PREVENTION OF FERTILIZATION *IN VITRO* BY AN ACROSIN INHIBITOR FROM RETE TESTIS FLUID OF THE RAM

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One of the enzymes probably involved in sperm penetration through the outer layers of the ovum is a trypsin-like enzyme, called acrosin because of its location in the acrosome. This enzyme is inhibited by substances found in semen and in the secretion of seminal vesicles of several mammalian species (see Werle & Zickgraf-Rüdel, 1972). It is generally thought that the spermatozoa mix with these inhibitors during ejaculation, and a complex between the inhibitors and the acrosin is formed at that time (Zaneveld, Polakoski, Robertson & Williams, 1971; Werle & Zickgraf-Rüdel, 1972). Removal of these inhibitors from the acrosome is supposed to be an essential part of the process of capacitation and the acrosome reaction (see Bedford, 1970). Several attempts have been made to prevent fertilization with synthetic or natural trypsin inhibitors, and most of these experiments have been successful (Stambaugh, Brackett & Mastroianni, 1969; Zaneveld, Polakoski, Robertson & Williams, 1971; Zaneveld, Robertson, Kessler & Williams, 1971).

We have found a high concentration of trypsin inhibitor in the fluid collected from the rete testis of rams and boars (Suominen & Setchell, 1972), and it would appear therefore that the spermatozoa are exposed to trypsin inhibitors from the moment they leave the testis. This inhibitor from the rete testis fluid (RTF) of the ram has been purified and characterized; it can be separated into three fractions (I, II and III) by CM-cellulose chromatography, but all three inhibitor fractions have similar characteristics. They do not inhibit any other common proteolytic enzyme apart from trypsin (i.e. chymotrypsin, plasmin, kallikrein, thrombin or urokinase), their molecular weight is about 6500, they are very stable and they are probably polypeptides (Suominen & Setchell, in preparation).

The present experiments were carried out to see whether these inhibitors were able to prevent fertilization *in vitro*. The inhibitors were purified from the RTF of the ram as described by Suominen & Setchell (in preparation). The effect of the RTF inhibitors on fertilization was tested *in vitro* using mouse ova and epididymal spermatozoa (Kaufman, 1973) incubated in a defined medium (Whittingham, 1971). To this culture medium, different amounts of RTF inhibitor II were added; this inhibitor fraction was chosen for experiment because it contained the highest inhibitor activity (88.7 inh. mU/ml; 1 inhibitor

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Table 1. The effect of inhibitor from ram rete testis fluid on fertilization *in vitro* in mice

Experiment no.	Approximate sperm concentration (sperm./ml $\times 10^{-6}$)	Concentration of inhibitor (μ g protein/ml)	Concentration of inhibitor (μ g protein/ 10^6 sperm.)	Inhibitor activity (inh. mU/ml)	Inhibitor activity (inh. mU/ 10^6 sperm.)	Fertilized ova/total no. of ova (percentage)	
						Control	Inhibitor
1	2.5 0.700	4.0 4.0	1.6 5.8	13.6 13.6	5.5 19.4	24/33 (73) 41/53 (77)	29/58 (50) 23/55 (42)
2	2.5 1.25 0.625 0.30	7.0 7.0 7.0 7.0	2.8 5.6 11.2 23.4	23.8 23.8 23.8 23.8	9.5 19.0 38.1 79.4	31/43 (72) 35/40 (88) 26/40 (65) 23/33 (70)	9/67 (13) 2/47 (4) 2/43 (5) 1/27 (4)

inh. mU—see text.

milliunit is the amount of inhibitor which reduces the hydrolysis of *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPA) by trypsin by 1 nmol/min at +37° C). Its protein concentration was 26 µg/ml. The controls contained an equivalent volume of 0.9% NaCl; in other experiments, other non-inhibitory fractions from the CM-cellulose chromatography were shown to have no effect on fertilization.

When the concentration of the inhibitor was similar (23.8 inh. mU/ml) to that in RTF (24.1 to 27.2 inh. mU/ml), fertilization was effectively blocked (Table 1). The results also suggest that fertilization was inhibited more effectively when the number of spermatozoa used was smaller and the inhibitor concentration was kept at the same level, i.e. when the amount of the inhibitor per spermatozoon was higher. At lower inhibitor concentration (one sixth of that occurring in the RTF), a slight but distinct reduction in fertilization rate was also noted.

Our results confirm the earlier findings of Stambaugh *et al.* (1969) on the effect of trypsin inhibitors on fertilization *in vitro*. These authors, however, used trypsin inhibitors derived from soybean and ovomucoid, and a high concentration (0.5 to 1.0 mg/ml) of these inhibitors was needed before fertilization was prevented. Zaneveld, Polakoski, Robertson & Williams (1971) and Zaneveld, Robertson, Kessler & Williams (1971), using an *in-vivo* technique, found that fertilization was effectively reduced by inhibitors purified from rabbit seminal plasma or from seminal vesicles at low concentrations, about the same as those used by us, 1.0 to 2.5 µg/10³ spermatozoa. Schumacher, Swartwout & Zuspan (1971) failed to block fertilization with polyvalent trypsin-kallikrein inhibitor (Trasylol®) or with soybean trypsin inhibitor when incubated with ejaculated spermatozoa or given systemically into rabbits. Their negative result could be partly explained by our finding that inhibitors purified from the RTF are more effective against acrosin than is trypsin-kallikrein inhibitor (Trasylol®). This may be true also in the case of inhibitors occurring in semen or in the secretion of the seminal vesicles.

It is clear, therefore, that the inhibitor in the RTF is effective in blocking fertilization, and the testis may be the source of the decapacitation factor in epididymal seminal plasma. It is now necessary to determine why these testicular inhibitors are less tightly bound to the spermatozoa than the inhibitors in ejaculated seminal plasma.

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