Marcelja, S. (1974) *Biochim. Biophys. Acta 367*, 165-174.
Marsh, D. (1974) *J. Membr. Biol. 18*, 145-162.
Nagle, J. F. (1973a) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3443-3444.
Nagle, J. F. (1973b) *J. Chem. Phys. 58*, 252-264.
Pink, D. A., & Carroll, C. E. (1978) *Phys. Lett. A 66*, 157-160.
Pink, D. A., & Chapman, D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1542-1546.
Salem, L. (1962) *J. Chem. Phys. 37*, 2100-2113.
Schindler, H., & Seelig, J. (1975) *Biochemistry 14*, 2283-2287.
Seelig, A., & Seelig, J. (1974) *Biochemistry 13*, 4839-4845.
Snyder, R. G. (1967) *J. Chem. Phys. 47*, 1316-1360.
Snyder, R. G. (1970) *J. Mol. Spectrosc. 36*, 222-231.
Snyder, R. G., & Schachtschneider, J. H. (1963) *Spectrochim. Acta 19*, 85-116.
Spiker, R. C., & Levin, I. W. (1975) *Biochim. Biophys. Acta 388*, 361-373.
Tardieu, A., Luzzati, V., & Reman, F. C. (1973) *J. Mol. Biol. 75*, 711-733.
Tasumi, M., Shimanouchi, T., & Miyazawa, T. (1962) *J. Mol. Spectrosc. 9*, 261-287.
Theimer, O. H. (1957) *J. Chem. Phys. 27*, 408-416.
Wulf, A. (1977) *J. Chem. Phys. 67*, 2254-2266.
Yellin, N., & Levin, I. W. (1977a) *Biochemistry 16*, 642-647.
Yellin, N., & Levin, I. W. (1977b) *Biochim. Biophys. Acta 468*, 490-494.
Yellin, N., & Levin, I. W. (1977c) *Biochim. Biophys. Acta 489*, 177-190.

Isolation, Physicochemical Properties, and Macromolecular Composition of Zona Pellucida from Porcine Oocytes†

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ABSTRACT: Oocytes released en masse from pig ovaries were isolated in large quantities by using sieving techniques. The isolated ooctyes were gently homogenized, and the largely intact zona pellucida "ghosts" were purified by using sieving techniques. Sufficient amounts of zonae were recovered to permit, for the first time, adequate characterization of the zona pellucida in chemical, physical, and macromolecular terms. The isolated zonae were >93% pure as determined by chemical, enzymatic, and microscopic criteria. The zonae were completely solubilized by a variety of conditions that do not break covalent bonds. The extent of solubilization was a function of pH, ionic strength, temperature, and the presence of various solubilizing agents such as detergents and urea. Chemically, the zonae were composed predominantly of protein (71%) and carbohydrate (19%). After acid hydrolysis of the zonae, no unusual amounts or types of amino acids were detected. The monosaccharides present after hydrolysis were those typically found in animal glycoproteins (Fuc, Man, Gal, GalNAc, and GlcNAc). Sialic acid in glycosidic linkage and sulfate and phosphate esters were present and were considered to be true constituents of the zona pellucida. Other substances detected, but considered contaminants rather than true constituents, included fatty acids (esterified and free) and uronic acids. The binding by several fluorescein-conjugated plant lectins to the in situ zona pellucida was determined by using light microscopy. The binding of the lectins to the zona pellucida was not uniform, indicating that the carbohydrate moieties of the zona pellucida were asymmetrically distributed. The zona pellucida was composed of at least three macromolecules as indicated by immunodiffusion and sodium dodecyl sulfate gel electrophoresis experiments. Determination of the number of macromolecules composing the zona pellucida was compromised by the aggregation and/or microheterogeneity of its constituent macromolecules. We conclude that the zona pellucida is composed of several glycoprotein macromolecules; interaction of these macromolecules to form supramolecular complexes and the integral zona pellucida is dependent on noncovalent forces.

The chemical and physical properties of the zona pellucida, the extracellular envelope surrounding mammalian oocytes and eggs adjacent to the plasma membrane, are of particular interest because of its biological importance. In the fertilization process, sperm penetration through the zona pellucida has been postulated to involve a preliminary sperm binding to the zona pellucida [Hartman et al., 1972; Hartman & Hutchison, 1976; for a discussion, see Gwatkin (1977)]. This is followed by a restricted hydrolysis of the zona pellucida with the assistance of sperm enzymes such as acrosin, thereby permitting the sperm access to the egg plasma membrane [Srivastava et al., 1965; Stambaugh & Buckley, 1969; Polakowski et al., 1972; Schleuning et al., 1973; for a discussion, see McRorie & Williams (1974) and Hartree (1977)]. After the fertilizing sperm has gained access to the egg and triggered the cortical reaction, the zona pellucida is chemically and physically modified to prevent supenumerary sperm penetration (Austin & Braden, 1956; Barros & Yanagimachi, 1971, 1972; Gwatkin et al., 1973). In addition to the above, the zona pellucida is currently of intense interest in terms of its immunological properties, as antibodies directed against zona pellucida components prevent sperm binding to the zona pellucida and fertilization of eggs both in vitro and in vivo (Shivers et al., 1972; Ownby & Shivers, 1972; Garavagno et al., 1974; Glass & Hanson, 1974; Jilek & Pavlok, 1975; Oikawa & Yanagimachi, 1975; Tsunoda & Chang, 1976a,b; Yanagimachi et al., 1976; Gwatkin & Williams, 1978).

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Attempts to provide a molecular basis for these biological phenomena by determining the chemical and physical properties of the zona pellucida have been frustrated by the limited availability of eggs. As stated by Jeanloz (1971), "The very small size of the mammalian egg and the difficulty in collecting a sufficient quantity of eggs are without doubt the main reasons for our very limited knowledge of the chemical structure of components of the zona pellucida."

In view of the biological importance and interest in the zona pellucida, we set out to isolate large quantities of this extracellular structure for the purpose of determining its chemical and physical properties. Our experimental approach was patterned after that used in isolating the analogous structures, the vitelline and fertilization envelopes, surrounding eggs from the amphibian *Xenopus laevis* (Wolf et al., 1976). Recognizing the limitation of obtaining sufficient numbers of eggs, we focused instead on the isolation of oocytes directly from ovaries. For reasons of economy and the numbers of ovaries needed, we considered the use of laboratory rodents as unfeasible. Of the domestic animals whose tissues are routinely available through slaughterhouses, the pig seemed the best choice due to its ready availability, the lack of seasonality in terms of ovulation, and the large numbers of oocytes expected due to the large number of eggs normally ovulated. Accordingly, we report here the large-scale isolation of pig oocytes and the physicochemical characterization of the purified zona pellucida.

