Inhibition of Fertilization in Frog Eggs by Univalent Fragments of Rabbit Antibody.* (27525)

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Previous studies(1,2) have shown that fertilization in the frog, *Rana pipiens*, can be inhibited by treatment of eggs with antibody prepared against egg-jelly coat material. This suggests that jelly receptor sites neutralized by the antibody perform an essential role in the fertilization reaction. However, antiserum treatment has additional effects on the eggs. These include dissociation of eggs (partial breakdown of the jelly mass) and precipitation of at least some of the egg-jelly layers. In view of these effects it is possible that the fertilization inhibiting action of the antiserum results from formation of a cross-linked lattice among the jelly molecules which could act as a mechanical barrier to the fertilizing sperm(3).

One means of eliminating the latter possibility is by rendering the multivalent antibody non-precipitating by converting it to the univalent form(4,5). A convenient method for preparing such antibody is by digestion with the proteolytic enzyme papain(6). It is found that treatment of eggs with such non-precipitating antibody results in an inhibition of fertilization.

Materials and methods. Antibodies were prepared in rabbits against the egg-jelly material of the frog, *Rana pipiens*(1). Antisera of high titer obtained in a number of bleedings of several animals were pooled. A gamma globulin fraction was prepared by a modification of Kekwick's method(7). This consisted of 2 precipitations with 18% sodium sulphate. Proteolysis by papain (obtained as twice-crystallized preparations from Worthington Biochemical Co., Freehold, N. J.) was accomplished as described by Porter(6) at a pH of 7.5 in a 0.1M phosphate buffer containing 0.01M cysteine and 0.002M versene. Digestion was carried out for 18 hours at 37°C. 0.02M iodoacetamide was added at the end of digestion to inactivate the enzyme and prevent reoxidation of antibody fractions to form a multivalent, precipitating antibody fragment(8). Following digestion all sera were dialyzed against Holtfreter's solution and frozen until ready for use.

Thirty to 40 eggs, obtained after pituitary hormone induced ovulation, were stripped onto glass slides and treated with the appropriate sera. The experiment was performed 4 times using eggs from a different female in each case. Eggs were treated for 5 minutes, then washed with a large volume of the Holtfreter's solution to remove any unreacted antibody. Insemination was accomplished with normal spermatozoa. Results were recorded as percentage of eggs which cleaved.

Precipitin tests were carried out in agar diffusion plates (Ouchterlony) in which 1% ionagar was employed. Plates were cut so that 2 wells were on each side of a center trough. Each well received a total of 0.75 ml of the appropriate sera. Plates were stored at room temperature for about 10 days and photographed for a permanent record.

Results. Inhibition of precipitation. The design of the agar plate (Fig. 1) was chosen so that one-half of the plate could be saturated with papain-digested control serum, and the other half with papain-digested, anti-jelly serum. Two series of applications were made into the plate as follows:

1) Wells 1 and 2 were first filled with digested, anti-jelly serum (univalent antibody). At the same time wells 3 and 4 received digested, control serum. No material was introduced into the center trough at this time. After 72 hours serum of all wells had diffused into the agar.

2) Wells 1 and 3 were then filled with undigested, anti-jelly serum (multivalent antibody). Wells 2 and 4 received undigested,

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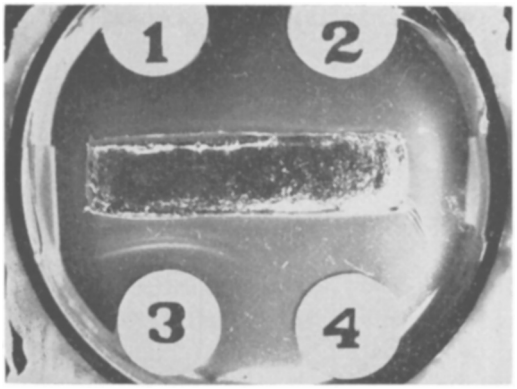

FIG. 1. Agar diffusion plate showing inhibition of precipitation by univalent antibody. Wells received the following serum:
1) digested anti-jelly and undigested anti-jelly
2) digested anti-jelly and undigested control
3) digested control and undigested anti-jelly
4) digested control and undigested control.
Center well received a solution of the jelly coat material. For explanation see text.

control serum. During this second series of treatments the center trough was filled with a solution of jelly coat material.

As seen in wells 2 and 4 (Fig. 1) no precipitin bands appeared between digested or undigested control serum and the jelly antigen. As expected (well 3) specific precipitin bands representing antigenic components from the jelly material formed with the undigested immune serum in the presence of digested control serum. Finally, as seen in well 1, no sharp bands formed when undigested immune serum diffused from the well that had been pretreated with digested immune serum. A single faint band did appear opposite well 1 after 7 days. Clearly pretreatment of the plate with digested immune serum inhibited formation of antigen-antibody precipitates. Presumably, the univalent, non-precipitating antibody fragments in the digested immune serum reacted with the diffusing jelly antigens and thereby protected the reactive sites of these antigens from combination with the multivalent precipitating antibody.

