The plant kingdom has abundant chemical compounds that elicit pronounced effects in animals and humans. A number of these compounds have become pharmacologic tools, and about one-hundred plant-derived compounds serve as drugs in the United States. According to the National Prescription Audit, 25% of all prescriptions dispensed from community pharmacies between 1959 and 1973 contained one or more active principles extracted from higher plants (63). In 1973, the American public spent an estimated $3 billion on plant-derived drugs (63), and in 1980 the expenditure was estimated to be $8 billion (58).

Although the prescription market for drugs made from plants is lucrative, plant-derived contraceptive agents have not been investigated extensively, and no such agents have found general acceptance for male fertility regulation (58,63). Djerassi suggests that the enormous development costs, and the considerable time required for approval by the U.S. Food and Drug Administration (FDA), limit the initiation of new contraceptive drug development programs in the United States, especially if continuous long-term dosing is required (55). Drug development programs involving plants are further discouraged because when a plant serving as a starting material for a drug is unavailable in the United States, controlling the source of supply becomes complicated. Other difficulties involve securing strong patent protection for natural products and eliminating biological variability in plant material from batch to batch (59,177).

The scientific community in developing countries has greater interest than does the U.S. pharmaceutical industry in programs to develop new drugs from plants. In most developing countries, plant drugs are familiar and culturally acceptable to the general populace; they are already a part of many indigenous medical systems, and they represent an available and inexpensive starting material, should industrial development of a plant drug become feasible.

One advantage in developing a male antifertility agent from a plant source, rather than through the complete synthesis of a new drug, is that a plant used as a contraceptive agent in an indigenous medical system is likely to have a long folkloric history and an established low toxicity potential.

An impetus for research is that chemical templates to guide a chemist in the synthesis of active male contraceptives are rare, and even if contraceptive research on plants serves only to produce compounds with novel structures, good activity, and some side effects, new molecular models will have been identified for further synthetic studies.

There are no simple procedures to identify plants with biologically active principles (24,59). Most of the useful plant-derived drugs have been discovered as a result of follow-up studies of ethnomedical (folkloric) information, or when investigators have sought to identify the active principle in a plant extract reported to have biological ac-
tility. Reliable ethnomedical data concerning the activity of plants for male contraception are few, and experimental data for plant extracts with effects on the male reproductive system are often unremarkable or irreproducible. A number of investigators have expressed negative opinions about the value of this type of research, but these opinions may represent personal biases rather than actual attempts to confirm the reported data.

Although no plant has been thoroughly studied for its effects on male reproduction, serious scientific investigation of plant-derived agents having antisperm effects is underway in a number of laboratories, and has been for some years. In 1974, our group reviewed the potential value of plants as sources of new antifertility agents (60,61), and we subsequently assessed the problems associated with biological assays to determine the antifertility activity of plants and natural substances (24,62).

In this report, we will consider the status of research on plant-derived agents that prevent sperm production if taken orally by the male, or that incapacitate or kill sperm on contact if used vaginally by the female. Problems involved in searching for plant-derived fertility regulating agents will also be highlighted.

**ANTIFERTILITY STUDIES USING PLANTS**

The male reproductive system is made up of tissues and structures involved in sperm production, storage, and transport to the female genital tract; interference with reproduction means interrupting any of these activities. Although hormones or hormone antagonists and steroids or steroid antagonists have all proven capable of regulating male fertility, they also exert their effects on non-reproductive tissues, as is the case with the steroid oral contraceptives in women. Therefore, it would be of great value to develop fertility inhibitors that are totally selective for reproductive systems and enzymes, and it is possible that a plant-derived drug may have this effect.

Some of the plants that have been studied for their fertility inhibiting effects in the male, and some of the more promising and interesting investigations of those plants, are described and briefly evaluated in the following section.

Aristolochia indica L. (Aristolochiaceae). This species has been widely employed in ethnomedicine as a fertility regulating agent in women, and the fertility effects have been substantiated in various laboratories.

To study the effects of *A. indica* in males, investigators administered a water soluble extract from a chloroform fraction of *A. indica* roots orally to male mice at a dose of 75 mg/kg every 3 days for seven doses. Varying degrees of degenerative change were found in the seminiferous germinal cell components, with prominent nuclear degeneration in all cell types. Reduction in the fluid content of accessory sex organs in *A. indica*-treated mice was prominent and seemed to indicate a decrease in endocrine function of the testis (133).

After one of the constituents of *A. indica* responsible for its biological activity was shown to be *trans-p-coumaric* acid (PCA), Pakrashi and co-workers studied the action of PCA in postmenopausal females to 134) and in testosterone- or prolactin-treated orchietomized rats as well (131). They concluded [in our opinion unconvincingly] that this substance was a prolactin inhibitor. Later, these workers reported that daily oral administration of PCA to male rats for 56 days produced complete loss of libido (132).

The roots of *A. indica* are known to contain significant amounts of aristolochic acid and a variety of related compounds. Aristolochic acid has antitumor and cytotoxic properties (106), which may explain at least some of the effects observed in the above experiments.

Azadirachta indica A. Juss (Meliaceae). A freshly prepared water extract of crushed green *A. indica* leaves was administered orally to male mice at a dose of 1.0 ml/animal daily for 1 month. Half of the animals were then sacrificed and the testes were subjected to histological examination. The remaining treated animals were mated and the resulting pregnancies and litter sizes were noted. Following mating, the males were no longer given the test extract. Forty-five days later they were again mated with healthy females (49).

The data from this study indicated an 80% reduction in pregnancies produced by *A. indica*-treated males, compared with pregnancies in controls. The males regained 100% fertility after 45 days of no treatment. Histologic examination of the testes from treated animals revealed no evidence of an antispermatogenic effect (49).

Although the data suggested that an antifertility effect was experienced by animals treated with the plant extract, too few details were presented in the report to preclude the possibility that the effect was due either to male functional sterility or to an unsuccessful mating. In a later report, the same research group suggested that the activity could be due to a loss of libido in *A. indica*-treated animals (50).

Balanites roxburghii Planch. (Zygophyllaceae). Preliminary data suggested that oral administration of an ethanol extract of fresh, ripe, dried *B. roxburghii* fruit pulp induced infertility in male gerbils, with an associated 3- to 6-fold increase in blood glucose levels (53). Based on these findings, a study was initiated using healthy male dogs. An ethanol extract of *B. roxburghii* fruit pulp was given orally to five dogs at a dose of 35 mg/kg for 60 days. Another group of five dogs received only alloxan. After 60 days, all animals were sacrificed and the testes and epididymides from the animals were removed, weighed, and examined histologically. A significant de-
crease in testis and epididymis weight in both groups of animals was seen (53). The authors concluded that the atrophic changes found in the testes of *B. roxburghii*-treated dogs were secondary effects of hyperglycemia. Further study is warranted to determine whether the hyperglycemic effect could be achieved separately from the effect on the testes of the treated dogs.

_Calotropis procera* (Ait.) R.Br. (Asclepiadaceae). An ethanol extract prepared from *C. procera* flowers, and administered orally every other day for 30 days to Indian desert male gerbils (*Meriones hurrianae* Jerdon), at a dose of 20 mg/animal, produced marked testicular necrosis. Spermatogonia, spermatocytes, and Sertoli cells showed severe degenerative changes, and the lumen of the epididymis decreased in size and lacked spermatozoa. Protein, RNA, and sialic acid contents in tissues were also reduced. Acid and alkaline phosphatase, serum transaminases, cholesterol, and total lipids were increased. Liver damage was evidenced by tumor-like structures on the lobes, and the diameters of seminiferous tubules and Leydig cell nuclei were reduced (68).

_Calotropis procera_ contains cytotoxic and antitumor cardenolides (80). The results described above are similar to those expected when cytotoxic agents such as vinblastine, vincristine, and related agents are administered to laboratory animals (see *Catharanthus roseus*).

A plant extract may have toxic effects as well as potentially useful ones. Normally in such cases, the next step is to further characterize the active extract, in the hope of separating the useful from the toxic effect. This approach seems appropriate in investigating the antispermatogenic potential of *C. procera*.

_Carica papaya L.* (Caricaceae). Sun-dried seeds from ripe papaya fruits (*C. papaya*) were ground and suspended in water, and the suspension was administered orally to fertile adult male rats at a dose of 20 mg/animal for 8 weeks. At the end of the dosing period, the treated males were mated with females of proven fertility and sacrificed. The papaya-treated male rats had a 40% reduction in sperm motility and semen pH was examined. The testes, epididymides, seminal vesicles, and ventral prostate were dissected from each animal and weighed. The papaya-treated male rats had a 40% reduced ability to fertilize the females. The weights of the genital organs and the motility pattern of spermatozoa of the treated animals were not significantly different from controls (46).

While these data are suggestive of a marginal antifertility effect, one must take into account that ground whole seeds were administered to the test animals at a fairly low dose level (20 mg/animal). In order to establish that these data represent a significant male antifertility effect, further testing is required, using concentrated solvent extracts from *C. papaya* seeds.

_Catharanthus roseus* (L.) G. Don (Apocynaceae). The total alkaloid fraction prepared from *C. roseus* leaves, when administered to male rats by intraperitoneal injection, led to graded degenerative changes in the spermatogenic elements of the testis. Further, the testes of treated rats were flaccid and weighed less than those of control animals (88).

These effects can be attributed to the vinblastine and vincristine in *C. roseus* leaves; the former alkaloid is a major constituent of the total bases. As with most cytotoxic agents, vinblastine and vincristine are antispermatogenic in laboratory animals (32, 39, 108, 136). Vinblastine causes oligospermia in humans as well (183).

_Dieffenbachia seguine* (Jacquin) Schott. (Araceae). The forced feeding of *D. seguine* to sterilize male and female prisoners in concentration camps during World War II was documented in 1949 in the accounts of the "Trials of War Criminals Before the Nuremberg Military Tribunals" (4). Apparently the active constituents of the plant lack selectivity, and *D. seguine* must be considered an irreversible sterilant, not a contraceptive agent. Both males and females lose their reproductive capability following ingestion of the plant.

The plant's sterilant effect was first reported in laboratory animals (123) following reports that it was used for sterilization purposes by natives in Brazil. Although the toxic effects of this plant have been well documented (20, 48, 56, 65, 103, 189, 190), the toxic or sterilant elements have not been identified. There may be two types of compounds present in *D. seguine*, one causing the sterilant effect and the other a toxic one. Further work appears warranted.

_Ecballium elaterium* A. Richard (Cucurbitaceae). The sole documentation that this plant may be useful as an antispermatogenic agent is a United States patent issued for this claim (130). The experimental evidence offered in support of male antifertility activity is confusing. For example, the patent states, "Thus, the use of the flower in producing a new contraceptive is highly surprising" (36). But this statement follows: "In order to prepare the claimed invention, the plant is collected, dried for two weeks, and the leaves and stems are ground up in a mortar and pestle until they become a powder" (130).

The extract (1.0 mg) was administered by gastric intubation to mature male rabbits, and 1 hour later, the animals were ejaculated by mechanical stimulation. Normal values for the control rabbit ejaculate were compared with those of the ejaculate collected 1 hour following dosing of each rabbit with 1.0 mg of the plant extract (*Table 1*). A reduction in sperm motility and semen pH was reported. Addition of the extract to ox semen produced complete liquefaction, with a 90% drop in motility in *vitro* (130).
Administration of 1.0 mg of the plant extract orally to men ranging in age from 18 to 40 years resulted in a drop of pH in the ejaculate from a normal 7.2-7.6 to a 1 hour post-treatment value of 1.5-2.0. Normal sperm counts were 80 to 120 million/ml, and 1 hour post-drug values were 5 to 15 million/ml. In 24 hours, all values in treated men returned to normal (130). Only time will tell whether these claims can be verified.

Gossypium species (Malvaceae). Gossypol is a yellow, phenolic, dimeric, sesquiterpene pigment derived primarily from various cotton (Gossypium) species. It presents an exciting lead for a potentially useful orally active male contraceptive agent.

Human studies on gossypol were initiated in China in the early 1970s, but it was not generally known that this compound had a potential application as an orally effective male contraceptive until a paper written in English was published in 1978 (6). Since then, the published data based on human, animal, and in vitro studies have increased rapidly. Most studies have been published in Chinese, however, and thus have not been widely read. Zatuchni and Osborn summarized the status of gossypol as a male contraceptive as it was known in 1980 (214). A more recent review, in which unpublished data from China are included, has been published by Prasad and Diczfalusy (139). A World Health Organization (WHO)-sponsored symposium in 1980 also summarized recent information on gossypol (64).

Other reviews on gossypol are also available (8, 107, 117, 118, 208). Studies on various aspects of gossypol are listed in Table 2.

There are several versions of the serendipitous discovery of the effects on spermatogenesis by gossypol (116, 141, 214). The earliest version we encountered was published in a Chinese journal in 1957. The following account is given (116):

"In between 10-25 years before the war, there was a small village in Jiang-su called Wang Estate. A friend of mine and myself had visited that village in 1929. There were about 30 families living there. They were all wealthy farmers, but were very thrifty. They always ate the least expensive food. At that time, cottonseed oil was far cheaper than bean oil so all of the families used cottonseed oil for cooking. During a period of more than 10 years when everyone ate cottonseed oil, there was not a single child born. At that time, the fact that there were no children born in a wealthy family was regarded as a strange thing. In such a wealthy village where no family had any children born in such a long period, people just thought this was the deed of an evil spirit. The villagers tried everything, even marrying widows who had borne children. This produced no results; once the women came to the village, they never got pregnant. This was a very frustrating thing in that village.

"In that period of about 10 years when everyone ate cottonseed oil, there was not a single child born. At that time, the fact that there were no children born in a wealthy family was regarded as a strange thing. In such a wealthy village where no family had any children born in such a long period, people just thought this was the deed of an evil spirit. The villagers tried everything, even marrying widows who had borne children. This produced no results; once the women came to the village, they never got pregnant. This was a very frustrating thing in that village.

"In that period of about 10 years, the news about that village had travelled very far. People just thought that this was a very strange thing. About 5-6 years before the war, there was a large crop of soy bean in the Northeast provinces. Large amounts of soy bean oil were sold to the South. The price of soy bean oil became much cheaper than cottonseed oil for cooking. A strange thing happened, every family in this village started to raise children.

"My proposition is based on this observed fact. It seems that cottonseed oil can be used for birth control.
ability of gossypol to inhibit oocyte penetration (95,179)
acrosin inhibition in vitro (95,179)
antispermatogenic and/or antifertility effects on
hamster (34,186)
male rats (9,34,42,44,75,76,86,92,115,164,194,196,204)
mice (40)
monkeys (160)
rabbits (34)
chronic toxicity in
dogs and monkeys (151)
rats (43,219)
digestion with pancreatin of microcapsules containing

gossypol (212)
distribution in the subcellular fractions of testes (112)
effects on
adrenal cortex function (7)
androgen-dependent organs in mice and rats (163)
posterior lobe of pituitary of castrate hogs (218)
autonomic nervous system of rodents (122)
DNA content of sperm from male subjects (202)
exfoliated cells in human semen from subjects taking
gossypol (162)
fructose metabolism by sperm (138)
growth and function of Leydig and Sertoli cells in culture (221)
isolated bovine sperm plasma membrane
Ca++-ATPase (198)
LH levels (113,192)
Na+ K+-ATPase of rat kidney (23,66,205,216)
Na+ K+-ATPase of guinea pig kidney (205)
rat liver (10,111,135)
rat liver enzymes (10)
Sertoli cell junction of the guinea pig (137)
serum progesterone levels (191)
testes, cytological (209)
testes, morphological (45)
testicular isozymes (108)
testosterone levels (113,114,150,192)
tumor promotion and/or initiation (79)
ultrastucture of human sperm in vitro (78)
ultrastucture of rat sperm in vitro (29,129)
effects of sister chromatid exchange on
CHO K-1 cells in vitro (110)
human peripheral lymphocytes (67,203)
mice (215)
effects on the K+ depleting effect on
rabbit heart (144)
rats (140,143,166,210)
skeletal muscle in vitro (207)
electron microscopy of
effects on cardiac muscle in monkeys (119)
gossypol toxic effects in rats (206)
histological effects on pituitary, adrenal cortex and
hypothalamus of
rabbits (150)
rats (113,192,211)
human studies
in Brazil (41)
in China (6,8,35,109,117,118,120,139,141,204,208)
hypokalemia in humans (22,139,142)
non-mutagenicity (38,193)
pharmacokinetics and metabolism in rats
(101,165,167,173,195)
recovery of spermatogenesis in chronically dosed rats (75)
spemcidal effects
in vitro (91,146,147,175,187,188)
in vivo (33,185,201)
synergism of gossypol antifertility effects in rats with
WIN-1844 (11)

Table 2. Varieties of gossypol studies, with references.

The fact that when stopping eating cottonseed oil the birth control effect automatically disappears, shows that it is most economical, convenient and natural."

In 1971, Chinese investigators in Nanjing determined that the active principle was gossypol, and toxicity experiments and preliminary dose evaluations were carried out. semen analyses were carried out on five male subjects. After administration of gossypol for 35 to 42 days, at a dose of 60 to 70 mg daily, four of the males were azoospermic and one was necrospermic. These data led to the clinical testing of gossypol as a male antifertility agent in 1972 (141).

More than 8,000 men have now been treated with gossypol in the 14 provinces of the People's Republic of China (120, 139). Some have received gossypol itself, some gossypol acetic acid (a more stable form), and a few have received gossypol formic acid. To date, there does not appear to be any significant difference in the action of these three materials. The usual dose administered is 20 mg daily for 60 to 70 days, followed by a maintenance dose of about 60 mg/week (107, 120, 139). At the present time, the only evidence of an antifertility effect in humans is a marked decrease in sperm counts, usually to 4 million/ml or less after dosing for 2 to 3 months in 99% of cases.

Concern has been expressed over the fertility recovery rate of men who have been using gossypol. Preliminary data suggest that 74% of subjects receiving gossypol for periods of 6 months to 4.5 years, whose sperm count was greater than 4 million/ml, recovered normal fertility (139). Correlations of recovery rates with duration of treatment have not as yet been published, but it is apparent that chances of recovery are greater in subjects who have not achieved azoospermic levels.

Side effects attributed to gossypol treatment in China are fatigue (12%), gastrointestinal symptoms (7%), decreased libido (5%), dizziness (4%), dryness of mouth (3%), minor occurrences of sleepiness, palpitation, eyelid edema, decreased perspiration, skin rash, and hypokalemia (139).

Hypokalemia was reported in 66 of 8,806 men (0.75%) using gossypol in clinical trials (120, 139). In China, the
incidence of hypokalemia has been associated with regional differences, which could be related to dietary intake. The familial thyrotoxicosis sometimes seen in Chinese males produces a periodic paralysis and other symptoms that are indistinguishable from those of hypokalemia due to gossypol (14, 126, 127). Treatment with potassium chloride (1 gm/day) reduces the fatigue, lassitude, and electrocardiogram changes in subjects with hypokalemia, and also prevents hypokalemia paralysis (139, 140, 142).

In animals, gossypol seems to have varying toxicity, based on species differences. Dogs appear to be more sensitive and monkeys least sensitive to its general toxic effects. Gossypol is not mutagenic in the Ames test (38, 193), but has been shown to be tumor-inducing or tumor-producing in mouse skin painting tests (79), a finding that requires further investigation.

The loss of fertility in rats and hamsters due to gossypol administration has been reported in several laboratories (34, 75, 76, 186). One study in non-human primates also demonstrated the ability of gossypol to decrease sperm concentration when administered to cynomolgous monkeys (Macaca fascicularis) (160). Species variability in response to gossypol has also been demonstrated in rats, hamsters, and mice (34).

The effects of gossypol on serum testosterone, FSH, and LH are inconsistent between species and in the same species. Hadley and co-workers reported a drop in serum testosterone and LH levels with no change in FSH in rats orally treated with gossypol, 30 mg/kg/day for 5 weeks (75). A slightly lower effective dose (20 mg/kg) was reported to decrease serum testosterone with no decrease in LH (163). Doses as low as 1 mg/kg daily for 1 week have also been reported to cause a lowering of serum testosterone (114). In non-human primates, however, no change in serum testosterone was observed, after oral dosing for 6 months with 10 mg/kg/day (160). Leydig cells isolated from gossypol-treated rats, as well as normal rat Leydig cells, in the presence of added gossypol, demonstrated a reduced production of testosterone when incubated in vitro with LH (75, 114).

Although there is no question that gossypol inhibits fertility, the site of action is not clear; most evidence suggests that it damages spermatids and spermatocytes. In semen obtained from gossypol-treated animals, usually spermatozoae are decreased and some are abnormal (75, 160). Damage to the sperm frequently involves the mitochondrial sheath and degenerative changes in the early portions of the tail (75). Experiments with ligated epididymides suggest that the sperm are damaged before they reach the epididymis (42).

Damage to the seminiferous tubules of hamsters and rats after treatment with gossypol is not evident (139, 164), but close examination of sperm in the rat testes reveals damage primarily to spermatids and spermatocytes (43, 164). These findings concur with those of Chinese investigators, and suggest that gossypol produces its primary effect at the later stages of sperm maturation in the testes (6), probably at the late spermatocyte stage. Testicular atrophy may also follow the direct effects on sperm after several months of exposure (219). However, effects on sperm occur much earlier than testicular effects, suggesting that testicular atrophy is secondary to accumulation of dead sperm in the tubules (43).

Electron microscopic examination of rat testes after low doses (5 mg/rat for 4 weeks) of gossypol showed dilation of mitochondria of the mid-piece in spermatids (45). Animals dosed orally with 30 mg/kg/day experienced slight damage of the spermatids, with nuclear vacuolation, wrinkly nuclear membrane, change in chromatin-staining reaction, and swelling or displacement of the cap and acrosome. Spermatozoa obtained from the cauda epididymidis of gossypol-treated rats have the same characteristics (29, 129). Continued treatment results in the appearance of exfoliated spermatids and spermatocytes from germinal epithelium, as well as spermatozoa with detached heads in the lumen of the tubules (209). Spermatogonia appear unaffected morphologically by gossypol administration. A single dose of 100 mg/kg results in injury similar to that seen with low-dose, chronic administration. Nine to 11 days after treatment, only Sertoli cells and germ cells remain (209). Evaluation of the biochemical activity of developing sperm confirms the morphologically described site of damage. Inhibition of incorporation of nucleic acid and protein precursors has been reported, with the greatest effect seen in spermatids and pachytene spermatocytes (209).

Although gossypol appears to affect the late spermatocyte stage, the biochemical mechanism for this action is unknown. Studies on the inhibition by gossypol of sperm enzymes, such as LDH (108), Ca ++ -ATPase (198) and acrosin (95, 179), as well as fructose metabolism (138), have not helped in the elucidation of the biochemical mechanisms of action. Many of the effects on biochemical assay systems are probably related to the antioxidant and chelating abilities of the chemically reactive gossypol molecule, resulting in non-specific inhibition of enzyme systems.

Gossypol, as isolated from Gossypium species, is an optically inactive molecule. Attempts have been made to separate the (+)- and (-)- forms of gossypol, but none have as yet been successful. (+)- Gossypol has been found in Thespesia populnea (Malvaceae) (100).

A recent experiment with hamsters (184) confirmed the lack of effect of (+)-gossypol on male fertility initially identified in rats by Wang (194). This lack of male antife...
tility effect by the (+)-form of gossypol may be a result of two possible mechanisms. First, it is possible that the physiological disposition of the (+)-isomer is different from that of the (-)-isomer. This is unlikely, since the major metabolite of gossypol is a glucuronide, and glucuronidation enzymes are non-specific. Second, the actions may be mediated by a stereospecific receptor site. The extremely small amount of gossypol that actually enters the testes to exert its action is good evidence that the anti-fertility effect is very specific.

A review of these experiments clearly indicates that male infertility occurs before there is a decrease in sperm counts, the criterion utilized in assessing gossypol effects on humans and non-human primates. It is quite possible that the doses currently being utilized (Brazil, China) may be in excess of that required for infertility. More work is needed to accurately characterize the actions of gossypol. Doses may be substantially decreased, with an associated decrease in toxicity, if better criteria for gossypol-induced infertility are developed in animals and then extended to men. Future experiments on the actions of gossypol must distinguish between nonspecific toxic effects and the potential specific actions of gossypol.

Hibiscus rosa-sinensis L. (Malvaceae) The first evidence that extracts of H. rosa-sinensis flowers produced an antispermatogenic effect in laboratory animals was published in 1972 by Kholkuie and co-workers (97). These investigators administered an ethanol extract of H. rosa-sinensis flowers to male rats orally for 30 days at dose levels of 50, 150, and 250 mg/animal. Definite histologic evidence for an effect on male reproduction was seen on day 14 of dosing. On day 30, the group of rats receiving the 250 mg/animal dose showed shrinkage of the seminiferous tubules, and complete disorganization of the testicular tissue and destruction of spermatogonial cells. Germinal cells were also affected and Leydig cells were absent. Sertoli cells were least affected, and the seminal vesicles and prostate were also unchanged. There was no effect in rats dosed at 50 and 150 mg/animal (97). The extract did not have androgenic or anti-androgenic activity.

Later, the same investigators published data suggesting that the initial results found by them were caused by inhibition of gonadotropin release or synthesis (97, 99). A benzene extract of flowers from this plant was similarly evaluated in male rats, with essentially the same results (168).

A single 7.5 mg dose of H. rosa-sinensis flower extract was injected subcutaneously in a group of reproductively active male bats (Rhinopoma kinneari), and the six testicular LDH isozymes were measured daily. It was reported that LDH4 disappeared from the isozymograms on days 2 to 4, but reappeared on day 5 (98).

Hibiscus rosa-sinensis is a member of the Malvaceae, the same plant family as the cotton plant (Gossypium species) from which gossypol is obtained. To date, an analysis of H. rosa-sinensis for gossypol has not been reported in the literature.

Hippophae salicifolia D. Don (Elaeagnaceae) A water soluble portion of an ethanol extract prepared from H. salicifolia bark was administered subcutaneously each day for 7 days to mature male rats. The animals were found to have degenerative changes in the seminiferous epithelium of the testes. Further, administration of the extract to another group of castrate rats receiving testosterone showed an inhibition of seminal vesicle development (89). The authors concluded that the extract of H. salicifolia produced an antispermaticogenic and an antiandrogenic effect. [From the experimental data presented, we consider the latter effect questionable.]

Leucaena glauca (L.) Benth. (Leguminosae). In Hawaii, where L. glauca grows abundantly as a weed, it is known as koa haole. Because the leaf meal of this plant has been found to contain more than 25% protein (dry weight), livestock producers and poultrymen use it for feed. However, some animals fed koa haole leaves have lowered fertility rates, and sows and rabbits develop alopecia (199). Ruminants do not seem to be affected in the latter respect (200).

Male rats fed with a ration containing 15% koa haole leaf meal for 2 weeks prior to mating had a significant decrease in pregnancies (121).

Leucaena glauca is known to contain significant amounts of the cytotoxic amino acid mimosine, about 3% in the leaves and 5% in the seeds. Mimosine may be responsible for the lowered male fertility in the previously mentioned study, since a mimosine-inactivating agent (sodium iron pyrophosphate) was added to the koa haole ration, which when fed to male rats did not impair fertility (121).

This constitutes another example of the presence of a cytotoxic agent in plant material showing antifertility effects in the male.

Lonicera ciliosa Polr. (Caprifoliaceae). Male and female mice were dosed daily with 10 to 20 mg of a 50% ethanol-water extract prepared from L. ciliosa leaves. The extract was given 2 weeks prior to, and for 3 weeks during, cohabitation of the mice. This resulted in a pronounced decrease in litters by the females in the study (19).

From the design of the study, it was not possible to ascertain whether the effect of the plant extract was on the males or the females, or both. We believe that the plant should be reevaluated, using a more appropriate experimental design.
**Lupinus termis** Forsk. (Leguminosae). Male rats were fed a ration containing 27% untreated or debrithered (hot water treatment) **L. termis** seeds for 9 weeks. The animals were then sacrificed and the seminal fluid and testes were examined. Although the histologic evidence revealed a decreased sperm production in Lupin-treated animals, the biochemical evidence did not rule out the possibility of an indirect effect through decreased production of testosterone (174). This is another study suggesting the need for further experiments to determine if the antispermatogenic effect is worth pursuing.

**Malavaviscus conzattii** Greem. (Malvaceae). An ethanol extract prepared from the dried flowers of **M. conzattii** was administered orally for 25 days to male gerbils (**Meriones hurrianae**) at a daily dose of 25 mg/kg, and to male rats at a dose of 50 mg/kg. On day 20, the animals were sacrificed and the testes, prostate, epididymides, and seminal vesicles were removed and weighed. The animals had no body weight loss relative to controls, but testicular weights decreased drastically in both species, as did the weights of the accessory sex glands. The weight of the adrenal glands increased significantly. The investigators suggest that both antispermatogenic and antiandrogenic effects were mediated by antigenadotropin activity (51). Similar results have been reported for an extract administered to bats (**Rhinopoma kirneri**) (52) and mice (87, 182).

**Malavaviscus conzattii** is closely related to **Gossypium** and **Hibiscus** species, and may contain gossypol. However, this species apparently has not been evaluated for the presence of gossypol, which would seem to be necessary before additional studies are carried out to substantiate the preliminary data.

**Momordica charantia** L. * (Cucurbitaceae). An ethanol extract of **M. charantia** fruits administered orally or subcutaneously to adult male gerbils (**Meriones hurrianae**) for a period of 2 weeks reduced testicular weights and disrupted spermatogenesis without significantly affecting the seminal vesicles or prostate. Doses ranged from 200 to 400 mg/kg (54). Dogs were dosed orally with the same extract at 1.75 mg/animal daily for 60 days, producing similar results (54).

Additional critical studies of this plant appear warranted.