Materials and Methods

Isolation of Oocytes and Zona Pellucida "Ghosts". Ovaries were collected from freshly slaughtered gilts (market weight of 200–240 lb) and were kept on ice until processed (3–7 h). Only ovaries showing mature follicles were used. Ooctyes could also be prepared from frozen ovaries by using the same procedure as for fresh ovaries. The zona pellucida (referred to as zona or zonae) prepared from such oocytes gave equivalent results to zona prepared from nonfrozen tissue. All isolation procedures for ooctyes and zonae were carried out at 0 °C. Ovaries were submerged in a 130 mM NaCl solution which also contained 10 mM sodium phosphate, 2 mM EGTA,[1] and 11 mM sodium citrate at pH 7.0. Ovarian follicles were ruptured with either a multineedled flower base (florist's or pin frog) or a series of 14-ganged razor blades separated from each other by galvanized washers. The ganged razor blades were the most satisfactory device. The device was mounted on a drill press so that the ovaries could be lightly pressed in the buffered salt solution on two opposite surfaces and the resulting flaccid ovary easily removed with a pair of forceps. The ovaries were pressed on a perforated polyethylene plate as shown in Figure 1, so that the released ooctyes dropped through the plate and were removed from contact with the ovary. Failure to use the perforated plate resulted in large losses of oocytes as they stuck to the flaccid ovary and were removed from the medium. The oocytes collected in the plugged bottom of the polyethylene funnel were washed through a series of nylon screens with meshes of 200, 150, and 74 μm, and the oocytes retained on the 74-μm screen were recycled through the 150-μm screen and collected on the 74-μm screen from 2 to 5 times. The oocytes were washed from the screen into a plastic beaker with a minimum of buffered salt solution. Substances smaller than the oocytes, e.g., erythrocytes, single cumulus cells, and soluble macromolecules, passed through the 74-μm screen whereas larger pieces of tissue and aggregated cumulus cells were retained by the 150-μm screen. The presence of the chelating agents plus the physical sieving of the oocytes very effectively stripped off the cumulus cells and yielded virtually cumulus-free ooctyes.

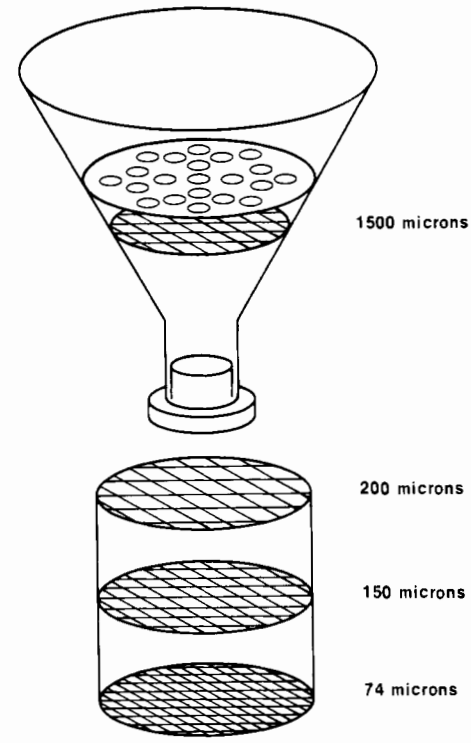

FIGURE 1: Arrangement of the porous plate and screens used for rupturing ovarian follicles and for separation of the released ooctyes.

Zona pellucida "ghosts" were prepared by gently homogenizing isolated oocytes in a Potter–Elvehjem homogenizer using the same buffered salt solution as in the oocyte isolation. The largely intact zonae were collected on a 50-μm screen and repeatedly washed with the buffered salt solution to wash away the cellular and soluble debris.

The same procedure was used to isolate oocytes and zonae from bovine ovaries. However, the yields of cow ooctyes from an equivalent number of ovaries were less than in the case of oocytes from pig ovaries.

Electron Microscopy. Ovaries, ooctyes, and isolated zonae were fixed in 4% glutaraldehyde and 0.1 M sodium phosphate, pH 7.0, at 4 °C for 2 to 3 h, rinsed with phosphate buffer, and postfixed in 1% osmium tetroxide. Subsequent procedures were as previously described (Grey et al., 1974).

Enzyme Analyses. Analysis for malate dehydrogenase activity was according to Ochoa (1955), for glucosaminidase activity was according to McGuire et al. (1972) and for arginine naphthylamidase activity was according to Suszkiw & Brecher (1970).

Protein and Carbohydrate Analyses. Protein was determined by using fluorescamine according to Udenfriend et al. (1972) with lysozyme or bovine serum albumin as a standard protein. The method of Lowry et al. (1951) was also used with bovine serum albumin or lysozyme as a standard. The fluorescamine method was the most sensitive, and lysozyme was chosen as the best standard protein as its lysine content was similar to that of the zona pellucida; accordingly, this method was the most frequently used.

Neutral sugar determination was by the phenol–H_2SO_4 method (Dubois et al., 1956) using galactose as a standard.

Solubility Studies. Solubilization of zonae under a variety of conditions was determined by protein analysis of the su-

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

pernatant solution after removal of particulate matter by centrifugation using a Beckman microfuge (10000*g*, 5 min). Complete (100%) solubilization using the fluorescamine method was defined as the total amount of reactive material present (pellet plus supernatant solution) after 6 M HCl hydrolysis at 100 °C for 24 h. In general, 10–30 μg of zona protein was used in any one experiment. Solubilization studies were normally carried out in a 400-μL plastic centrifuge tube.

Chemical Analyses. Amino acid analysis was performed after 6 M HCl hydrolysis by using a Durrum amino acid analyzer. Monosaccharide analyses after acid hydrolysis were of the alditol acetate derivatives by using gas–liquid chromatographic procedures (Porter, 1975). Fatty acid analysis after hydrolysis was of the methyl ester derivatives by using gas–liquid chromatography (Stumpf & Boardman, 1970). Uronic acids were determined by the method of Bitter & Muir (1962) using glucuronic acid as a standard. Sulfate analysis was according to Terho & Hartiala (1971). Phosphate analysis was by the colorimetric method of Penney (1976) and by the fluorometric method of Holzbecher & Ryan (1973).

Immunological Methods. Antisera to isolated zonae were prepared by injecting two female New Zealand white rabbits with a combination of intact zonae, zonae solubilized by heating at 70 °C for 30 min, and zonae solubilized with 1% sodium dodecyl sulfate and 0.07 M Na_2SO_3 at 50 °C for 16 h. Samples were emulsified in complete Freund's adjuvant and injected into multiple intradermal and subscapular sites. Approximately 10 000 zonae or 330 μg of protein was injected per rabbit. After 3 weeks, booster injections (5000 zonae) in incomplete Freund's adjuvant were given. Two control rabbits were treated with Freund's adjuvant alone. Bleeding and collection of antisera were done by conventional techniques (Williams & Chase, 1967). Two-dimensional immunodiffusion used 0.9% agarose containing 150 mM NaCl and 5 mM sodium phosphate, pH 7.0.

Lectin Binding Studies. Fluorescein-conjugated lectins (E-Y Laboratories) were used to determine the sugar moieties associated with the zona pellucida. Pig and cow oocytes were incubated for 15 min at room temperature in 10 mM Tris-HCl, 150 mM NaCl, and 10 mM $CaCl_2$, pH 7.8, with a lectin concentration of 5 μg/mL. Oocytes were collected on a 50-μm nylon screen and thoroughly washed with the above solution without the lectin and then allowed to sit in buffer for 2 h to remove the unbound lectin. Observations on the location of the fluorescein-conjugated lectin were made with a Zeiss microscope equipped with epifluorescent optics using a BG12 excitation filter and a 53/54 emission filter.

