a) Trägergebundene Aminosäuren. Als fester Träger dient vernetztes Polystyrol. Die daran gebundene Aminoschutzgruppe ist ein Benzhydryloxycarbonyl-Analogon, bei dem ein Phenylring durch den des Polystyrols ersetzt ist.

Vernetztes Polystyrol wird benzoyliert[7], mit Natriumborhydrid zum Carbinol reduziert[7,8] und zum entsprechenden gemischten Kohlensäurephenylester umgesetzt[9]. Durch Reaktion mit der entsprechenden Aminosäure erhält man das Derivat[9]. Der Reaktionsverlauf wird auf jeder Stufe durch Elementaranalyse und IR-Spektrum verfolgt. Die Beladung liegt bei 1,8–2,5 Aminosäurereste pro 10 Monomereinheiten.

b) Kupplungsreaktion. Äquimolare Mengen von Prolinpicolylester[10] und trägergebundenes, an der Aminogruppe geschütztes Alanin werden in DMF mit DCCI und Hydroxybenzotriazol[11] gekuppelt. Der Umsatz wird mit der Durchflussanalyse verfolgt[6]. Wenn die Aminokomponente nicht vollständig verbraucht ist, wird nochmals eine entsprechende Menge des Alaninderivates zugesetzt. Nach beendeter Reaktion (über Nacht bei 0 °C) wird gewaschen, bis das Filtrat beim Eindampfen keine Rückstände mehr enthält und ninhydrin-negativ ist.

c) Freisetzung des Peptids. Sie erfolgt durch Zugabe von 10%iger Trifluoressigsäure in Methylenchlorid. Nach 10 Min wird abfiltriert und mehrfach mit DMF gewaschen, bis das Filtrat ninhydrin-negativ ist. Darauf wird das Filtrat im Hochvakuum eingedampft und mit DMF aufgenommen. Die Abtrennung der Aminosäure erfolgt durch Ionenaustausch an SE-Sephadex oder Gelpermeations-Chromatographie an Sephadex G-10 mit DMF als Elutionsmittel. Hierauf kann die Kupplung mit der nächsten trägergebundenen Aminosäure erfolgen.

Nach vollendeter Synthese wird die Carboxylschutzgruppe durch katalytische Hydrogenolyse abgespalten. Das Peptid ist chromatographisch rein[12].

Das Peptid wurde auch unter Verwendung von Polyäthylenglykolmonostearyläther als Carboxylschutzgruppe[5] snythetisiert. Der einzige Unterschied zum geschilderten Verfahren ist die Abtrennung der freien Aminosäure; sie erfolgt hier durch Ausfällen des Peptidesters mit Äthanol oder durch Ultrafiltration[2].

Summary. A new strategy for peptide synthesis on polymeric supports is described. This alternating liquid-solid phase peptide synthesis procedure facilitates the ready separation of reacted and unreacted amino component at the end of each coupling reaction. Incomplete coupling reactions and incomplete deprotection reactions, the most serious problems in previously described methods of peptide synthesis on polymeric supports, have in this procedure no influence on the success of a synthesis. The principle is exemplified by the synthesis of the pentapeptide Gly-Val-Gly-Ala-Pro.

H. Frank und H. Hagenmaier

Chemisches Institut der Universität,
Auf der Morgenstelle, D-74 Tübingen 1
(Bundesrepublik Deutschland, BRD), 6. September 1974.

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Studies on the Maintenance of Porcine Graafian Follicles in Organ Culture

It is now well established for a variety of mammalian species that the changes characteristic of pre-ovulatory maturation in the Graafian follicle are dependent upon an adequate supply of gonadotrophic hormones, and especially on the so called 'LH-surge' which occurs in the pig about 40 h before ovulation. It is conceivable, however, that two of the processes involved (resumption of meiosis by the oocyte and synthesis of steroid hormones by the somatic tissues of the follicle), require different endocrine control mechanisms (see Neal and Baker[1]). The precise rôle played by FSH and LH remains unknown and is difficult to study in vivo. The purpose of the present investigation was therefore to develop a suitable technique for maintaining porcine Graafian follicles in organ culture so that the effects of gonadotrophic hormones administered during the phases of pre-ovulatory maturation could be assessed. If successful, these techniques would also provide a means of studying the interrelationships between the germinal and somatic components of the follicle.