**Ocimum sanctum** L. (Labiatae). Oral daily dosing for 15 days with a benzene extract prepared from the dried leaves of **O. sanctum** at levels of 100, 150, and 200 mg/kg decreased testes weight with no effect on the epididymides, seminal vesicles, prostate, or vas deferens of male rats (94, 156). A decreased sperm count in the rats receiving 150 mg/kg was also reported (94). These data should be confirmed in new studies using larger numbers of animals and dosing for more than 15 days.

<table>
<thead>
<tr>
<th>PLANT FAMILY</th>
<th>GENUS AND SPECIES</th>
<th>PLANT PART</th>
<th>TYPE SPERM* RAT</th>
<th>ACTIVE PRINCIPLE(S) REFERENCE(S)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AMARANTHACEAE</strong></td>
<td>Deeringia amaranthoides Merr.</td>
<td>Aerial parts</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>ARALIACEAE</strong></td>
<td>Scheflera capitata Harms</td>
<td>Aerial parts</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>ASCLEPIADACEAE</strong></td>
<td>Pycnostelma Paniculatum K. Schum</td>
<td>Not stated</td>
<td>+ † nt</td>
<td>-</td>
</tr>
<tr>
<td><strong>BALSAMINACEAE</strong></td>
<td>Impatiens duthiei Hook. F.</td>
<td>Whole plant</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>CARYOPHYLLACEAE</strong></td>
<td>Gysophila Cerasoides D. Don</td>
<td>Whole plant</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>GYSOPHILA</strong></td>
<td>G. Paniculata L.</td>
<td>Roots</td>
<td>nt</td>
<td>+</td>
</tr>
<tr>
<td><strong>SAPONARIA OFFICINALIS</strong></td>
<td>L.</td>
<td>Roots</td>
<td>nt</td>
<td>+</td>
</tr>
<tr>
<td><strong>VACCARIA SEGETALIS</strong></td>
<td>(NECK.) Garcke</td>
<td>Not stated</td>
<td>+ † nt</td>
<td>Vacegosside (Triterpene saponin)</td>
</tr>
<tr>
<td><strong>COMBRETACEAE</strong></td>
<td>Terminalia Bellera Roxb.</td>
<td>Fruit</td>
<td>+</td>
<td>nt</td>
</tr>
<tr>
<td><strong>TERMINALIA HORRIDA</strong></td>
<td>Stein.</td>
<td>Fruit</td>
<td>nt</td>
<td>+</td>
</tr>
<tr>
<td><strong>COMPOSITAE</strong></td>
<td>Calendula officinalis L.</td>
<td>Whole plant</td>
<td>+</td>
<td>nt</td>
</tr>
<tr>
<td><strong>CENTRANTHERUM ANTHELMINTICUM</strong></td>
<td>(WILLD.) Runtze</td>
<td>Flowers</td>
<td>nt</td>
<td>+</td>
</tr>
<tr>
<td><strong>SOLIDAGO VIRGAUREA</strong></td>
<td>L.</td>
<td>Whole plant</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>GIAMINEAE</strong></td>
<td>Dimebia gracilis Nees ex Steud.</td>
<td>Whole plant</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>LABIATAE</strong></td>
<td>Anisomeles Malabarica R. Br. ex Sim.</td>
<td>Aerial parts</td>
<td>+</td>
<td>+</td>
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<tr>
<td><strong>CALAMINTHA UMBROSA</strong></td>
<td>Fisch. &amp; Mey.</td>
<td>Whole plant</td>
<td>nt</td>
<td>+</td>
</tr>
<tr>
<td><strong>LEGUMINOSAE</strong></td>
<td>Acacia concinna DC.</td>
<td>Bark</td>
<td>nt</td>
<td>+</td>
</tr>
<tr>
<td><strong>AESCHYNOMENE INDICA</strong></td>
<td>L.</td>
<td>Whole plant</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>ALBIZIA LEBBEK</strong></td>
<td>Denth.</td>
<td>Seed pod</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 3. Plants reported to be spermicidal (*in vitro*), shown with their active principles.
<table>
<thead>
<tr>
<th>PLANT FAMILY</th>
<th>GENUS AND SPECIES</th>
<th>PLANT PART</th>
<th>TYPE SPERM*</th>
<th>ACTIVE PRINCIPLE(S)</th>
<th>REFERENCE(S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALBIZIA</td>
<td>ALBIZIA LEBBEK BENTH.</td>
<td>Roots</td>
<td>+ +</td>
<td>Lebbekanin E (Triterpene saponin)</td>
<td>16,158,181</td>
</tr>
<tr>
<td>ALBIZIA</td>
<td>ALBIZIA PROCERA BENTH.</td>
<td>Roots</td>
<td>nt +</td>
<td>Oleanolic acid saponin</td>
<td>16-18,181</td>
</tr>
<tr>
<td>ALBIZIA</td>
<td>ALBIZIA PROCERA BENTH.</td>
<td>Seeds</td>
<td>nt +</td>
<td>Oleanolic acid saponin</td>
<td>16-18</td>
</tr>
<tr>
<td>ENTEROLOBIUM</td>
<td>ENTEROLOBIUM CYCLOCARPUM GRISB.</td>
<td>Leaves</td>
<td>nt +</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>GLEDISTIA</td>
<td>GLEDISTIA SINENSIS LAMK.</td>
<td>Aerial parts</td>
<td>nt +</td>
<td>–</td>
<td>16-18,158</td>
</tr>
<tr>
<td>GLYCRRHIZA</td>
<td>GLYCRRHIZA GLABRA L.</td>
<td>Aerial parts</td>
<td>nt +</td>
<td>–</td>
<td>157,158</td>
</tr>
<tr>
<td>MELILOTUS</td>
<td>MELILOTUS SICULA VITMAN</td>
<td>Seeds</td>
<td>nt +</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>OUGENIA</td>
<td>OUGENIA DALBERGIOIDES BENTH.</td>
<td>Stem bark</td>
<td>+ –</td>
<td>–</td>
<td>158</td>
</tr>
<tr>
<td>PITHECELLOBIUM</td>
<td>PITHECELLOBIUM DULCE (ROXB.) BENTH.</td>
<td>Seeds</td>
<td>+ +</td>
<td>Oleanolic and echinocystic acid saponins</td>
<td>18</td>
</tr>
<tr>
<td>PTEROLOBIUM</td>
<td>PTEROLOBIUM INDICUM A. RICHARDS</td>
<td>Aerial parts</td>
<td>+ +</td>
<td>–</td>
<td>157,158</td>
</tr>
<tr>
<td>SAMANEA</td>
<td>SAMANEA SAMAN (JACQUIN) MERR.</td>
<td>Aerial parts</td>
<td>+ +</td>
<td>–</td>
<td>158</td>
</tr>
<tr>
<td>LILIACEAE</td>
<td>TRIGONELLA FOENUM-GRAECUM L.</td>
<td>Seeds</td>
<td>+ +</td>
<td>–</td>
<td>158</td>
</tr>
<tr>
<td>RUSCUS</td>
<td>RUSCUS HYPOGLOSSUM L.</td>
<td>Aerial parts</td>
<td>nt +</td>
<td>–</td>
<td>217</td>
</tr>
<tr>
<td>MYRSINACEAE</td>
<td>ARDISIA MERICIFOLIA WALLICH</td>
<td>Aerial parts</td>
<td>nt +</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>MAESA</td>
<td>MAESA INDICA VAR. ANGSTIFOLIA HOOK F. &amp; THOMS.</td>
<td>Aerial parts</td>
<td>nt +</td>
<td>–</td>
<td>158</td>
</tr>
<tr>
<td>PHYTOLACCACEAE</td>
<td>PHYTOLACCA ACINOSA ROXB.</td>
<td>Aerial parts</td>
<td>nt +</td>
<td>–</td>
<td>158</td>
</tr>
<tr>
<td>PHYTOLACCA</td>
<td>PHYTOLACCA AMERICANA L.</td>
<td>Aerial parts</td>
<td>nt +</td>
<td>–</td>
<td>159</td>
</tr>
<tr>
<td>PHYTOLACCA</td>
<td>PHYTOLACCA ODODECANDRA L'HERIT.</td>
<td>Fruit</td>
<td>nt +</td>
<td>Oleanolic acid saponin</td>
<td>171</td>
</tr>
<tr>
<td>PITTOSPORACEAE</td>
<td>PITTOSPORUM NEELGERENSE WIGHT &amp; ARNOTT</td>
<td>Aerial parts</td>
<td>+ –</td>
<td>Pittoside A; Pittoside B (Triterpene saponin)</td>
<td>83</td>
</tr>
<tr>
<td>POLEMONIACEAE</td>
<td>POLEMONIUM CAERULEUM L.</td>
<td>Aerial parts</td>
<td>nt +</td>
<td>–</td>
<td>5</td>
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<tr>
<td>PRIMULACEAE</td>
<td>ANAGALLIS ARVENSIUS L.</td>
<td>Whole plant</td>
<td>nt +</td>
<td>Anagalligenone saponin</td>
<td>16,18</td>
</tr>
<tr>
<td>RANUNCULACEAE</td>
<td>CALTHA PALUSTRIS L.</td>
<td>Whole plant</td>
<td>nt +</td>
<td>–</td>
<td>69,158</td>
</tr>
<tr>
<td>SAPINDACEAE</td>
<td>BLIGHIA SAPIDA KOENIG</td>
<td>Fruit</td>
<td>nt +</td>
<td>Oleanolic acid and hederagenin saponins</td>
<td>18,69,157</td>
</tr>
<tr>
<td>SAPIUM</td>
<td>SAPIUM MUKOROSI GAERTN.</td>
<td>Aerial parts</td>
<td>nt +</td>
<td>Hederagenin saponin</td>
<td>69,158</td>
</tr>
<tr>
<td>SAPIUM</td>
<td>SAPIUM MUKOROSI GAERTN.</td>
<td>Fruit</td>
<td>nt +</td>
<td>–</td>
<td>69,158</td>
</tr>
<tr>
<td>SAPIUM</td>
<td>SAPIUM TRIFOLIATUS L.</td>
<td>Aerial parts</td>
<td>nt +</td>
<td>–</td>
<td>69</td>
</tr>
<tr>
<td>SAPOTACEAE</td>
<td>MADHUCU BUTYRACEA (ROXB.) MACBRIE</td>
<td>Seeds</td>
<td>nt +</td>
<td>Basic acid saponin</td>
<td>16,18</td>
</tr>
<tr>
<td>MADHUCU</td>
<td>MADHUCU LATIFOLIA (ROXB.) MACBRIE</td>
<td>Seeds</td>
<td>nt +</td>
<td>Basic acid saponin</td>
<td>16,18,157</td>
</tr>
<tr>
<td>MIMUSOPS</td>
<td>MIMUSOPS ELENGI L.</td>
<td>Seeds</td>
<td>nt +</td>
<td>Basic acid saponin</td>
<td>16,18</td>
</tr>
<tr>
<td>MIMUSOPS</td>
<td>MIMUSOPS HEXANDRA ROXB.</td>
<td>Seeds</td>
<td>nt +</td>
<td>Basic acid saponin</td>
<td>16,18</td>
</tr>
<tr>
<td>MIMUSOPS</td>
<td>MIMUSOPS LITTORALIS KURZ</td>
<td>Seeds</td>
<td>nt +</td>
<td>Basic acid saponin</td>
<td>15,18</td>
</tr>
<tr>
<td>SCROPHULARIACEAE</td>
<td>CELSIA COROMANDELINA VAHL</td>
<td>Whole plant</td>
<td>nt +</td>
<td>–</td>
<td>158</td>
</tr>
<tr>
<td>HYDROCOTYLE</td>
<td>HYDROCOTYLE SAVANICA THUNB.</td>
<td>Whole plant</td>
<td>nt +</td>
<td>Bacoside (Triterpene saponin)</td>
<td>157</td>
</tr>
<tr>
<td>SOLANACEAE</td>
<td>SOLANUM XANTHOCARPUM SCHRAD. &amp; WENDL.</td>
<td>Whole plant</td>
<td>nt +</td>
<td>–</td>
<td>158</td>
</tr>
<tr>
<td>SYMPOLOCACEAE</td>
<td>SYMPOLOCOS GARDNERIANA WIGHT</td>
<td>Aerial parts</td>
<td>+ –</td>
<td>–</td>
<td>158</td>
</tr>
<tr>
<td>UMBELLIFERAE</td>
<td>UMBELLIFERAE CENTELLA ASIATICA URBAN</td>
<td>Whole plant</td>
<td>+ –</td>
<td>–</td>
<td>157</td>
</tr>
<tr>
<td>HYDROCYTLE</td>
<td>HYDROCYTLE SAVANICA THUNB.</td>
<td>Whole plant</td>
<td>+ –</td>
<td>–</td>
<td>158</td>
</tr>
<tr>
<td>PIMPINELLA</td>
<td>PIMPINELLA DIVERSIFOLIA DC.</td>
<td>Whole plant</td>
<td>+ nt</td>
<td>–</td>
<td>158</td>
</tr>
<tr>
<td>VERBENACEAE</td>
<td>VERBENACEAE CLERODENDRUM SERRATUM SPRENG.</td>
<td>Aerial parts</td>
<td>+ +</td>
<td>–</td>
<td>158</td>
</tr>
<tr>
<td>ZYGOPHYLLACEAE</td>
<td>ZYGOPHYLLACEAE BALANITES ROXBURGHI PLANCH.</td>
<td>Fruit</td>
<td>+ +</td>
<td>Diosgenin saponin</td>
<td>18</td>
</tr>
</tbody>
</table>

* +, spermicidal; –, not spermicidal; nt, not tested (see original reference for concentrations used and test conditions)
† bull sperm was used
‡ reported used as a vaginal contraceptive in 100 women for up to 1 year at a concentration of 1:1,000, with no side effects and with complete protection.
**Prunus emarginata** Walp. (Rosaceae). This plant was tested for antifertility activity at the same time as was *Lonicera ciliosa* (19), but should be retested using more orthodox procedures to determine antifertility effects in the male.

*Withania somnifera* (L.) Dunal (Solanaceae). *Withania somnifera* root powder was fed to male and female mice at a dose of 25 mg daily for 10 days. Males were then mated with the females and the feeding with *W. somnifera* root was continued during the mating period, until pregnant animals littered. Administration of this plant seemed to delay mating, decrease pregnancies, and result in smaller litter sizes (70). However, since both males and females received the test drug, it is not possible to determine whether the effect was on the male or the female, or both. Additional studies must be carried out using appropriate protocols to ascertain the effects on males.

**SPERMIDES OF PLANT ORIGIN**

Most of the currently marketed spermicides, such as nonoxynol-9, act by disruption of the spermatozoon plasma cell membranes. This action is only one of numerous

<table>
<thead>
<tr>
<th>COMPOUND NAME</th>
<th>CHEMICAL CLASS</th>
<th>EFFECTIVE CONC.</th>
<th>SPERM SOURCE</th>
<th>REF.</th>
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</thead>
<tbody>
<tr>
<td>DL-ALANINE</td>
<td>Amino acid</td>
<td>200 mMol</td>
<td>Rabbit</td>
<td>154</td>
</tr>
<tr>
<td>BENZOQUINONE</td>
<td>Benzoquinone</td>
<td>1-5,1,000</td>
<td>Guinea pig</td>
<td>73</td>
</tr>
<tr>
<td>BERBERINE</td>
<td>Isoquinoline alkaloid</td>
<td>1-333</td>
<td>Bull</td>
<td>149</td>
</tr>
<tr>
<td>CATECHOL</td>
<td>Phenol</td>
<td>1-200</td>
<td>Guinea pig</td>
<td>73</td>
</tr>
<tr>
<td>CHIKUSETUSAPONIN IV</td>
<td>Triterpene saponin</td>
<td>20-100ug/ml</td>
<td>Human</td>
<td>171</td>
</tr>
<tr>
<td>3,4-DIHYDROXYCHALCONE</td>
<td>Chalcone</td>
<td>1-100</td>
<td>Rat</td>
<td>172</td>
</tr>
<tr>
<td>EMETINE</td>
<td>Isoquinoline alkaloid</td>
<td>1-10,000</td>
<td>Human</td>
<td>30,36</td>
</tr>
<tr>
<td>GLYCINE</td>
<td>Amino acid</td>
<td>100 mMol</td>
<td>Rabbit</td>
<td>154</td>
</tr>
<tr>
<td>GOSSYPOL</td>
<td>Dimeric sesquiterpene</td>
<td>0.02-20 mg/ml</td>
<td>Human</td>
<td>91,146,147,187,188</td>
</tr>
<tr>
<td>GUAIACOL</td>
<td>Phenol</td>
<td>1-1,600</td>
<td>Guinea pig</td>
<td>73</td>
</tr>
<tr>
<td>LEBBEKANIN E</td>
<td>Triterpene</td>
<td>Not stated</td>
<td>Human</td>
<td>181</td>
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<tr>
<td>LEMMATOXIN</td>
<td>Triterpene</td>
<td>80 µg/ml</td>
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<td>LEMMATOXIN C</td>
<td>Triterpene</td>
<td>20 µg/ml</td>
<td>Human</td>
<td>171</td>
</tr>
<tr>
<td>L-LYSINE HCL</td>
<td>Amino acid</td>
<td>200 mMol</td>
<td>Rabbit</td>
<td>154</td>
</tr>
<tr>
<td>2-METHOXY-5-METHYL-BENZOQUINONE</td>
<td>Benzoinone</td>
<td>1,6,000</td>
<td>Guinea pig</td>
<td>73</td>
</tr>
<tr>
<td>2-METHOXY-1,4-NAPHTHOQUINONE</td>
<td>Naphthoquinone</td>
<td>1,100</td>
<td>Rat</td>
<td>171</td>
</tr>
<tr>
<td>MYRICETIN</td>
<td>Flavonoid</td>
<td>2 mg/ml</td>
<td>Human</td>
<td>172</td>
</tr>
<tr>
<td>NIMBIN (NA SALT)</td>
<td>Triterpene</td>
<td>1-1,000</td>
<td>Rat</td>
<td>161</td>
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<tr>
<td>NUHVAR LUTEUM ALKALOID</td>
<td>Alkaloid</td>
<td>No: stated</td>
<td>Human</td>
<td>2</td>
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<tr>
<td>OLEANOGLYCOTOXIN A</td>
<td>Triterpene saponin</td>
<td>10-100 µg/ml</td>
<td>Human</td>
<td>171</td>
</tr>
<tr>
<td>DL-ORNITHINE HCL</td>
<td>Amino acid</td>
<td>200 mMol</td>
<td>Rabbit</td>
<td>154</td>
</tr>
<tr>
<td>OUABAIN</td>
<td>Cardiac glycoside</td>
<td>0.01-0.001 M</td>
<td>Boar</td>
<td>25-27</td>
</tr>
<tr>
<td>PITTOSIDE A</td>
<td>Triterpene saponin</td>
<td>1-1,250</td>
<td>Human</td>
<td>83</td>
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<tr>
<td>PITTOSIDE B</td>
<td>Triterpene saponin</td>
<td>1-1,250</td>
<td>Human</td>
<td>83</td>
</tr>
<tr>
<td>PAEONOL</td>
<td>Acetophenone</td>
<td>Not stated</td>
<td>Bull</td>
<td>12</td>
</tr>
<tr>
<td>PYROGALLOL</td>
<td>Phenol</td>
<td>1-50</td>
<td>Guinea pig</td>
<td>73</td>
</tr>
<tr>
<td>QUERCETIN</td>
<td>Flavonoid</td>
<td>1-1,000</td>
<td>Rat</td>
<td>172</td>
</tr>
<tr>
<td>QUININE</td>
<td>Quinoline alkaloid</td>
<td>1-100,00</td>
<td>Human</td>
<td>30,36,159</td>
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<tr>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td>1-1,000</td>
<td>Rat</td>
<td>172</td>
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<td>1-6,400</td>
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Table 4. Plant principles reported to have spermicidal properties (in vitro).
mechanisms to kill spermatozoa. In the past, mercuric compounds were used as spermicides and acted as general protoplasmic poisons, but the marketing and use of mercury-containing preparations has been discontinued.

Numerous enzyme systems in viable sperm are susceptible to inhibition, for example, those involving glycolysis, energy production, and myosin contraction. The local application of spermicides allows for less specificity of action, when compared with the specificity required for oral or injectable contraceptive agents. Although development of specific inhibitors of sperm metabolic pathways may be difficult, sperm-specific isozymes, such as LDHx, are being identified.

It is quite possible that future investigations into the biochemistry of spermatozoa will provide new and more specific sites for their destruction.

A large number of plants have been randomly selected and screened for spermicidal activity in vitro and several appear promising. Those species found to be active, and the nature of the active principle(s), when known, are presented in Table 3. A list of plant-derived chemical substances of known or partially known structure, reported to be spermicidal in vitro, appears in Table 4. The active compounds represent a variety of chemical structures, many of which appear to be too toxic for consideration as spermicidal agents in humans.

A majority of plant-derived spermicides are triterpene saponins of several structure types; steroid saponins are less frequently encountered.

Combinations of sugars, attached in glycosidic linkage to the aglycones, include arabinose, fructose, fucose, galactose, glucose, glucuronic acid, rhamnose, and xylose. At the present time, just which structural features within this class of spermicides are required for optimal activity is open to speculation. A study comparing the spermicidal effects of various triterpene saponins of known structure could reveal information that might be helpful in this respect.

Mechanistic studies have not been reported relative to the spermicidal action of the triterpene saponins. Since all saponins exhibit surface-active properties, the most likely mechanism for their action is a disruption of the sperm plasma cell membrane, in a manner similar to that of nonoxynol-9.

Plants are an untapped resource for inexpensive and effective spermicide preparations. The initial screening of plant extracts for spermicidal activity is simple and does not require elaborate facilities. In countries where research costs limit the type of experimental studies that can be conducted, and where flora is abundant, programs to develop inexpensive vaginal spermicides should prove productive.

SEMEN COAGULATION AND LIQUEFACTION

Human semen coagulates immediately following ejaculation, trapping and immobilizing spermatozoa in a tight fibrin matrix. Under normal circumstances, liquefaction occurs within several minutes and is usually complete after 5 to 20 minutes. The semen of some species (guinea pig and rat) coagulates but does not liquefy; rather, a plug is formed from the ejaculate. Semen from other species (dog and bull) does not coagulate at all; the coagulum temporarily immobilizes spermatozoa and allows a resting phase before the sperm become motile and travel through the female reproductive tract.

Components of the coagulation system are added to the ejaculate via secretions from the seminal vesicles. The liquefaction factors, primarily plasminogen activators, originate from the secretions of the Cowper and prostate glands. Modification of Cowper and prostate gland fluids could eliminate or greatly reduce the rate of liquefaction of semen and thereby decrease fertility. This effect on the liquefaction rate has been observed in men with pathological conditions that result in delayed liquefaction of semen. A long-lasting coagulum could cause a significant reduction in fertility in the human, but whether increased coagulation time alone would result in consistent complete loss of fertility is doubtful. Agents that extend the coagulation period could be utilized in conjunction with agents acting on other spermatozoan properties, and might result in a synergistic effect to reduce fertility.

Currently, the coagulation system in semen has not been carefully examined biochemically to identify differences from other coagulation systems. Until specific differences are discovered, the use of coagulation-inducing substances will be restricted to vaginal contraceptives.

With the exception of a few plants with lectins that coagulate semen, plants have not been studied for this effect until recent years; in 1977, about 1600 Indian plants were screened, and 90 showed positive semen coagulating properties (158). Initial testing involved preparation of ethanol/water (1:1) extracts of dry plant material, and testing of these (after removal of solvent) at a 2% concentration on human semen and rat vasal or epididymal contents (196). All 90 positive extracts coagulated rat vasal or epididymal contents, whereas only 49 coagulated human semen. In ten active extracts, further study suggested that the active principles were tannins. Most likely, these extracts had a denaturing effect on one or more proteins involved in the semen coagulation-liquefaction process.

SPERM AGGULUTINATION

Spermatozoa normally bind several substances to their outer membrane; these substances, called sperm-coating antigens, are derived from the male genital tract fluid and are usually associated with sperm fertility inhibition. Ex-
amples of sperm-coating antigens are decapacitation factor and proteinase inhibitors. These binding substances normally block sperm fertility and are removed from the sperm head or are deactivated during the process of capacitation in the female genital tract.

Several agglutinins, such as plant lectins, have been shown to adhere to the plasma membrane of human spermatozoa and inhibit the normal processing of sperm. Tight binding of exogenous substances to spermatozoa may result in perturbations in the plasma membrane, which then prevent capacitation.

Antibodies against sperm-specific antigens, such as LDH, have successfully inhibited fertility in female animals. Although the results are inconclusive, the agglutination of sperm by means of sperm-specific antibodies may be possible.

Although investigators must exercise caution in evaluating the sperm-agglutinating properties of agents capable of interacting with the immune system, agents that cause sperm agglutination through direct interactions with the sperm membrane may be valuable adjuncts in vaginal contraception. As with the coagulation system, this mechanism may reduce fertility, but it is not likely to be 100% effective.

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<th>COMPOUND NAME</th>
<th>CHEMICAL CLASS</th>
<th>REFERENCES</th>
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<td>ASCORBIC ACID</td>
<td>Vitamin</td>
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<tr>
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<td>CIRANTIN</td>
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<tr>
<td>XANTHORHAMNOL</td>
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Table 5. Plant-derived hyaluronidase inhibitors determined by in vitro and/or in vivo evidence.
CONCLUSION

Initially, we noted that plants are definitely a source of many useful and widely-employed drugs, and that practical fertility-regulating agents are likely eventually to be discovered from this source.

There is little folkloric information about the use of plants to regulate fertility in the male. Some published reports exist on the testing of crude plant extracts for male antifertility activity, but these represent only a fraction of the number of reports suggesting effects of plant extracts on female fertility.

There seems to be a lack of correlation between experimental results obtained by one group of investigators and another, in data obtained in vitro and in vivo, and in experimental results and information found in folklore. Factors complicating the adequate assessment of plants affecting male fertility are inadequate numbers of vehicle-treated controls, poor experimental design, problems related to insolubility of crude plant extracts, variation in routes of administration, and the diversity in reproductive function and control among various laboratory species.

Another significant problem involves the manner in which plant names are cited in scientific articles. Botanical nomenclature can be confusing when Latin binomials of plants are either so grossly misspelled as to be unrecognizable, or the misspelled name resembles another taxon. Translating into English much of the work published in Chinese that has been cited herein.

ACKNOWLEDGEMENTS

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With the demonstration and isolation of hypothalamic peptides that control the secretion rates of anterior pituitary hormones in the 1970s, a new era of identifiable physiologic control signals began (47). During the past ten years, hypothalamic peptides such as thyrotropin-releasing hormone (TRH), somatostatin, and gonadotropin-releasing hormone (GnRH) have also been identified in extrahypothalamic brain tissue and in the gut (29, 36), and GnRH-specific receptors have been found in the gonads themselves (31). The placenta has been found to be quite rich in unexpected peptides, such as GnRH, TRH, somatostatin, adrenocorticotropic hormone (ACTH), thyroid-stimulating hormone (TSH), and prolactin (35).

Ovarian follicular fluid has also been examined for peptides exerting a variety of activities. For many years, physiologists have known that follicular fluid (FF) of human, bovine, porcine, and equine origin is a rich source of ovarian steroids (18), and more recently, the fluid has also been shown to contain three pituitary hormones: follicle-stimulating hormone (FSH), luteinizing hormone (LH), and prolactin (42). Within the past five years, numerous other peptides have been identified in FF: oocyte maturation inhibitor (OMI), FSH receptor-binding inhibitor (FSHRBI), luteinization stimulator and inhibitor (LS and LI), ovarian inhibin or folliculostatin (FST), and gonadocin and gonadostatin. The latter terms are generic names for factors that stimulate or inhibit secretion of both FSH and LH from the pituitary gland.

The discovery of these novel peptides has engendered excitement and controversy, and a number of books have been written about them (24, 32, 53). This article is a progress report on this rapidly moving field, including a description of a new "morphology" of the ovarian follicle, and a review of the interrelationships of ovarian microscopic morphology and gonadotropin receptors, and ovarian secretion of steroids (34, 52).

LIFE HISTORY OF THE FOLLCLE

The morphologic changes that occur during primordial, primary, and early secondary follicle growth are illustrated in Figure 1. When a primordial follicle begins to grow, a series of ultrastructural and biochemical changes is initiated by the dictyate oocyte, culminating in the formation of a fully grown and physiologically differentiated ovum, enclosed in a glycoprotein coat called the zona pellucida, and single layer of cuboidal granulosa cells (20). This follicular unit, called a primary follicle, is approximately 150 µ in diameter, and steadily increasing granulosa cell mitosis causes the primary follicle to grow to a diameter of approximately 200 µ.

Meanwhile, the granulosa cells develop FSH, estrogen, and testosterone receptors and become physiologically coupled by gap junctions, and the fully differentiated primary follicle migrates into the medulla, where it acquires a theca tissue. In response to FSH, the development of the secondary follicle begins (20).

The follicles that contain oocytes become atretic, for the most part, and fewer than 1% of all such oocyte-follicle complexes ovulate (20, 34, 52). The morphologic sequence, which culminates in ovulation, is coordinated with gonadotropin secretion rates, and steroid secretion, as well as with specific hormonal receptors in the follicle cells. Information about specific receptors, by which circulating hormones act on ovarian cells, explains why some follicles fail to grow to maturity until near the time of ovulation.
menopause, while others grow and ovulate at the time of puberty, even though all follicles within the ovary are exposed simultaneously to the same hormones in the capillary blood (46).

During each menstrual cycle, some follicles increase in size by proliferation of granulosa cells, and begin to secrete small amounts of estrogen (15) (Figure 2). It appears that the granulosa cells in proliferating follicles start with a small complement of FSH membrane receptors and estradiol cytosol receptors, and that progressive development changes occur in the response of granulosa cells to hormones (Figure 3).