Electrophoresis. Sodium dodecyl sulfate–polyacrylamide electrophoresis used the conditions of Laemmli (1970). Electrophoresis grade acrylamide (Bio-Rad Laboratories) was used; 6% acrylamide gels gave optimal resolution of zona polypeptides. Gels were stained with Coomassie Brilliant Blue R or periodic acid–Schiff base according to the procedure of Fairbanks et al. (1971) and processed as previously described (Wolf et al., 1976).

Results

Isolation of Zonae. The isolation scheme developed here resulted in the isolation of sufficient quantities of oocytes and zonae to permit the analysis of their chemical and physical properties. Yields of both oocytes and zonae were high, particularly so with pig ovaries. Some 40 000–50 000 oocytes were obtained from 200 ovaries in 3 to 4 man hours. The oocytes had a diameter of 157 ± 4 μm (SD) inclusive of the zona. From the uniform size of the isolated oocytes, we infer that they were collected from a restricted number of oocyte developmental stages. In addition, as the average size of isolated oocytes and the average width of the zona pellucida approximated that of ovulated eggs, the oocytes were probably in phase B of their growth as described by Brambell (1928) [see also Mauleon & Mariana (1977)], although some were probably undergoing atresia. Phase B oocytes have essentially attained their mature size and increase in size very slowly whereas the follicle is rapidly increasing in size. Approximately 80% of the zonae were recovered from the homogenized ooctyes. Application of these methods to other ovaries, e.g., cow, rabbit, and sheep, also resulted in the isolation of large numbers of ooctyes and zonae (B. Dunbar, unpublished experiments). The zonae were "sticky" and tended to adhere to surfaces, particularly to glass. Hence, it was important to work with plasticware as much as possible.

Table I: Enzymatic Activities in Oocyte Homogenate, Follicular Fluid, and Isolated Zonae

enzyme act.	units/mg of protein[a] oocyte homogenate	follicular fluid	isolated zonae	(zonae/homogenate) × 100 (%)
MDH	1.46	0.051	0.097	6.6
amidase	430	3.3	12	3
glucosaminidase	0.52	0.061	0.034	6.5

[a] A unit of activity for malate dehydrogenase (MDH) was defined as 1 ΔA_{340nm}/min, for amidase activity using argininenaphthylamide as 1 Δ(fluorescent unit)/min with excitation at 335 nm and emission at 410 nm, and for glucosaminidase using *p*-nitrophenyl-β-D-*N*-acetylglucosaminide as 1 ΔA_{400nm}/min.

Purity of Isolated Oocytes and Zonae. The cellular and subcellular purity of the oocytes and zonae was established by light and electron microscopic methods. On a cellular basis, less than 5% of the cells present in the oocyte preparation were other than oocytes. Cumulus cells were the major cellular contaminant. However, due to their much smaller size compared to the oocyte, their contamination on the basis of weight was less than 5%. Electron microscopic examination of the zona preparation revealed villar processes embedded in the zona matrix contributed by both the oocytes as well as by the cumulus cells (Figure 2). The embedded villar processes, which are retracted from the zona prior to ovulation of a mature oocyte, to a large extent were pulled out of zona matrix and to a small extent were sheared off by the isolation procedure employed. The fibrillar nature of the zona is very apparent, as is the morphological "sidedness" or asymmetry of the zona. The residual space formerly occupied by the ooctye villar processes embedded in the zona matrix had a uniform lacy or microvesicular appearance. The residual space formerly occupied by the cumulus villar processes had a nonuniform, macrovesicular appearance. The microvesicles were viewed at higher magnification, and the residual electron-dense material at the periphery of the vesicles was clearly membranous in structure and was presumably remnants of the villar membranes. No other significant contamination at the subcellular level was evident.

Chemical purity of the zona preparations was established by enzymatic analysis as well as chemical analysis (fatty acid composition to be presented later). Table I presents the results of enzyme assays for malate dehydrogenase activity, exopeptidase activity using argininenaphthylamide as substrate, and glucosaminidase activity using *p*-nitrophenyl-β-D-*N*-acetylglucosaminide as substrate. Malate dehydrogenase activity was chosen due to its ubiquitous cellular distribution, and the other two activities were chosen by analogy with their presence in eggs of *X. laevis* (L. C. Greve and J. L. Hedrick,

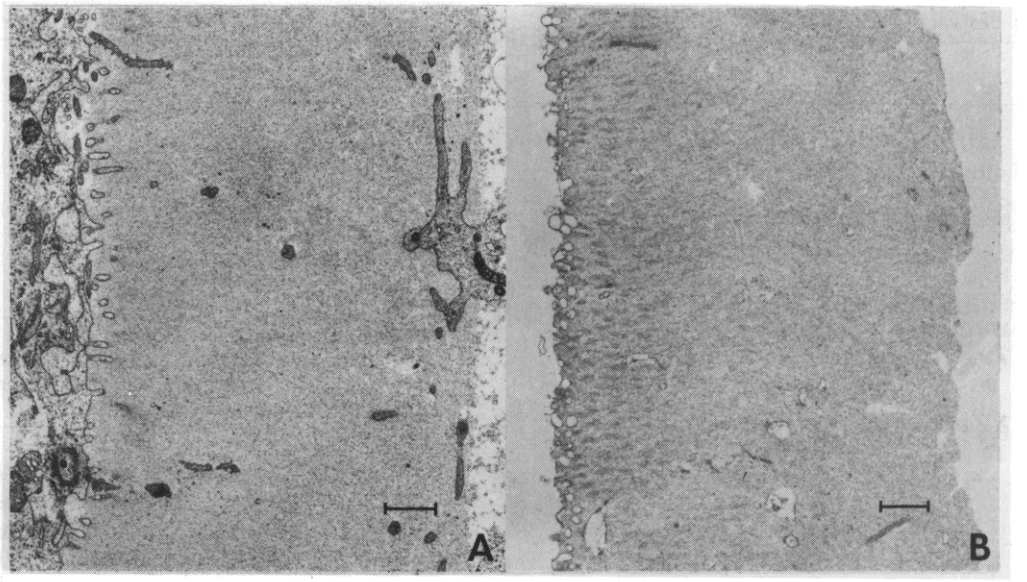

FIGURE 2: Transmission electron micrographs of a pig oocyte with associated cumulus cells (A) and an isolated zona pellucida (B). In both instances the zona pellucida is oriented with its oocyte surface to the left. The bar represents 1 μm.

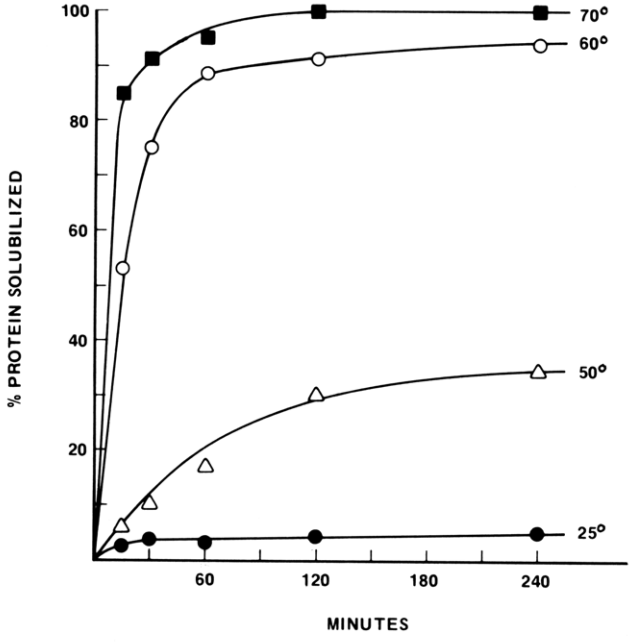

FIGURE 3: Effect of temperature and time on zona solubilization in 0.11 M sodium borate, pH 10.