Materials and methods. 16 sexually mature pigs, either purebred Large Whites or Large White crosses maintained at the pig unit of the Edinburgh School of Agriculture, were used in this study. Their ovaries were removed during mid-ventral laparotomy at known stages of the oestrous cycle (on days 17 to 21). Approximately 80 follicles with antra, each follicle measuring 4 to 10 mm in diameter, were cleanly dissected from the ovarian tissue within 1 to 2 h of surgery. Some of the follicles were fixed for histology to serve as controls, while the remainder were set up for organ culture using one of the following procedures: a) the follicles were placed 'free floating' in culture medium within plastic Petri dishes containing Eagle's minimal essential medium supplemented with calf serum (20% v/v) and glutamine. Racks of 4 of these dishes (each with 2 to 5 follicles) were placed in modified 'Kilner' preserving jars (see Baker and Neal[2]), which were gassed at 0 to 0.703 kg/cm^2 with 5% CO_2 in either air or 55% oxygen; b) as for system (a), except that the follicles were supported by lens tissue on a stainless steel grid, such that the follicle only received nutrients which diffused through the tissue; c) method (b) was modified by the addition of a second piece of lens tissue which was draped over the follicle to act as a 'wick' which wetted the entire surface of the follicle with medium; d) the follicles, together with 2 ml of nutrient medium, were placed in roller culture tubes which were rotated at 4 rpm within the incubator. The same gas mixtures were used as in (a) above, but the tubes were not gassed at the high pressures.

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Some of the cultures were supplemented with insulin (50 μg/ml), while others received gonadotrophins. The latter consisted of human chorionic gonadotrophin (HCG: 'Pregnyl'; Organon Laboratories, London, at a dose of 1–10 IU/ml), NIH-FSH-S9 (0.01–1.0 mg/ml), or NIH-LH-S18 (0.01–1.0 mg/ml), either singly or in combination.

The culture medium was completely replaced with fresh nutrient on the 4th day of culture and the explanted follicles were fixed with Bouin's aqueous fluid for histology on the 8th day. The follicles were serially sectioned at 5–8 μm and stained with haematoxylin and eosin. Comparisons were made of the histological appearance of the control (non-cultured) follicles and of those cultured according to the various procedures listed above.

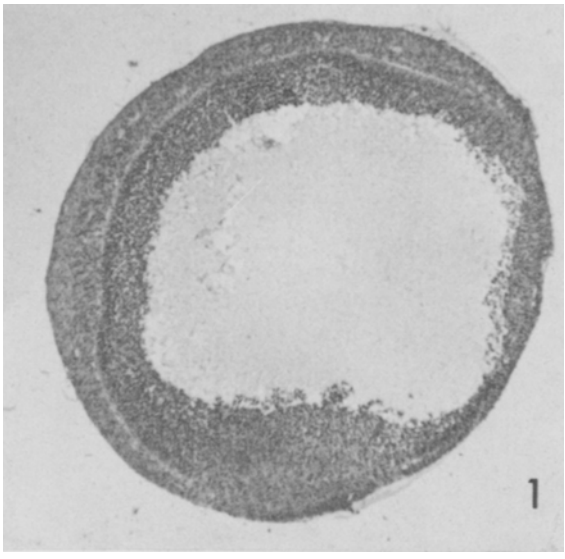

Fig. 1. 4 mm Graafian follicle cultured for 4 days using method (c) and with insulin in the nutritive medium. Ovary recovered on day 17 of the oestrous cycle. ×70.