The current hypothesis proposed to explain the "two cell theory" of follicle estrogen production is that in response to LH stimulation, the steroid secreting cells in the theca interna secrete androstenedione. As shown in Figure 4, the androstenedione diffuses across the basement lamina of the follicle and enters the granulosa cells; here it is aromatized into estrogen by the aromatase enzymes induced in the granulosa cells by FSH, and the newly synthesized estrogen is released into the follicular fluid and peripheral circulation (20). Thus, estradiol synthesis and secretion probably require LH acting on the LH receptors in theca cells, which make androstenedione and testosterone. These precursors are converted to estradiol under the influence of FSH acting on granulosa cells (17). Thus, as each small follicle begins to synthesize and secrete very small amounts of estradiol, the local estradiol and FSH, acting on receptors, cause granulosa cell division, with enhanced numbers of FSH receptors within the follicle; each granulosa cell appears to have an unvarying number of FSH receptors. A given follicle will start to enlarge, and if its rate of enlargement coincides with the follicular phase of the menstrual cycle, it may enlarge to a great enough size that estradiol secretion, and estrogen and FSH acting together, will increase the

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**Figure 1.** Life history of the follicle (Reproduced, with permission, from Erickson GF: Normal ovarian function. Clin Obstet Gynecol 21:31, 1978).

**Figure 2.** Part of follicle showing sites of action of gonadotropins, and production and action of steroids. (Reproduced, with permission, from Dorrington JH: Pituitary and placental hormones. In Austin CR, Short RV (eds): Mechanisms of Hormone Action. Cambridge, Cambridge University Press, 1979, pp 53-80).

**Figure 3.** Illustration of hypothetical changes in response of granulosa cells to hormones. $\text{RH}, \text{RLH}, \text{RE}$: Receptors for FSH, LH, and estradiol; $T$: Testosterone; $E$: Estradiol. (Reproduced, with permission, from Richards' chapter in Jones RE (ed): The Vertebrate Ovary: Comparative Biology and Evaluation. New York, Plenum Press, 1978, p 336).
number of LH receptors in theca cells and finally in granulosa cells (Figure 3). This further enhances estrogen production.

By this time, the follicle we have followed becomes the dominant follicle in the cycle; it secretes enough estrogen to trigger the release of the LH surge. This LH, acting on the LH receptors scattered throughout the follicle, sets into play the steps leading to the resumption of meiosis in the oocyte (4) and ovulation within the dominant follicle, but no other (48). Any follicle in the ovary that had begun to grow, but did not reach critical maturity at the time when LH and FSH levels were optimal, now becomes atretic and disappears. The thecal and granulosa cells of the ovulated follicle luteinize, producing progesterone as the dominant steroid of the luteal phase.

At the point when a follicle exceeds 0.2 mm in diameter, fluid begins to accumulate within the follicle (Figure 5). The contents suggest that follicular fluid is derived partly from the plasma and partly from secretory products of the granulosa cells themselves (18). Morphologically, granulosa cells are loosely packed and show gap junctions, which permit free passage of materials throughout the interior of the follicle. The specialized membrane junctions first appear between the granulosa cells shortly after the oocyte completes its growth in the primary follicle. They facilitate cellular interchange of small molecules, the size of cyclic adenosine monophosphate and steroid hormones, and are thought to play an important role in coordinating hormonal stimulation of granulosa cell function during follicle development (Figure 6). There is also easy access to the interior of the oocyte itself, through its surrounding cumulus of granulosa cells, which connects to the interior of the oocyte (26) (Figure 7). The adaptive significance of the fluid micro-environment of the cells within the follicle is clear: the fluid compartment provides access for FSH and LH molecules to cell receptors, while providing a local area of amplified steroid and other signals. Thus, in the ovary, contiguous follicles may be at very different growth stages, but all cells within a given follicle are bathed in the same environment.

Figure 5. Sequence of hormone present in follicular fluid during growth of follicle: Increasing concentration (E) and peak concentration (E) of estradiol. Follicular progesterone (P) is at very high concentrations in preovulatory follicle. Early (EF), middle (MF), late (LF), and very late follicular (VLF) growth phases of menstrual cycle in women. (Reproduced, with permission, from: McNatty KP: Follicular Fluid. In Jones RE (ed): The Vertebrate Ovary. New York, Plenum Press, 1978, p 215).
"Plasma proteins"
Steroid binding protein
Enzymes
- Intracellular
- Extracellular
  - “Ovulating” — plasminogen; proteases
Mucopolysaccharides (proteoglycans)
  - Hyaluronic acid
  - Chondroitin sulfate acid
Steroids
- Estrogens
- Progestins
- Androgens
Pituitary protein hormones
- FSH
- LH
- Prolactin
Non-steroidal ovarian factors
- Oocyte meiosis inhibitor (OMI)
- FSH secretion suppressing factor
  (inhibin or folliculostatin)
- Luteinization stimulator (LS)
- Luteinization inhibitor (LI)
- FSH-receptor-binding-inhibitor (FSHBI)
- Gonadocrinin
- Gonadostatin

Table 1. Interesting substances found in follicular fluid.

If one accepts this argument for the adaptive significance of follicular fluid, one can understand why the discovery of a number of putative peptide signals or factors in easily obtainable follicular fluid has aroused excitement and has suggested that these factors may take part in the process of follicular individualization.

**FOLLICULAR FLUID**

Follicular fluid provides an easy sample of intrafollicular interstitial or extracellular fluid, and is a rich source of many substances. A partial list of the fluid contents is provided in Table 1 (8, 18, 41).

Since follicular fluid is at capillary pressure, the follicular basement membrane is not a barrier to water movement (18, 41). Concentration of serum proteins is somewhat lower in follicular fluid than in blood. Serum proteins above 1,000,000 MW are excluded from the follicle.

Preovulatory gonadotropin surges initiate rupture of the follicular wall, which occurs after the preovulatory swelling of the largest follicle. Activation of a protease in follicular fluid is usually assumed to be necessary for reduction of wall strength, with subsequent rupture. Under the influence of FSH, plasminogen is converted to plasmin, a protease that Beers, Strickland, and associates believe may cause ovulation (6, 55). Espey has suggested other candidates as the critical protease (22).

Follicular fluid is a very viscous liquid, and the stickiness that plagues everyone who has worked with it is due to mucopolysaccharides, or proteoglycan molecules, composed of sugars attached in chains of polypeptides (3). These molecules have a high viscosity, and exert a kind of matrix structure in solvents. Proteoglycans, which have been identified in FF, include chondroitin sulfate, hyaluronic acid, dermatan sulfate, and heparin. FSH appears to enhance the synthesis of these materials by granulosa cells (2). These substances are involved in cumulus expansion (19) and are correlated with antrum formation (41). The concentration of chondroitin sulfate and heparin sulfates decreases as follicular maturation occurs, being more concentrated in fluid from small pig follicles than from large ones (3).
The exact role of these molecules in oocyte-cumulus changes and ovulation remains to be elucidated, but they do appear to be secretion products of granulosa cells per se. FSH can enhance synthesis of these molecules by granulosa cells, and apparently can cause cumulus expansion (around the oocyte), for which hyaluronic acid synthesis is necessary. While LH is usually considered to be the "ovulating hormone" (48), FSH undoubtedly participates importantly in determining the composition and volume of FF as well as the follicular morphology.

Follicular fluid is rich in steroids, and steroid concentration in follicular fluid greatly exceeds that in blood. Not surprisingly, more estradiol is seen in follicles with fluid that also contains FSH since, as we saw above, FSH plus estradiol enhances granulosa cell mitosis and thus enlarges follicles (46) (Figure 5). When follicles of different sizes and at different stages of the menstrual cycle are compared (41), the highest levels of estradiol are found in the very late follicular phase large follicles, which are preovulatory. Progesterone is found in fairly low levels, except in the late follicle. Follicles also contain measurable amounts of FSH, LH, and prolactin, always, of course, at concentrations lower than are found in the blood (41). FSH is found in all follicles that have started to form an antrum, while LH is seen only in follicles of very late phase. LH and progesterone appear together in these preovulatory follicles. Prolactin levels tend to fall with follicular maturation.

Thus, follicles have their own hormonal environment (Figure 5), and a mechanism appears to restrict diffusion of steroids from the follicular fluid into the blood, as can be seen from the extraordinarily high estrogen levels achieved. Movement of steroids into follicular fluid from nearby follicular structures is also restricted, as can be seen by the low progesterone levels detected in follicles during the luteal phase, in the presence of an ipsilateral corpus luteum (41).

Prostaglandins, which may be involved in ovulation, are also found in follicular fluid from preovulatory follicles, after the LH surge (22, 41).

Table 1 lists the non-steroid "novel" factors found in follicular fluid. Investigators seeking steroids and gonadotropin hormones in FF are also seeking local factors to account for the heterogeneity of morphology, secretory potential, and destiny of contiguous follicles within each ovary.

To understand the process by which a follicle becomes "chosen" for a given cycle, one must see what is different among follicles, rather than what is the same. While preferential vasculature has been one explanation of differences, local factors within the follicles probably play nivotal roles in differentiation.

**OOCYTE MATURATION INHIBITOR (OMI)**

After a variable number of years of rest, meiosis begins and continues to resume meiosis within an hour or so of reappearance in the blood of the LH surge (4). If an entire follicle is shelled out of the ovary before the LH surge has taken place, the oocyte remains arrested, unless the LH is placed within the explant medium. If an oocyte (with cumulus) is removed from a follicle and explanted, it undergoes meiosis without addition of hormones to the culture medium (56). This suggests that within the protected environment of the follicle, completion of meiosis is prevented until the LH preovulatory surge removes an inhibitor or renders it inactive.

Tsafriri and associates have shown that FF from several species contains a factor that can prevent oocyte maturation in vitro (56, 57, 58). The investigators named the factor Oocyte Maturation Inhibitor, or OMI. Since maturation is inhibited when oocytes are co-cultured with granulosa cells, granulosa cells appear to secrete OMI into the medium or into follicular fluid in situ. Small follicles seem to secrete more OMI than large follicles, and FF from small and medium-sized follicles contains more OMI activity than FF from large follicles. FF also inhibits progesterone secretion by cumulus-enclosed oocytes (9), presumably via OMI.

We have already seen that the oocyte is connected by processes to granulosa cells and the cumulus layer (26) (Figure 7). OMI may be delivered to such oocytes from the mass of granulosa cells in situ, so that when the oocyte-cumulus mass is cultured alone, lack of OMI delivery accounts for "spontaneous" maturation. The LH surge causes a break in connections between cumulus and oocyte, and thus may initiate meiosis by preventing OMI from reaching the oocyte (26).

Purification of OMI has been difficult, possibly because of the uncertainties of a bioassay system that depends on assessment of oocyte germinal vesicle breakdown. The OMI, a protein not a steroid, with a molecular weight between 1,000 and 10,000, has been identified in bovine, porcine, and human FF.

**LUTEINIZATION INHIBITOR AND STIMULATOR**

Luteinization is a process that converts the granulosa cell-theca unit remaining after ovulation to cells with 1) a large cytoplasm/nucleus ratio, 2) a profusion of LH receptors, and 3) the capacity to secrete high rates of progesterone. Among mammalian species, dependence of the corpus luteum on a particular luteotropic hormone to "rescue" it at the onset of pregnancy varies considerably, but all species appear to depend on progesterone from the corpus luteum to sustain pregnancy, at least at
the beginning. Whether a local signal or more than one local signal prevents luteinization of cells within the preovulatory follicle and then permits these cells to luteinize to form a corpus luteum is not known.

While luteinization depends on LH, FSH, and/or prolactin, or on estrogen (this varies with the species), it also may depend on intrinsic factors. If granulosa cells from large preovulatory follicles are cultured in vitro, they undergo "spontaneous" luteinization in the absence of added luteotropic signals like LH; on the other hand, granulosa cells from small follicles will not luteinize unless gonadotropins are added to the culture medium. These observations suggest that small follicles might contain a "luteinization stimulation" factor, whereas large follicles might contain a "luteinization inhibition" factor, whereas large follicles might contain a "luteinization stimulation" factor (12, 24, 37).

If FF from small antral follicles or from atretic large follicles is added in vitro to granulosa cells harvested from large follicles, it can prevent luteinization. If this luteinization inhibitor (LI) is added to granulosa cells from small follicles, it will prevent the induction of LH receptors by FSH that must precede luteinization. On the other hand, follicular fluid from large follicles can enhance luteinization and progesterone secretion in granulosa cells harvested from small follicles and tested in vitro. The fluid can also enhance the induction of LH receptors by FSH in these granulosa cells. The factor from large follicles has been named luteinization stimulator (LS). The immature follicles appear to contain a factor that inhibits luteinization (LI) and the accumulation of LH receptors. Follicles destined to become responsive to gonadotropins, and eventually to ovulate, acquire a higher ratio of LH receptors to LI; follicles destined to become atretic do not acquire LS in sufficient quantity. What controls the acquisition of these factors is not known at present, nor is their mechanism of action understood. Their identification has provided yet another set of local factors distinguishing contiguous follicles.

These factors have not been isolated. They appear not to be steroids, since charcoal-extraction does not remove activity. They both appear to have a molecular weight greater than 10,000.

FOLLICULOOSTATIN (OVARIAN INHIBIN)

Folliculostatin is a factor in follicular fluid that suppresses the rate of FSH secretion by the anterior pituitary gland. It was discovered simultaneously by investigators in two laboratories searching for such a factor for two different reasons. DeJong and co-workers (13) were searching for a new source of inhibin, and reasoned that granulosa cells were analogous to Sertoli cells, and thus might secrete an inhibin (24). At Northwestern University, we were looking for an ovarian feedback signal with pre-

Figure 8. Effect of porcine follicular fluid (charcoal extracted) on secretion of FSH by rat pituitary. In vitro study utilized a dispersed monolayer of pituitary cells, exposed to pFF for 48 hours. In vivo study utilized an acutely ovariectomized rat, injected intravenously with pFF and autopsied 5.5 hours post-injection. FSH was measured by RIA either in culture medium (in vitro) or in serum (in vivo). Porcine serum was used as control for pFF.

differential negative effects on FSH, since we had shown that estradiol negative feedback is much more effective on LH than on FSH (51).

Porcine follicular fluid that has been charcoal-extracted to remove steroids exerts suppression of FSH secretion into the plasma of rats in vivo (50), and also suppresses FSH secretion when applied to pituitary cells in vitro (12) (Figure 8). LH secretion is not inhibited in vivo (50) nor in vitro under basal conditions (with no added GnRH) (12, 28).

The FSH-inhibiting or suppressing follicular factor has been found in FF from bovine, sheep, porcine, equine, and human sources, and it suppresses FSH in rats, mice, horses, hamsters, and monkeys (49). About 2 hours are needed for it to suppress FSH significantly in vivo, and the effect of a single intravenous injection disappears about after 10 hours. It cannot block ovulation if given before the ovulatory surges of LH or FSH, but it can prevent growth of smaller follicles dependent on elevated FSH (30, 50). FF taken from small and medium porcine follicles contains a higher concentration of folliculostatin activity than FF from large follicles (39). FF from human follicles, taken during the follicular phase, has more folliculostatin activity than follicles sampled during the luteal phase (10).

Folliculostatin appears to be a protein, and is associated with a molecule (or complex) with a molecular weight over 70,000 (28, 33, 39). All its known characteristics suggest that it may be identical to testicular inhibin but more potent in FF than in testicular sources.
Unlike the other factors discussed (OMI, LS, and LI), folliculostatin must act via the bloodstream to suppress pituitary function, i.e., it must be a hormone. It is secreted by granulosa cells in culture (21); it may be found in ovarian vein blood of rats and monkeys (1,9,38). Until a more sensitive assay for its measurement is achieved, the verification of its hormonal status will be unclear. Whether folliculostatin also has an effect on the follicle itself is not known, since the FSH-suppressing activity is found in FF not only containing OMI, LS, and LI, but also showing some activities that alter receptor-binding of LH and FSH. Until these factors are isolated, their individual characteristics cannot be assessed. The high potency and specificity of folliculostatin in suppressing FSH but not pituitary LH secretion has focused much attention on its possible hormonal status.

**GONADOTROPIN BINDING INHIBITORS**

As indicated earlier, ovarian cell receptors are of central importance to LH and FSH in determining the ability of blood LH and FSH levels to influence selective follicles. Also, the induction of such receptors is an important method for controlling ovarian function. There appears to be another level of control of receptor function; substances have been identified in ovarian tissue extracts and FF that interfere with the binding of FSH and LH to their respective ovarian receptors.

Reichert and co-workers have identified a factor they call FSH binding inhibitor (FSHBI) in bovine FF and in human serum (11,45). The material is assessed by its ability to inhibit binding of radio-labeled FSH to granulosa cells in vitro. The concentration of FSHBI increases with increasing size of the bovine follicles from which FF is harvested. The inhibitor appears to be a peptide with a molecular weight of less than 5000, and may exist in several component molecular weight fractions.

Ward and associates have discovered a factor that inhibits the binding of LH to its ovarian receptors, and have designated the activity as LH receptor binding inhibitor (LHRBI) (59,60). The factor has been identified in corpora lutea from rat and porcine ovaries. Not only does it inhibit LH binding, but it also prevents LH-stimulated ovarian steroidogenesis. It is a peptide present in a non-dializable fraction (MW less than 10,000) and a dializable fraction (MW less than 3800). It appears not to bind to the same site on the LH receptor as LH itself (i.e., it is not a competitive binder). This factor has not been identified in FF, and unlike inhibin and GnRH, it has not been identified in testicular preparations. Reichert has identified a factor in bovine FF that stimulates LH binding (LHSA) (45).

**GnRH AND THE OVARY**

Perhaps the most unexpected findings relative to intra-ovarian peptides concern GnRH-related factors. The discovery that GnRH in humans and other species eventually caused ovarian and testicular factors (25) was at first ascribed to “down-regulation” of GnRH receptors in the anterior pituitary. However, GnRH was then shown to act locally on the ovary, preventing FSH-associated estradiol secretion and induction of LH receptors when placed in vitro with cultured granulosa cells (31). This action of GnRH can be blocked by specific antagonists of that peptide.

Simultaneous with the above observations was the discovery of specific receptors for GnRH in ovaries (and testes), which fits with the observation that GnRH can act on the ovary (31). Yet, the levels of GnRH in peripheral blood (outside the pituitary portal system) are not high enough to activate the gonadal GnRH receptors. This suggests that GnRH or a related peptide might be produced locally, and thus could modulate the effects of gonadotropins locally. Guillemin and co-workers have claimed that a GnRH-like peptide (called gonadocrinin) can be found in FF, and can stimulate LH and FSH secretion when applied to isolated pituitary cells (the basic bioassay for hypothalamic GnRH itself) (62). Whether gonadocrinin really exists or merely represents GnRH contamination is not clear. Gonadocrinin has not been shown to increase in medium when granulosa cells are incubated, as folliculostatin has been shown to be secreted.

Ying and Guillemin have also found a substance in FF that they have called “gonadostatin” (61). This factor inhibits the secretion of both LH and FSH by pituitary cell cultures that have been challenged with exogenous GnRH. This material appears to have a molecular weight below 10,000. In contrast, the activity we have identified as folliculostatin, or ovarian inhibin (Figure 8), has a molecular weight greater than 10,000 and specifically inhibits FSH secretion in basal pituitary cell culture. Since all of these activities are being detected within a common source, follicular fluid, various claims of different factors will not be resolved until some purification has been achieved.

**A SPECULATIVE VIEW OF THE ROLE OF PEPTIDE FACTORS IN THE LIFE HISTORY OF THE FOLLICLE**

Each follicle probably is initially provided with granulosa cells that have FSH receptors. These follicles may acquire circulating FSH and start granulosa cell mitosis. With mitosis and increasing numbers of cells, the follicle develops the capacity to synthesize estradiol. Increasing the estradiol enhances FSH effects, and the follicle grows further and antral fluid begins to collect.
At this stage, locally produced OMI continues to hold the oocyte, contained within the follicle, in the dictyate stage of meiosis. Local LI prevents FSH from inducing LH receptors prematurely until a critical follicle size is reached. The critical size may be signaled locally by the concentration within the FF of an unknown factor. The thecal cells have acquired LH receptors under the influence of estrogen and FSH. Follicular secretion of estrogen increases in proportion to numbers of granulosa cells, and folliculostatin secretion also increases. The folliculostatin is responsible for falling FSH values during the late follicular phase. Gradually, LI is lost and LS concentrations rise, permitting induction of LH receptors within the granulosa cells.

When estrogen levels are high enough to trigger the precipitous pre-ovulatory LH and FSH surges, these surges set into play a cascade of local events in the dominant follicle. LH immediately causes breaking of the cumulus cell-oocyte connections and meiosis resumes, since OMI is not delivered to the oocyte. FSH causes beginning dispersal of the cumulus cells around the oocyte. Intra-follicular proteases begin to decrease the strength of the follicle wall. The size of the antrum increases, perhaps because osmotic pressure increases, leading to enhanced water transfer from the blood. Blood vessels begin to invade the granulosa cell layers. Eventually, the follicle ruptures at the narrowest point, and the cumulus is delivered to the fimbriae, along with the oocyte, which by now has thrown off a polar body.

The corpus luteum that has been left behind luteinizes morphologically and enzymatically, and secretes progesterone under the influence of LH. If pregnancy occurs, it is maintained under the continuing influence of chorionic gonadotropin.

Ascribing a role for the LH and FSH receptor binding inhibitors is difficult. They may represent artificial pieces of membrane receptors that impede binding, or they may represent fail-safe mechanisms that are necessary to block hormone binding to receptors, if something goes wrong in the chosen follicle. One possibly adaptive contingency for the latter mechanism might be the occurrence of oocyte damage. The aborting of such a follicle by atresia would be highly adaptive.

The local production of GnRH-like molecules and of GnRH receptors may represent accidental and unused nuclear translation and transcription of a genetic message, or it may involve a local neural mechanism (43). There are suggestive reports that neural representation occurs for the ovary. Recent evidence of noradrenergic participation in gonadotropin transduction and steroidogenesis (52) suggests that the ovary may be subject to the influence of efferent neural input and may influence CNS function by a neural pathway back to the nervous system. The whole issue of peptidergic neurons and neural tissue as targets for pituitary hormones as well as for steroids suggests that more surprises could develop concerning ovarian regulation.

**CONTRACEPTIVE AND CLINICAL IMPLICATIONS**

The specificity of the factors discussed, and their presumed localization of action, suggest a number of new targets for contraception. If the LH surge were prevented from inducing withdrawal of OMI from the oocyte in the dominant follicle, all of the endocrine events of the cycle would occur, but the oocyte would not be viable. Injection of OMI in such a way as to reach the oocyte during the time when the surge is occurring could prevent resumption of meiosis.

The ovarian GnRH receptors are a prime target for contraception; they appear to be the conduit through which GnRH and its agonists can suppress steroidogenesis, ovulation, and corpus luteum function (25).

Such agonists working via the pituitary gland can also lead to indirect suppression of ovarian function, through down-regulation of GnRH receptors within the pituitary and subsequent failure of LH and FSH secretion. Antagonistic analogues of GnRH can also block pituitary gonadotropin secretion, leading to suppression of LH and FSH. Obviously, timed administration of GnRH agonists or antagonists can block ovulation, follicular development, or corpus luteum function, depending on when during the cycle administration occurs. The promising data showing that luteolysis can be induced by GnRH agonists, resulting in contraception, have been modulated by demonstrations that hCG from an intervening implantation may be able to rescue the corpus luteum (24). While at the present time, gonadal receptors have proven interesting possible targets for contraception, we do not know as yet what these receptors normally recognize as ligands in the in situ ovary. If there is a local release of GnRH-like peptides, exerting some hitherto unsuspected local regulation, there will undoubtedly be other contraceptive targets. At present, the GnRH receptors in the ovary have been indistinguishable from those in the pituitary, except in the numbers of receptors per cell. If there are differences in recognition sites, analogues might be synthesized that could selectively act as agonists or antagonists at the gonadal level but not at the pituitary level, or vice versa.

While GnRH agonists can cause luteolysis via LH withdrawal from normal corpora, it might be possible to prevent the induction of LH receptors in the first place. Luteinization inhibitor, if it could be isolated and purified, might enter the dominant follicle via the circulation, and permit an ovulatory response to the LH surge, but no luteinization. Conversely, an antibody to luteinization
stimulator might prevent LH receptors from being induced in growing follicles, blocking ovulation in response to an LH surge, or preventing corpus luteum maturation. If, as has been proposed, luteinization occurs with adequate LS/LI ratios, then atresia of the dominant follicle might be achieved by treatment altering the ratio of these putative follicular factors.

Clearly, a complex cascade of events lies between secretion of GnRH and action of LH and FSH on the ovary. Just as antagonists to the decapeptide can compete with GnRH on its pituitary or ovarian receptors, the receptor-binding inhibitors to LH and FSH could provide local interfering agents that would prevent gonadotropin regulation of ovarian function. If the events of the cycle were permitted to proceed normally, and then timed injections of such binding inhibition factors were carried out, LH or FSH could be prevented from exerting target effects.

Folliculostatin is the most specific suppressing agent for FSH that has been used. Injected into rats or cattle, it can prevent or delay follicular recruitment after ovulation (50). In monkeys it can suppress FSH secretion, and this material, if given during the follicular phase, can cause a short luteal phase, which can be overcome with hMG (14, 54). Theoretically, if injected during the elevated FSH peak that occurs in primates at the time of menses, the folliculostatin should prevent growth by inhibiting FSH. The rodent data suggest, however, that it might just delay the cycle, and after cessation of treatment, a rebound of FSH would occur (50).

Folliculostatin can prevent FSH secretion in male recipients (40), and could be used as a block for spermatogenesis. The possible advantage of folliculostatin over steroids as a pituitary inhibitor lies in the possibility that such proteinaceous materials may have fewer targets. However, the unexpected distribution of many peptide receptors in gut and brain should prevent indiscriminate use of “specific” peptides in the expectation that no other targets exist.

The factors under discussion could offer important insights in cases of ovarian failure as well. Women with secondary amenorrhea caused by follicular loss or absence show higher serum FSH values than do women with follicles; while serum LH values overlap, serum FSH levels are inevitably higher (27). Inadequate formation or maintenance of corpora lutea might result from local over-production of LI. At present, the factor influencing the control of synthesis and release of the local peptides is unknown, but such information is being sought.

**CONCLUSION**

Seldom has a field grown as fast as has the investigation of local ovarian peptides. This research interest can be viewed as a part of the broader hunt for peptide secretion in neural tissue, the GI tract, and the placenta. The new class of regulators has revolutionized our views of cell to cell communication in the nervous system, and may have the same effect on our concepts of ovarian physiology. Like the nervous system, the ovary has contiguous structures that must be kept separated from each other in function, and peptides may provide an important part of the localizing mechanism for both organs.
REFERENCES


SPERM ENZYME INHIBITORS FOR VAGINAL AND OTHER CONTRACEPTION

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Ever since scientists identified the ejaculate, and specifically spermatozoa, as the male factor that causes pregnancy, attempts have been made to prevent the fusion of the male and female gametes. At present, physical methods to prevent sperm migration include permanent or reversible obstruction of the vas deferens or the fallopian tube, covering the external cervical os with a cap or diaphragm, and the penile condom. Chemicals are used to interrupt the functional activity of the spermatozoa; vaginal contraceptives presently on the market employ spermicidal agents (primarily nonoxynol-9) that immobilize the spermatozoa by their surfactant activity. Immunologic research is aimed at developing systemic or local antibodies against spermatozoa, resulting in their agglutination and immobilization.

Today, the only nonsurgical sperm-directed techniques in clinical use are the spermicidal agents, the cervical diaphragm and cap, and the condom. Used in combination with each other, the diaphragm and spermicide have a mean use-effectiveness of 13 unwanted pregnancies per 100 woman-years, whereas spermicides alone have a mean use-effectiveness of 20 unwanted pregnancies per 100 woman-years (59). Vaginal contraceptives have a variable success rate, which is due in part to the human factor, that is, the need for the spermicidal agent to be deposited directly into the vagina shortly before each intercourse, and for the diaphragm (or cap) to be placed correctly over the cervix.

Today's vaginal contraceptives also have physiologic limitations, particularly if spermicides are used without a diaphragm. Spermatozoa can enter cervical mucus within 30 to 60 seconds after being deposited vaginally, which may not be enough time for the spermicide to contact the gametes. Additionally, human semen coagulates immediately upon ejaculation, trapping the spermatozoa. Surfactants, the most frequently used spermicides, may have difficulty passing through the coagulum and contacting spermatozoa. The coagulum is often located very close to the cervical os, so that as liquefaction occurs, the spermatozoa enter the cervical mucus immediately, without having contacted the spermicide.

The physiologic limitations of presently-marketed spermicides were shown by a primate study performed with one of the more effective vaginal contraceptive formulations on the market (76). Although the formulation was placed vaginally immediately before each coital act, half of the primates became pregnant within three to four menstrual cycles. Less than desirable contraceptive activity was also obtained in the rabbit with presently marketed vaginal contraceptives (9, 27, 46, 77).

Thus, the human factor alone apparently accounts only partially for the failure rate of vaginal contraceptives, and agents are needed that are more effective, either because they are more active spermicides, or because they prevent the functional activity of the spermatozoa by a different mechanism. Additionally, it would be beneficial if these agents could 1) readily pass into the seminal coagulum, and 2) enter cervical mucus, thereby hindering cervical passage of the spermatozoa.