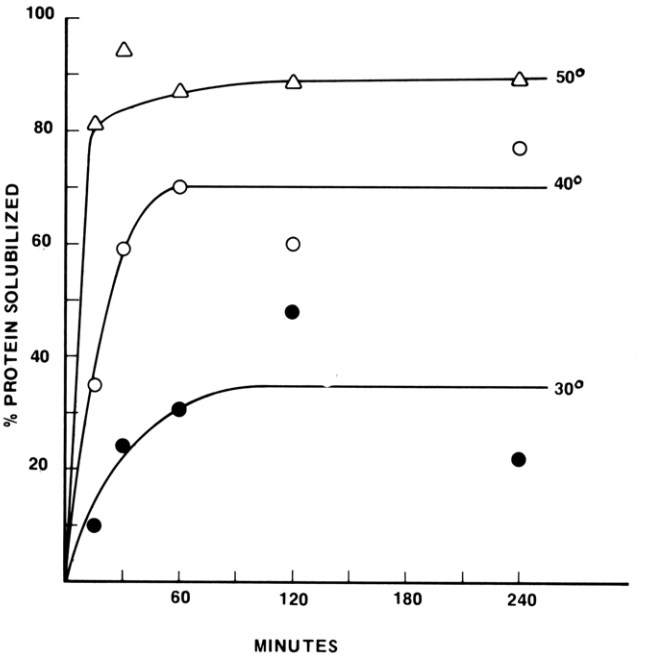

FIGURE 4: Effect of temperature and time on zona solubilization in 0.4 mM Na_2CO_3, pH 10.

unpublished experiments). Comparing the specific activities in the oocyte homogenate and the isolated zonae, there was between 3 and 7% of the specific activity remaining in the purified zonae. For any given enzyme by this criterion, the isolated zonae were 97–93% "pure". There is some degree of uncertainty in equating these enzymatic activities with chemical or macromolecular purity of the zonae, however. Since the zona is composed predominantly, if not entirely, of glycoproteins, certain of these enzymes may bind to the zona in the sense of an enzyme–substrate complex and hence give an overestimate of their presence. Nonetheless, a purity of the isolated zonae of 93% or greater is acceptable at this stage of the work but will limit the interpretation of some analyses in terms of chemical or macromolecular composition.

Solubilization. In order to study the zona composition in terms of its macromolecular constituents, it was necessary to solubilize the zona under conditions which did not disrupt covalent bonds or under conditions where covalent modification was strictly controlled, e.g., reduction of disulfide bonds. A systematic investigation of buffer specificity, ionic strength, pH, and the presence of various protein denaturants is reported in Table II. The isolated zonae were effectively solubilized under several conditions. Heating (50 °C) the zonae in the presence of borate buffer at pH 10 results in 89% solubilization. Since the zona is composed of glycoproteins, borate ions may exhibit a specific solubilizing effect due to complexing with the carbohydrate side chains. However, it is clear from the results of Table II that alkaline pH values increase the zona solubility as does low ionic strengths. $NaDodSO_4$ also effectively solubilizes the zona, but solubilization by $NaDodSO_4$ is improved when disulfide bonds are reduced.

The effect of temperature and time on solubilization with sodium borate buffer at pH 10 (Figure 3) and with distilled water adjusted to pH 10 with Na_2CO_3 (Figure 4) was evaluated. It is apparent from both studies that solubility is a function of both time and temperature, but time and temperature are not reciprocally related. It would appear that

Table II: Solubilization of Isolated Zonae

conditions[a]	% solubilized (±SD)[b]
sodium borate, $\gamma/2 = 0.1$	
0.11 M, pH 10.1	89
0.132 M, pH 9.6	72
0.20 M, pH 9.1	33
0.20 M, pH 8.1 ($\gamma/2 = 0.018$)	49
Tris-HCl, $\gamma/2 = 0.1$	
0.2 M, pH 8.1	9.7
sodium phosphate, $\gamma/2 = 0.1$	
0.2 M, pH 7.21	5.6
sodium acetate, $\gamma/2 = 0.018$	
0.036 M, pH 4.8	26
0.075 M, pH 4.3	15
0.2 M, pH 3.8	11
water[c]	
pH 10	98
pH 8	84
pH 7	98
urea	
8 M	51
6 M	68
2 M	63
detergents	
1% $NaDodSO_4$, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.0	69 ± 5
1% $NaDodSO_4$, 0.2 M sodium borate, 0.01 M mercaptoethanol, pH 9.2	82 ± 3
1% $NaDodSO_4$, 0.07 M Na_2SO_3, 0.04 mM $CuSO_4$, 0.2 M sodium borate, pH 9.2	92 ± 8
5% Triton X-100, 10% mercaptoethanol, 0.2 M sodium borate, pH 9.2	49 ± 8

[a] Heated at 60 °C for 120 min. [b] After centrifugation, solubilized protein and insoluble pellet were hydrolyzed in 6 M HCl for 24 h at 100 °C, and amino substances were analyzed by using the fluorescamine method. Protein solubilized is expressed as the percentage of total protein present in both fractions. [c] Water was adjusted to pH indicated with Na_2CO_3.

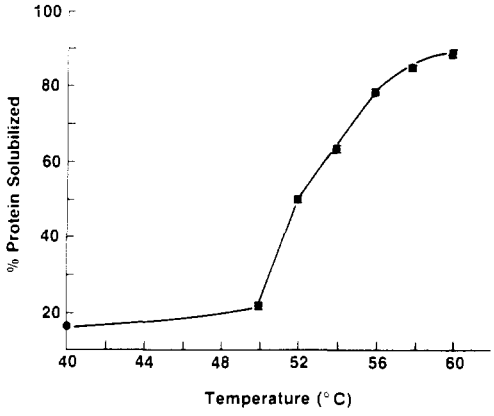

FIGURE 5: Zona solubilization as a function of temperature in 0.11 M sodium borate, pH 10. Samples were heated at the temperatures indicated for 2 h.

both rate (activation energy) and equilibrium (thermodynamic considerations) phenomena enter into the solubilization behavior of the zona (see macromolecular composition). However, the zona "ghosts" were virtually 100% solubilized under both conditions and remained soluble after returning the solution to room temperature. By analogy with the temperature-dependent transition of the double-stranded helix of DNA to a random coil, a melting temperature, T_m, can be determined for the transition of particulate zonae to solubilized zonae. Figure 5 illustrates zona solubility as a function of temperature in sodium borate buffer, pH 10. Under these

Table III: Chemical Properties of Isolated Zonae

constituent	ng/zona	μg/mg of protein	% of total
protein[a]	33	1000	71
neutral hexose[b]	8.7	264	19
uronic acids	1.1	34	2.4
sialic acid	1.3	38	2.7
acyl fatty acids	1.3	38	2.7
sulfate	0.59	18	1.3
phosphate	0.50	15	1.1
total	46.5		100.2

[a] Based on fluorescamine assay using lysozyme as a standard. [b] Corrected to a standard with a color value corresponding to the hexose composition determined by gas–liquid chromatographic analysis (color value = 0.99 with glucose = 1.00).

conditions, the inflection point of the curve occurred at ~52 °C. A T_m of 52 °C is sufficiently low that it seems highly unlikely that covalent bonds are being broken in the solubilization process. Accordingly, we conclude that the structural integrity of the zona pellucida is maintained by noncovalent forces.

