arise from differences between hospital and nonhospital births. Also, a recent analysis of the reporting of spina bifida at the New York State Department of Health (*6*) reveals at least 85 percent complete reporting of the defect on birth and stillbirth certificates. There is no reason to assume that the reporting of anencephalus is any worse, since this defect is so easily recognized.

It could be argued that a family with two malformed infants would be more likely to be ascertained than a family with one malformation. Granting this possibility, the difference between the population incidence of anencephalus and spina bifida and the incidence in sibs would still be significant.

A bias does exist in that some of these sibships are still incomplete. Sibs born after the index cases are therefore underestimated. Similarly, families which have moved out of the reporting jurisdiction while in the childbearing period will also produce an underestimate of cases. Ten of 308 siblings affected may therefore be a low estimate.

The findings of this study are in close agreement with those of Polman and Penrose. They suggest the importance of familial factors, genetic or environmental, in the causation of anencephalus and spina bifida (*7*).

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Experimental Study of the Developing Mammalian Egg: Removal of the Zona Pellucida

Abstract. The zona pellucida may be removed from all stages of the mouse egg by digestion with pronase. Cumulus and corona cells are also dispersed by the enzyme. No change in membrane disgestibility occurs at fertilization. In tests thus far of two-cell eggs and later stages, development continues. Blastocysts exhibit "hatching" behavior despite absence of the zona. Functions of the zona include maintenance of the normal cleavage pattern and prevention of egg fusion.

The fact that the mammalian egg has thus far served as an experimental subject only to a relatively limited extent, despite its great potential interest, can be ascribed at least in part to persistent technical difficulties. We have successfully overcome a number of these difficulties in the case of the mouse egg; this report describes the procedure for removing the zona pellucida. Among the problems now more accessible to investigation are the mechanisms of sperm penetration and fertilization, the causes of polyspermy, the effects of large molecules or disease entities which the zona might ordinarily exclude (*1*), and the role of the parts of the developing egg in morphogenesis.

The zona pellucida is slightly elastic and can be peeled off with a pair of glass needles, but the process is tedious and, like other kinds of manipulation toward the same end, often results in injury to one or more of the early blastomeres. According to Braden the membrane is composed of neutral or weakly acid mucoprotein (*2*). Though it can easily be dissolved by a variety of treatments, viability has been seriously impaired in all cases in which egg survival has been observed. Effective agents, with some species differences among laboratory mammals, include low *p*H (<5), denaturants, strong oxidizing or reducing compounds, and a number of proteolytic enzymes (trypsin, chymotryspin, ficin) (*2*). Use of a mold protease was also mentioned by Braden in the rat (*2*), but its influence on the egg was not noted. In preliminary tests we verified earlier work on the mouse and added lysozyme, elastase, collagenase, and hyaluronidase to the list of products ineffective in this species. The assembled information does not, however, support any clear conclusions regarding the chemical nature of the membrane.

More recently, we have discovered that the enzyme pronase (*3*) is highly efficient at removing the zona pellucida of the mouse egg at all stages of development. It is, moreover, the only substance to date which apparently does not damage the egg during the brief exposure required (*4*). The enzyme was first isolated in Japan from a strain of *Streptomyces griseus* and was of interest because of its wide substrate specificity (*5*).

Digestion of the mouse egg zona with pronase occurs readily under a wide range of conditions. The following standard procedure has been adopted. A 0.5-percent solution of the enzyme is made up in bicarbonate-free Hanks balanced salt solution. Insoluble mycelial impurities are removed during sterile filtration. The solution remains stable for at least a week under refrigeration. Shortly before use, it is warmed to 37°C under 5 percent CO_2 until the *p*H, which is initially high, is approximately neutral. Rinsing the eggs in protein-free medium before transfer is unnecessary. Further incubation is at room temperature. Though the solution cools, digestion time is very constant at close to 3 minutes. A medium for in vitro cultivation of two-cell or older eggs has been devised (*6*). The eggs are washed four times in this medium, and their development is observed after further incubation.

The so-called zona reaction has often been discussed as a possible means of rendering polyspermy less likely in some species, including the mouse (*7*). According to this hypothesis, a change in the zona pellucida occurs upon fertilization and inhibits penetration by more than one sperm. It was therefore of interest to compare the rates of hydrolysis of the zona in the one-celled egg before and after fertilization. Rates of digestion by pronase in the two stages were, however, identical. The follicle cells of the cumulus oophorus and corona radiata which still surround the uncleaved egg are instantaneously dispersed by pronase, and their intercellular matrix is dissolved. Thus the occurrence of a protein constituent in addition to the hyaluronic acid known to be present is confirmed. Fertilized eggs denuded before cleavage are being tested for normalcy of development by reimplantation into pseudopregnant females.

Two-cell eggs usually show abnormal cleavage patterns in vitro after the zona is removed; this appears to be due to inadequately developed cell adhesiveness, as a result of which the blastomeres form loosely bound flat plaques or random arrangements (*8*). They may, however, round up and form a morula which continues development. Eight-cell eggs deprived of the zona progress in vitro in a com-

pletely normal fashion to blastocyst, when they may be reimplanted into the uterus of a foster mother for further development. Blastomeres of naked eggs during all stages of cleavage tend to remain in contact with their neighbors, though they may be separated with versene (*6*).