During the last three decades, much has been learned about the biochemistry of the spermatozoon and the role of some of its chemical entities in the fertilization process. For instance, we know that certain sperm enzymes, several of which are specific to the spermatozoon, have a key role in the maintenance of sperm motility and in the ability of the spermatozoon to penetrate into and fuse with the egg. Inhibition of these enzymes will lead to infertility.
Utilizing this inhibition is an attractive approach to the development of new contraceptive agents, particularly if specific inhibitors can be developed. Such sperm enzyme inhibitors may be useful not only as vaginal contraceptives, but also for enhancing the effectiveness of the intrauterine device (IUD), or even as systemic contraceptive agents. Although the idea of using sperm enzyme inhibitors is by no means novel, and was already suggested in the 1940s, recent data show that this approach is feasible in developing a new class of compounds for contraceptive purposes.

FUNCTIONAL ACTIVITY OF THE SPERMATOZOOON IN FERTILIZATION

Morphologically, the spermatozoon consists of a head and a tail. The human sperm head measures approximately 2 x 3 x 5 μ, and the tail is about 45 μ in length. Most of the sperm head comprises a large nucleus that contains tightly packed chromosomal material (Figure 1).

A lysosome-like organelle, the acrosome, surrounds the anterior portion of the nucleus. An inner and outer acrosomal membrane limit the acrosome proper.

The first portion of the sperm tail is the midpiece, which contains a mitochondrial sheath surrounded by a small amount of cytoplasm. The mitochondrial sheath encircles the tail fibers (axonemal complex). The entire spermatozoon, including the midpiece cytoplasm and acrosome, is surrounded by a plasma membrane.

Basically, the spermatozoon possesses two enzyme systems that are essential for its activity. The first system is associated with the midpiece and tail, and includes the enzymes involved in the membrane transport of compounds, glycolysis, citric acid cycle, oxidative phosphorylation, and other metabolic activities. These processes serve primarily to generate the energy required for the sliding of the axonemal microtubules, that is, for sperm motility. Interference with the midpiece enzyme system results in sperm immobilization.

The second enzyme system is associated with the acrosome, and includes enzymes such as hyaluronidase and acrosin. The acrosomal enzymes have an essential role in the ability of a spermatozoon to fertilize the egg (38, 44).

At the time of fertilization, the egg is surrounded by several investments: 1) the follicle cell layer, consisting of the cumulus oophorus only, or the cumulus oophorus and corona radiata; and 2) the zona pellucida, a mucopoly-


Figure 2. Schematic representation of the fertilization process. The two round bodies between the zona pellucida and the egg are the polar bodies. (From Zaneveld LJD: The biology of human spermatozoa. In Wynn RM (ed): Obstetrics and Gynecology Annual, Vol 7. New York, Appleton-Century-Crofts, 1978, p 33)
gametes (28, 53). When the follicle cell layer is removed from mouse eggs, the hyaluronidase inhibitors no longer prevent fertilization. This is not the case with hamster gametes, since the one hyaluronidase inhibitor that was tested also blocked the penetration of spermatozoa through the zona pellucida (48). The inhibitor did not prevent sperm passage through the vitelline membrane, however.  

Table I lists, in chronologic order, various studies showing the in vivo and antifertility activity of synthetic hyaluronidase inhibitors. Already in the late 1940s and the 1950s, investigators were testing hyaluronidase inhibitors as contraceptives. Those inhibitors receiving the greatest attention were derivatives of hyaluronic acid and certain flavonoids, particularly phosphorylated hesperidin. In laboratory animals, the hyaluronidase inhibitors decreased the conception rate, either when added to spermatozoa before artificial insemination, or when placed vaginally before coitus.

A controversial study performed at that time with humans is of particular interest (62). Phosphorylated hesperidin was administered orally to both men and women of 300 married couples over a period of 3 to 30 months. Only two pregnancies occurred, owing to a failure to take the hyaluronidase inhibitor. No side effects were noted, and the contraceptive effect was completely reversed within 48 hours of halting inhibitor administration. However, other investigators were unable to confirm the antifertility activity of this hyaluronidase inhibitor when they administered it orally or intraperitoneally to rodents (Table I). Even though no argument was raised regarding the vaginal contraceptive potency of the inhibitors, further research was halted at that time, in part because of the controversy that had developed, and in part because of the advent of the steroidal contraceptive techniques.

During the past decade, two types of antihyaluronidases have been evaluated for their contraceptive activity: hyaluronidase antibodies, and chemical inhibitors. Antibodies prepared against sperm hyaluronidase inhibit the lytic activity of this enzyme toward synthetic substances and the follicle cell layer (41, 78), and can prevent the in vitro fertilization of spermatozoa (14). Since the hyaluronidases of the spermatozoa and testis are identical, and sperm hyaluronidase appears to be specific to the male genital tract, that is, it differs from that of other tissues and organs (41, 78), these antibodies should not crossreact with other tissues. Thus, an immunologic approach with sperm hyaluronidase as antigen could be applied to both men and women to prevent conception, without causing unwarranted side effects.

In vivo antifertility studies need to be performed after immunization with pure hyaluronidase to ensure that hyaluronidase antibodies can enter the genital tract in
high enough concentrations to form a complex with the hyaluronidase of the spermatozoa and prevent fertilization. Such studies appear to have been attempted by only one group of investigators so far (7). Using purified bovine testicular hyaluronidase as antigen, infertility could not be induced in female rabbits, but a significant decrease in sperm count occurred in male rabbits on immunization. The effect was completely reversible, and the males became fertile 9 to 15 weeks after the last antigenic stimulus. The effectiveness of the antibodies in the male rabbit is somewhat surprising, since bull and rabbit hyaluronidase crossreact poorly, if at all (78). It is of interest that a number of women with unexplained infertility possess antibodies against sperm hyaluronidase, in addition to the usual sperm agglutinating and sperm immobilizing antibodies (41). The ability of chemical hyaluronidase inhibitors to prevent fertilization was confirmed recently (29, 30). Phosphorylated hesperidin and PSs 3 both prevented conception on vaginal placement in rabbits before coitus (Table 1). These results, as well as those reported in the 1940s and 1950s, encouraged us to perform some detailed studies evaluating the practical use of the hyaluronidase inhibitors as

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*Hydroquinone-sulfonic acid formaldehyde polymer.

Table 1. *In vivo* antifertility activity of synthetic hyaluronidase inhibitors.
contraceptives. Little was known about the toxicity of the compounds that had been used so far, but we felt that agents could be found that were inhibitory toward hyaluronidase, and were already used clinically as non-contraceptive pharmaceuticals. Since such compounds are already on the market, their side effects should be minimal and, if they are contraceptive, they should be rapidly available for clinical use. This approach appeared realistic when we found that myocrisin (disodium aurothiomalate), an anti-inflammatory agent, prevented the penetration of mouse and hamster spermatozoa through the layers surrounding the egg (48, 53).

A number of marketed compounds were screened for their ability to inhibit hyaluronidase. Of these, five were effective (Table 2). Their enzyme inhibitory activity toward some typical acrosomal enzymes was established and, with one exception, all were specific for hyaluronidase. The antifertility activity of these compounds was tested by mixing them with capacitated mouse spermatozoa before adding the treated spermatozoa to oocytes in vitro. All the compounds, except for one, prevented fertilization (Table 2). No effect on sperm motility or forward progression was noted at the concentration of the inhibitors used. Several of the inhibitors, again at concentrations that did not affect sperm motility, were also contraceptive when placed vaginally in rabbits before coitus (Table 2). Phenylbutazone and oxyphenbutazone, which are presently both used clinically as anti-inflammatory agents, were particularly effective even when used at concentrations as low as 0.02 mg/ml and 0.1 mg/ml, respectively.

These results are encouraging, especially considering that under exactly the same conditions in rabbits, presently marketed vaginal contraceptives were reported to reduce the conception rate only partially, usually by only 50% to 70% or less (9, 27, 46, 72, 77). Several of these vaginal contraceptives contain nonoxynol-9 at concentrations of approximately 50 mg/ml. Thus, at least in the rabbit, phenylbutazone and oxyphenbutazone appear to be several orders of magnitude more potent than nonoxynol-9, the spermicide most frequently used in present-day vaginal contraceptives.

### ACROSIN INHIBITORS

#### Vaginal Contraceptive Action.

Acrosin, a sperm proteinase, is probably involved in several aspects of the fertilization process. Inhibitors of this enzyme were reported to prevent the sperm acrosome reaction, sperm-binding to the zona pellucida, and sperm passage through the zona pellucida and the vitelline membrane (40, 56, 57, 64, 73). Thus, treatment of spermatozoa with acrosin inhibitors prevents their fertilizing capacity.

Acrosin has received a significant amount of attention during the past decade, and its properties have been fairly well characterized (6, 38, 45). As with other proteinases, the enzyme can be inhibited by both naturally occurring and synthetic agents. One naturally occurring acrosin inhibitor is present in seminal plasma and can, on addition to capacitated rabbit spermatozoa, prevent their in vivo fertilization when the treated spermatozoa are inseminated into the oviduct (80). Since this inhibitor is naturally

<table>
<thead>
<tr>
<th>COMPOUND (COMPANY)</th>
<th>ENZYME</th>
<th>Hyaluronidase</th>
<th>Acrosin</th>
<th>β-glucuronidase</th>
<th>N-acetyl glucosaminidase</th>
<th>β-galactosidase</th>
<th>ANTI-FERTILITY ACTIVITY IN VITRO</th>
<th>ANTI-FERTILITY ACTIVITY ON VAGINAL PLACEMENT IN RABBITS</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYOCRISIN (MERCK, SHARP &amp; DOHME)</td>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>±</td>
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<tr>
<td>FENOPROFEN Ca++ (LILLY)</td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PENICILLAMINE (MERCK, SHARP &amp; DOHME)</td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>OXYPHEN- BUTAZONE (CIBA-GEIGY)</td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PHENYL- BUTAZONE (CIBA-GEIGY)</td>
<td></td>
<td>+</td>
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<td>+</td>
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</tbody>
</table>

Table 2. Enzyme inhibitory spectrum and antifertility activity of certain pharmaceutical agents that inhibit hyaluronidase.
present, it must be of low toxicity, and would be an attractive contraceptive agent. However, the seminal inhibitor is mostly removed from the spermatozoa during capacitation and/or the acrosome reaction (24, 81). Therefore, it cannot be used as a vaginal contraceptive, but it may be potentially useful as an agent that can be released from an IUD to enhance the effectiveness of this device (see next section).

The same type of reversible binding to spermatozoa probably occurs with other naturally occurring proteinase inhibitors. A number of these inhibitors, particularly the Kunitz pancreatic trypsin inhibitor (Trasylol), were shown to be contraceptive in *in vitro* fertilization systems, or when mixed with spermatozoa before the treated gametes were inseminated into oviducts (74). However, they do not appear practical as contraceptive agents, unless they are released directly into the uterus or fallopian tubes, or are administered systemically at high concentrations (see further). For example, Schumacher and associates (58), treating rabbit spermatozoa with soybean trypsin inhibitor or Trasylol (4 to 22.5 mg/ml) prior to insemination into the vagina, noted no effect on conception. Similarly, 0.9 mg/ml of antipain, a bacterial proteinase inhibitor, placed in the vagina of primates before coitus, did not cause a significant reduction in conception (75).

In contrast to the naturally occurring inhibitors, certain synthetic agents can bind irreversibly or pseudo-irreversibly to proteinases, and should have more immediate clinical potential as contraceptives. A number of synthetic acrosin inhibitors have been tested for their ability to prevent conception *in vivo* (Table 3). Of these, N-[p-tosyl-L-lysine chloromethyl ketone (TLCK) and p-nitrophenyl-p'-guanidinobenzoate (NPGB) were particularly potent when tested in rodents and rabbits. After vaginal application to primates, NPGB (at 0.09 mg/ml) was much more effective than TLCK (10 mg/ml), and equally or slightly more effective than Delfen Vaginal Cream (Ortho Pharmaceutical Co.) in preventing pregnancy (75).

Although these results showed clearly that the vaginal placement of acrosin inhibitors prevents or decreases conception, a problem was foreseen with their clinical applicability. For instance, TLCK is an alkylating agent (potentially carcinogenic), and NPGB releases nitrophenol when it reacts with acrosin. For the acrosin inhibitors to be practical, the compounds must be of low toxicity.

Hall and co-workers synthesized N-carbobenzoxy amino acid esters that showed contraceptive activity when placed vaginally in mice (26). The N-carbobenzoxyglycine vinyl ester was particularly potent and had a low acute toxicity with an LD50 > 500 mg/kg (the LD50 is the dose at which half of the animals die immediately when a compound is administered). Subsequently, these investigators prepared a number of N-protected glycine activated esters, all of which decreased fertilization when placed vaginally at 10 mg/kg/day for 4 weeks in mice (13). N-carbobenzoxyglycine vinyl ester, and N-carbobenzoxyglycine 1,2-dibromoethyl ester, were most effective, resulting in 0% fertilization rates. The acrosin inhibitory activity of the compounds correlated positively with the vaginal contraceptive potency.

A different approach was used by our laboratory. Since NPGB was an effective contraceptive agent with an LD50 of 180 mg/kg in mice (75), but it released nitrophenol, we replaced the nitrophenol by a phenol already on the market and approved by the U.S. Food and Drug Administration for human use. The guanidinobenzoic acid portion of the molecule is probably of low toxicity, and similar compounds have been used clinically. Nine such phenol derivatives of guanidinobenzoic acid (arylguanidinobenzoates) were synthesized (31) (Table 4). All of them were found to be potent inhibitors of human acrosin with IC50 values (the concentration at which half of the enzyme is inhibited) of 10-7 to 10-10 M.

The arylguanidinobenzoates blocked fertilization at concentrations that did not affect sperm motility when added to capacitated mouse spermatozoa before the treated gametes were mixed with eggs in *vitro*. Subsequently, the *in vivo* contraceptive efficacy of the inhibitors was screened by placing them at 0.1 mg/ml concentrations in the vagina of rabbits before coitus (Table 4). All compounds except two inhibited fertilization. The arylguanidinobenzoates containing the phenols acetoinophen, ethylparaben, and methylsalicylate were most active. Similar to the hyaluronidase inhibitors, these arylguanidinobenzoates appear to be orders of magnitude more potent than nonoxynol-9 (see section on Hyaluronidase Inhibitors).

Since none of the acrosin inhibitors had ever been tested to determine if they could prevent the fertilization of human spermatozoa, we mixed the arylguanidinobenzoates (at 10-4 to 10-6 M) with human spermatozoa before the gametes were capacitated and added to denuded hamster oocytes, to assess their fertilizing capacity (69). No effect on sperm motility was observed, and all compounds tested decreased the fertilizing capacity of the human spermatozoa. Thus, inhibitors of acrosin are also contraceptive toward human gametes.

Toxicologic studies with the arylguanidinobenzoates are presently in progress in our laboratory. Acute studies in mice and rats showed that the compounds are of very low toxicity in regard to the amount needed for contraception. The LD50 of the compounds, on intraperitoneal injection, varied in most cases from 500 to 1000 mg/kg and was occasionally even higher. By comparison, nonoxynol-9, when tested for its acute toxicity in our laboratory under...
<table>
<thead>
<tr>
<th>INHIBITOR*</th>
<th>STUDY</th>
<th>TEST METHOD</th>
<th>SIGNIFICANT OBSERVATIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLCK, TPCK, NPGB, EPGB</td>
<td>Zaneveld et al., 1970 (79)</td>
<td>Capacitation assay; inhibitors added to ejaculated rabbit spermatozoa before uterine insemination; or vaginal application of inhibitors to rabbits before coitus</td>
<td>TLCK but not TPCK (5 to 15 μg/10^9 sperm), blocked fertilizing ability of capacitated spermatozoa; TLCK and NPGB but not EPGB (10 μg/10^9 sperm) decreased fertilization when added to ejaculated spermatozoa before uterine insemination; TLCK (3 mg/ml in K-Y jelly) inhibited conception when deposited vaginally before coitus</td>
</tr>
<tr>
<td>TLCK</td>
<td>Zaneveld et al., 1971 (77)</td>
<td>Vaginal application to rabbits before coitus</td>
<td>Mixing TLCK (5 mg/ml) with Delfen Vaginal Cream (Ortho Pharm. Co., containing 50 mg/ml nonoxynol-9) greatly increased the contraceptive properties of Delfen</td>
</tr>
<tr>
<td>TLCK, GPB, APB, NPGB, L-arginine, poly-L-lysine, benzamidine</td>
<td>Newell et al., 1972 (46)</td>
<td>Inhibitors applied vaginally to rabbits before coitus</td>
<td>TLCK (3 mg/ml in K-Y jelly) depressed fertilization to a larger extent than GPB, APB, or L-arginine; a mixture of TLCK, GPB, APB, NPGB, poly-L-lysine, benzamidine, and L-arginine (each 2 to 3 mg/ml in K-Y jelly) possessed greatest antifertility activity; addition of TLCK (3 mg/ml) and GPB (2 mg/ml) to Delfen Vaginal Cream enhanced the contraceptive activity of Delfen</td>
</tr>
<tr>
<td>TLCK, TPCK</td>
<td>Miyamoto &amp; Chang, 1973 (42)</td>
<td>Uterine insemination of inhibitor-treated ejaculated hamster spermatozoa</td>
<td>Both inhibitors significantly decreased the fertilizing capacity of spermatozoa at concentrations of 0.003% to 0.005% with only slight impairment of motility. No effect on fetal weight or other anatomic aspect of the fetus was noted</td>
</tr>
<tr>
<td>TLCK, TPCK</td>
<td>Dabich &amp; Andary, 1974 (11)</td>
<td>Uterine injections or slow release of inhibitor from capsules placed in uterus in mice 2 to 4 days after coitus</td>
<td>Both inhibitors (3 mg/ml) prevented blastocyst implantation when added to uterus; combined capsule and injection was the most effective mode of administration</td>
</tr>
<tr>
<td>NPGB, TLCK</td>
<td>Zaneveld et al., 1979 (75)</td>
<td>Vaginal application of inhibitors to stump-tailed macaques (Macaca arctoides) before coitus</td>
<td>In K-Y jelly, NPGB (0.09 mg/ml) possessed significantly higher contraceptive effect than TLCK (10 mg/ml) and was equally as effective as Delfen Vaginal Cream</td>
</tr>
<tr>
<td>Activated N-carbobenzoxy amino acid esters, TLCK, TPCK</td>
<td>Hall et al., 1979 (26)</td>
<td>Vaginal application or intraperitoneal injection into mice</td>
<td>Most compounds (10 mg/kg/day) decreased or completely inhibited fertilization. The carbobenzoxy-glycine, -leucine, and -proline esters were most effective. TLCK and TPCK also inhibited conception</td>
</tr>
<tr>
<td>*TLCK, NPGB</td>
<td>Joyce et al., 1979 (29)</td>
<td>Rabbit spermatozoa treated with inhibitors before vaginal insemination, or the vaginal application of inhibitors to rabbits before coitus</td>
<td>The inhibitors effectively prevented fertilization in both systems (NPGB at 100 μg/ml; TLCK at 200 μg/ml)</td>
</tr>
<tr>
<td>N-protected glycine activated esters, TLCK, TPCK</td>
<td>Drew et al., 1981 (13)</td>
<td>Vaginal application or intraperitoneal injection into mice</td>
<td>At 10 mg/kg/day, N-carbobenzoxy-vinyl ester and N-carbobenzoxy-glycine 1,2-dibromoethyl ester completely prevented pregnancy when applied vaginally. All other esters also showed contraceptive activity. The compounds were less active when applied intraperitoneally. Acrosin inhibitory activity of the esters correlated with their vaginal contraceptive potency. TLCK and TPCK prevented fertilization when placed vaginally</td>
</tr>
<tr>
<td>Benzamidine, aminobenzamidine, NPGB, MUGB</td>
<td>Beyler &amp; Zaneveld, 1982 (5)</td>
<td>Release from subcutaneously placed minipumps in mice before blastocyst implantation</td>
<td>Aminobenzamidine (97.1 mg/lg/day). NPGB (0.8 mg/kg/day) and MUGB (1.0 mg/kg/day) caused a 50% decrease in the fertilization rate. Benzamidine had no effect</td>
</tr>
<tr>
<td>Phenol derivatives of guanidino benzoic acid (arylguanidinobenzoates)</td>
<td>Kaminski et al., 1981 (31)</td>
<td>Vaginal insemination into rabbits</td>
<td>A number of compounds possessed high contraceptive activity at 0.1 mg/ml. (see Table 4)</td>
</tr>
</tbody>
</table>

Table 3. In vivo antifertility activity of synthetic acrosin inhibitors.
exactly the same conditions, had an LD_{50} of 180 mg/kg, worse than that of any of the arylguanidinobenzoates.

In regard to acrosin inhibition and vaginal contraception, it is also worthwhile to consider certain ions, particularly zinc. Most of the acrosin present on ejaculated spermatozoa is in a zymogen form, called proacrosin. Proacrosin conversion to acrosin is probably a necessary step for the fertilization process. At least in some species, acrosin activity and proacrosin conversion can be inhibited by zinc ions. Zinc ions also inhibit the acrosome reaction of hamster spermatozoa (40). Deposition of certain zinc salts in the vagina of rabbits before coitus decreases their fertilization rate (72). Such zinc salts also enhance the effectiveness of presently marketed vaginal contraceptives, at least in the rabbit (72).

**Action in Association with an Intrauterine Device (IUD).** Besides being useful for vaginal contraception, acrosin inhibitors may also be employed in conjunction with the IUD. The IUD causes several undesirable side effects, among them menorrhagia, dysmenorrhea, and pelvic inflammatory disease (PID). These side effects are due at least in part to the shape of the IUD, which makes the device irritating to the endometrium. However, such irritation is presently necessary for the IUD to be effective, since the device probably acts primarily by inducing a chronic inflammatory response of the endometrium, so that blastocyst implantation is prevented. One could decrease the irritating properties of the IUD by altering its design so that it fits the uterus better, while retaining its contraceptive activity by releasing antifertility agents. For instance, the release of copper from the IUD enhances its contraceptive effectiveness. Other agents, including the enzyme inhibitors, are likely to be much more potent than copper in preventing conception.

### PHENOL MOIETY OF THE ARYLGUANIDINOBENZOATE* | PHARMACOLOGIC ACTIVITY OF PHENOL | CONTRACEPTIVE POTENCY OF PHENOL WHEN PLACED VAGINALLY IN RABBITS BEFORE COITUS (0.1 mg/ml)†
--- | --- | ---
ACETOMINOPHEN | Analgesic, antipyretic | ++++
ETHYLPARABEN | Preservative | +++
METHYLPARABEN | Preservative | ++
SALICYLAMIDE | Analgesic | 0
8-HYDROXYQUINOLINE | Antimicrobial | ++
METHYLSALICYLATE | Counter-irritant, flavoring agent | +++
EUGENOL | Dental obtundant, topical anesthetic | ++
HEXYLRESORCINOL | Antiseptic | ++
THYMOL | Antibacterial, antifungal | 0

*All the arylguanidinobenzoates are potent inhibitors of human acrosin (31).
† ++++ = highly contraceptive (0-10% fertilization)
+++ = contraceptive (10-30% fertilization)
++ = not contraceptive (80-100% fertilization)

Table 4. Phenol derivatives of guanidinobenzoic acid (arylguanidinobenzoates) as vaginal contraceptives in rabbits.

The use of acrosin inhibitors to enhance the contraceptive potency and to decrease the side effects of the IUD is particularly attractive for three reasons. First, the agents are potent inhibitors of the sperm acrosome reaction and fertilization. Second, certain acrosin inhibitors can prevent blastocyst implantation, further assuring the contraceptive activity of the inhibitors when released in utero (11) (implantation appears to involve a blastocyst proteinase that has a number of properties in common with acrosin, and can be inhibited by similar agents) (12). Third, some side effects of the IUD can be minimized if certain antifibrinolytic agents (inhibitors of plasminogen activation, and/or plasmin) are deposited into the uterus (61, 67).

The IUD appears to cause an activation of fibrinolytic activity in the uterus (60), which in turn results in decreased clot formation and enhanced menstrual flow and pain. When injected into the uterus, the Kunitz pancreatic trypsin inhibitor (Trasylol) is particularly effective in reducing such pain and bleeding (67). Release of antifibrinolytic agents from IUDs in women reduces the excessive menstrual blood flow to approximately physiologic levels (51, 66). Thus, release of certain proteinase inhibitors from the IUD may not only increase the effectiveness of this device, but should also prevent some of the side effects commonly associated with IUD use, particularly if the shape of the IUD can be altered so that it is less irritating to the uterus.

Since the three enzymes (acrosin, the blastocyst proteinase, and plasmin) have a number of properties in common, and can be inhibited by similar agents, it is very likely that inhibitors of acrosin and the blastocyst proteinase can be used that also have antifibrinolytic activity. For instance, Trasylol is not only an antifibrinolytic agent

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but is also a potent inhibitor of the blastocyst proteinase and acrosin, and prevents fertilization when mixed with spermatozoa before oviductal insemination (80). Since NPGB inhibits acrosin, plasmin, and the blastocyst proteinase, it is likely that the arylguanidinobenzoates synthesized by our laboratory will be useful in conjunction with the IUD.

Several patents were recently awarded to Eli Lilly Co. (U.S.) for the controlled release of certain acrosin inhibitors from silicone into the uterus (U.S. patents 4264-575, 4264-576, 4264-577, 4264-578). The compounds covered included sulfonate salts of the formula \( RSO_3M \) (where \( R \) is a cation), sulfoalkyl alkanoate salts of the formula \( RCOO(CH_2)_nSO_3M \), salts of the formula \( ROSO_3M \), and sterol sulfates derived from cholesterol, campesterol, \( \beta \)-sitosterol, lanosterol, erosterol, dehydrocholesterol or \( \beta \)-hydroxycholesterol acid.

**Systemic Action.** Acrosin inhibitors may also be applied systemically to induce infertility. Long-term intraperitoneal injection of \( N \)-carbobenzyloxy amino acids, particularly \( N \)-protected glycine activated esters (10 mg/kg/day), into female mice decreases the fertilization rate by about 50%. This finding was especially encouraging because NPGB and MUGB were applied at different time points to mice also decreases conception (32), but no antifertility activity was observed when the Kunitz pancreatic trypsin inhibitor (Trasylol) was administered systemically to rabbits and mice (6 to 20 mg/kg/day) (58).

When agents are administered for prolonged periods of time, one cannot differentiate whether the inhibitors prevent fertilization, implantation, or fetal development. In order to determine if systemically administered acrosin inhibitors can specifically prevent the processes that occur before implantation, we released benzamidine (88.6 mg/kg/day), aminobenzamidine (97.1 mg/kg/day), NPGB (0.8 mg/kg/day) and the arylguanidinobenzoate containing methyllumbelliferone as phenol (MUGB) (1.0 mg/kg/day) from subcutaneously implanted minipumps in mice during the preimplantation period only (5). Except for benzamidine, all three inhibitors decreased the fertilization rate by about 50%. This finding was especially encouraging because NPGB and MUGB were applied at low concentrations.

The systemic application of inhibitors needs to be studied in greater detail, however, before it can be used clinically. Preferably, the inhibitors should be specific for acrosin. Since acrosin is biochemically and immunologically different from trypsin, the enzyme most similar to acrosin (6, 45), this should be possible. However, until now, only certain monosaccharides have been found to selectively inhibit acrosin rather than trypsin (2).

Antibodies prepared against acrosin inhibit the activity of this enzyme toward high molecular weight substrates, and do not crossreact with trypsin, that is, they appear to be specific (6, 45). Such antibodies may be useful from a contraceptive standpoint. Antibody studies have been complicated, because of difficulty in preparing enough pure acrosin as antigen so that high antibody titers can be produced. Some data are available, however, showing that rabbits immunized with acrosin and possessing high antibody titers are not likely to become pregnant when mated, whereas fertility was not prevented when the rabbits showed lower acrosin antibody titers (65). Also, treatment of capacitated rabbit spermatozooa with anti-acrosin immunoglobulins before tubal insemination of the gametes renders the rabbits infertile (15).

**GOSSYPOL**

Gossypol, a disesquiterpene aldehyde, obtained primarily from the cotton plant, is an antispermatogenic agent when given orally to men and to several other animal species. This was first established in China (3), and has since been confirmed by others. The clinical use of gossypol has been argued, however, because of the possible toxicity of this compound. One of the first effects noted after administration of gossypol is a decrease in sperm motility. For this reason, our laboratory and other research groups investigated the spermicidal activity of gossypol. When it is mixed with polyvinylpyrrolidone (PVP), which solubilizes gossypol, or even in the absence of PVP, gossypol has in vitro spermicidal activity approaching that of existing vaginal contraceptive formulations (50, 54, 70). When placed vaginally in primates before coitus, gossypol-PVP causes a dose-dependent decrease in the motility of the spermatozoa (8). The antimotility effect of gossypol is caused possibly by its ability to inhibit a number of midpiece metabolic enzymes, such as lactate dehydrogenase and several enzymes involved in oxidative phosphorylation (1, 37, 43, 50).

Its spermicidal activity makes gossypol potentially useful as a vaginal contraceptive; however, its potency may not be high enough to compete with presently marketed vaginal contraceptives. Thus, based strictly on its spermicidal properties, gossypol would probably be useful only in those parts of the world that do not have ready access to marketed formulations. We therefore initiated a study to see if low, nonspermicidal dose levels of gossypol could prevent fertilization. Preliminary experiments by Williams in rabbits had shown that such low levels of gossypol were contraceptive when placed vaginally before coitus (72). The effect of gossypol on the fertilizing capacity of human spermatozoa was evaluated by mixing low amounts of the compound with whole semen for 5 minutes, removing the seminal plasma and excess gossypol by washing the spermatozoa, capacitating the gametes in medium, and adding them to hamster oocytes from which the outer investments had been
removed. At concentrations ranging from 5.5 to 16.5 \(\mu g/ml\) semen, gossypol showed no effect on sperm motility, but a dose-dependent inhibition of fertilization took place (33). Additionally, there was a parallel, dose-dependent decrease in the ability of proacrosin (the zymogen form of acrosin that is prevalent in ejaculated spermatozoa) to convert to acrosin. Apparently, the antifertility mechanism of gossypol at low, non-stermicidal dose levels is due in part to its ability to prevent proacrosin activation.