Chemical Properties. The chemical properties of isolated zonae are indicated in Table III. Since greater than 90% of the material can be accounted for as protein and carbohydrate, we conclude that the zona pellucida is composed predominantly of glycoproteins.

As all protein methods are relative, we analyzed the isolated zonae by a variety of protein methods using different standards. The following values relative to the fluorescamine method using lysozyme as a standard and defined as 1.0 were found: Lowry with bovine serum albumin as standard, 1.19; Lowry with lysozyme as a standard, 0.89; amino acid residue weight from amino acid analysis, 1.43.

The absorbance of zonae dissolved in 5 mM Na_2CO_3, pH 10, was determined. Absorptivity values were calculated by using these data and protein concentrations determined by other methods. The values were $A_{280nm}^{1\%,1cm} = 18.7$ relative to lysozyme (fluorescamine method) and $A_{280nm}^{1\%,1cm} = 15.8$ relative to bovine serum albumin (Lowry method).

The presence of uronic acids can be interpreted as an indication that the zona is also composed of proteoglycans or that the zona is contaminated to a small extent by proteoglycans. In view of the paradigm that the cumulus cells are held together by hyaluronoic acid and, therefore, some hyaluronic acid is to be expected as a contaminant and from the monosaccharide composition of the zona preparations (Table IV), we favor the interpretation that the uronic acid present is a contaminant rather than a constituent of the zona.

Sialic acid is present in small amounts consistent with the findings of others based on cytochemical staining (Soupart & Noyes, 1964).

Fatty acids were present in the zona preparations in both esterified (detectable only after hydrolysis) and nonesterified forms. Presumably the nonesterified (free) fatty acids were present as contaminants probably absorbed when the ooctyes were broken and the zona "ghosts" were exposed to the contents of the cell. The pig oocyte has a large number of yolk bodies present (Norberg, 1972a,b), but the chemical composition of these yolk organelles has not been described. The average of two determinations for free fatty acids in the zona preparations was 1.3% relative to protein weight with a composition of 16:0, 39%; 18:0, 16%; 18:1, 18%; 18:2, 8%; and unidentified, 19%. The ratio of saturated to unsaturated fatty acids was 68:32. The esterified fatty acids were 3.8% relative to protein weight with a composition of 16:0, 40%; 18:0, 20%;

Table IV: Amino Acid[a] and Monosaccharide Composition of Zonae

amino acid	mol %	mol/10^5 g of protein
Ala	6.0	48
Arg	4.8	39
Asx	8.5	68
1/2-Cys[b]	3.2	26
Glx	9.5	76
Gly	7.7	61
His	3.6	29
Ile	3.1	25
Leu	9.1	73
Lys	3.9	31
Met[b]	1.4	12
Phe	3.2	26
Pro	7.9	63
Ser[g]	7.4	59
Thr[g]	9.3	74
Trp[c]	1.7	13
Tyr[c]	1.8	14
Val	7.8	63

monosaccharide	wt % of total carbohydrate[h]	mol/10^5 g of protein[e]
Fuc	3.7	8
GlcNAc[d]	34.6	54
GalNAc[d]	4.8	8
Man	9.4	18
Gal	33.8	65
Glc	2.9	6
sialic acid[f]	10.9	12

[a] Average of four analyses. [b] Performic acid oxidation followed by acid hydrolysis. [c] Determined spectrophotometrically according to Edelhoch (1967). Tyr value is the same as those by automated amino acid analysis and by spectrophotometry. [d] Hexosamines are presumed to be acetylated. [e] Relative to protein determined by fluorescamine method with lysozyme as standard. [f] Determined by the method of Warren (1959). [g] Corrected to zero time of hydrolysis. [h] By use of gas–liquid chromatography, 310 μg of neutral hexose and hexosamine was recovered per 1000 μg of protein.

18:1, 29%; and 18:2, 10%. The ratio of saturated to unsaturated fatty acids was 61:39. The finding of esterified fatty acids was anticipated as the electron micrographs of zona ghosts revealed residual villar membranes embedded in the zona matrix (Figure 2).

The finding of phosphate and sulfate after hydrolysis indicates their presence as organic esters. It is possible that sulfate is present as a contaminant (proteoglycan) and it is also possible that phosphate is present as a contaminant (phospholipids), but it is difficult to account for all of these esters in this fashion. The presence of sulfate esters in the zona was previously suggested from cytochemical staining studies and [^{35}S]SO_4 incorporation [for a review, see Piko (1969) and also Seshachar & Bagga (1963)].

Amino Acid and Monosaccharide Composition of the Zona. As indicated in Table IV, the amino acid composition of the zona, in terms of types and amounts, was not distinctive. The ratio of polar to nonpolar amino acids is 50:50, also not distinctive.

The monosaccharide composition was typical of glycoproteins. Glucosamine and galactose accounted for two-thirds of the monosaccharides present. We have a low degree of confidence in the observation that glucose is present. We have observed from time to time with different samples of biological material the appearance of false glucose peaks by the methods used here. It has recently been reported that glucose peaks can be attributed to contaminants in dialysis tubing (Coleman & Yates, 1978). In addition, one unidentified peak was observed but it contained less than 1% of the total monosaccharides.

Table V: Plant Lectin Reactivity of the Zona Pellucida

origin	abbreviation	specificity[a]	binding	regional location
Canavalia ensiformis	ConA	α-Man > α-Glc > α-GlcNAc	+	uniform
Glycine max	SBA	α-GalNAc > β-GalNAc > α-Gal	+	inner
Ricinus communis	RCA_1	β-Gal	+	outer
Triticum vulgaris	WGA	[β-(1–4)-GlcNAc]$_2$	+	inner
Arachis hypogea	PNA	[Gal-β-(1–3)-GalNAc] > GalNAc = α-Gal	–	
Dolichos biflorus	DBA	α-GalNAc	–	
Ulex europus	UEA_1	α-L-Fuc	–	

[a] All sugars are of the D configuration except for Fuc.

