and examined histologically. For the time being only a preliminary survey of results will be given.

The majority of grafts (90%) developed into masses of normal embryonic tissues ranging in shape and size from small discs to big tumorous masses reminiscent of the grafts of the entire rat egg cylinders. The only exception was the isolated endoderm which developed in only 2 out of the total 12 grafts.

The *ectoderm* differentiated regularly into typical ectodermal derivatives (neural tissue, epidermis). The mesodermal tissues (adipose tissue, cartilage, bone, muscle) were also present, but their incidence was not as high as in the grafts of the ectoderm + mesoderm combination. Derivatives of the endoderm were never found in ectodermal grafts.

The isolated *endoderm* did not differentiate at all. In the 2 successful endodermal grafts mentioned above, some mesodermal derivatives (cartilage, muscle) were also found. It was obvious that in these cases the endodermal layer was 'contaminated' with some adherent mesodermal cells. In the grafts of the endoderm and mesoderm, endodermal derivatives (intestine, respiratory epithelium, glands) differentiated regularly.

In grafts of the isolated *mesoderm*, the brown adipose tissue was the most constant differentiation. In some grafts, however, the derivatives of the ectoderm and the endoderm were also present. If grafted in combination with the ectoderm or the endoderm, the mesoderm differentiated into all characteristic derivatives which can be found in grafts of the entire egg cylinder.

The data reported show that the common method of 'splitting off' of epithelial layers from the underlying mesenchyme by the enzymatic digestion of their basement membranes can be applied with success to the separation of germ layers in early rat embryos. This procedure does not affect either the viability of embryonic cells or their ability to differentiate into normal tissues in homotypic grafts.

The main obstacle to a complete isolation of the *entire* germ layers was the existence of restricted areas of intensive migration of cells (primitive streak, Hensen's node) in which the germ layers being still in dynamic continuity with one another have not yet attained their individuality. In these areas a 'contamination' of the particular germ layer with adherent cells of the neighbouring one could not be excluded. Under these circumstances, the histodifferentiation in grafts of single germ layers cannot be considered as an adequate result of their auto-differentiative capacities.

In the present experiment, the principal aim of grafting the isolated germ layers was to test their viability and general ability for further development. In the future work special care will be taken to isolate and graft only the areas in which the formation of definite germ layers has already occurred.

Résumé. Les feuillets du cylindre-œuf du rat ont été disjoints par une solution composée de deux enzymes: 0,5% de trypsine et 2,5% de pancréatine. La séparation a été achevée par une aiguille de tungstène. Les feuillets ainsi détachés retiennent leur pouvoir de croissance et de différenciation comme homogreffes, sous la capsule rénale.

B. LEVAK-ŠVAJGER, A. ŠVAJGER and N. ŠKREB

Institute of Biology, University of Zagreb, Department of Biology and Department of Histology and Embryology, Faculty of Medicine, Zagreb, p.p 422 (Yugoslavia), 28 July 1969.

The Role of the Diffusible Factor Released by the Egg Jelly in Fertilization of the Toad Egg

It has previously been reported that the gelatinous envelopes around the oocytes of the toad *Bufo arenarum* contain a diffusible substance, readily extractable by distilled water or balanced salt solution, which seems to be essential for fertilization[1]. Since this diffusible factor is quickly released from the jelly, it has been assumed that this principle would activate free spermatozoa before they penetrate the jelly[2]. On the other hand, some evidence indicates a small molecular size and a basic character[3]. Taking into account these properties, it was desirable to investigate if the role played by the diffusible factor in fertilization could be the result of an alkalizing effect on the insemination medium. Some experimental results supporting this assumption are reported in the present paper.

Material and methods. Bufo arenarum oocytes were obtained from the ovisacs of females treated with suspensions of homologous hypophysis preserved according to PISANÓ[4]. Sperm suspensions (about 10^6 cells/ml) were prepared by dilacerating the testes in 10% amphibian Ringer solution without bicarbonate.

Results were estimated using the fertilization rate method, which consists, essentially, in interrupting fertilization at different intervals of time by means of lauryl sulfate[5]. After insemination, egg cords were immersed for 5 sec in 0.1% lauryl solution followed by a quick wash in tap water. Control experiments showed that this treatment has no harmful effects on egg development. Further details will be found in the description of each experiment.

Results and discussion. As a first step, it was necessary to ascertain whether, under normal conditions, the diffusible factor had some influence on the pH of the inseminating medium. In fact, since various products, including appreciable amounts of proteins, are simultaneously released from the jelly after immersion in water, the presence of the diffusible factor is not enough to predict any pH effect on the surrounding medium. This was investigated by extracting the diffusible factor following the stepwise procedure as is routinely performed in this laboratory, and measuring the pH values of washing solutions by means of a Zeromatic pH meter. About 2000 oocytes were extracted by 3 consecutive washes with 200 ml of Ringer solution of 15 min each, with occasional gentle shaking. Under these conditions, fertilizability of washed oocytes decreases progressively being completely lost at the end of the last extraction

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as seen from the washed controls in a further series of experiments. The results recorded in the Table show the variation of pH produced by the presence of oocyte strings of *B. arenarum*, which exhibited an alkalizing capacity. These pH changes were detected within the first minute and kept constant as long as every extraction step lasted. It is evident from the data obtained that the alkalizing capacity of the strings decreases step by step as they are submitted to repeated extractions.