Vaginal contraceptive experiments in rabbits, using low gossypol doses, are presently in progress, and appear to confirm the results of Williams (72). Thus, gossypol may have potential from a vaginal contraceptive standpoint. If its vaginal absorption is also low, toxic effects should be minimal or nonexistent.

**LACTATE DEHYDROGENASE-X ANTIBODIES**

Lactate dehydrogenase (LDH) is one of the metabolic enzymes of the sperm midpiece. The testis and spermatozoa possess a form of this enzyme (LDH-X or LDH-C4) that is specific to the male genital tract. In the female, LDH-X is absent from all tissues, including those of the reproductive tract (34). LDH-X antibodies do not bind to LDH-1 or LDH-5, and do not inhibit these isozymes, but they do inhibit the activity of LDH-X (19). Immunologically, LDH-X is attractive for the development of a contraceptive method that is specifically directed toward spermatozoa, without affecting any other tissues. The reality of this approach was shown by Goldberg and coworkers, who immunized female mice, rabbits, and primates with LDH-X and noted a significant decrease in their conception rate (20, 23, 34). However, the passive immunization with LDH-X had no effect on the fertility of female mice (17). Immunization of males with LDH-X also had contraceptive effects, at least in the rabbit (22) and the guinea pig (71).

As with acrosin and hyaluronidase, use of LDH-X as an antigen for contraceptive purposes poses problems of both supply and homogeneity. These limitations can be overcome if one or more peptides can be obtained that are small enough for chemical synthesis and have antibodies that cross-react with the native antigens. Such peptides have been prepared from LDH-X (21) and their antibodies were shown not only to cross-react with LDH-X but also to prevent the fertilization of mouse gametes (18).

**OTHER ENZYME INHIBITORS**

For successful fertilization, spermatozoa require the activity of a number of other enzymes not yet discussed (6, 38, 40, 44, 56). Inhibition of these enzymes would lead to infertility. For instance, numerous compounds can inhibit the glycolytic and respiratory enzymes of the midpiece, thus inducing sperm immobilization. Many respiratory enzyme inhibitors are available, and a number of these have been shown to prevent sperm motility, including hydrazine, hydroxyl amine, sodium azide, cyanide, carbon monoxide, and malonate. Inhibitors of glycolytic enzymes that were shown to prevent sperm motility include iodoacetate, iodosobenzoate, iodoacetamide, p-chloromercuribenzoate, hydrogen peroxide, phenylmercuric acetate, p-carboxyphenoxyarsine and the metal ions copper, cadmium, mercury, lead, sodium arsenite, and selenite. Several of these enzyme inhibitors are already used in presently marketed vaginal contraceptives; however, they are rarely the primary ingredient. Most are too toxic for clinical use, because they affect the metabolic processes of other tissue cells, as well as those of spermatozoa.

The response of spermatozoa to these midpiece enzyme inhibitors varies tremendously among different species, and differs also according to the genital tract origin of the spermatozoa tested, that is, from the epididymis or the ejaculate. If a compound inhibits the motility of spermatozoa of one species we cannot assume that it will inhibit the motility of spermatozoa of another. Before we can conclude that such compounds may be of clinical use, their immobilizing activity toward human spermatozoa needs to be investigated.

The inhibition of several acrosomal enzymes besides acrosin and hyaluronidase can prevent capacitation and/or the acrosome reaction, and thus fertilization. For instance, glycosidase inhibitors were reported to inhibit the capacitation of hamster spermatozoa (25) although in our laboratory, the addition of \(\beta\)-glucuronidase and N-acetylglucosaminidase inhibitors to capacitated mouse spermatozoa did not prevent their in vitro fertilizing capacity (28). A phospholipase inhibitor, p-bromophenacylbro- mide, inhibits the acrosome reaction of hamster spermatozoa (35). The acrosome reaction is also prevented by inhibitors of enzymes involved in protein phospholipid transmethylation (39). So far, no in vivo contraceptive studies have been performed with these inhibitors. Certain seminal plasma components that have enzyme inhibitory properties can prevent the fertilizing capacity of spermatozoa. One of these is an acrosin (proteinase) inhibitor (see Acrosin Inhibitors). Another is a glycoprotein with a molecular weight varying from 200,000 to 370,000, depending on the species (16, 52). This glycoprotein is often called “decapacitation factor,” because it
renders capacitated spermatozoa infertile. The rabbit glycoprotein can be broken down to low molecular weight peptides (containing about 10 amino acids) that retain their antifertility activity (38, 55). The exact mechanism of action of the glycoprotein is not known, but the glycoprotein prevents penetration of spermatozoa through the investments surrounding the egg (52) either by inhibiting the acrosome reaction (16), by inhibiting an acrosomal hydrolase called "corona penetrating enzyme" (CPE) (82), or by an as yet undiscovered mechanism.

Another glycoprotein (fetuin) present in serum also inhibits CPE and prevents fertilization when added to capacitated rabbit spermatozoa (63). Such naturally occurring factors, particularly if active in a low molecular weight, synthesizable form, are interesting, because they should be of low toxicity. However, at least the glycoprotein from seminal plasma appears to be removed from the spermatozoa during capacitation, so that the compound will probably not be useful as a vaginal contraceptive, unless an irreversibly binding form can be produced.

"Suicide" inhibitors, which become inactive after they react with a specific sperm enzyme, can also be developed. The phenol derivatives of guanidinobenzoic acid (arylguanidinobenzoates) that inhibit acrosin are an example. As vaginal contraceptives, the enzyme inhibitors can be used either alone or in combination with spermicides, so that the benefit of both activities can be obtained. Some acrosomal enzyme inhibitors are also spermicidal, and may be dually active as spermicides and antifertilization agents. Since several of the hyaluronidase and acrosin inhibitors have already been shown to be more effective and less toxic than nonoxynol-9, one can expect that enzyme inhibitors will appear on the market in vaginal contraceptive preparations in due time.

Enzyme inhibitors can also be used to enhance IUD effectiveness. Although the IUD already has a very low failure rate, it causes menorrhagia, dysmenorrhea, and pelvic inflammatory disease. Some of these side effects can be minimized by designing IUDs with shapes that are less irritating to the endometrium, but retain their contraceptive potency by releasing enzyme inhibitors. Additionally, certain acrosin and/or fibrinolytic inhibitors are also capable of decreasing the menorrhagia and possibly the dysmenorrhea associated with IUD use.

Enzyme inhibitors can be employed systemically to prevent conception. At present, no good chemical inhibitors are available for this purpose, because none is as yet specific enough toward spermatozoa; thus, they also inhibit certain enzymes from other tissues. However, the immunologic approach seems feasible. In the past, immunizing animals with testis, testicular extracts, spermatozoa, and sperm extracts, has resulted in some degree of infertility. However, such a heterogeneous antigen preparation is much less attractive from a clinical standpoint than a homogeneous preparation consisting of a sperm-specific enzyme, such as lactate dehydrogenase, acrosin, or hyaluronidase. In this regard, the results with LDH-X have been very encouraging.

The research so far has focused on only a very small number of the possible enzymes that can be inhibited to prevent fertilization, primarily because we know too little about the biochemical properties of the sperm enzymes and their role in the fertilization process. Investigators should give considerable attention to this important area of contraceptive research during the coming decades. The use of gamete-directed agents holds great promise in leading to generally acceptable, non-toxic, highly effective contraceptive methodology.

ACKNOWLEDGEMENT

Most of the research on this topic done in our laboratory was supported by the Program for Applied Research on Fertility Regulation (PARFR-204) and by the National Institutes of Health (NIH HD 09868).
REFERENCES


The contraceptive vaginal ring (CVR) is a clinically tested contraceptive steroid delivery system that provides 1) more constant circulating drug levels than oral formulations; 2) minimal subject-physician contact; and 3) acceptable use-effectiveness and continuation rates.

The rationale for this mode of drug delivery combines the knowledge that steroids (as well as other agents) can be rapidly absorbed into the circulation from the vagina, and the observation that the steroids contained in dimethylpolysiloxane (Silastic) devices can be released for long periods of time without producing local discomfort or significant systemic or local side effects.

This article reviews the development of the CVR, with special emphasis on the device developed by the Population Council, and summarizes the clinical and metabolic studies that have been performed to date. The device being developed by the World Health Organization, designed to release levonorgestrel (LNG) at a rate of approximately 20 µg/day, does not consistently inhibit ovulation, and will not be discussed here because no publications about this device are as yet available (4).

CHARACTERISTICS OF SILASTIC DEVICES

Devices made of dimethylpolysiloxane are non-toxic (26), they release the steroids they contain at rates proportional to their surface area (7) and inversely proportional to the thickness of the outer wall of the devices (11), and the amount of steroid contained within the device determines the duration of action (6).

Size

Determination of the optimum size of the toroidal CVRs must take into account subject comfort as well as the possibility of spontaneous expulsion of the device with activities that increase intra-abdominal pressure. The initial studies were performed with devices 70 and 80 mm in outer diameter and 10 mm thick (14). Because of concern about possible expulsion, devices 75 mm in diameter contained a flat spring much like that in a conventional diaphragm. These rings were abandoned because, although none of the subjects complained of slippage, vaginal erosions were produced in over half the subjects (15). A study of rings with an outer diameter of 65 mm and a thickness of 7 mm demonstrated that these thinner rings produced even more erosion than did the thicker rings (16). Subsequently, rings with an outside diameter of 61 mm (18, 19), 60 mm (3, 13, 17, 30-33), 58 mm (27-29), 55 mm (11), and 50 mm (27-29), and a thickness of 9 to 9.5 mm have been well tolerated.

From the results of these early prototype studies, it appears that CVRs with an outer diameter of about 50 to 58 mm and a thickness of 7 mm to 9.5 mm are well tolerated by most women. One ring is suitable for all women, and unlike the diaphragm, the device does not have to be fitted or placed in a certain position, although it generally becomes oriented around the cervix as long as the outer surface of the CVR is in contact with the vaginal epithelium, systemic absorption of the steroids will occur. Vaginal mucosal erosion occurs very infrequently, and heals spontaneously following simple
removal of the CVR (15, 16, 19). Spontaneous expulsion of the device can be easily corrected by having the user reinsert the device. Upon colposcopic examination of women following 2 years of use, no changes of the vaginal epithelium adjacent to the CVR have been noted (25).

**Ring design**

**Homogeneous.** The initial design of the CVR was a ring made of a homogeneous mixture of steroid and Silastic (14) (Figure 1A). This device was found to release high initial dosages of steroid resulting in high circulating blood levels, followed by a rapid reduction of steroid levels (13, 30) coinciding with episodes of breakthrough bleeding or breakthrough spotting (BTB/BTS).

**Core.** In order to eliminate the initial high levels followed by the rapid decline, the core ring was developed (Figure 1B). Rings 9 mm thick, with steroids contained within a 3.5 mm central core, were tested and produced fairly uniform serum levels while in situ, except for an initial period, and had sufficient steroid capacity to allow for several cycles (3).

**Shell.** In an effort to develop devices with more uniform release rates, rings with a shell design were developed (Figure 1C). These shell rings have a steroid and Silastic layer applied around an inner core of inert Silastic (16, 19, 31). The active layer is covered with another layer of inert Silastic tubing, thus providing an almost uniform distance through which the steroid must travel in order to be absorbed (11, 12, 18, 19, 31, 32).

**Collagen band.** In an effort to develop a device that would potentially use less steroid and would be easier to fabricate, the collagen band device was developed (Figure 1D). Collagen bands containing steroid were designed to lie in a groove on an inert inner core of Silastic. These devices were abandoned, because the bands broke or were displaced and unpredictable blood levels of steroid occurred (33).

**Drugs tested**

Studies have been performed utilizing the following progestogens alone or in combination with estrogens: medroxyprogesterone acetate (MPA), chlormadinone acetate (CMA), norethindrone (NET), R2323, dl-norgestrel (dl-Ng), levonorgestrel (LNG), LNG plus estradiol benzoate (EB), and LNG plus estradiol (E). The 21-carbon compounds, e.g., MPA, which suppressed ovulation while being released in doses of 520 to 1219 µg per day from a homogeneous ring, and CMA released from a shell ring, produced blood levels similar to those achieved after oral administration; however, following reports of mammary tumors in beagle dogs (14-16, 17), these compounds were no longer studied.

Homogeneous NET rings that release 850 or 1529 µg of the drug per day produced unacceptable episodes of breakthrough bleeding and spotting associated with an offensive odor, and ovulation occurred in one-quarter of the cycles studied (17). R2323 administered in a core device releasing 150 to 400 µg per day produced no BTB/BTS and blocked ovulation. Following removal of the device, withdrawal bleeding occurred promptly (3). Further tests with this promising agent were abandoned when men ingesting 100 mg of R2323 per week developed increased transaminase levels (3).

In tests of rings releasing dl-Ng at rates of 120 to 350 µg per day, the major problem was BTB/BTS, which occurred in 63% of cycles studied. Ovulation also occurred in approximately 15% of cycles with this device in place (31).

In an effort to reduce the incidence of BTB/BTS, a collagen band device was developed, into which LNG plus EB was incorporated. However, BTB/BTS was observed in 64% of cycles studied, suggesting that no
benefit accrued from the addition of EB to the band, since
the amounts (50 to 100 μg) of estradiol benzoate available
for vaginal absorption were very small, and once
absorbed, the drug did not alter circulating serum
estriol levels (33). Shell rings releasing LNG alone
demonstrated higher sustained circulating serum LNG
levels (1.6 to 2.4 ng/ml) than those shell rings releasing
dl-Ng (1.2 to 1.7 ng/ml, over a 6-month period (18, 31).
These LNG shell rings were associated with BTB/BTS in
33%, lack of withdrawal bleeding in 5%, and ovulation in
3% of cycles studied.
Investigators considered the possibility that adding E2
to the LNG ring would more effectively control bleeding and
suppress ovulation. In the first trial with rings releasing
LNG plus E2, significantly improved bleeding control was
observed. BTB/BTS occurred in only 7% of cycles
studied. Withdrawal bleeding after ring removal occurred
within 1 to 5 days, with a mean of 2.6 days, and lasted
from 3 to 7 days, with a mean of 4.5 days. There was no
failure of withdrawal bleeding, and no ovulation occurred
in any of the cycles studied (19). These rings released an
average of 289 μg of LNG per day, and 212 μg of E2 per
day, and produced fairly constant serum LNG levels of 1
to 3 ng/ml and an initial peak level of E2 of about 100
pg/ml, which rapidly declined but presumably stimulated
the endometrium sufficiently to provide improved bleed-
ing control.

Use schedule

The original use schedule was similar to that recom-
mended for oral contraceptive use. The CVR was
inserted on day 5 of the cycle and left in place for 21 days.
During the first studies with medroxyprogesterone
acetate rings in women following this schedule, a high
incidence of BTB/BTS occurred, suggesting that insuffi-
cient amounts of endogenous estrogen were being
secreted during use of this progestogen-only containing
CVR (14, 15). Therefore, a trial was performed in which
the CVR was inserted on day 10, when more endogenous
estradiol would be secreted by the ovary. Since the
incidence of BTB/BTS was not reduced with this
schedule, the approach was abandoned (16).

With another regimen, a bleeding signal was tested; each
subject was instructed to leave the ring in place and to
remove it for 5 days only if she experienced BTB/BTS
(32). In studies with this regimen, the incidence of
bleeding and spotting was usually lower than that ex-
pected during a comparable period of untreated cycles.
The bleeding was also scantier than that during the
subject's ordinary menstruations, but prolonged amen-
orrhic episodes occurred. In almost all instances, the
ring was reinserted within 5 days after removal, and no
ovulations were observed. Although no vaginal erosions
occurred during this study, the vaginal rugae were
flattened. The fact that the patient did not need to
remember when the ring had to be removed was thought
to counterbalance the unpredictable occurrence of bleed-
ing episodes with this schedule. When questioned,
however, subjects preferred the fixed time schedule to
this erratic schedule.

Thus, the protocol now recommended for the use of the
vaginal ring is to have the woman insert it herself on day 5
of the cycle and wear it for 3 weeks, then remove it for a
7-day period to allow withdrawal bleeding. Should the
CVR interfere with coitus, it may be removed for up to 3
hours and then reinserted postcoitally without reducing
the contraceptive effect.

Pharmacodynamics

When the self-administered CVR containing both
levonorgestrel and estradiol is placed in the vagina, the
steroids are released from the surface and absorbed
through the vaginal epithelium into the circulation at a
fairly constant rate (Figure 2). Patients using the rings
had mean levonorgestrel levels of 2.5 μg/ml for the first
cycle and 1.3 μg/ml during the sixth cycle (19). These
levels are sufficient to inhibit ovulation, but are several
times less than the 4 to 8 μg/ml peak levels obtained with
daily ingestion of tablets containing 500 μg norgestrel
(250 μg levonorgestrel) (5). Ovulation continues to be
inhibited during the week that the rings are not in place.
Although midcycle gonadotropin peaks are abolished,
Figure 2. Serum estradiol and levonorgestrel levels on
log-scale and progesterone levels during six treatment
cycles with vaginal rings. Rings were inserted on day 1
and removed on day 21 during each cycle. Open bars at
top of graph indicate 3-week treatment cycles with rings
in place. Black bars indicate bleeding days (full height for
bleeding and half height for spotting). (Reproduced, with
permission, from Am J Obstet Gynecol 130:58, 1978.)
gonadotropin peaks have been observed during the 1-week interval when the ring is removed, or soon after its re-insertion (Figure 3).

There are three differences accounting for the variation in estrogenic effect produced by the CVR and oral contraceptive steroids: first, a natural estrogen, estradiol, is released from the CVR instead of the more potent (in terms of hepatic effects) synthetic estrogen, ethinyl estradiol, found in the oral tablets. Second, a smaller amount of estrogen is absorbed from the CVR, and this absorption occurs only during the first few days of each treatment cycle (Figure 2), because of the relatively lower solubility and diffusion of estradiol in comparison to levonorgestrel in dimethylpolysiloxane. Third, the CVR route of administration initially bypasses the liver, while the steroids absorbed orally pass directly to the liver after absorption in the gut. For these reasons, the CVR has less of an effect on hepatic alterations than do combination oral contraceptives, which may increase angiotensinogen and steroid binding globulins while decreasing antithrombin III (9, 21).

Shell rings used for six or seven consecutive 21-day cycles, with a 7-day non-use interval between cycles, have been analyzed for average steroid loss (12). In vivo, rings 58 mm in diameter were found to release levonorgestrel and estradiol at mean rates of 293 ± 54 μg per day and 183 ± 34 μg per day, respectively. Rings of 50 mm

Figure 3. Three times a week assay of serum LH (solid line) and FSH (dotted line) during six treatment cycles. Open bars at top of graph represent the 3 weeks rings were in place. Values for LH and FSH are for same subject as in Figure 2. (Reproduced, with permission, from Hafez ESE, Van Os WAA (eds): Biodegradables and Delivery Systems for Contraception, p 165. Lancaster, England, MTP Press, Limited, 1980.)

Type of termination

Table 1. One-year termination and continuation rates per 100 acceptors by regimen and reason for termination (all segments of use).

* P<0.01
† P<0.001

diameter had mean levonorgestrel and estradiol release rates of 252 ± 34 μg per day and 152 ± 21 μg per day, respectively. Sufficient steroid was present in each of these rings so that they could be used for at least six treatment cycles before the drug was depleted.

**CLINICAL STUDIES**

In order to assess the contraceptive effectiveness and acceptability of the shell CVR containing LNG and E₂, devices of two sizes — 50 mm and 58 mm in outer diameter and a thickness of 9.5 mm — were fabricated and users' experience was compared to experience of users of an oral contraceptive containing 150 μg LNG with 30 μg E₂ (Nordette).

These rings were compared to Nordette in a multicenter study involving data from eight clinics — in Brazil, Chile, Denmark, Dominican Republic, Finland, United States (Los Angeles), Nigeria, and Sweden (27-29). A total of 547 women using the 50 mm CVR, 556 women using the 58 mm CVR, and 553 women using the Nordette oral contraceptive participated in the study.

Among the CVR users in all segments of use, 1-year net pregnancy rates were less than 3 per 100, approximately the same as the pregnancy rates observed among users of Nordette (Table 1). Continuation rates at 1 year were 50 per 100 users of the rings (all segments of use). This rate was significantly higher than, or equal to, the rate observed among the users of Nordette — 38.2 or 55.4 per 100 users, depending upon whether the lost to follow-up rates of these subjects were considered a termination or not, respectively. The profile of women terminating was similar for the users of the two sizes of rings, but it differed significantly from that of the Nordette acceptors. Gross 1-year rates of termination for medical reasons ranged from 25 to 29 per 100 for the three regimens, without a significant difference. However, ring users were more likely to terminate use for vaginal problems and pill users for headaches, nausea, and other systemic symptoms. Problems relating to use of the CVR regimen accounted for a significantly higher discontinuation rate among CVR than among Nordette users. Terminations for use-related reasons were coded into seven categories: frequent ring expulsion, interference of the ring with coitus, users' dislike of or difficult with insertion or removal of the ring, unpleasant ring odor, difficulties in storage of the ring or pill, ring loss, and problems associated with use of the pill. Lost rings, insertion and removal difficulties, or users' dislike of insertion or removal, accounted for the majority of CVR use-related terminations. These trials indicate that CVRs of this design are as effective in use and have continuation rates at least equal to, and possibly superior to, Nordette under the same study conditions.

In addition, side effects of the rings and Nordette were evaluated by noting spontaneous complaints, by recording additional medications taken by users, and by physical examination (28). Inquiries about changes in the frequency of specific conditions were made at the end of the subjects' participation in the first year of the study (Table 2). The incidence of spontaneous complaints was similar among users of the two different-sized rings and of Nordette.

<table>
<thead>
<tr>
<th>PROBLEM</th>
<th>50 mm CVR</th>
<th>58 mm CVR</th>
<th>NORDETTE</th>
<th>SIGNIFICANCE (3 REGIMENS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAGINAL</td>
<td>34.1</td>
<td>37.8</td>
<td>9.6</td>
<td>*</td>
</tr>
<tr>
<td>LOWER ABDOMINAL PAIN</td>
<td>10.5</td>
<td>14.6</td>
<td>9.6</td>
<td>NS</td>
</tr>
<tr>
<td>HEADACHE</td>
<td>7.7</td>
<td>7.1</td>
<td>19.4</td>
<td>*</td>
</tr>
<tr>
<td>NERVOUSNESS</td>
<td>2.8</td>
<td>3.6</td>
<td>5.9</td>
<td>NS</td>
</tr>
<tr>
<td>DEPRESSION</td>
<td>1.7</td>
<td>2.1</td>
<td>0.7</td>
<td>NS</td>
</tr>
<tr>
<td>MENSTRUAL</td>
<td>34.7</td>
<td>22.0</td>
<td>21.7</td>
<td>*</td>
</tr>
<tr>
<td>ACNE OR OTHER SKIN PROBLEMS</td>
<td>7.7</td>
<td>8.0</td>
<td>5.3</td>
<td>NS</td>
</tr>
<tr>
<td>MASTALGIA</td>
<td>0.3</td>
<td>0.9</td>
<td>1.6</td>
<td>NS</td>
</tr>
<tr>
<td>OTHER</td>
<td>21.0</td>
<td>21.4</td>
<td>31.6</td>
<td>†</td>
</tr>
<tr>
<td>ALL FIRST PROBLEMS</td>
<td>120.5</td>
<td>117.6</td>
<td>105.4</td>
<td>NS</td>
</tr>
<tr>
<td>WOMEN-YEARS</td>
<td>351.8</td>
<td>335.8</td>
<td>335.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS = not significant
* P<0.001
† P<0.01


Table 2. First problems mentioned in response to question, "How have you been feeling since last visit?" By regimen; all segments of use; incidence per 100 women-years during first 12 months.
Vaginal complaints obviously were far more frequent among ring users, while only users of the 50 mm ring had significantly greater menstrual complaints than users of either the 58 mm ring or Nordette. Headaches, dizziness, and nausea were reported more frequently by users of Nordette. All the regimens were associated with weight gain of about 1 kg and increased hemoglobin levels of about 0.5 gm/dl. Nordette, but not the CVR, was associated with small but significant increases in both mean diastolic (0.9 mm Hg) and systolic (1.5 mm Hg) blood pressure.

A more detailed examination of the menstrual events, based on diaries, demonstrated that the CVRs, used continuously for 3 weeks and then removed for 1 week, and Nordette produced approximately the same total number of bleeding and spotting days during six cycles of use (27-29) but the small (50 mm outer diameter) ring was associated with somewhat more spotting (Table 3). This ring was also associated with somewhat more prolonged bleeding and spotting runs and with more prolonged nonbleeding intervals than reported by users of the larger (58 mm outer diameter) ring or Nordette. On the average, CVR users experienced about 1 day per month of bleeding or spotting with the ring in place. Evidence from menstrual diaries indicates that the 58 mm ring provides control over the menstrual cycle comparable to that of Nordette (29).

In an effort to determine acceptability of these rings in a rural setting, an investigation was undertaken in rural, small town, and urban slum clinics in four locations, two in Brazil and two in the Dominican Republic (8). The CVR was offered as a new method in the clinics and described as similar to the pill but placed in the vagina for 3 weeks each month followed by a 1-week rest interval. Follow-up surveys were carried out in the four locations at the end of the experimental period. Of the total contraceptive acceptors in each of the four locations, 3%, 8%, 9%, and 12.5% chose the CVR. The acceptance rate was higher in three rural clinics, where the nurses themselves used the ring. The follow-up surveys showed that the user's ability to place and keep the ring in the vagina without removal for a prolonged period was the

<table>
<thead>
<tr>
<th>DAYS</th>
<th>50 mm CVR</th>
<th>58 mm CVR</th>
<th>NORDETTE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-30 (from acceptance)</td>
<td>5.90</td>
<td>5.57</td>
<td>6.42</td>
</tr>
<tr>
<td>31-60</td>
<td>4.78</td>
<td>4.75</td>
<td>4.51</td>
</tr>
<tr>
<td>61-90</td>
<td>4.80</td>
<td>4.54</td>
<td>4.54</td>
</tr>
<tr>
<td>91-120</td>
<td>4.60</td>
<td>4.28</td>
<td>4.30</td>
</tr>
<tr>
<td>121-150</td>
<td>4.76</td>
<td>4.30</td>
<td>4.37</td>
</tr>
<tr>
<td>151-180</td>
<td>4.66</td>
<td>4.41</td>
<td>4.43</td>
</tr>
<tr>
<td>N = days 1-30</td>
<td>500</td>
<td>493</td>
<td>422</td>
</tr>
<tr>
<td>N = days 151-180</td>
<td>356</td>
<td>335</td>
<td>270</td>
</tr>
</tbody>
</table>

PERCENTAGE DISTRIBUTION OF BLEEDING AND SPOTTING, DAYS 1-168
(6x28 DAY REFERENCE PERIOD)

<table>
<thead>
<tr>
<th>No. women</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;12</td>
<td>4.0 ± 12.01</td>
</tr>
<tr>
<td>13-24</td>
<td>39.0 ± 41.7</td>
</tr>
<tr>
<td>25-36</td>
<td>35.8 ± 42.0</td>
</tr>
<tr>
<td>37-48</td>
<td>14.4 ± 7.6</td>
</tr>
<tr>
<td>49-60</td>
<td>5.3 ± 3.9</td>
</tr>
<tr>
<td>&gt;60</td>
<td>1.3 ± 0.8</td>
</tr>
<tr>
<td>No. women</td>
<td>374</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>28.67 ± 12.01</td>
</tr>
</tbody>
</table>

MEAN NUMBER OF BLEEDING AND SPOTTING DAYS EXPERIENCED IN DAYS 169-336

<table>
<thead>
<tr>
<th>No. women</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>246</td>
<td>25.30 ± 12.26</td>
</tr>
<tr>
<td>220</td>
<td>24.55 ± 10.60</td>
</tr>
<tr>
<td>169</td>
<td>24.42 ± 6.95</td>
</tr>
</tbody>
</table>


Table 3. Bleeding and spotting days by regimen (data from menstrual diaries).
most important attribute of the method and played a large role in the woman's reaction to it. Anticipated use-related problems were the most prominent reason given by pill acceptors for not choosing the ring; however, ease of use was named as the "most liked" characteristic by 55% of the ring users. Women tended to remove the ring for intercourse and to wash it frequently, often with detergents, indicating their concern with "cleanliness" of an object kept within a body cavity for long periods.

**METABOLIC STUDIES**

**Carbohydrate and liver function.** A prospective, long-term study undertaken to compare the metabolic effects of the contraceptive vaginal ring and Nordette in two groups of women (n = 22 and 20, respectively) has also been reported (1). An intravenous glucose tolerance test (IV-GTT), including determination of the insulin response to glucose, and liver function tests (bilirubin, alanine amino transferase, asparagine amino transferase, and alkaline phosphatase) were performed pretreatment and after 2, 6, and 12 months of treatment, and at about 1 month post-treatment (Table 4). Both the glucose tolerance and fasting values of glucose were unaltered. The early insulin response to glucose increased by 50% in the CVR group after 1 year of treatment, but not in the oral contraceptive group. All other insulin values were unchanged.

The effect on the peak insulin is not related to or indicative of any change of peripheral insulin sensitivity, but shows, rather, the sensitivity of the pancreas to the glucose stimulus. Since there is evidence of progesterone receptors in the beta cells (10), at least the initial insulin response to a glucose load might be directly influenced by progestogens. The unchanged fasting and 60 minute insulin levels in this study, as well as the normal glucose tolerance, indicate that levonorgestrel, on its own, does not cause either impairment of glucose tolerance or peripheral insulin resistance.