Lectin Reactivity. By analogy with the work on lectin reactivity of the zona pellucida surrounding rodent eggs (Oikawa et al., 1973; Nicolson et al., 1975), we investigated the lectin reactivity and regional location of seven different fluorescein-conjugated plant lectins with the cow and pig zona (Table V). Four of the seven lectins tested bound to both the cow and pig zona in an equivalent manner. The specificity of binding was consistent with the established monosaccharide specificity of the lectins as addition of the appropriate monosaccharide inhibited binding. As previously demonstrated by Oikawa et al. (1974) for rodent zona, the pig and cow zona exhibited an asymmetric distribution of sugars bound by the plant lectins. We also observed that cumulus cells bound the same four lectins. However, the possible contamination of the zona with cumulus cells or cumulus cell products cannot adequately explain the lectin binding of the zona as, for instance, SBA bound preferentially to the oocyte side of the zona rather than to the cumulus side of the zona. It should also be noted that lectins which possess overlapping specificities, e.g., SBA and DBA, showed marked differences in binding. The definition of specificity for lectins based on monosaccharide inhibition is an incomplete description of lectin specificity.

Immunodiffusion. The antigenic composition of the zona was determined by using rabbit antisera to zona in two-dimensional immunodiffusion studies. The antisera were tested at several dilutions of serum and antigen (zona). Under the conditions used, the zona was not highly immunogenic as precipitin reactions could not be detected when antisera were diluted beyond 8–16-fold. Results were also highly dependent on the nature of the antigen used. A distinction between intact or insoluble zona, solubilized zona, and solubilized-dissociated zona must be made. As will be discussed later (see $NaDodSO_4$ Gel Electrophoresis), simple heating or melting of the zona (as done in the solubility studies) solubilized the zona, presumably by disrupting noncovalent forces, into large supramolecular complexes. These supramolecular complexes (detectable by gel filtration and gel electrophoresis) were so large that they did not effectively diffuse into an agarose gel. On the other hand, reduction of disulfide bonds and the presence of $NaDodSO_4$ effectively solubilized and dissociated the zona into its constituent macromolecular parts. These detergent–glycoprotein complexes diffused into an agarose gel, but the presence of $NaDodSO_4$ can alter the antigenic reactivity of the macromolecules, as it is well-known that the antigenicity of many proteins is dependent on their conformation. In addition, we observed that $NaDodSO_4$, when present in the

Table VI: Immunodiffusion Results

rabbit antiserum to[a]	antigen tested[b]	max no. of lines obsd	identity reaction[c]
preimmune serum	pig zona[d]	0	0
complete Freund's adjuvant	pig zona[d]	0	0
pig zona	pig zona[d]	3	ND
pig zona	pig zona[e]	3	ND
pig zona	cow zona[e]	1	complete identity with pig zona line
pig zona	pig serum	0	0
pig zona	pig serum albumin	0	0
pig zona	pig liver[f]	1	partial identity with pig zona line
pig zona	pig kidney[f]	2	one partial and one nonidentity with pig zona lines
pig zona	pig cumulus cells[f]	2	complete identity with pig zona lines
pig zona (adsorbed with pig liver)	pig liver[f]	0	0
pig zona (adsorbed with pig liver)	pig zona[d]	1	ND
pig zona (adsorbed with pig kidney)	pig kidney[f]	0	0
pig zona (adsorbed with pig kidney)	pig zona[d]	2	ND

[a] In most instances serum was tested at several dilutions. [b] Several different antigen concentrations were tested. [c] ND = not determined. [d] Solubilized by heating (50 °C for 16 h) in 1% $NaDodSO_4$ and 0.07 M Na_2SO_3, pH 9.2 (see Table II). Maximum number of precipitin lines observed was corrected for the $NaDodSO_4$–antibody line. [e] Heat solubilized at 70 °C for 30 min. [f] Cell homogenate.

antigen well, gave a false precipitin line close to the antibody well. One further limitation of the immunodiffusion results presented in Table VI was that the antiserum produced by a given animal was dependent on the state of zona antigens injected into the animal, i.e., solid, solubilized, or dissociated zona (B. Dunbar, unpublished experiments). In order to maximize the immunogenic response, we injected all three forms of the zona. With these limitations, interpretation of immunodiffusion studies using the zona pellucida both in this report and in the reports of others must be done with caution.

The largest number of pig zona precipitin lines realized in this study was three (Table VI). As the technique of immunodiffusion in two dimensions demonstrates the minimum number of antigens present, we concluded that the zona was composed of at least three antigenic substances (macromolecules). At least one of these antigenic substances was the same in the pig and cow zona. The isolation procedure apparently removed any serum components from the zona (serum components are known to be major constituents of follicular fluid) as no precipitin line was observed with either serum albumin or whole serum. The zona antiserum was not organ specific as a precipitin line(s) was observed with liver and kidney extracts. Presumably the lack of organ specificity was caused by injection of undissolved zona (B. Dunbar, unpublished experiments; Gwatkin & Williams, 1978). The zona antiserum exhibited two lines of reaction with a cumulus cell extract. This could be due to cumulus cell contamination of the isolated zonae, as already mentioned, or it could be due to the presence of zona antigens in the cumulus cells. The cellular site of zona biosynthesis has not been unequivocally established, but reports have appeared suggesting that cumulus cells are involved [for a discussion, see Piko (1969) and Baker (1971)]. If this were the case, cross-reaction between zona antiserum and cumulus cells would be expected. Adsorption of the antibodies in zona antiserum which are not organ specific (kidney and liver) still leaves the antiserum immunologically reactive with dissociated zona antigens. This is consistent with the reports of others that the zona pellucida contains unique antigenic determinants (Sacco & Shivers, 1973; Sacco & Palm, 1977).

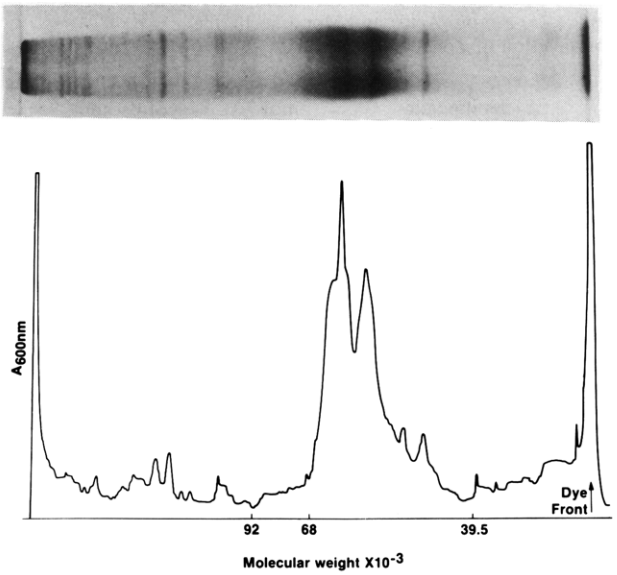

FIGURE 6: $NaDodSO_4$ gel electrophoresis of zona. Separation of zona macromolecules was in a 6% acrylamide gel. The separated components were stained with Coomassie Blue, and the stained gel was photographed and scanned by using a wavelength of 600 nm.