The next step was to investigate whether fertilizability of oocytes lacking the diffusible factor could be restored by replacing this component of normal fertilization by some other basic substance. To test this, the recovery of oocytes extracted with Ringer solution was attempted by adjusting the pH of the inseminating medium by means of inorganic salts. Definite pH values were obtained by simple addition of 0.05*M* $NaHCO_3$ or

pH changes following immersion of oocyte cords in Ringer solution

Experiment No.	1st extraction Initial pH	1st extraction Final pH	2nd extraction Initial pH	2nd extraction Final pH	3rd extraction Initial pH	3rd extraction Final pH
1	5.3	7.1	5.3	6.4	5.3	6.0
2	5.3	6.9	5.3	6.2	5.3	5.8
3	5.3	6.9	5.4	6.1	5.6	5.8

Initial pH refers to pH values of Ringer solution as determined immediately before each extraction.

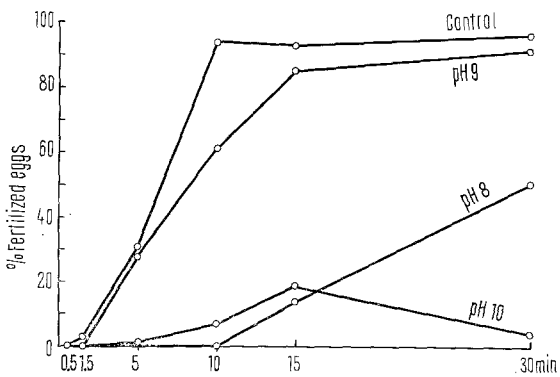

Fig. 1. Rates of fertilization of washed oocytes inseminated in Ringer solution at different pH values (as determined before each experiment). 'Washed controls' and oocytes inseminated at pH 7 gave 0% fertilized eggs after 30 min of insemination.

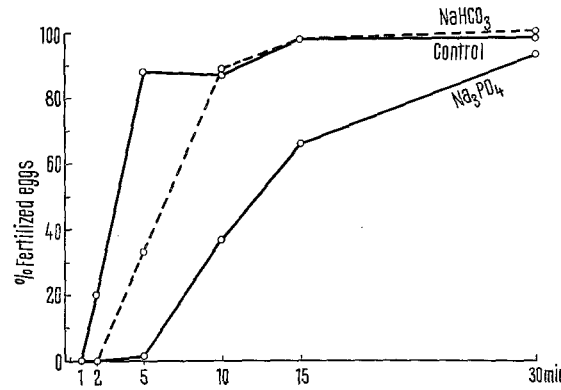

Fig. 2. Rates of fertilization of washed oocytes inseminated in Ringer solution at pH 9 adjusted either with 0.05*M* $NaHCO_3$ or 0.05*M* Na_3PO_4 and 0.05*M* Na_2HPO_4. 'Washed controls' gave 0% fertilized eggs after 30 min of insemination.

0.05*M* Na_2CO_3 to Ringer solution. Oocyte cords were washed as previously described, and then immersed in Ringer solution at a definite pH value for 5 min. Insemination was performed in a separate beaker containing saline at the same pH, the cord and a minimum volume of sperm suspension being simultaneously added. After stopping fertilization at different intervals by means of lauryl sulfate, each string was washed, immersed in Ringer solution, and kept at room temperature until cleavage occurred. As controls, normal and washed oocytes were inseminated in ordinary Ringer solution (pH 5.4–6.2), under identical conditions. Curves represented in Figure 1 show the recovery of oocytes lacking the diffusible factor, as well as the dependence of the rate of fertilization upon the pH of the inseminating medium. In no case could washed oocytes be recovered by inseminating in Ringer solution at pH 7. Though considerable differences were found between different batches of gametes, relative values were consistently observed.

In order to prove that the results reported above were solely due to a pH effect, it was desirable to attempt the recovery of washed oocytes by means of some other salt. Following the same experimental procedure, a batch of oocytes was inseminated in saline brought up to pH 9 with 0.05*M* Na_3PO_4 and 0.05*M* Na_2HPO_4 as compared with a second batch inseminated in saline brought up to the same pH value with 0.05*M* $NaHCO_3$. Controls were the same as reported above. Insemination in the presence of phosphate also resulted in marked recovery of washed oocytes as indicated by the curves expressing the rates of fertilization (Figure 2).

Taking into account the fact that the diffusible factor is readily released from the oocyte cords, as well as its basic character in conjunction with the results reported above, a preliminary explanation concerning its role in fertilization may be attempted. As a high concentration of the diffusible factor is to be expected immediately around the oocyte cords as soon as they are shed into the water, a sudden rise of pH should occur very close to the surface of the jelly material. Assuming that this pH change is required for a very short period of time only, just to assure the attachment of free spermatozoa to the surface of the jelly coat, the diffusible factor would be able to act before its subsequent dilution in the inseminating medium.

It has been reported that *Ciona intestinalis* spermatozoa in the presence of sea water at pH 9–10 exhibit effects similar to those produced by a fertilizin solution. This fact, however, does not seem to have been considered in connection to the fertilization reaction[7].

Resumen. Los experimentos descritos indican que el factor difusible de las cubiertas gelatinosas de los ovocitos de *Bufo arenarum* activaría a los espermatozoides a través de un efecto alcalinizante sobre el medio de inseminación.

F. D. Barbieri and M. Cabada[8]

Instituto de Biología,
Facultad de Bioquímica, Química y Farmacia,
Universidad Nacional de Tucumán,
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[8] Present address: Departamento de Biología, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata (R. Argentina).