<table>
<thead>
<tr>
<th>CVR</th>
<th>OC</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 months</td>
<td>26*</td>
</tr>
<tr>
<td>6 months</td>
<td>32†</td>
</tr>
<tr>
<td>12 months</td>
<td>50†</td>
</tr>
</tbody>
</table>

* P<0.05  
† P<0.01  
Calculated on paired data.


Table 4. Mean increase of peak insulin in per cent of pretreatment values.

With the oral contraceptive, there was no effect on the fasting glucose concentration or on glucose tolerance. The insulin concentrations, particularly in fasting states and at 60 minutes of the IV-GTT, showed a tendency to higher values. This is in accordance with earlier findings of peripheral insulin resistance when estrogens of the synthetic type are used in combination with levonorgestrel (24).

All liver function values remained within normal range in all subjects. There was a small significant decrease in alkaline phosphatase in both groups, which is in contrast to the elevation noted with higher dosage combined oral contraceptives.

It was concluded that neither of these two contraceptive methods, the effects of which are predominantly gestagenic, seems to cause impairment of glucose tolerance or hepatic function.

**Globulins.** A study comparing the combination CVR to various combination oral contraceptives has demonstrated that the CVR produces no changes in corticosteroid binding globulin-binding capacity (CBG-BC), angiotensinogen, or antithrombin III, in contrast to oral contraceptives, which produce significant increases of the first two and a significant reduction in the last. These globulins respond to estrogen-dominant preparations and suggest that the combination CVR-releasing levonorgestrel and estradiol is a relative progesterone-dominant preparation (21). Indeed, the CVR produces a significant reduction of sex hormone binding globulin-binding capacity (SHBG-BC) and no change in the total serum norgestrel, but significantly greater non-SHBG-bound norgestrel (both per cent and mass, µg/ml) when compared to an oral contraceptive containing 300µg norgestrel and 30 µg ethinyl estradiol (9). The overall effect of such a preparation is to produce no estrogen-mediated effects, for reasons already described (see Pharmacodynamics section), while producing relative progesterone dominance (see Lipids and lipoproteins section, following).

**Lipids and lipoproteins.** Because of the concern that gestagen-dominant contraceptive steroids may adversely affect lipids and lipoproteins, several studies investigating the effect of the CVR on lipids, lipoproteins, serum lipoproteins, and apolipoproteins have been undertaken. In a study in the United States, lipids and lipoproteins, as determined by analytic ultra-centrifugation, were studied in 5 controls and 10 women using 58 mm CVRs releasing 290 µg per day of LNG and 180 µg per day of E2. The groups were comparable for race, age, parity, and obesity indices, alcohol ingestion, smoking, diet, and exercise. Fasting blood samples were obtained twice before CVR treatment, after 2 and 7 weeks of treatment and 1 week.
thereafter (Table 5). The women using the CVR had a significant incremental reduction of cholesterol from baseline to treatment (15%), which was distributed among all the lipoprotein classes — especially HDL-C (27%). The cholesterol/HDL-C ratio was significantly increased with treatment. All mean changes were within the reference range. The reduction in HDL (21%), especially in the subclasses HDL 2a (48%) and HDL 2b (71%), was significant, and for the subclasses HDL 2a and HDL 2b, reduction was outside the reference range. The HDL/HDL ratio increased significantly (44%), while the LDL/HDL ratio increased significantly outside the reference range (131%) with treatment. Of the lipid and lipoprotein measurements that changed significantly with treatment, HDL-C, HDL, HDL 2a, LDL/HDL, and LDL/HDL 2a + 2b changed significantly toward baseline in 1 week when the subjects were off treatment (23).

A 1-year, prospective Swedish study compared the effects on lipoproteins, lipids, and apolipoproteins of a combined oral contraceptive (30 μg ethinyl estradiol and 150 μg levonorgestrel) and a CVR-releasing estradiol (about 180 μg per day) and levonorgestrel (about 290 μg per day) (Table 6). The two treatments induced significantly different effects. In the OC group, the lipoprotein-lipid concentrations showed only minor changes, but apolipoprotein B and A-I increased by about 15%. In contrast, during treatment with the CVR, there was a 25% decrement of cholesterol in high density lipoprotein and a 10% decrement in low density lipoprotein cholesterol, with only minor effects of apolipoprotein B and A-I. The ratio of LDL and HDL cholesterol increased in the CVR group, but not in the oral contraceptive group. The results also indicate a change in the composition of the LDL and HDL particles, with an altered lipid/protein ratio, during both contraceptive treatments. Despite the impressive relative increase in the LDL/HDL ratio in the contraceptive ring group, the average absolute value of this ratio did not reach the mean for healthy men (2).

The effects of the 50 mm combination levonorgestrel and estradiol ring, which release 250 μg per day and 150 μg per day, respectively, of these steroids, were studied in 10 healthy, normally menstruating women attending a family planning clinic in Santo Domingo (20). A schedule of 21 days of use followed by 7 days of non-use was followed for 6 cycles (Table 7). During the first two cycles of use, concentrations of cholesterol, HDL cholesterol, triglycerides, and LDL cholesterol declined significantly from control levels — as much as 20% for cholesterol, 18% for HDL cholesterol, 25% for triglycerides, and 13% for LDL cholesterol. There were no subsequent changes with continued use. These declines are similar in direction but of lesser magnitude than those reported from the clinics in the United States and Sweden, where pretreatment plasma levels of the same lipids were considerably higher. There was no significant change in the total cholesterol/HDL cholesterol ratio during treatment. The differences observed among these studies may be due to the different ethnic groups, their dietary practices, or to the different (lower) release rate of steroids in the latter study.

A possible potential reduction of predicted incidence of myocardial infarction with use of the CVR suggested by a reduction in total cholesterol appears to be counterbalanced by a reduction in HDL-C and increases in the cholesterol/HDL-C and LDL/HDL ratios. The potential clinical implications of these findings, if any, remain to be determined.

Vaginal flora. The CVR is a foreign body that is placed into the vaginal vault for an extended period of time. Therefore, concern has been expressed whether any

<table>
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* Significant changes
† Percentage change from pretreatment
‡ Outside the reference range


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* Percentage change from pretreatment
† Significant changes
‡ Arbitrary units


Table 5. Average values of selected lipids and lipoproteins (mg/dl) in 10 women using 58 mm LNG + E2 CVRs.

Table 6. Average values of selected lipids and lipoproteins (mmol/l) in 22 women using 58 mm LNG + E2 CVRs.
changes in the flora of the vagina occur with CVR usage. A variety of studies with different agents (MPA, CMA, dl-NG, LNG-E2) have shown that although vaginal secretions are increased with CVR usage, which in some instances were the result of pathogenic organisms such as Candida, the resulting vaginitis could be treated while the woman continued to use the CVR (11, 16, 19, 31).

To more carefully study this issue, a prospective study was undertaken in which premenopausal women seeking a steroid contraceptive method were allowed to choose between a CVR containing levonorgestrel and estradiol used in a 3 week in, 1 week out regimen (n = 20) and an oral contraceptive containing levonorgestrel (150 μg) and ethinyl estradiol (30 μg) in a 28-day regimen (n = 10). Cultures from the posterior vaginal fornix were obtained prior to therapy in both groups, and monthly for 6 months for the CVR group, and after 1, 3, and 6 months for the OC group. These cultures were streaked on specific media to provide quantitative aerobic and anaerobic organisms, Lactobacillus sp., Candida sp., Gardnerella vaginalis and Neisseria gonorrhoeae counts in micro-organisms per ml. A comparison of the number and types of organisms isolated from vaginal cultures obtained initially and after 6 months of use demonstrated no statistically significant differences in colony counts between CVR and OC users. The results of this study suggest that the use of the CVR is not associated with a greater growth of pathogens than is oral administration of a progestin and estrogen combination (24).

<table>
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* Percentage change from pretreatment
† Significant changes


Table 7. Average values of selected lipids (mg/dl) in 10 women using 50 mm LNG - E2 CVRs.

CONCLUSION

The shell ring with an outer diameter of 58 mm and thickness of 9 to 9.5 mm, releasing LNG (280 μg per day) and estradiol (180 μg per day), which is self-administered and used for 3 continuous weeks and then removed for 1 week, is associated with acceptable rates of breakthrough bleeding and spotting, almost complete inhibition of ovulation, and acceptable rates of withdrawal bleeding upon ring removal, and it may be re-used for at least 6 months.

These rings are as effective as, and have continuation rates at least equal to, oral contraceptives. Additionally, since headache and nausea are less common with ring use, this method of contraception may be preferred by women who complain of these or other symptoms while taking the oral steroid.

The ring produces few, if any, of the metabolic effects that are associated with oral estrogen administration, such as an increase in angiotensinogen and steroid binding globulins, and a decrease in antithrombin III. Thus, the side effects of hypertension and thrombosis, which can occasionally occur with oral contraceptives, would most likely not be increased with CVR usage. Studies from some, but not all, centers indicate that women wearing vaginal rings have a decrease in total cholesterol, LDL cholesterol, and HDL cholesterol. The changes in the commonly used "risk ratios" (total cholesterol/HDL or LDL/HDL) are small in most women and the values usually do not approach those found in normal male or female patients with cardiovascular disease. The relevance of these observations is currently being investigated.

A concern that the ring would not be used by women in developing countries has not materialized. About 10% of women who were offered the ring as a new method without having received any previous information regarding the possible advantages of the method demonstrated that they would choose the method and use the rings with relatively high continuation rates. Thus, this new method of steroid contraception is likely to be an effective alternative for women who cannot or will not use the other currently available contraceptive methods.
REFERENCES


We are fast approaching an era in which "zero population growth" will be a vital necessity rather than merely a desirable alternative. Uncontrolled population growth in many parts of the world has contributed to grave economic, political, and social problems. Ironically, the poorer, less developed nations have a faster rate of population increase and are unable to control it to a significant extent. Reducing the millions of unplanned and unwanted pregnancies worldwide would solve many economic and health problems and would enable developing countries, particularly, to improve their standard of living (54). This need for regulating human fertility in order to slow the rate of population increase has been recognized for decades by various sociologists, but only in recent years, and to a lesser degree, by political leaders and governments. Presently available methods of contraception are either inadequate, inconvenient, or unacceptable to some groups and individuals (79). Better control of human fertility will require the development and testing of new methods of contraception that will be effective and, at the same time, will be safer, simpler and more convenient than the methods now available (79). This task can be achieved through a greater commitment to contraceptive research.

A series of complex processes is involved in human reproduction. In the human female, a delicate qualitative and quantitative interplay between the hypothalamus, pituitary, and ovaries and luteinizing hormone (LH), follicle-stimulating hormone (FSH), and ovarian steroids is essential for follicular maturation, ovulation, implantation, nidation, and maintenance of gestation (70). Development of contraceptive methods has been and can continue to be based on interference with some of these processes. Many agents, including the hormones themselves, depending on the time they are administered, interfere with these mechanisms and thus produce antifertility effects.

Among several contraceptive approaches being considered and developed is one based on analogs of the hypothalamic hormone controlling secretion of both LH and FSH from the anterior pituitary gland (Figure 1). This approach was made possible by the isolation, determination of structure, and synthesis of this hormone in 1971 (55, 56, 69, 71, 75, 77). The hypothalamic hormone is called the LH- and FSH-releasing hormone, abbreviated as LH-RH/FSH-RH, or simply gonadotropin-releasing hormone (Gn-RH) (70, 73). While LH-RH is also accepted as the main FSH-releasing hormone, for reasons of convenience and historical continuity the abbreviation LH-RH is generally used for naming its analogs.

LH-RH is the main link between the brain and the pituitary as far as reproductive function is concerned (Figure 2). Much work indicates that this link can be disrupted by various means. Consequently, approaches based on the use of suitable LH-RH analogs should, in the foreseeable future, lead to the development of a method of contraception at the central level that might be easy to apply and free of undesirable side effects (Figure 2). Some selected background information, as well as the very latest findings on the topic of antagonistic analogs of LH-RH, will be reviewed here.

THEORETICAL CONSIDERATIONS FOR ANTAGONISTS OF LH-RH

The design of modified structures that might compete with a biologically active compound for the same receptor sites, and yet exhibit little intrinsic activity, is a classical concept that has been used to develop a number of...
Figure 1. The molecular structure of luteinizing hormone-releasing hormone (LH-RH).

Figure 2. Representation of central site(s) of action of LH-RH antagonists and ensuing blockade at the gonadal level.

For example, certain micro-organisms require p-aminobenzoic acid to form folic acid. Sulfanilamide, a structural analog of p-aminobenzoate, blocks folic acid synthesis by substrate competition, and is thus an effective inhibitor of growth of susceptible bacteria (41). Sulfanilamide is used for treatment of various infections. These findings on sulfanilamide were a milestone in the history of chemotherapy and initiated a new era in medicine (41). Another classical example of an antagonist is 5-fluorouracil, a pyrimidine antimetabolite used for cancer chemotherapy.

Classical competitive inhibition occurs at the substrate binding or receptor site, the receptor being defined as a cellular transducer of hormone action that binds the hormone in a reversible interaction. Receptors for peptide hormones are found in the cell membrane. The chemical structure and stereochemistry of an inhibitory analog of a peptide must resemble that of the substrate or the hormone.

This approach has only recently met with any degree of success in the peptide field. When we proposed the development of inhibitory analogs of LH-RH in 1971 (74), the concept of a peptide endowed with anti-LH-RH activity was based purely on theoretical considerations. Knowledge of the structural features that constitute the active site in a peptide is the primary step for the synthetic development of specific antagonists. We correctly forecast that replacement or deletion of some
LH-RH RECEPTOR INTERACTION WITH
LH-RH ANTAGONISTS

amino acids in LH-RH might result in an analog possessing the structural features requisite for effective binding with the receptor, but lacking those necessary to elicit a physiological response, i.e., the stimulation of LH and FSH release (74). Such analogs could be competitive inhibitors of LH-RH; that is, they would be devoid of intrinsic LH-RH activity, but by competing for attachment to the receptor site with endogenous LH-RH, they might reduce LH and FSH secretion (Figure 3).

Synthesis of non-competitive inhibitors of LH-RH, such as chlorambucil-LH-RH, which might react irreversibly with the pituitary receptor by alkylating it, has also been attempted (10), but these compounds appear to be less potent than the modern inhibitors.

It must be emphasized that competitive LH-RH antagonists were and are intended primarily for female contraception. It is important to realize that abolishing the basal secretion of LH and FSH is not necessary. On the contrary, complete inhibition of LH and FSH release would be harmful, since it would interfere with ovarian steroidogenesis, which in turn might have undesirable effects on women. Instead, the task of LH-RH antagonists would be to block the midcycle surge of LH and FSH necessary for ovulation. To be more practical, a contraceptive polypeptide would probably have to be given by a route of administration other than parenteral. Results from clinical experiences with LH-RH and its stimulatory analogs indicate that intranasal administration may be the most efficacious. An inhibitor could also be conveniently administered every 1 to 3 months in the form of injectable biocompatible microcapsules of a biodegradable polymer that would allow its slow release.

Competitive LH-RH antagonists appear to be ideal candidates for the development of a new contraceptive method, since they should disrupt the reproductive cycle relatively safely.

RELATIONSHIP BETWEEN STRUCTURE AND
BIOLOGICAL ACTIVITY OF LH-RH

Various studies with many LH-RH analogs indicate that certain amino acids can be replaced in the LH-RH molecule without a major loss of activity (70, 73). It appears that amino acids in positions 1 and 4 to 10 may be involved only in binding to the receptors and/or in exerting conformational effects.

However, histidine and tryptophan are likely to exert a functional effect in addition to providing receptor-binding capacity, since simple substitutions or deletions in positions 2 or 3 greatly decrease or abolish LH-RH activity (70). Indeed, the His-Trp sequence appears to constitute an “active center” in LH-RH. Thus, the imidazole group of histidine in position 2 possesses features, such as acid-based character and hydrogen bonding capacity, that render it necessary for expression of activity; and replacement or deletion of histidine drastically reduces agonist activity. Similarly, the deletion or replacement of tryptophan in position 3, or even a change in its configuration, also results in nearly complete loss of activity. This crucial role of Trp may be linked in some way to the electron-donating capacity of the indole nucleus of tryptophan. The functional character of histidine and L-tryptophan in positions 2 and 3 of LH-RH has been further confirmed by synthesis of many inhibitors of LH-RH with changes in these positions (see section below). It is interesting that while most alterations in the 2 and/or 3 position(s) of LH-RH destroy the gonadotropin-releasing effect, some of these modifications may preserve or even increase the binding affinity of the analog to the receptors.

In the synthesis of LH-RH antagonists, virtually all investigative groups have taken advantage of the information gained from work with the superactive agonists that replacement of glycine in the 6 position of LH-RH by D-Ala, D-Leu, D-Phe, D-Trp, or other D-amino acids leads to a major increase in biological activity (21, 58). This phenomenon is attributed to better binding conformation than in LH-RH and is also indicated by empirical energy calculations of Momany (57). The activities of the D-6-amino acid analogs appear to increase with the size of the side chain, which suggests that lipophilicity may also be a factor. The most active analog seems to be D-Trp6-LH-RH, which also shows prolonged activity (28). Clinical results indicate that the potency of D-Trp6-LH-RH in human beings is 50 to 100 times greater than that of LH-RH.
The incorporation of ethylamide (EA) in the 10 position and a D-amino acid in the 6 position produces some analogs 30 to 100 times more potent than LH-RH, and they cause prolonged release of LH and FSH (22, 35). D-Leu<sub>6</sub>,desGly<sub>10</sub>-LH-RH EA and D-Ser(Bu)<sub>6</sub>-LH-RHEA (H766) were reported to be 50 and 100 times more potent, respectively, than LH-RH (51, 70). Thus, the changes in the 6 and 10 positions reinforce each other and produce superactive, long-acting analogs. These superactive analogs of LH-RH are active when administered intravenously, subcutaneously, intramuscularly, orally, intravaginally, intranasally, or even rectally (70, 73).

**DEVELOPMENT OF LH-RH ANTAGONISTS**

One of the early analogs, desHis<sub>2</sub>-LH-RH, was reported to competitively antagonize LH-RH in monolayer cultures of rat anterior pituitary cells when present in concentrations 10,000 times greater than LH-RH (85). However, in our hands, desHis<sub>2</sub>-LH-RH did not show significant anti-LH-RH activity in various *in vivo* tests (76). We tried to increase the weak antagonist activity of this analog by adding superactive modifications in positions 10 and 6 of the LH-RH sequence. We synthesized desHis<sub>2</sub>,desGly<sub>10</sub>-LH-RH EA and showed that in ovariectomized, steroid-treated rats, it inhibited LH-RH-induced LH release (21, 89). This was the first peptide that significantly reduced LH secretion in response to LH-RH in *in vivo* (89). Tryp­tophan in position 3 could also be replaced to give peptides with some inhibitory activity, such as Leu<sub>3</sub>-LH-RH (70). A whole series of potential antagonists was then synthesized and assayed in immature male rats. This assay permitted us to accurately compare the effectiveness of LH-RH antagonists by measuring the inhibition of response to LH-RH. Among the analogs tested, desHis<sub>2</sub>Leu<sub>3</sub>desGly<sub>10</sub>-LH-RH EA; desHis<sub>2</sub>D-Ala<sub>6</sub>desGly<sub>10</sub>-LH-RH EA; desHis<sub>2</sub>D-Ala<sub>6</sub>LH-RH; desHis<sub>2</sub>Leu<sub>3</sub>D-Ala<sub>6</sub>desGly<sub>10</sub>-LH-RH EA caused a significant inhibition of LH-RH-induced release of LH and FSH. However, desHis<sub>2</sub>D-Ala<sub>6</sub>desGly<sub>10</sub>-LH-RH EA and desHis<sub>2</sub>Leu<sub>3</sub>D-Ala<sub>6</sub>desGly<sub>10</sub>-LH-RH EA also showed some intrinsic LH- and FSH-releasing activities (90). It appeared that, for a given antagonist, inhibitory effect was improved by incorporating either the D-amino acid in the 6 position, or a C-terminal ethylamide group. However, incorporation of both these modifications within the same analog often increased the inherent agonist activity (90). The substitution of D-leucine or D-phenylalanine in position 6 further increased antagonist activity. For instance, in immature male rats, desHis<sub>2</sub>D-Phe<sub>6</sub>-LH-RH caused inhibition of response to LH-RH for 4 hours (70).

Another important discovery was made by Rees and associates, that replacement of His in position 2 by D-Phe, rather than its deletion, produces a better antagonist (63). D-Phe<sub>2</sub>-LH-RH was a far more effective inhibitor than desHis<sub>2</sub>-LH-RH. D-Phe<sub>2</sub>D-Leu<sub>6</sub>-LH-RH and D-Phe<sub>2</sub>D-Phe<sub>6</sub>-LH-RH inhibited 50% of LH release induced by LH-RH at a molar ratio (MR<sub>90</sub>) of 150 and 25, respectively. In general, the D-Phe<sub>2</sub>-analogs were about three times more active than the corresponding desHis<sub>2</sub>-analogs *in vitro* and *in vivo* (70). D-Phe<sub>2</sub>D-Phe<sub>6</sub>-LH-RH produced significant inhibition of LH and FSH for 6 to 8 hours after injection in immature male rats. Subsequently, the assay based on blockade of ovulation in 4-day cycling rats became the most reliable method for testing antagonists (20, 30). At first, multiple doses of antagonist were given, since their duration of action was not sufficient to produce complete inhibition of the LH surge that takes place over an 8-hour period in the rat. Analogues such as D-Phe<sub>2</sub>D-Ala<sub>6</sub>-LH-RH and D-Phe<sub>2</sub>D-Leu<sub>6</sub>-LH-RH were able to block ovulation and the preovulatory gonadotropin surge in cycling rats when multiple doses of 12 mg/kg of body weight were given during the afternoon proestrus (20, 30). In 4-day cycling rats, D-Phe<sub>2</sub>D-Leu<sub>6</sub>-LH-RH caused 83% blockade of ovulation after three injections of 2 mg on the proestrus day (30). D-Phe<sub>2</sub>D-Phe<sub>6</sub>-LH-RH was effective in single doses of about 6 mg/kg in the rat. The antagonist D-Phe<sub>2</sub>D-Phe<sub>6</sub>Aza-Gly<sub>10</sub>-LH-RH containing an Aza-Glycine residue in position 10 was reported to block ovulation induced by LH-RH in androgen-sterilized constant estrus rats (33).

Attempts were then made by modifying position 3 to synthesize peptides with lower inherent LH-RH activity that retained the desirable inhibitory properties of D-Phe<sub>2</sub>D-Phe<sub>6</sub>-LH-RH. D-Phe<sub>2</sub>Phe<sub>3</sub>D-Phe<sub>6</sub>-LH-RH and D-Phe<sub>2</sub>D-Leu<sub>6</sub>-LH-RH were able to block ovulation and the preovulatory gonadotropin surge in cycling rats when multiple doses of 12 mg/kg of body weight were given during the afternoon proestrus (20, 30). At first, multiple doses of antagonist were given, since their duration of action was not sufficient to produce complete inhibition of the LH surge that takes place over an 8-hour period in the rat. Analogues such as D-Phe<sub>2</sub>D-Ala<sub>6</sub>-LH-RH and D-Phe<sub>2</sub>D-Leu<sub>6</sub>-LH-RH were able to block ovulation and the preovulatory gonadotropin surge in cycling rats when multiple doses of 12 mg/kg of body weight were given during the afternoon proestrus (20, 30). In 4-day cycling rats, D-Phe<sub>2</sub>D-Leu<sub>6</sub>-LH-RH caused 83% blockade of ovulation after three injections of 2 mg on the proestrus day (30). D-Phe<sub>2</sub>D-Phe<sub>6</sub>-LH-RH was effective in single doses of about 6 mg/kg in the rat. The antagonist D-Phe<sub>2</sub>D-Phe<sub>6</sub>Aza-Gly<sub>10</sub>-LH-RH containing an Aza-Glycine residue in position 10 was reported to block ovulation induced by LH-RH in androgen-sterilized constant estrus rats (33).

The replacement of Trp by D-Trp in position 3 appeared to increase the potency of inhibitory peptides significantly (29). In tests for inhibition of ovulation, D-Phe<sub>2</sub>D-Trp<sub>3</sub>D-Phe<sub>6</sub>-LH-RH (*Figure 4*) was about twice as effective as D-Phe<sub>2</sub>Phe<sub>3</sub>D-Phe<sub>6</sub>-LH-RH and longer acting (29). D-Phe<sub>2</sub>D-Trp<sub>3</sub>D-Phe<sub>6</sub>-LH-RH was the first inhibitory analog found to be active in human beings (36). Other replacements of positions 3 and 6 of the LH-RH sequence also produced potent inhibitory analogs. D-Phe<sub>2</sub>Pro<sub>1</sub>D-Trp<sub>3</sub>D-Phe<sub>6</sub>-LH-RH, infused by a minipump at the rate of 375 µg/day for 4 days to cycling rats, inhibited ovulation (49).

An improvement in antagonistic activity resulted from the replacement of L-pyroGlu by the D-pyroGlu group (66). Another approach that has been used for increasing
Replacement of D-Phe by D-p-Cl-Phe in position 1 when injected in propylene-glycol-saline solution and at Phe-reptides were about equally effective. Ac-L-Trp3,D-Trp3,D-Trp6-LH-RH were also potent inhibitors, the best of the series being the isophthaloyl and succinoyl dimers (80, 82). One of the most active compounds in this series was the isophthaloyl dimer of D-pyroGlu,D-Phe2,-LH-RH that had greater inhibitory activity than D-Phe2,D-Trp3,D-Lys6-LH-RH on a weight basis, despite its higher molecular weight. This increased activity was probably due to the presence of two N termini that could interact with two receptor sites simultaneously. However, a peptide with three N-termini had decreased activity, presumably because of steric problems between chains.

Dimers of D-Phe2,D-Trp3,D-Lys6-LH-RH were also potent inhibitors, the best of the series being the isophthaloyl and succinoyl dimers (80, 82). One of the most active compounds in this series was the isophthaloyl dimer of D-pyroGlu,D-Phe2,D-Trp3,D-Lys6-LH-RH, which gave significant blockade of ovulation in the rat at a dose of 0.5 mg (27, 80). This dimer was also found to be active in human beings (72). Some modifications in the peptide backbone, e.g., by introduction of the χCH-CH3-S group, have also led to active antagonists, probably because of resistance to enzymatic degradation (82).

**MORE MODERN ANTAGONISTS**

Subsequent work was continued on positions 1 and 2, particularly on the effects of various substituents on the benzene ring of the D-Phe residue, and it was found that the p-Cl-peptides had improved inhibitory activities (14, 25, 26, 34). Channabasavaiah and Stewart (14) reported that substitution of acylated D-amino acids, including D-Phe, in position 1 led to good inhibitors. In our hands N-Ac-D-Phe1,D-p-Cl-Phe2,D-Trp3,D-Trp6-LH-RH produced good blockade of ovulation at a low dose of 62 µg when injected in propylene-glycol-saline solution and at 31 µg in corn oil (25, 26).

Replacement of D-Phe by D-p-Cl-Phe in position 1 improved the inhibitory activity about two-fold (26). p-F-Phe-reptides were about equally effective. Ac-D-Trp1,3,D-p-Cl-Phe2,D-Phe4-LH-RH was also very active (26, 34). When D-Ala was incorporated into one of the more active sequences in position 10 to give [Ac-D-Phe1,D-p-Cl-Phe2,D-Trp3,6,D-Ala10]-LH-RH, another two-fold increase in activity was found (26). [Ac-D-p-Cl-Phe1,2,D-Trp3,D-Phe6,D-Ala10]-LH-RH was effective in blocking ovulation in rats when given in 10-15 µg doses at 12 noon on proestrus (34). Prolonged blockade of ovulation, which occurred when 150 µg doses of this analog were given, was associated with a 90% reduction in pituitary binding sites for LH-RH (59, 72). A similar improvement in activity occurred in N-Ac-D-Trp1 analogs containing D-Ala10 (34). N-Ac-D-Trp1,3,D-p-Cl-Phe2,D-Phe6,D-Ala10 was active at a dose of 10 µg in blocking ovulation in rats (26, 34, 72).

Other groups used different structural substitutions in position 1. Spatola and Agarwal reported that Ac-Gly-L-D-p-Cl-Phe2,D-Trp4,6-LH-RH inhibited ovulation in rats in doses of 25 µg/rat (81).

Rivier and co-workers studied several analogs containing Ac-dehydro-Pro1(Δ3-Pro) substitution in position 1, combined with D-p-Cl-Phe in position 2 (65). Ac-dehydro-Pro1,D-p-Cl-Phe2,D-Trp3,6-LH-RH and Ac-dehydro-Pro1,D-p-F-Phe2,D-Trp3,6-LH-RH inhibited ovulation in doses of 7.5 µg and 5 µg respectively. Rivier’s group also made some analogs with β(2-Naphthyl-D-Ala) substitutions in positions 3 and 6 (65). Ac-dehydro-Pro1,D-p-F-Phe2,β-(2-Naphthyl)-D-Ala1,6,8-LH-RH was highly active at 2.5 µg/rat in the ovulation test. Both N-Acetyl-dehydro-Pro and β(2-Naphthyl)-D-Ala appeared to be good substituitions.

Bowers and Folkers’ group synthesized various series of LH-RH antagonists with D-amino acid substitutions in positions 2,3,6 as well as 4,5,8 (9). Of these analogs, N-Ac-D-Thr1,D-Phe2,D-Trp3,6,D-Ser4,D-Tyr5,D-Arg8 was active at doses of 12.5 µg/rat and showed no changes in conformation.