$NaDodSO_4$ Gel Electrophoresis. The macromolecular composition of the zona was investigated by using $NaDodSO_4$ gel electrophoresis. Conditions for solubilization of the isolated zonae and for electrophoresis were those described by Laemmli (1970). The results are shown in Figure 6. A relatively complex pattern of protein bands was obtained with considerable "smearing" or electrophoretic microheterogeneity. The resolution obtained was not satisfactory, but we have not as yet been able to alter the electrophoretic conditions to obtain better resolution of the zona components. The stained pattern obtained after electrophoresis when mercaptoethanol was not included in the solubilization medium was only subtly different, e.g., some higher molecular weight components and some broadened bands. Greater than 60% of the protein applied was within the 40 000–68 000 molecular weight range which also stained for carbohydrate by using the periodic acid–Schiff base stain and, therefore, is presumably composed of glycoproteins. The cow zona gave a similar electrophoretic pattern to the pig zona. The number of macromolecular components in the zona is unclear from these results. However, three or four major molecular weight classes of glycoproteins are clearly present with nine or more minor components. The material at the origin may be an undissociated macromolecular aggregate, although it was always present no matter what solubilizing conditions were employed.

Discussion

Previous studies on the chemical and physical properties of the zona from various mammals have been compromised in terms of accurate and precise analyses for two reasons. (1) The isolated zonae were not routinely subjected to any type of purity tests other than visual observation with the light microscope (Oikawa, 1978). Thus, chemical analyses reflected the properties of contaminating substances as well as the in-

trinsic properties of the zona. (2) The small amounts of isolated zonae available precluded many types of analyses because of sensitivity limitations and required microanalytical procedures in most instances. As a result, little chemical information of a highly accurate nature is available. Indeed, many of the conclusions concerning the chemical nature of the zona have been based on histochemical data [see, for instance, Tadano & Yamada (1978)]. As stated by Jeanloz (1971), "Although histochemical reagents can be invaluable tools for studying the distribution of known well-defined compounds, they may lead to incorrect interpretations when the chemical structure of the visualized components is unknown." From microscopic and enzymatic analyses, the zona isolated in these studies is at least 93% pure; i.e., there is less than a 7% contamination of other subcellular and macromolecular constituents. Nonetheless, interpretation of some of the analytical results presented here must take into account the fact that the isolated zona preparations were not entirely pure.

From the solubility results reported here, we conclude that the structural integrity of the zona pellucida is dependent upon noncovalent interactions between constituent macromolecular components. The zona was effectively solubilized by conditions that would not break covalent bonds. The pig zona pellucida and *X. laevis* vitelline envelope are the same in this respect (Wolf et al., 1976). Several observations on the solubility properties of the zona pellucida have been reported (Hall, 1935; Braden, 1952; Gwatkin, 1964; Cholewa-Stewart & Massaro, 1972; Inoue & Wolf, 1974a,b, 1975; Gwatkin & Williams, 1978). In all of these reports, zona solubility was determined visually with the light microscope, and in all but the most recent report the observations were made on zona in situ around the egg. In this study, we observed that in some instances the zona suspension appeared to dissolve visually, but when the solution was analyzed for zona protein after centrifugation there was little present. Thus, changes in the hydration state of the zona which lead to changes in the optical properties (refractive index) of this structure can be misinterpreted as solubilization. Solubilization under conditions that do not lead to dissociation of the constituent macromolecules, e.g., melting in water adjusted to pH 10, resulted in a solution that contained supramolecular complexes of zona macromolecules as evidenced by its electrophoretic and diffusional behavior. Dissociation into individual macromolecules is aided by reduction of disulfide bonds and requires the presence of some dissociating agent such as $NaDodSO_4$. The same observations were reported for the *X. laevis* vitelline envelope (Wolf et al., 1976). Accordingly, physicochemical and immunological characterization of zona solubilized under nondissociating conditions must take into account the fact that zona constituents are present as supramolecular complexes.

The presence of uronic acids, esters of sulfate and phosphate, and fatty acids in the zona must be interpreted in view of the fact that the zonae are contaminated. It is reasonable to assume that some hyaluronic acid and proteoglycans present in the cumulus mass and follicular fluid surrounding the oocytes will contaminate the zona preparations. A report by Yanagishita et al. (1979) details the isolation and characterization of proteoglycans from porcine ovarian follicular fluid. From the chemical characteristics of the most highly purified proteoglycan fraction, denoted D1, a ratio of uronic acid/protein of 4.90 μmol/mg can be calculated. The ratio of uronic acid (glucuronic acid)/protein found in the zona was 0.175 μmol/mg. On a weight basis, contamination of the zona preparation with the D1 proteoglycan fraction to the extent of 3.6% could account for all the uronic acid detected in the zona preparations. This is well within the purity limits of the zona preparations established here. Therefore, it seems unlikely that uronic acids are a normal constituent of the pig zona pellucida.

The presence of sulfate in the zona preparations could also result from contamination by follicular fluid proteoglycans. Again, from Yanagishita et al. (1979), proteoglycan fraction D1 was reported to be 50% dermatan sulfate and to have a molar ratio of sulfate/uronic acid disaccharide of 1:1. A weight ratio of sulfate/uronic acid calculated from these data gives a value of 0.11 g of sulfate per 1.0 g of uronic acid. The weight ratio of the pig zona pellucida reported here was 0.53 g of sulfate per 1.0 g of uronic acid. This fivefold higher weight ratio of the zona suggests that sulfate is present in more than just the proteoglycan contaminant. In addition, to account for the sulfate entirely as proteoglycan contamination would require a weight contamination of the zona pellucida by proteoglycan fraction D1 of 62%. This level of contamination seems unreasonable. We conclude that sulfate esters are probably true constituents of the pig zona pellucida.

Ultrastructural examination of the isolated zonae revealed the presence of membranous structures embedded in the matrix of the zona, undoubtedly representing remains of villar processes (Figure 2). From the membrane contamination, the presence of fatty acids and phosphate from phospholipids was anticipated. Comparison of the fatty acid composition of the isolated zona with the composition of whole pig ovary and isolated pig Graffian follicles reported by Holman & Hofstetter (1965) showed them to be very different. Therefore, there is not a gross contamination of the isolated zona with tissue or cellular lipids. By the assumption that the contaminating plasma membrane is composed of approximately one-third fatty acids, then on a weight basis the membrane contamination is ~8%. This is approximately the same as the estimates of zona purity, e.g., 92%. By the assumptions that all the fatty acids are derived from phospholipid, that the fatty acids comprise 70% of the weight of an average phospholipid, and that phosphate is 12% by weight of the average phospholipid, the phosphate associated with phospholipid contamination can be calculated. By use of these assumptions, a phosphate level of 6.5 μg of phosphate per 1000 μg of protein was calculated; the phosphate found by analysis of the zona was 15 μg of phosphate per 1000 μg of protein. Thus, less than one-half of the phosphate can be accounted for by phospholipid contamination. It seems likely that phosphate esters are true constituents of the pig zona pellucida.

The amino acid composition of the pig zona was not distinctive. The amino acid composition of the hamster zona pellucida was reported (Gwatkin, 1978). No evidence as to the purity of the starting material was included, however, and the amino acid composition reported for the hamster zona bears little resemblance to that reported here for the pig zona. In addition, it would appear that the hamster zona data were misinterpreted in terms of the absence of tryptophan, asparagine, and glutamine as these amino acids are destroyed during acid hydrolysis. In addition, calculation of the net charge of the reputed single macromolecule composing the hamster zona calculated from the number of acidic and basic amino acids present is meaningless without knowing the relative proportions of asparagine–aspartic acid and glutamine–glutamic acid.