The mouse blastocyst exhibits a striking spontaneous "hatching" out of the zona pellucida in vitro (*9*). One or more spherical projections, each with the wall composed of a single-cell, extend from the thinly stretched trophoblast through what appear to be small pores in the zona pellucida. The remainder of the blastocyst then emerges through a widened crack, and the zona is left behind but does not disappear. This is also presumed to occur in vivo prior to implantation. The possibility suggests itself that blastocyst emergence might result simply from mechanical pressure against the zona as the internal cavity expands with fluid. Eggs were therefore observed in culture after the membrane had been stripped at an earlier stage. Lobes were still "extruded" from the surface in a simulation of hatching. It was also noted that the zona of the morula is more easily ruptured by pipetting than in earlier stages. These data suggest that the zona becomes modified in some way during late cleavage and that some active process is involved in the passage of the blastocyst out of the zona.

The surfaces of eggs from which the zona pellucida has been removed are considerably stickier than they are when the membrane is still present, and the eggs tend to adhere to each other. The fusion is greatly accelerated at 37°C, and this fact has served as the basis of a method for synthesizing genetic mosaics (*4*, *8*). Accidental loss of the zona pellucida when two or more eggs are present together in vivo could therefore lead to formation of mosaic individuals. An example of this in the human might be the XX/XY case described recently by Gartler *et al.* (*10*).

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Totipotency of Cells from Fruit Pericarp Tissue in vitro

Abstract. Callus derived from avocado fruit pericarp grown in vitro for several generations developed roots with stele, endodermis, cortex, epidermis, and root cap. No correlation between environment and root production was demonstrated.

Plant tissue cultures generally isolated from vascular cambium, such as carrot root phloem, or from stem or root pith have been widely used for morphogenetic studies.

Attention has been given recently to basic requirements for the induction of proliferation of specific fruit-tissue explants in vitro. Pericarp and mesocarp tissues from such diverse fruit types as apple (*1*), citron (*2*), peach (*3*), pear, quince (*4*), and tropical fruits such as avocado, banana, and cherimoya (*5*) have been shown capable of cell division and enlargement when provided with a suitable nutrient medium. However, most of these fruit tissues have not been established in continuous culture. Exceptions thus far are apple (*1*), juice vesicle stalks from lemon (*6*), the pericarp of citron (*Citrus medica* L.), and the pericarp of avocado (*Persea americana* Mill.). The latter is of particular concern in this report because of the high degree of differentiation that occurred over a period of 1 year, resulting in the appearance of roots. That such a tissue as fruit pericarp should form roots is significant in demonstrating the totipotency of the tissue.

The explants used in these studies were obtained from pericarp of a nearly mature Hass avocado fruit. Tissue disks 8 mm in diameter and 2 mm thick were cut, under aseptic conditions, from a surface-sterilized fruit by means of a stainless steel slicer and borer. The disks were placed in screw-top vials containing Nitsch's basic solution (*7*) modified by the use of iron in the form of chelate No. 138 HFe (Geigy Company) (0.5 part per million) instead of ferric citrate, and by the addition of indoleacetic acid (10 parts per million) and 0.8 percent agar. The cultures were kept in a storage box, with no particular control of temperature or light, and allowed to grow for 6 weeks. Newly formed callus was then removed and cut into several pieces and subcultured on fresh medium. Cultures thus established were increased through routine subtransfers every 6 to 8 weeks. Anatomical observations were made on fresh and prepared materials.

The explants grew actively in the medium in the first 4 weeks; their fresh weight nearly doubled. Cell proliferation and cell enlargement occurred, forming abundant callus. Active proliferation was observable as white spongy growth which later developed into more compact callus masses. These became enlarged brown, nodular, corked areas. Friability was not observed to the degree attained by us in citron callus (*8*) or reported by other workers on pea (*9*), spruce (*10*), and cactus (*11*). Visible surface proliferation ceased after 4 to 6 weeks, although the cultures remained viable.

After 1 year, roots appeared in several of the subcultures, which were four to six generations removed from the original explants (Fig. 1*A*). No correlation could be made between conditions of the environment and the high degree of differentiation. Limited experiments in which concentrations of indoleacetic acid, kinetin, gibberellin, or thiamine were varied in the medium did not induce root development in comparable materials.

The roots penetrated into the agar or appeared on the upper exposed callus surface. The longest root was 4 mm long and 0.5 mm in diameter at the time it was collected. The roots were typically cylindrical, constricted somewhat at the point of emergence. A definite root cap developed around the ellipsoidal tip (Fig. 1*B*). Morphologically, the roots consisted of a central primary stele in tetrarch arrangement, a well-defined endodermis, and a cortex of starch-filled parenchyma cells delimited by a distinct epidermis with thick, suberized outer cell walls (Fig. 1*C*).

Microscopic observations of 9- to