study *in vivo*. *Journal of Reproduction and Fertility* **46** 49–54
- Yanagimachi R, Yanagimachi H and Rogers BJ** (1976) The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of human spermatozoa *Biology of Reproduction* **15** 471–476
- Yanagimachi R, Lopata A, Odom CB, Bronson RA, Mahi CA and Nicolson GL** (1979) Retention of biologic characteristics of zona pellucida in highly concentrated salt solution: the use of salt-stored eggs for assessing the fertilizing capacity of spermatozoa *Fertility and Sterility* **31** 562–574
- Yoshimatsu N, Yanagimachi R and Lopata A** (1988) Zonae pellucidae of salt-stored hamster and human eggs: their penetrability by homologous and heterologous spermatozoa *Gamete Research* **21** 115–126
- Zamboni L** (1970) Ultrastructure of mammalian oocytes and ova *Biology of Reproduction Supplement* **2** 44–63