It has recently been demonstrated in our laboratory that insertion of D-lysine, or better still, D-arginine, in the position of LH-RH antagonists results in greater anti-ovulatory activity as compared to that found in the corresponding D-Phe or D-Trp analogs (23). For instance, [Ac-D-p-Cl-Phe1,2,D-Trp3,D-Arg6,D-Ala10]-LH-RH (Figure 5) exhibited antiovulatory activity at a dose of 3 µg in 40% propylene glycol/saline and at 750 ng in corn oil per rat (23, 72). This was the first competitive LH-RH antagonist with activity in the range below 1 µg (23, 72). In addition, this antagonist is soluble in saline and active when given orally (61).

(N-Ac-D-p-Cl-Phe1,2,D-Trp3,D-Arg6,D-Ala10) -LH-RH

**Figure 5. Structure of Arg8 antagonist.**
The antiovulatory activity of various antagonists of LH-RH is illustrated in Table 1. It can be seen that during the past decade at least a 1000-fold increase in inhibitory activity has been achieved.

<table>
<thead>
<tr>
<th>LH-RH Analog</th>
<th>Dose* (mg)</th>
<th>Blockade of Ovulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Phe-D-Leu6</td>
<td>2.0 (x 3)</td>
<td>82</td>
</tr>
<tr>
<td>D-Phe-D-Phe1-D-Phe6</td>
<td>1.5</td>
<td>100</td>
</tr>
<tr>
<td>D-Phe-D-Trp3-D-Phe6</td>
<td>1.0</td>
<td>90</td>
</tr>
<tr>
<td>D-Phe-D-pCl-Phe2.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Trp-D-Phe6</td>
<td>0.25</td>
<td>82</td>
</tr>
<tr>
<td>Ac-D-Phe1-D-pCl-Phe2,D-Trp1,6</td>
<td>0.052</td>
<td>100</td>
</tr>
<tr>
<td>Ac-D-pCl-Phe1,2,D-Trp1,6</td>
<td>0.015</td>
<td>70</td>
</tr>
<tr>
<td>Ac-D-pCl-Phe1,2,D-Trp3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Phe6-D-Ala10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ac-D-pCl-Phe1,2,D-Trp1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ac-D-pCl-Phe1,2,D-Trp3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Arg-D-Ala10</td>
<td>0.003</td>
<td>50</td>
</tr>
<tr>
<td>D-Arg-D-Ala10</td>
<td>0.003</td>
<td>78</td>
</tr>
</tbody>
</table>

*Most analogs were injected in 40% propylene glycol/saline.


Table 1. Antiovulatory activity of various LH-RH antagonists in 4-day cycling rats.

In conclusion, although additional improvements in inhibitory activity are likely to be achieved, several recently synthesized antagonists are already potent enough to be practical for clinical tests. Plans have been made to subject these antagonists of LH-RH to systematic clinical trials as soon as necessary pharmacological and toxicological tests are completed.

**BIOLOGICAL EVALUATION OF LH-RH ANTAGONISTS**

Both in vivo and in vitro methods were used by nearly all investigative groups to test inhibitory activity of various analogs of LH-RH made by synthetic peptide chemists (9, 29, 65, 70, 72). Although scientists in our laboratory possessed considerable experience in in vitro studies with isolated pituitary tissue dating back more than two decades (67), we decided to base the evaluation of antagonists on the results of in vivo tests. This was prompted by the realization that in order for these antagonists to form the basis of an effective contraception method, they would have to perform under in vivo conditions where various effects, such as enzymatic degradation in the blood stream, would have to be taken into account. A high in vitro activity of an antagonist might be of little relevance if it is rapidly degraded in the blood stream. Nevertheless, some groups used in vitro tests extensively. We used in vitro tests occasionally to guide early attempts to synthesize LH-RH antagonists, to obtain evidence for an effect on the pituitary gland, and to shed light on the mechanisms of action. More recently, various groups have carried out measurements of the binding of antagonists to receptors in order to obtain information for designing new antagonists.

**IN VITRO ASSAYS**

Since LH-RH and its analogs stimulate the release of LH and FSH from cultured rat pituitary cells (84, 86), methods have been developed to measure suppression of response to LH-RH by inhibitory analogs in this system. After 4 or 5 days in culture, cells were washed and incubated for 5 hours at 37°C in Dulbecco-modified Eagle’s medium in the presence α: absence of 3 x 10^-9 M LH-RH. Antagonistic LH-RH analogs added 10 minutes prior to LH-RH inhibited LH and FSH release (24, 52). In our hands, in vitro activities of some LH-RH antagonists were sometimes different from in vivo potencies (24, 52). However, Bowers and co-workers found that for most of the antagonistic analogs, in vitro and in vivo potencies closely paralleled each other (9).

**STUDIES WITH PITUITARY MEMBRANE RECEPTORS**

The investigation of binding affinity of antagonists of LH-RH with adenohypophysial plasma membranes should help in designing better inhibitory analogs. Various groups have studied the interaction of some early inhibitory analogs with pituitary receptors for LH-RH. Spona showed that the early antagonist desHis2,desGly10-LH-RH bound to receptors in order to obtain evidence for an effect on the pituitary gland. However, Bowers and co-workers found that for most of the antagonistic analogs, in vitro and in vivo potencies closely paralleled each other (9).

In our initial studies, rat pituitary homogenate was incubated with ([125I]LH-RH) in the presence of different concentrations of either cold LH-RH or D-Phe2,D-Trp3,D-Phe6-LH-RH. LH-RH competed for receptor sites with the tracer, but the antagonist displaced the tracer from receptor sites to a greater degree than did the parent hormone (70, 72). Similarly, the displacement of [125I]LH-RH by cold LH-RH in adenohypophysial plasma membrane preparations was also smaller than that produced by D-Phe2,D-Trp3,D-Phe6-LH-RH (70, 72).

There is now excellent evidence that LH-RH and agonistic analogs bind to specific receptors in the anterior pituitary gland. These receptors are located on the surface of the gonadotrophs. Several groups worked out the optimized conditions for in vitro receptor binding studies (17, 43, 44, 45, 47, 53). Both low (K_a = 10^8 M^-1) and high (K_a = 10^6-10^9 M^-1) affinity sites for LH-RH have been demonstrated in pituitary membrane preparations (12, 17, 53). Modifications in the 6 and 10 positions, which produce super-
active agonists, also increase binding affinity to LH-RH receptors (17). In the rat, significant variations in the LH-RH receptors occur under several physiological conditions, including estrus cycle and pregnancy (12, 18, 68). The affinity of modern antagonists for LH-RH receptors is as high as that of some of the most potent agonists, the equilibrium association constants (K_a) being 4-6 x 10^8 M^-1, i.e., 8 to 10 times higher than that of LH-RH (K_a = 6.6 x 10^4 M^-1) (15, 17, 59). The post-castration rise in pituitary LH-RH receptors can be prevented by treatment with LH-RH antagonist N-Acetyl-Ala,D-p-Cl-Phe,D-Trp,D-Lys,LH-RH (16). When measured in the unprocessed homogenate, this reduction in receptors could be accounted for in part by the receptor occupied by the antagonist. The dissociation rate of this antagonist from pituitary membrane preparations was also four times slower than the dissociation rate of a potent agonist (46). The inhibition of ovulation by LH-RH antagonist is also accompanied by a reduction in pituitary LH-RH binding sites (59).

Conn and co-workers demonstrated recently that a typical antagonist, D-pyro-Glu-D-Phe,D-Trp,D-Lys,LH-RH, can become an LH-RH agonist if it is cross-linked to form a dimer and conjugated to an antibody to D-Lys,LH-RH (19). The stimulatory effect might be caused by receptor microaggregation, i.e., bringing two receptor molecules within a critical distance.

**IN VIVO EVALUATION OF LH-RH ANTAGONISTS**

inhibition of LH and FSH release and blockade of ovulation in rats. In early studies, various in vivo methods, for instance inhibition of LH-RH induced LH release in ovariec tomized rats treated with steroids, were used to follow the anti-LH-RH activity of the LH-RH antagonists (89). Only the most significant studies will be discussed here.

One of the most convenient assays consisted of blockade of LH and FSH release in immature male rats (88, 90). Animals were injected subcutaneously with antagonists or with vehicle alone. Two hundred nanograms of synthetic LH-RH or saline solution were injected subcutaneously at the same time or at different times thereafter. Blood was collected 30 minutes after LH-RH or saline administration. Active antagonist had the ability to reduce LH and FSH release. Typical results are illustrated in Figure 6. The anti-LH-RH activity was calculated as a percentage of inhibition of gonadotropin release over a 1 to 6 hour period.

However, the most meaningful data were based on blockade of ovulation in proestrus rats. Antiovulatory tests were performed with adult female rats weighing approximately 200 g. Daily vaginal smears were taken and only rats that showed at least two successive regular 4-day cycles were used. Inhibitory analogs dissolved in propylene glycol/saline solution or corn oil were injected subcutaneously, usually at 12 noon on the proestrus day. Control groups were given vehicle alone. On the following morning, the oviducts of the animals were inspected for ova under a dissecting microscope (20, 30, 88). The most convenient way to express the potency was to compare the percentage of blockade of ovulation in response to a certain dose of inhibitory analog. Earlier studies in proestrus rats showed only a partial blockade of ovulation after repeated administration (6 times) of large doses of antagonist D-Phe,D-Ala,LH-RH (20). It was found later that a single administration of better antagonists could reduce or depress the preovulatory surge of LH and FSH and block ovulation (30, 70, 72) (Figure 7).

A single subcutaneous injection of 1.5 mg of D-Phe,D-Phe,LH-RH given at noon on the proestrus day resulted in 95% reduction of preovulatory LH surge and 84% reduction of FSH surge (30). Modern antagonists such as N-Ac-D-p-Cl-Phe,D-Trp,D-Arg,D-Ala,LH-RH can block ovulation in rats in much smaller doses — 3 μg in propylene glycol/saline or 0.75 μg in oil (Table 1).
Figure 7. Effect of single subcutaneous administration of D-Phe^2^-Phe^3^-D-Phe^6^-LH-RH (1.5 mg) on the preovulatory surge of LH in proestrus rats. (From de la Cruz A, Coy DH, Vilchez-Martinez JA et al: Science 191:195, 1976.)

The LH-RH antagonist Ac-D-p-Cl-Phe^1^-2,D-Trp^3,D-Arg^6,D-Ala^10^-LH-RH is also active when given orally (61). Administration by gavage of 2 mg antagonist at 2 pm on proestrus day blocked the preovulatory surge of LH and ovulation (61). Ovulation was also delayed for at least one day in some animals given 1 or 0.5 mg antagonist orally (61). The demonstration that antagonists of LH-RH can exert contraceptive effects when given orally enhances their therapeutic potential.

Pharmacologic doses (1000 µg) of this LH-RH antagonist N-Ac-D-p-Cl-Phe^1^-2,D-Trp^3,D-Arg^6,D-Ala^10^-LH-RH were also reported to inhibit ovulation induced by hCG in pregnant mare serum (PMS)-pretreated hypophysectomized rats (60). Although these doses were about 300 times larger than those needed to block ovulation at the pituitary level in proestrus rats, these studies suggest that some of the effects of LH-RH antagonists, in analogy to agonists, can be exerted in part at the ovarian level. Previous studies by Hseu and associates (48, 50) support the idea that antagonists of LH-RH may exert some direct action on the gonads.

Disruption of the estrus cycle and mating behavior in rats. Various investigators reported previously that larger doses of early antagonists of LH-RH could disrupt the estrus cycle in rats (20, 70). Recently, Rivier and associates (64) reported that Ac-dehydro-Pro^1,D-p-Cl-Phe^2,D-Trp^3^-6,Me-Leu^7^-LH-RH disrupted the estrus cycle of female rats when given in daily doses of 200 µg. Cycles resumed 6 to 9 days after cessation of treatment. Another potent antagonist, Ac-dehydro-Pro^1,D-p-Cl-Phe^2,D-Trp^3^-6,LH-RH(3), produced a marked decrease in lordosis behavior when infused into the third ventricle (32). These results suggest that antagonist analogs of LH-RH reduce sexual receptivity in the female rat (32).

D-Phe^2^-D-Ala-LH-RH has been rigorously tested by Corbin and Beattie (7, 20). They found that high doses (6 mg) of this peptide could prevent pregnancy when administered precoitally, during proestrus, to rats which were then subjected to mating (7, 20). Rivier and associates (64) also reported recently that Ac-dehydro-Pro^1,D-p-Cl-Phe^2,D-Trp^3^-6,Me-Leu^7^-LH-RH administered daily on days 7 to 12 of pregnancy in doses of 1 mg caused a decrease in plasma progesterone levels.

Golden Hamsters. In golden hamsters, ovulation was more difficult to block with inhibitory analogs than it was in rats (70).

In cycling hamsters, repeated administration of D-Phe^2^-D-Leu^6^-LH-RH on the afternoon of proestrus decreased LH surge by 83% (70) but this suppressed ovulation by only 30%. The reason for this was discovered later, when it was shown that hamsters can ovulate fully with only about 10% of the gonadotropins normally released during proestrus (70). We also examined various early analogs for their ability to suppress LH-RH-induced ovulation in
phenobarbital-blocked hamsters. Only D-Phe\textsuperscript{2},D-Leu\textsuperscript{6}, LH-RH blocked ovulation when it was injected 60 minutes before 75 ng LH-RH. For reasons explained above, subsequent studies in hamsters were abandoned (70).

**RABBITS.** LH-RH antagonist can also inhibit ovulation caused by mating or induced by LH-RH in mature female rabbits (62). When multiple large doses of D-Phe\textsuperscript{2},Phe\textsuperscript{3}, D-Phe\textsuperscript{6}-LH-RH were administered 3 to 5 times at half-hour intervals beginning 30 minutes prior to mating, the rise in plasma LH was reduced, and three out of five treated rabbits showed partial or complete blockade of ovulation. Similar results were obtained with D-Phe\textsuperscript{2},D-Trp\textsuperscript{3},D-Phe\textsuperscript{6}-LH-RH.

Corbin and Beattie were the first to report that 25 mg of D-Phe\textsuperscript{2},D-Ala\textsuperscript{6}-LH-RH could block LH-RH-induced ovulation in rabbits (20). We showed that a peak in the plasma LH level and full ovulation, routinely induced in all does by intravenous injection of LH-RH, could be suppressed by pretreatment with D-Phe\textsuperscript{2},Phe\textsuperscript{3},D-Phe\textsuperscript{6}-LH-RH (62).

**STUDIES WITH LH-RH ANALOGS IN NON-HUMAN FEMALE PRIMATES**

Various problems were encountered in initial studies with antagonistic analogs in primates until suitable models were found (70). Initial tests demonstrated that large doses of early LH-RH antagonists, D-Phe\textsuperscript{2},D-Phe\textsuperscript{6}-LH-RH and D-Phe\textsuperscript{2},D-Trp\textsuperscript{3},D-Phe\textsuperscript{6}-LH-RH, inhibited responses to LH-RH in baboons, rhesus monkeys, marmosets, and chimpanzees (40, 42, 70). Chimpanzees respond to LH-RH in a manner closely similar to women. D-Phe\textsuperscript{2},D-Trp\textsuperscript{3}, D-Phe\textsuperscript{6}-LH-RH, in doses of 35 mg, inhibited the LH response of chimpanzees given 10 \( \mu \)g of LH-RH without altering basal gonadotropin levels (40). Wilks and associates reported modification of preovulatory gonadotropin \( \Delta \) peaks estrogen peaks in cycling rhesus monkeys treated with 300 mg doses of D-Phe\textsuperscript{2},Pro\textsuperscript{3},D-Phe\textsuperscript{6}-LH-RH (92). However, even at these large doses, the analog did not completely inhibit gonadotropin surges and luteinization (92).

Recent investigations, particularly by Asch and his associates (1-6, 8) provided proof that LH-RH antagonists, developed primarily on the basis of antiovulatory tests in rats, are active in female primates (1-8).

In a series of studies, Asch and co-workers demonstrated inhibitory effects of modern LH-RH antagonists on gonadotropin production and ovulation in rhesus monkeys and cynomolgous monkeys (1-6, 8). In the first study, it was shown that daily administration of 1 mg of Ac-D-Trp\textsuperscript{1,3},D-P-CI-Phe\textsuperscript{2},D-Phe\textsuperscript{6},D-Ala\textsuperscript{10}-LH-RH prevented the castration-induced rise of LH and FSH in female rhesus monkeys (3). Controls showed significant elevation of gonadotropins 3 to 4 days after castration. After discontinuation of treatment with the antagonist, gonadotropin levels rose to elevated levels observed in control animals (3). Acute intramuscular administration of 1 mg of this antagonist to ovariectomized rhesus monkeys decreased LH and FSH levels within 2 to 4 hours (4). The gonadotropin levels were reduced for at least 24 hours. The rise in LH and FSH in response to administration of 150 \( \mu \)g LH-RH was significantly inhibited in ovariectomized animals by pretreatment 18 hours before with 1 mg antagonist (5). In another study, daily administration of 1 mg doses of the same antagonist to 7 normally cycling rhesus monkeys from days 10 to 14 of the cycle significantly delayed the preovulatory LH surge and ovulation in 5 animals (6). The length of the cycle was proportionately increased in these animals, ovulation occurring 6 to 10 days later. In the two remaining animals, LH and estrogen peaks occurred while they were receiving LH-RH antagonist, but in both cases, estrogen values were already ascending when treatment was started (6). One of these animals did not ovulate, as determined by laparoscopy, and did not form a visible corpus luteum (6). This and another study showed that a marked decrease in postovulatory LH levels induced by LH-RH antagonist does not alter the length of the luteal phase (6). It was also demonstrated that 1 mg doses of N-Ac-D-Trp\textsuperscript{1,3},D-p-CI-Phe\textsuperscript{2},D-Phe\textsuperscript{6},D-Ala\textsuperscript{10}-LH-RH decreased the amplitude of LH pulses in rhesus monkeys, while 2 mg of this antagonist abolished both the frequency and amplitude of LH pulses. Other investigations indicated that LH-RH antagonists can suppress positive feedback and potentiate negative feedback of estradiol benzoate on gonadotropin secretion in ovariectomized rhesus monkeys (1, 2). Animals that received N-Ac-D-Trp\textsuperscript{1,3},D-p-CI-Phe\textsuperscript{2},D-Phe\textsuperscript{6},D-Ala\textsuperscript{10}-LH-RH in doses of 1 mg or Ac-D-p-CI-Phe\textsuperscript{1,2},D-Trp\textsuperscript{3},D-Arg\textsuperscript{6},D-Ala\textsuperscript{10}-LH-RH in doses of 0.2 mg, in addition to 40 \( \mu \)g of estradiol benzoate, showed a more pronounced and prolonged suppression of gonadotropin levels during the first 24 hours than animals treated with estradiol benzoate alone. Animals injected with the antagonist also showed a suppression of LH and FSH peaks typically observed 26 to 48 hours after estradiol benzoate administration (1, 2). The results of this study indicate that LH-RH is an essential prerequisite for the mechanism of positive feedback of estrogen on gonadotropin secretion.

Work in cynomolgus monkeys (Macaca fascicularis) also showed, in analogy to results in rhesus monkeys, that antagonist N-Ac-D-Trp\textsuperscript{1,3},D-p-CI-Phe\textsuperscript{2},D-Phe\textsuperscript{6},D-Ala\textsuperscript{10}-LH-RH, given subcutaneously, in doses of 1 mg, for 25 days from the first day of the menstrual cycle, suppressed ovulation (8). The animals remained anuvulatory during administration of the antagonist. Four animals ovulated 7 to 15 days following discontinuation of the drug and 1 monkey ovulated 125 days later. These
studies in rhesus and cynomolgous monkeys showed clearly that chronic administration of LH-RH antagonist can abolish ovulation in primates (6, 8).

These investigations also demonstrated that a non-human primate model can be used to test the efficacy of LH-RH antagonists as potential contraceptive agents in the human female. LH-RH antagonists were found to be potent, long-acting inhibitors of LH and FSH secretion in nonhuman primates, with potential clinical application in fertility control.

**CLINICAL TRIALS WITH ANTAGONISTS OF LH-RH IN WOMEN**

In the course of development of LH-RH antagonists, as inhibitory activity improved, we felt it would be of critical importance to show that these analogs were active in women.

The demonstration that large doses of D-Phe²,D-Trp³,D-Phe⁶-LH-RH diminished the gonadotropin response to LH-RH in normal men led us to investigate whether this inhibitory analog had the same effect in normal ovulatory women (36) (Figure 8). Eight woman physicians aged 20 to 28 years, who were normally menstruating, cyclic, and ovulatory, volunteered for the investigation (11). Intramuscular administration of 90 mg of D-Phe²,D-Trp³,D-Lys⁶-LH-RH, administered in doses of 50 mg to climacteric women and to women with ovarian amenorrhea lowered high LH and FSH levels for a period of 6 to 8 hours (72). N-Ac-D-Phe¹,D-p-C-(Phe²,D-Trp³,D-Cl-Phe⁶)-LH-RH and N-Ac-D-Phe¹,D-p-Cl-Phe²,D-Trp³⁶,D-Cl-Phe⁶,D-Ala¹⁰-LH-RH, in doses of 10 mg and 5 mg respectively, lowered LH and FSH in climacteric women for more than 24 hours (72).

Both of these analogs were then tested in normal women with the aim of inhibiting ovulation. N-Ac-D-Phe¹,D-p-Cl-Phe²,D-Trp³⁶,D-LH-RH blocked ovulatory LH peak and ovulation in 8 out of 14 normal women when given on day 12 of the cycle in doses of 10 mg. Six of these women menstruated 12 to 20 days later and two experienced earlier uterine bleeding (72, 93). The other six women

![Figure 8. Mean response in serum LH levels of 4 men to 25 µg LH-RH before (control) and after intramuscular administration of 90 mg D-Phe²,D-Trp³,D-Phe⁶-LH-RH. Asterisks indicate values significantly (p < 0.01) different from the value at that time during the control period. (From Gonzalez-Barcena D, Kastin AJ, Coy DH et al: Lancet ii:997-998, 1977).](image-url)
showed some attenuation of both LH and FSH surges but ovulated. All 14 patients showed normal ovulation and resumed regular menses in the next cycle. This proved that the effect of the antagonist are reversible (93).

In another study, a still more powerful antagonist, N-Ac-D-p-Cl-Phe<sub>1</sub>,<sup>2</sup>D-Trp<sub>3</sub>,D-Phe<sub>6</sub>,D-Ala<sub>10</sub>LH-RH, administered on day 12 in a dose of 5 mg, blocked ovulation based on LH, FSH and progesterone levels in 6 out of 9 normal women. No breakthrough bleeding occurred. In some women, the menstrual period was delayed up to 10 days but in others, menstruation occurred within the normal limits of the cycle (72, 93).

N-Ac-D-p-Cl-Phe<sub>1</sub>,<sup>2</sup>D-Trp<sub>3</sub>,D-Phe<sub>6</sub>,D-Ala<sub>10</sub>LH-RH was then tested in smaller doses in ten normally menstruating ovulatory women aged 20 to 30 years (94). Intramuscular administration of the antagonist in doses of 2 mg on day 12 of the menstrual cycle inhibited midcycle surge of LH and FSH and ovulation in 6 out of 10 women (Figure 9). Serum progesterone levels and urinary pregnanediol values in these 6 women were consistently low, and corresponded to anovulatory cycles (94). These women menstruated 12 to 26 days after injection of the antagonist. Two patients in whom the analog did not abolish the LH mid-cycle surge showed a short luteal phase and premature menstruation. An early decline in progesterone levels suggested that luteolysis took place. Two women ovulated, but no pregnancies occurred. Endometrial biopsies revealed moderate proliferative endometrium in women in whom ovulation was blocked. No untoward side effects of this antagonist were recorded, but the injection was reported to be painful by all patients because of 40% propylene glycol/saline solution used as a carrier. All women resumed regular menses and ovulation in the subsequent 2 to 4 cycles. No episodes of breakthrough bleeding occurred in treated and post-control cycles (94).

So far only one other group has reported clinical studies on LH-RH antagonist (13). A powerful LH-RH antagonist, Ac-dehydro-Pro<sub>1</sub>,D-p-F-Phe<sub>2</sub>,D-Trp<sub>3</sub>,LH-RH, administered intravenously in doses of 80 µg/kg, lowered serum levels of LH and FSH by 43% and 18% respectively within 4 hours in 5 hypergonadotropic women, and decreased the pulses of both gonadotropins (13). Subcutaneous or intramuscular injection of this antagonist suppressed LH levels by 39% and FSH by 13% to 27% at 6 to 8 hours, gonadotropin levels returning to normal within 24 hours. When this antagonist was given during an intravenous infusion of LH-RH (0.2 µg/min) at a time of maximal gonadotropin stimulation, it reduced LH levels by 49% and FSH by 13% within 2 hours. These studies confirmed our previous findings (11, 36, 38, 39, 93, 94) that LH-RH antagonists can suppress endogenous or LH-RH-induced gonadotropin release.

No data are available so far on the clinical efficacy of the powerful antagonist N-Ac-D-p-Cl-Phe<sub>1</sub>,<sup>2</sup>D-Trp<sub>3</sub>,D-Phe<sub>4</sub>,D-Ala<sub>10</sub>LH-RH, which is soluble in water or saline. Evaluation of effects of chronic administration of this antagonist and other powerful modern antagonists on ovulatory patterns in women will have to await the completion of long-term toxicity studies.

**THE STATUS OF FEMALE CONTRACEPTION BASED ON LH-RH ANTAGONISTS**

Results of our studies described above indicate that a single administration of LH-RH antagonists can abolish the mid-cycle surge of LH and FSH and inhibit ovulation in most women, or induce luteolysis in others. Consequently, the contraceptive effect of LH-RH antagonists could be exerted through two possible mechanisms. These findings confirm the view that LH-RH antagonists can be used for the development of a new contraceptive method.
The use of antagonists of LH-RH may, however, be connected with some problems. The irregular bleeding after a single administration of the antagonists seems to be at present one of the inconveniences (93, 94). It will be necessary to administer the antagonists for several cycles or even a more prolonged period of time in order to evaluate the anovulatory action and chronic effects, as well as the morphological changes in the endometrium. Since the antagonists were designed for inhibition of LH and FSH secretion, they should not cause any transient stimulatory “flare-ups” and should lead to a better ovarian suppression than the agonists, which were originally intended for treatment of infertility. Thus, the incidence of endometrial hyperplasia and unopposed estrogen secretion is expected to be low after treatment with the antagonists. It is also hoped that antagonists will offer other advantages over the agonistic analogs of LH-RH in fertility control. For example, it may be possible to administer the antagonist from day 5 to day 25, as in the case of the oral contraceptive pills, or only a few times near mid-cycle, to inhibit ovulation, in contrast to daily administration of agonists. It would be desirable to preserve a normal menstrual cycle, if possible, during antagonist administration.

Some minor problems that became apparent during the acute trials with the antagonists can be solved without major difficulties. The discomfort associated with the injection of 40% propylene glycol as vehicle could be easily eliminated by the use of suitable carriers such as gelatin-mannitol solution for the more lipophilic antagonists. In addition, the powerful antagonist N-Ac-D-p-Cl-Phe¹,²,D-Trp⁴,D-Arg⁶,D-Ala¹⁰-LH-RH is soluble in water and saline solution. Evaluation of various routes of administration of these antagonists will also have to be carried out and suitable routes devised. Frequent parenteral administration would be inconvenient and, hence, impractical. Oral administration of LH-RH agonists in rats or humans required doses at least 1000 times larger than those causing a comparable elevation of gonadotropins by the parenteral route (31, 37, 70, 72, 73). The same appears to be true for the antagonists on the basis of oral administration tests in rats (61). Thus, in human beings, oral administration of LH-RH antagonists would probably have a very low degree of effectiveness, of the order of about 1/1000, as compared with the parenteral route. Hence, a practical route of administration for the antagonists appears to be intranasal. Intranasal application has been found to be a convenient and practical way of administering LH-RH and its agonists, H766 and D-Trp⁶-LH-RH (91), and the same should be possible for more lipophilic LH-RH antagonists. In addition to intranasal sprays, recent studies indicate that injectable microcapsules with LH-RH agonists can be prepared from biodegradable biocompatible polymer poly (d,l-lactide-co-glycolide) for once-a-month administration (78, 87). These microcapsules with D-Trp⁶-LH-RH or D-Nal(2)⁶-LH-RH were designed for constant controlled release of the agonists over a 30-day period. The efficacy of these release systems in rhesus monkeys and rats has been demonstrated (87).

Similar or related formulations of polymers for microencapsulation of LH-RH antagonist are certain to be tried and developed. Since such microcapsules need to be administered only every 30 to 90 days, this method would further facilitate the development of contraceptive approaches based on LH-RH antagonists. Periodic intramuscular administration of microcapsules with the antagonist would avoid the pregnancies that would result from the failure by some women to follow other regimens properly. Such a method would also be appropriate for women in developing countries.

In case the analogs already developed should not have the efficacy required for clinical trials, success in this project may be ensured by synthesis of additional LH-RH antagonists by peptide chemists and subsequent testing by reproductive biologists and clinicians.

ACKNOWLEDGMENT

The feasibility of creating, by synthetic approaches, LH-RH analogs that inhibit LH and FSH release and ovulation, which was forecast in 1971, has been clearly proved. LH-RH agonists already have sufficient potency to permit extensive trials on blockade of ovulation in normal women. In these trials, the analogs could be injected intramuscularly in a special vehicle or as microcapsules for controlled release over a longer period. More convenient routes of administration, such as nasal spray, will also have to be investigated. It is probable that antagonists of LH-RH could form the basis of new contraceptive methods. These methods would be based on blockade of the ovulatory mid-cycle peak of LH by antagonists of LH-RH or on induction of luteolysis. A peptide contraceptive that is free of the side effects of antifertility steroids would be a welcome addition to presently available methods for the control of human fertility.