Inhibition of fertilization. Eggs which had been treated with digested antibody showed some characteristics similar to those of eggs treated with undigested antibody, *i.e.*, failure of eggs to undergo the usual rotation of orientation upon insemination, and later the failure of eggs to undergo cleavage. However, the extent of separation of eggs treated with the digested antibody was not as great as those treated with undigested antibody. Precipitation of jelly layers by the digested antibody was slight as compared to that of those treated with undigested antibody. Fig. 2 shows per cent cleavage obtained when eggs were treated with digested control serum as compared to those obtained in treatments with digested and undigested, anti-jelly serum. These results indicate that the digested, anti-jelly serum inhibits the fertilizability of eggs just as does the undigested, anti-jelly serum.

Discussion. The results presented here concern the effects of papain-digested, anti-jelly serum on eggs of the frog, *Rana pipiens*. Previous studies on egg jellies of the frog have shown that the jelly contains a number of antigenic components which are species-specific as well as components which are common between closely related species of *Rana*. Antibodies prepared against either the species-specific or common components of the jelly significantly inhibit the cleavage of eggs of *R. pipiens*. These results will be presented later.

Such cleavage inhibition probably represents inhibition of fertilization since other fertilization events such as rotation of eggs fail to occur. It is known in the sea urchin

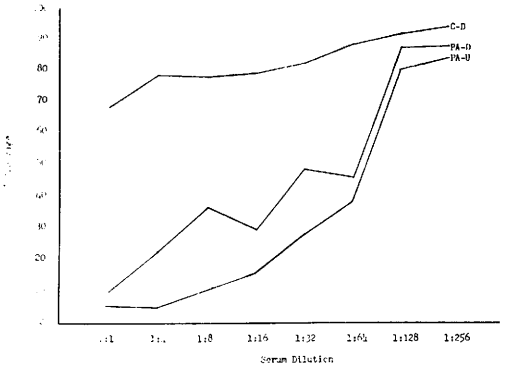

FIG. 2. Cleavage of eggs of *Rana pipiens* (inseminated with normal spermatozoa) treated prior to fertilization for 5 min with digested control serum (C-D), digested anti-jelly serum (PA-D), undigested anti-jelly serum (PA-U). Each point represents the averaged 4 experiments giving a total of approximately 160 eggs. For explanation see text.

that anti-jelly serum inhibits cleavage of fertilized eggs.

As shown in this experiment, the agar diffusion technic serves as a convenient method for demonstrating the conversion of a precipitating, multivalent antibody to a non-precipitating, univalent form by papain digestion. These univalent non-precipitating antibody fragments do inhibit the fertilizability of eggs presumably by blocking the receptor molecules. The exact site of the inhibitory action has not been determined. It is hoped that studies employing fluorescein-labeled antibody will give a visual means of localizing the specific receptor sites.

Summary. Papain digested (univalent) anti-jelly serum inhibits the fertilizability of eggs of *R. pipiens* just as does the undigested anti-jelly serum. This observation, combined with the precipitation inhibiting action, strongly suggests a direct blocking of receptor sites in the egg jelly that perform a significant role(s) in fertilization.

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Comparison of Bile Acids in Intestinal Contents of Germfree and Conventional Rats.*† (27526)

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After administration of cholic acid-24-C^{14} to conventional rats the isotope is excreted *via* feces and the half-life time of cholic acid estimated to 2-3 days(1,2). The main labeled excretory products have been identified as 3,12-dihydroxy-7-ketocholanic, deoxycholic and 3-hydroxy-12-ketocholanic acids(3,4). The microbial transformation mainly occurs in cecum, where almost all conjugated bile acids are hydrolyzed. In the small intestine the bile acid composition is mainly the same as in the bile.

Germfree rats and rats treated with chemotherapeutics have much longer half-life time of cholic acid-24-C^{14} than conventional rats(5,6). The labeled excretory products consist of taurocholic acid and a small amount of glycocholic acid. The amount of bile acids excreted by germfree rats is less than half of that excreted by conventional rats (1.9 and 5.1 mg per day respectively)(7). The intestinal flora thus cause an increased elimination of bile acids from the enterohepatic circulation. The rate of cholate excretion in feces by rats is also influenced by the composition of the diet(2). The possibility exists that these dietary influences are also related to changes in the intestinal flora.

The aim of the present investigation was to further elucidate the difference of bile acid turnover in germfree and conventional rats by comparing the physical state of bile acids in intestinal content present in small intestine, cecum and colon.

Experimental. The material comprised 2 germfree rats reared according to Gustafsson (8,9) on his semi-synthetic diet D 7, 4 conventional rats of the same strain and on diet D 7 and 4 conventional rats of a strain of

* Bile acids and steroids 121.

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