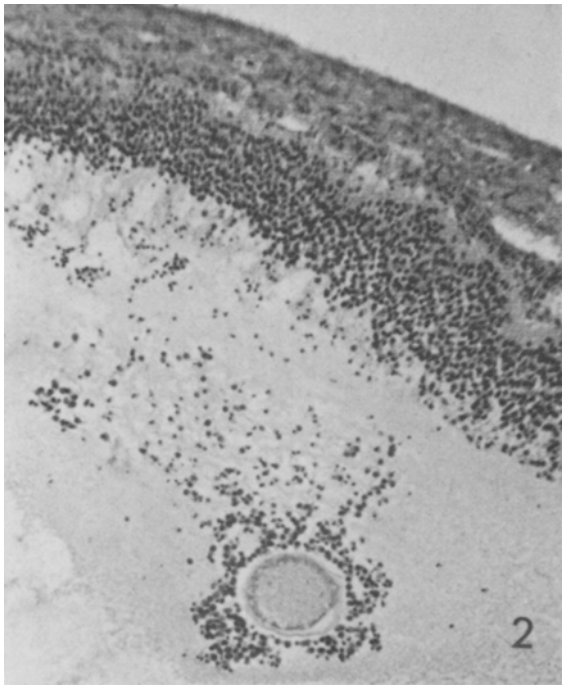

Fig. 2. 8 mm follicle cultured 'free-floating' in medium containing HCG. The oocyte, granulosa and theca have cultured fairly well, but the cumulus cells are undergoing pyknosis. ×400.

Results. Culture of Graafian follicles 'free floating' in the medium within plastic Petri dishes or in the roller tubes (methods a and d) gave poor results: the stratum granulosum became detached from the theca interna of the follicle, and both layers showed varying degrees of pyknosis. The oocyte and its surrounding cumulus cells consistently showed degenerative changes (Figure 2). By the 8th day of organ culture, degeneration was advanced and the histology of the cultured follicles was markedly different from that of the controls.

The most consistently good results were obtained when follicles were cultured on stainless steel grids, especially when lens tissue was draped over the follicle as in method (c) above (Figure 3). Furthermore, gassing at 0.703 kg/cm² with 5% CO_2 in 55% oxygen was more effective than with air at the same pressure (Figure 3). Follicles cultured under these conditions possessed few pyknotic granulosa cells and the stratum granulosum rarely showed marked signs of becoming detached from the theca interna. However, a small localized area of damage, which seemed to occur in that part of the follicle wall which was in contact with the grid, was found in most of the follicles: it was characterized by a thinning of the granulosa layer and a mild degree of pyknosis. The oocytes in these follicles generally responded well to culture, and sometimes progressed from the dictyate stages of meiotic prophase to metaphase I or II: by the 8th day many had become mildly eosinophilic (Figure 4).

The addition of insulin to the culture medium gave variable results, although in general terms it was considered beneficial. None of the gonadotrophic hormones improved the histological appearance of the cultured follicles: the luteinization which was detected in some of the follicles seemed to be dependent upon the hormonal status of the animal providing the follicles, rather than on the addition of HCG, LH or FSH to the medium.

Discussion. Most of the methods of organ culture employed in the present study were unsuccessful, and the treated follicles showed extensive signs of degeneration. However, those cultured at high pressure with 5% CO_2 and 55% oxygen were well maintained if supported by stainless steel rafts and draped with lens tissue. Pyknotic changes were largely confined to a small area of the wall of the follicle which had been in contact with the grid. The remainder of the granulosa and theca layers, together with the oocyte within its cumulus, generally cultured well. The methods used and the results obtained were thus strikingly similar to observations for sheep (MOOR et al.[3]) and human (BAKER and NEAL[4]) follicles. The apparent lack of any beneficial effect in vitro due to addition of gonadotrophic hormones was also recorded in these studies. However, MOOR et al.[3], obtained their best results with sheep follicles removed from animals at oestrus, or from those which had been pretreated with PMSG prior to removal of the ovaries for organ culture. We have not so far assayed the medium recovered at the end of the culture period for progesterone and thus are not able to confirm the observation that levels of this hormone increase when the follicle shows early signs of luteinization (see MOOR et al.[3]).