CONCLUSION

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The immunogenicity of spermatozoa manifests itself in a variety of clinical conditions. Autoimmune orchitis is the result of an immune response to either spermatozoa or constituents of the testes. Idiopathic infertility of both males and females is often associated with antibodies to spermatozoa. In addition, antisperm immunoglobulins are a consequence of vasectomy that may compromise restoration of fertility. These pathologic conditions are consistent with the observations that testes and spermatozoa are antigenic in both males and females. Analysis of the nature of the antigenic stimulus is the basis for treatment and prevention of disease, as well as for restoration of fertility. If these uncontrolled pathologic infertilities were understood, the underlying mechanism could be manipulated to produce controlled infertility. Indeed, the fertility in females of several species is reduced following immunization with sperm or testes extracts. A well-defined sperm antigen could be applicable in immunization of both sexes. Thus, it may be possible to exploit the antigenicity of spermatozoa in the development of a new contraceptive technology.

The antigenicity of spermatozoa and testes has been known since the early 1900s; nevertheless, a precise description of the immune response to male reproductive tissues requires the isolation and characterization of specific antigens. There have been many attempts to identify the antigenic constituents of testes and spermatozoa, and to detect immune reactions and infertility following immunization with a variety of sperm "fractions" (27). More recently, there have been attempts to isolate and characterize specific sperm antigens (40). Considerable progress has been made in understanding the structure, localization, and antigenic properties of one enzyme, lactate dehydrogenase C4 (LDH-C4; LDH-X), which serves as a useful model for developing a contraceptive vaccine (63).
AUTOIMMUNE DISEASE

The pathogenesis of experimental allergic orchitis (EAO) was reviewed recently by Tung (54). A complex immunopathology is revealed by the detailed studies of EAO in the guinea pig. Furthermore, there are examples of naturally occurring orchitis and of immune response to testis-specific antigens in some vasectomized men (54).

An interesting model of naturally occurring orchitis has been discovered in a colony of dark mink in which 20% to 30% of the males were infertile (56). Orchitis and/or aspermatogenesis with testicular sperm antigen-antibody complexes were unequivocally demonstrated in many of these animals. Primary infertility was observed in some mink soon after puberty. These animals had low levels of antisperm antibodies. Aspermatogenesis, but rarely orchitis, was the main testicular abnormality. Tung observed that mink with primary infertility were defective in gonadotropin releasing hormone (GnRH) secretion, presumably due to abnormal hypothalamic function or its control mechanisms (55). Secondary infertility occurred in animals after a period of proven fertility. In these mink, there were significantly higher levels of antisperm antibodies, many testes with severe orchitis and/or aspermatogenesis, and presumptive evidence of immune complex deposition. The symptoms of secondary infertility increased during the breeding season. Ultrastructural studies revealed breakdown of tight junctional complexes between adjacent Sertoli cells (55). These findings suggest that the blood-testis barrier becomes defective during seasonal regression of the testis. Autoimmunization may lead to the development of orchitis, although the exact role of immune complexes in pathogenesis of secondary infertility remains to be defined.

Obviously, precise analysis of the immune response and of immune pathogenesis in males would be greatly facilitated by definition of the aspermatogenesis antigen(s). Previous studies on isolation and characterization are cited by Tung (54).

Teuscher and associates have isolated and partially characterized a polypeptide from guinea pig testis that induces EAO (53). This peptide, designated AP3, has an apparent molecular weight of 12,500 and induced severe EAO in 100% of the guinea pigs tested. Neither the subcellular location nor the functional role of AP3 is known at present.

IMMUNOLOGIC CONSEQUENCES OF VASECTOMY

Tung has summarized the immunopathologic complications of vasectomy and evidence of postvasectomy orchitis (54). Essentially, vasectomy creates a pool of spermatids that becomes accessible to the immune system as a result of the surgical procedure. Autoantibody responses to sperm-specific antigens appear in vasectomized animals of all species studied. Also, postvasectomy orchitis develops in rabbits and guinea pigs (57). However, only 50% to 60% of the vasectomized subjects, regardless of species, develop such antibodies (51). Apparently, the autoimmune response is strain-dependent and controlled by a single gene in the guinea pig model. Takami and associates demonstrated that in vasectomized rats, autoantibody production to sperm is not influenced by genes in the major histocompatibility complex or by genes on the Y chromosome (50).

After vasectomy, some serum antibody reacts with cell surface antigens, while other antibody is directed to internal antigens of testicular cells and sperm (57). Presumably, the latter reflects release of sperm degradation products. The cell surface antigens recognized postvasectomy include at least three major polypeptides, however the contribution of each to antigenicity remains unclear. Autoantibodies isolated from the sera of vasectomized guinea pigs react with three major sperm plasma membrane antigens of 69,000, 42,000 and 20,000 daltons, respectively (51). These peptides are significantly larger than the AP3 antigen of EAO. Identification and characterization of the antigenic stimulus is essential to achieve an understanding of orchitis and postvasectomy immunopathology.

IMMUNOLOGIC INFERTILITY

Serum antisperm antibodies have been implicated in cases of unexplained infertility. In fact, such observations have been the basis of attempts to manipulate the immune response to spermatozoa, both to alleviate undesired infertility and to develop a contraceptive.

A variety of assays for spermagglutination, immobilization, and cytotoxicity have been used in attempts to establish a quantitative relationship between infertility and antisperm antibodies. This work was recently reviewed by Menge (28). The incidence of antibodies detected by sperm agglutination and immobilization methods varies respectively from 5% to 30% and 2% to 16% for infertile women, and from 3% to 20% and 3% to 7% for infertile men (31). Moghissi and Wallach also implicate sperm antibodies either in sera, reproductive tract fluids, or both in couples with persistent unexplained infertility (34). These authors caution, however, that many published reports of an immunologic basis are inadequate in their evaluation of infertility.

Reduced penetration of cervical mucus by sperm is associated with serum titers of antisperm antibodies in both sexes and also with immobilizing antibody activity in cervical mucus (31). The incidence of subsequent
pregnancy in 376 infertile couples was reduced significantly if the man or the woman had antisperm antibodies in serum or in genital tract secretions. Agglutinating antibodies in both sexes occurred about three times more frequently than immobilizing antibodies (31). There have been no reports to date of immobilizing antibody activity in the sera of fertile individuals (31). In general, infertility in human couples may be caused by immunologic reactions in which sperm penetration of cervical mucus is impeded, transport and viability in the female reproductive tract is decreased, penetration of the ovum by the sperm is inhibited, or normal postfertilization development of the fertilized ovum is impaired (31).

Serious problems exist with most of the assay procedures used to detect antisperm antibodies in sera and reproductive tract fluids. These assays are complicated by Fc receptors on spermatozoa that cause a nonspecific binding of immunoglobulins in vitro (64). At the very least, both interpretation and quantitation of the various assays of spermagglutination are difficult, so there is great demand for improved methods.

Wolf and associates have recently described solid phase assays utilizing radiolabeled or enzyme-linked second antibody that may provide increased sensitivity and specificity to detect antisperm antibodies in infertile couples or vasectomized males (65).

Little is known about the specific antigens underlying these immunologic infertilities. When human serum samples containing antisperm antibodies were analyzed by a radioimmunoassay (24), unique sperm antigens that elicit these antibodies could be identified. Thus, sera from four patients reacted with a protein of about 30,000 daltons. Sera from two patients reacted with a protein band having a molecular weight of about 60,000. Sera from an additional nine patients did not react with unique peptides of sperm.

**ANALYSIS OF SPERM ANTIGENS**

The antigenic complexity of the male gamete undoubtedly confuses the analysis of these clinically significant conditions. The same pathology can reflect a variety of antigens, perhaps to different extents and with variation among individuals (24). It is necessary, therefore, to consider the total immunogenic inventory of the male reproductive system.

As noted above, many of the proteins that are synthesized during spermatogenesis represent potential autoantigens, since sexual maturation begins long after immunologic maturity. In addition to various testis-specific enzymes, plasma membrane components of developing germ cells could be antigenic.

Millette and Bellvé immunized rabbits with type B spermatagonia, 82% to 88% pure, from the mouse, and detected antigenic membrane components on germ cells at all stages of differentiation, ranging from primitive Type A spermatogonia to mature spermatozoa (32). Furthermore, certain antigenic components of the plasma membrane of early germ cells were partitioned selectively during spermiogenesis into that portion of the plasma membrane destined for the residual body.

The complexity of the plasma membrane components of developing mammalian spermatogenic cells is described by Millette and Moulding (33). At least 25 to 30 polypeptides were detected from pachytene primary spermatocytes and round spermatids, with no peptide unique to either stage.

Kramer and Erickson described stage-specific protein synthesis patterns during spermatogenesis (21). Pachytene spermatocytes were more active in protein synthesis than either early or late spermatids. Approximately 85% of the total extractable supernatant proteins were present in all three cell types. Twenty proteins were detected in both early and late spermatid fractions but not in other stages. Four proteins were found only in pachytene spermatocytes, three in spermatocytes and early spermatids, ten in early spermatids, and only one protein was found in late spermatids. Solubilized particulate proteins also showed stage specificity, although 75% to 80% were in all cell types (21).

Protein synthetic patterns in mouse spermatocytes and spermatids were examined in cultures of seminiferous tubules or isolated germ cells (3). Soluble polypeptides synthesized by middle to late pachytene spermatocytes and round spermatids were resolved as about 250 radiolabeled spots, compared to only 100 spots from intermediate spermatids. There was a drastic reduction in the number of newly synthesized polypeptides during spermiogenesis, with few new molecular species that are translated postmeiotically.

Stage-specific synthesis of peptides is clearly reflected in gene expression during murine spermatogenesis (7, 48, 49). Pachytene spermatocytes and round spermatids synthesized approximately equivalent numbers of polypeptides, with less synthesis in elongating spermatids and residual bodies (48). Stage specificity was detected in mRNA populations, both in terms of selective production and selective utilization of mRNA (7). It appears that much of the protein synthesis in the elongating spermatid is directed by mRNAs that had been stored in round spermatids (49).

**ISOLATION OF SPECIFIC PROTEINS**

Of the many peptides synthesized during spermatogenesis...
genesis, not all are antigenic. In most cases, the functional or metabolic role of these sperm-specific proteins has not been identified. Such identification would represent a major accomplishment in the study of autoantigenicity of testicular constituents by facilitating isolation procedures.

Proteins that have been purified from testes or spermatozoa include some of defined function, e.g., enzymes, and some that play an as yet undetermined structural role, e.g., plasma membrane constituents. Purification strategies for enzymic proteins can be relatively straightforward, and readily yield homogeneous products. In general, measurements of enzyme activity monitor the isolation protocol, and the steps are relatively mild, particularly in the case of soluble proteins. The primary limiting factor is the obtaining of sufficient starting material to yield a useful amount of product for further analysis. Once a homogeneous protein has been isolated, its auto- or isoantigenicity can be readily established. Thus, LDH-C\(_2\), PGK-2, cytochrome c, and possibly acrosin and hyaluronidase are testis-specific and autoantigenic (10).

Purification of testes or sperm peptides with known biological activity presents a much more formidable problem. Immunochemical techniques may be employed to monitor the isolation of antigenic peptides. The simplest experiment involves immunization with intact sperm and subsequent measurement of antibody binding to these cells. Lopo and Vacquier showed that rabbit antiserum to sperm of the sea urchin Strongylocentrotus purpuratus reacts with sperm from 28 species and from 7 phyla of the animal kingdom, including mammals (26). Antibody binding is not due to H-Y or Fc receptors or to cell surface tubulin. This report suggests the possible existence of common antigenic determinants on the surface of all animal sperm (26). It would be exceedingly useful to have a species from which sperm are abundantly available, to isolate a determinant common to human spermatozoa.

A number of investigators have immunized animals with sperm or testes extracts, or extracts of isolated populations of germ cells at various stages of spermatogenesis. Antisera obtained by these procedures may be used to purify specific antigenic peptides, to localize them on the sperm surface, or to follow their appearance and distribution during spermatogenesis.

In the case of the guinea pig, EAO induction has been used to follow the isolation of the autoantigens AP2 (52) and AP3 (53). AP2 is a soluble antigen of 9000 daltons M.W. This protein is released by the in vitro-induced acrosome reaction of guinea pig cauda epididymal sperm. Thus, this strategy defines an activity (EAO) and subjects it to a purification protocol.

O’Rand and Porter used an autoantisera immunoadsorbent column and preparative gel electrophoresis to purify small quantities of a rabbit sperm membrane autoantigen, designated RSA-1 (42). Subsequently, they described a procedure that greatly increased the yield of RSA-1 and also uncovered RSA-2 (43). Testis membrane pellets were fractionated by an SDS 7% to 15% polyacrylamide gradient prep-disc gel column. The investigators recovered RSA-1 in a yield sufficient to determine carbohydrate and amino acid content. From its average hydrophilicity, RSA-1 appears to be a relative nonpolar, asymmetric protein in the general category of fibrous or tropomyosin-like proteins (43). It is a sialoglycoprotein of 13,000 ± 1200 daltons intrinsic to the plasma membrane of spermatozoa and similar in several respects to the guinea pig testis autoantigens (4). However, neither AP3 (53), which is almost the same size as RSA-1, nor AP2 (52) appears to be a glycopeptide.

Lee and co-workers reported that antisera from rabbits immunized with homologous epididymal sperm reacted with three major protein bands on SDS gels, with corresponding molecular weights of about 70,000, 14,000 and 13,000 respectively (24). Whether one of these small peptides is homologous to RSA-1 remains to be determined.

RSA-1 was localized on the plasma membrane of spermatozoa and spermatogenic cells by fluorescein- and peroxidase-labeled antibodies. It appears first on the surface of pachytene spermatocytes and is present throughout spermatogenesis (42). On the mature spermatozoon, RSA-1 is concentrated in the postacrosomal and middle-piece regions, but may occur in isolated patches over the acrosome and tail regions. It would be interesting to demonstrate this antigen in other species.

Romrell and associates identified rabbit sperm surface autoantigens that appeared during spermatogenesis (44). Two distinct subclasses of autoantigens were detected: an early class appearing on pachytene spermatocytes, but not present on spermatozoa, and a late class first appearing on spermatids. The authors suggest that each stage of spermatogenesis contains a few so-called differentiation antigens whose function is of limited duration. Specific surface proteins may appear during formation of Sertoli-germ cell junctions and disappear during disruption of the junction occurring with sperm release.

Autoantibodies to guinea pig sperm autoantigens have been localized in plasma membrane over the entire sperm head, acrosomal contents, fibrous sheath, and outer dense fibers of the tail filament and the inner acrosomal membrane of some acrosome-reacted spermatozoa (58).

All these studies employed polyclonal sera against
complex immunogens. Thus, many antigens are detected, and the concentration of the specific antibodies and the affinities are variable. Restriction of the assay to a single antigen will greatly facilitate purification. Monoclonal antibodies add both sensitivity and specificity to probe the antigenic peptides of spermatozoa.

**MONOCLONAL ANTIBODY PROBES OF SPERM ANTIGENS**

Hybridoma technology allows the production of monospecific antibodies without the need for immunization with a purified antigen (20). Each clone from the fusion of a myeloma cell with a lymphocyte produces an antibody of unique specificity. Not only does this permit more precise discrimination in the analysis of sperm antigens, but such antibodies are also potentially useful in establishing a functional role for these peptides.

Feuchter and associates developed four monoclonal antibodies by hyperimmunization of male mice with murine sperm from the cauda epididymis (6). Each of the monoclonal antibodies bound to a specific region of the sperm surface. Three of these antibodies recognized topographically restricted antigenic determinants on the sperm head and also bound to sperm from rat, rabbit, hamster, and guinea pig. The fourth monoclonal antibody detected a determinant on the sperm tail, and appeared to be specific. None of these antibodies reacted with ejaculated human sperm. The antigenic determinants were first detectable on sperm from the corpus epididymis, suggesting that maturation in the epididymis involved unmasking or modification of preexisting surface components, release of molecules from the sperm cytoplasm, or attachment of new components to the sperm surface (6). The monoclonal antibody that binds to the sperm tail also binds to epithelial cells of the epididymis (59). This antigen is, therefore, probably secreted by principal cells occupying a short segment of the distal caput epididymis.

Similar results were obtained with antisera raised against purified epididymal-specific proteins from hamster and rabbit (35) and rat (5, 19, 22). Apparently, mammalian spermatozoa are coated with antigenic glycoproteins during epididymal transit (22). There is evidence to show, in the ram, a highly specific uptake of components from cauda epididymal fluid by testicular sperm (60). It should be possible to dissect this process with monoclonal antibodies and to define more precisely the nature and role of epididymal constituents in sperm maturation.

Monoclonal antibodies also provide a powerful tool to unravel the role of sperm-specific proteins in fertilization. Alexander (1) has developed several monoclonal antibodies by immunization of mice with human spermatozoa. Many of these are directed against evolutionarily conserved antigens that are especially valuable, since the effect of a particular antibody on fertilization can be tested in species other than man. Some of Alexander's monoclonals react with fresh but not capacitated sperm, an indication that those antibodies are to antigens associated with the acrosome reaction. One of these is specific to an antigen in the 20,000 dalton range. At least two monoclonals are directed against sperm-specific surface antigens that may be glycoproteins ranging from approximately 18,000 to 40,000 daltons.

Monoclonal antibodies prepared against mouse epididymal sperm by Schmell & associates indicated patterns of spatial differentiation of target antigens in localized regions of the sperm surface (46). Furthermore, the anti-tail binding monoclonal antibodies also appeared to be species-specific. These investigators combined radio-labeling, immunoprecipitation, and gel electrophoresis to detect 200,000, 68,000 and 40,000 dalton antigens of the sperm surface (47).

Monoclonal antibodies to syngeneic testis cells were prepared to define differentiation antigens of spermatogenesis in mouse testes (2). One such antigen appears at about the time meiosis begins and persists at least to the early spermatid stage. It may be present in relatively low concentration in epididymal sperm. A second monoclonal antibody recognizes a testis antigen that may first appear in spermatogonia or early prophase spermatocytes and that cannot be detected on epididymal sperm.

The monoclonal antibodies described above are directed to antigens whose function has not been identified. Hybridomas have also been generated following immunization with LDH-C. These monoclonal antibodies, described in detail below, have proven useful in assessing the evolutionary history of this sperm-specific enzyme and in relating protein structure to antigenicity (14).

Specific antigens identified by these chemical and immunological techniques will be useful in dissecting the various immunopathologies of sperm, as well as in describing the stages of gene expression in spermatozoa. They may also serve as the basis for a contraceptive vaccine.

**EXPERIMENTALLY INDUCED IMMUNOLOGIC INFERTILITY**

A contraceptive vaccine is suggested by the observed association between infertility and anti-sperm antibodies in both male and female human beings. Suppression of fertility is well documented in female laboratory animals immunized with sperm. The goal of present-day research remains that of identifying the responsible antigen(s) from extracts of sperm and testes.
Fab fragments that were capable of inhibiting sperm-naz and co-workers immunized mice with detergent-important role during penetration through the zona (41). Alternatively, RSA-1 may play an antibody blocks release of acrosomal enzymes required for zona penetration. Since RSA-1 cannot be detected on acrosome-reacted sperm by immunofluorescence (41), it is likely that the specific Fab fragment derived from antisera to RSA-1 were used to treat spermatozoa prior to artificial insemination (AI) of nonimmunized does abolished fertility. The antisera also blocked attachment of rabbit sperm to rat ova in vitro. Rabbits immunized with the LIS-extracted sperm pellet showed a normal frequency of fertilization but a reduced rate of implantation. Antibodies present in these sera did not inhibit attachment of rabbit sperm to rat ova in vitro, or fertilization by AI in nonimmune rabbits. There was a significant reduction in implantation of fertilized ova transferred from nonimmunized does to rabbits immunized against the LIS-extracted sperm pellet.

Saling and O’Rand prepared rabbit anti-mouse sperm Fab fragments that were capable of inhibiting sperm-zona interaction and sperm-egg fusion in vitro (45). This serum contained antibodies to four major and three minor antigens. As noted below, it may be that these antigens play an essential role in fertilization, but it is not possible to rule out mechanical blockage of sperm function by antibody binding.

Menge and Black described the effects of rabbit or rhesus monkey anti-human sperm sera on human sperm penetration of zona-free hamster ova (29). Treatment of sperm with normal sera tended to increase the percentage of ova penetrated, compared with basic medium alone. Antisera against sperm, sperm extract, and testes, as well as Fab preparations of these antisera, caused significant decreases in the number of ova penetrated. Pretreatment of zona-free ova with antisperm Fab preparations had no effect on the sperm penetration rate but antisperm serum did depress the rate of penetration. These authors suggest that antibodies against integral surface antigens of sperm membranes may either be covering receptor molecules for the egg membrane or causing a steric hindrance of the membrane interaction between the gametes. Unfortunately, the egg pretreatment results are consistent with a nonspecific blocking reaction.

Fab fragments derived from antisera to RSA-1 were used to treat spermatozoa prior to AI (41). There was a 68% reduction in the number of fetuses recovered. Sperm binding to the zona was reduced in vitro, and there was complete inhibition of egg penetration. Since RSA-1 cannot be detected on acrosome-reacted sperm by immunofluorescence (41), it is likely that the specific Fab antibody blocks release of acrosomal enzymes required for zona penetration. Alternatively, RSA-1 may play an important role during penetration through the zona (41).

Naz and co-workers immunized mice with detergent-solubilized rabbit epididymal sperm, and developed two hybridomas secreting monoclonal antibodies against sperm (36). Insemination of female rabbits with sperm treated with either of the monoclonal antibodies resulted in significant reduction in fertility, as seen by the percentage of 9-day embryo implants recovered. Although these antibodies inhibited in vitro binding of rabbit sperm with the zona pellucida of rat ova, fertilization in vivo was not significantly affected. Both of these monoclonal antibodies appear to recognize the same antigen of testicular origin and with an approximate molecular weight of 63,000 daltons.

Naz and Menge used an immunoaffinity column to isolate the antigen (37). The antigen was identified as a glycoprotein containing about 20% carbohydrate and was immunogenic in mice but not in rabbits (38). Nevertheless, the monoclonal antibodies reacted with rabbit, human, and murine sperm (37). The immunoreproductive significance of this antigen requires further study. However, the postfertilization effects of these antibodies and the self-recognition of the glycoprotein by the immune system of the rabbit suggest a somatic origin of the antigen: stimulus. This could lead to a cross-reactivity with tissues that would compromise the contraceptive potential of this antigen.

Naturally occurring antibody-mediated infertility should provide a useful model for development of a contraceptive vaccine. It seems reasonable to propose, for example, that enhancement of an immune response to male gametes in the female could indeed suppress fertility. It is obvious from the foregoing discussion that sperm antigens do exist and are immunogenic. In some cases, they also can provoke an immune response or development of antibody that renders sperm incapable of fertilizing the egg, of reaching the site of fertilization, or of inhibiting postimplantation development of the embryo.

The most important requirement for development of a contraceptive vaccine is identification of a highly specific and immunogenic sperm antigen. In addition, the antigen must be available in relatively large quantities, and this is unlikely if it is to be obtained from natural sources (i.e., testes or sperm). Therefore, the antigen must be amenable to chemical synthesis. The major problem with the various antigens so far described is that useful quantities necessary for precise structural and immunochcmical characterization are not available, and may not be, without application of heroic isolation and purification procedures. At the present time, the most viable sperm-specific antigen for vaccine development is lactate dehydrogenase C4 (LDH-C4; LDH:1-X).

**DEVELOPMENT OF A CONTRACEPTIVE VACCINE; SPERM-SPECIFIC LDH-C4**

All vertebrate tissues contain LDH. This tetrameric
enzyme occurs in multiple molecular forms, or isozymes, which are randomly assembled from two types of subunits, A and B. A third subunit type, C, is synthesized only in testes during active spermatogenesis, and is distinct from the A and B peptides of somatic origin. LDH-C_4 first appears in the primary spermatocyte during mid-pachytene of the first meiotic division. The restriction of LDH-C_4 to the germinal epithelium is apparent in Figure 1. LDH-C_4 is demonstrated by immunofluorescent localization in primary spermatocytes and spermatids, while the spermatogonia and the non-germinal elements, such as interstitial and Sertoli cells, are clearly devoid of this isozyme. The appearance of LDH-C_4 in mid-pachytene primary spermatocytes is illustrated in the immature testes section in Figure 2. Synthesis of this isozyme continues, so that it becomes the most abundant form of LDH in spermatozoa. The unique developmental and biochemical properties of LDH-C_4 are of considerable interest and significance to testes and sperm metabolism. They were reviewed recently in detail by Wheat and Goldberg (63).

Since LDH-C_4 is not found in prepubertal testes or in any other tissue of the male, and is also completely absent from the female, it is antigenic in both sexes of homologous and heterologous species. Rabbit antiserum to mouse LDH-C_4 reacts with this isozyme from all mammalian species, but does not react with isozymes composed of A and B subunits (8). The availability of relatively large quantities (hundreds of milligrams) of mouse LDH-C_4 facilitates rigorous analysis of the effect of immunization with LDH-C_4 on fertility.

Female laboratory animals were immunized with LDH-C_4 and then mated. Fertility was significantly reduced in both mice and rabbits (9, 25). The study was extended to nonhuman primates (10), and nine adult female baboons (Papio anubis) of proven fertility were immunized with mouse LDH-C_4. Circulating antibody was produced in all animals and reacted with mouse, baboon, and human LDH-C_4. All animals showed normal ovulatory menstrual cycles during the immunization protocol. In a series of breeding experiments, 22 of 30 matings, or 74%,
Figure 3. Immunofluorescent localization of LDH-C4 to the spermatozoon principal piece of the tail. The pattern seen here for murine sperm is the same in human spermatozoa.

were infertile, as compared to 28% in control matings. The contraceptive effect of the vaccine containing LDH-C4 was associated with high antibody titer. Normal pregnancies ensued in baboons in which titer declined after termination of booster injections. The conceptions that did occur during the experiment were restricted to animals with poorly developed immune responses and did not yield normal fetuses. Thus, this treatment is effective, it does not impair embryonic development or damage the female reproductive system, and is reversible.

For LDH-C4 to provide the basis for a practical immunological contraceptive, two major problems must be solved. First, a synthetic substitute for the natural product antigen must be identified. Second, the contraceptive effect must be made more nearly complete. Knowledge of the mechanisms of immunocontraception can lead to improved effectiveness.

Antibodies to LDH-C4 may suppress fertility in at least two ways. Inhibition of intracellular enzyme activity by these immunoglobulins would impair energy metabolism and functional motility of the sperm. Antibodies to LDH-C4 do inhibit enzyme activity in vitro (8). Alternatively, antibody binding sites provided by LDH-C4 on the sperm surface would mediate spermagglutination, as well as complement dependent cytotoxicity (11) (Figure 3). Thus, a useful contraceptive effect of immunization with LDH-C4 can be achieved by the action of specific antibodies in the female reproductive tract at a sufficiently high concentration to provide an immunologic barrier preventing sperm from reaching and fertilizing the egg. Anti-LDH-C4 serum is transferred into the female reproductive tract (17) and sperm transport is markedly inhibited in the genital tract of immunized females (18).

These data suggest that immunization with LDH-C4 results in immunoglobulins that enter the female reproductive tract and prevent sperm from fertilizing the egg. Ensuring a high concentration of anti-LDH-C4 by stimulation of the secretory immune system in the female reproductive tract would make the contraceptive effect more complete. Female mice immunized with LDH-C4 placed directly into the uterus exhibited elevated levels of LDH-C4-specific IgA in their uterine fluids. In animals receiving a systemic immunization 7 to 10 days prior to intrauterine inoculation, LDH-C4-specific IgA and IgG could be detected in both uterine fluids and in serum (Shelton and Goldberg, unpublished). These results suggest that manipulation of the secretory immune system of the female reproductive tract could enhance the immunocontraceptive effects of LDH-C4.
Problems of both supply and homogeneity preclude use of a natural product antigen as a contraceptive vaccine. Our strategy in development of such a vaccine, therefore, has been to map antigenic determinants of LDH-C₄. The procedure essentially involves chemical or enzymic digestion of LDH-C₄ and isolation of the fragments. Those peptides that bind anti-LDH-C₄ are chemically characterized. Appropriate peptides are synthesized and conjugated to carrier molecules for immunization. The anti-peptide antibodies are tested for binding to LDH-C₄.

Finally, the immunocontraceptive properties of these synthetic determinants are assessed. Although these studies are still in progress, we have demonstrated that our strategy is sound. Thirteen antibody-binding peptides have been identified. A structural model of the LDH-C subunit indicates the positions of these peptides (Figure 4). Immunoglobulins that react with LDH-C₄ have developed in rabbits immunized with four different synthetic peptide fragments of the native protein (13, 15, 62). Furthermore, these antibodies agglutinate sperm and significantly inhibit in vitro fertilization (Beyler et al., unpublished).

CONCLUSION

Immunoc contraception has been suggested as a solution to the need for alternative and reversible contraceptives. Both pathologic and experimentally-induced infertility support this suggestion. The use of a well-defined, synthetic antigen is essential to progress in developing a vaccine for fertility control and to establish with certainty the utility of such technology. These studies with LDH-C₄ are consistent with the feasibility of a synthetic antigen for use in a vaccine to control fertility. LDH-C₄ remains the best-developed candidate for future studies. As noted above, immunization with LDH-C₄ does suppress fertility, and the complete biochemical characterization of this isozyme is well underway. These studies will provide a wealth of potential synthetic antigens to substitute for the natural product in experiments to determine whether immunologic contraception is appropriate and desirable for wide-scale application in human beings.

Although there are certainly a number of practical problems to be resolved, prospects are good that during the next decade, a vaccine to prevent pregnancy will become available.

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