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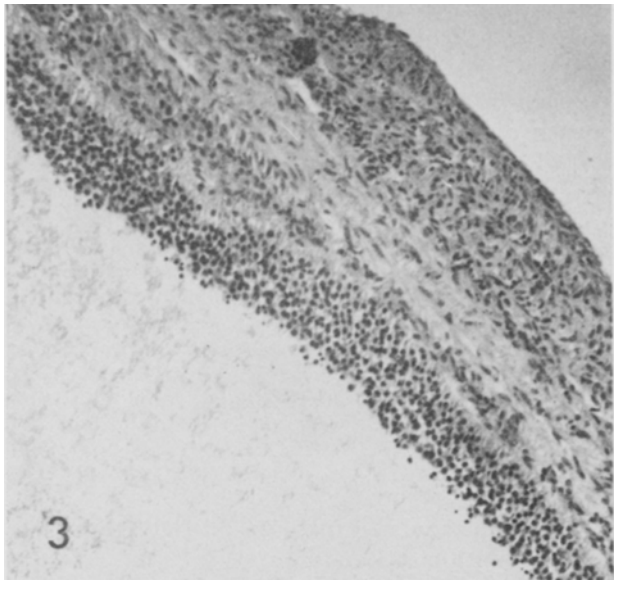
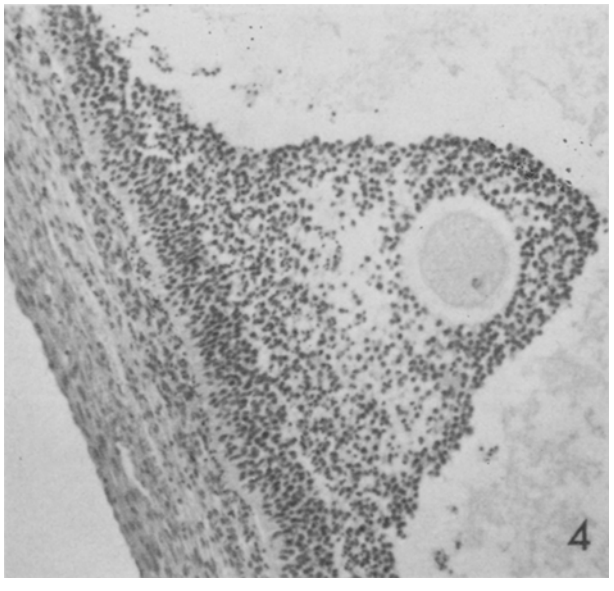


Fig. 3. Follicle recovered on day 18 and cultured for 4 days using method (c). Insulin and HCG were added to the medium. ×400.



Our observation that the most successful cultures were obtained when the levels of oxygen were highest is in keeping with the results of THIBAULT and GÉRARD[5]. These authors cultured rabbit follicles with a gas phase of 5% CO_2 in air and found that the best results were obtained with a pressure of 5 to 10 bars (ca. 5.1 or 10.2 kg/cm²). Similar results have been obtained by BAKER and NEAL[6] for Graafian follicles obtained from mouse ovaries. The increased gas pressure may result in a high partial pressure of dissolved oxygen while keeping the concentration in the gas phase below the toxic level. If this suggestion is true, it is probable that we will need to use elevated gas pressures if the present findings are to be improved upon. Nevertheless, the results of the present study have provided a technique for maintaining porcine Graafian follicles in organ culture, and studies currently underway involve investigations on the control of pre-ovulatory maturation in both the fluid and cellular components of the follicle[7].

Résumé. Nous avons étudié les follicules de Graaf dans différents milieux de culture organique classique, le but éventuel étant de voir comment le LH provoque la reprise de la méiose. Les follicules ont été prélevés de l'ovaire de 16 truies se trouvant à différents stades du cycle oestrien et mis en culture sous pression augmentée (0.703 kg/cm²). En général, les couches de cellules et même l'ovocyte supportèrent bien la mode de culture décrit.

T. G. BAKER, R. H. F. HUNTER and P. NEAL

Department of Obstetrics and Gynaecology, University of Edinburgh, Hormone Laboratory, Royal Infirmary, Edinburgh EH3 9ER (Scotland); and School of Agriculture, University of Edinburgh (Scotland), 24 June 1974.

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Fig. 4. Same specimen and treatment as in Figure 3. Apart from slight pyknotic changes in the cumulus cells, the follicle resembles non-cultured controls. ×400.