The monosaccharides present in the zona were those typically associated with glycoproteins. However, the monosaccharide composition is certainly compromised by any follicular fluid proteoglycan contamination. Table VII compares the monosaccharide composition relative to fucose of the pig

Table VII: Monosaccharide Ratios Relative to Fucose for Proteoglycan Fraction D1 and Pig Zona Pellucida

	Fuc	Glc-NAc	Glc-NAc	Man	Gal	Glc	NANA
proteoglycan[a]	1	21	141	5.1	34	5.5	24
zona pellucida	1	6.8	1	2.3	8.1	0.8	1.5

[a] Taken from Yanagishita et al. (1979).

follicular fluid proteoglycans reported by Yanagishita et al. (1979) and the pig zona pellucida reported here. It is readily apparent that the monosaccharide composition between the two is different. For instance, the proteoglycans are rich in GalNAc while the pig zona has a greater proportion of GlcNAc. The low level of GalNAc found in the zona could be accounted for by proteoglycan contamination; a 1.4% contamination of proteoglycan fraction D1 in the zona pellucida could account for all the GalNAc present. Thus, the monosaccharide composition reported here for the pig zona must be considered as a preliminary one.

Comparison of the lectin reactivity of the pig zona with that of the hamster zona showed marked differences (Yanagimachi & Nicolson, 1976). For instance, the hamster zona reacted with WGA on the outer (cumulus) periphery while the pig zona reacted with WGA on its innermost (oocyte) aspect. In addition, DBA reacted with hamster zona but did not react with the pig zona. This difference could be attributed to differences in the affinity of binding, however, as the concentration dependency of lectin binding was not determined. The asymmetric nature of the pig zona as determined by lectin reactivity is consistent with the asymmetric nature of the zona of various rodent eggs (Nicolson et al., 1975).

Attempts to determine the number of macromolecules composing the pig zona by immunological and electrophoretic methods were complicated by the physical and chemical nature of the zona. Immunodiffusion experiments indicated the presence of three macromolecular determinants. On the other hand, $NaDodSO_4$ gel electrophoresis separated three or four major glycoprotein components with nine or more minor components. The gel pattern, however, was not well resolved with much smearing and trailing evident. We conclude that the macromolecular composition of the pig zona, while limited in terms of the number of macromolecules present (perhaps 10 or more), is experimentally difficult to determine because of interaction between individual components or microheterogeneity. Different degrees of sulfation, phosphorylation, or sialylation of any given macromolecule could give rise to a microheterogeneity that would produce a smeared pattern on gel electrophoresis. Attempts to separate the zona components by electrophoresis in the absence of $NaDodSO_4$ were totally unsuccessful as supramolecular aggregates are present which do not enter the gel. This is in contrast with a report by Gould et al. (1971) on the microelectrophoresis of rabbit zona in the absence of $NaDodSO_4$. However, it is difficult to interpret the diagrams published by Gould et al. (1971), and no information as to the purity of the isolated zona or the amount of zona material that entered the gel was reported. A preliminary communication on the macromolecular composition determined by $NaDodSO_4$ gel electrophoresis of the mouse zona was published (Bleil & Wasserman, 1978). In contrast to the pig zona, the mouse zona appeared to be relatively simple, being composed of three macromolecules. However, the results obtained by Repin & Akimova (1976) suggested that the mouse zona was composed of five macromolecules.

While this paper was being written, a report on the macromolecular composition of the pig zona determined by $NaDodSO_4$ gel electrophoresis appeared (Menino & Wright, 1979). Their results are markedly different from the ones reported here. Menino & Wright (1979) did not isolate the zona pellucida for their studies but rather attempted to solubilize it in situ from around the oocytes using 6 M urea solutions. Unfortunately, no estimate of oocyte contamination was made and, from the results reported here, 6 M urea solutions would not completely solubilize the zona.

Additional definition of the macromolecular properties of the pig zona pellucida requires the application of techniques that yield better resolution of the constituent glycoproteins, such as additional types and altered conditions of electrophoresis, than those used here and more "powerful" immunological techniques such as crossed-immunoelectrophoresis. It may be necessary to partially purify some of the zona macromolecules in order to reduce their interaction and simplify the gel electrophoresis patterns in order to answer the seemingly simple question as to how many macromolecules compose the zona pellucida.

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References

Austin, C. R., & Braden, A. W. H. (1956) *J. Exp. Biol. 33*, 358–365.
Baker, T. G. (1971) *Adv. Biosci. 6*, 7–28.
Barros, C., & Yanagimachi, R. (1971) *Nature (London) 233*, 268–269.
Barros, C., & Yanagimachi, R. (1972) *J. Exp. Zool. 180*, 251–266.
Bitter, T., & Muir, H. M. (1962) *Anal. Biochem. 4*, 330–334.
Bleil, J. D., & Wasserman, P. W. (1978) *J. Cell Biol. 79*, 173a.
Braden, A. W. H. (1952) *Aust. J. Sci. Res., Ser. B 5*, 460–471.
Brambell, F. W. R. (1928) *Proc. R. Soc. London, Ser. B 101*, 391.
Cholewa-Stewart, J., & Massaro, E. J. (1972) *Biol. Reprod. 7*, 166–169.
Coleman, M. T., & Yates, A. J. (1978) *J. Chromatogr. 166*, 611–614.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350–356.
Dunbar, B. S., Wardrip, N. J., & Hedrick, J. L. (1978a) *Biol. Reprod. 18, Suppl. 1*, 22a.
Dunbar, B. S., Wardrip, N. J., & Hedrick, J. L. (1978b) *J. Cell Biol. 79*, 163a.
Edelhoch, H. (1967) *Biochemistry 6*, 1948–1954.
Fairbanks, G., Steck, T. G., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2616.
Garavagno, A., Posada, J., Barros, C., & Shivers, C. A. (1974) *J. Exp. Zool. 189*, 37–50.
Glass, L. E., & Hanson, J. E. (1974) *Fertil. Steril. 25*, 484–493.
Gould, K., Zaneveld, L. J. D., Srivastava, P. N., & Williams, W. L. (1971) *Proc. Soc. Exp. Biol. Med. 136*, 6–10.
Grey, R. D., Wolf, D. P., & Hedrick, J. L. (1974) *Dev. Biol. 36*, 44–61.
Gwatkin, R. B. L. (1964) *J. Reprod. Fertil. 7*, 99–105.
Gwatkin, R. B. L. (1977) *Fertilization Mechanisms in Man and Mammals*, Plenum Press, New York.
Gwatkin, R. B. L. (1978) *Birth Defects, Orig. Artic. Ser. 14*, 363–376.
Gwatkin, R. B. L., & Williams, D. T. (1978) *Gamete Res. 1*, 19–26.

Gwatkin, R. B. L., Williams, D. T., Hartmann, J. F., & Kniazuk, M. (1973) *J. Reprod. Fertil. 32*, 259–265.
Hall, B. U. (1935) *Proc. Soc. Exp. Biol. Med. 32*, 747–748.
Hartmann, J. F., & Hutchison, C. F. (1976) Ninth Annual Meeting, Society for Study of Reproduction, Abstract No. 2, Philadelphia, PA.
Hartmann, J. F., Gwatkin, R. B. L., & Hutchison, C. F. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2767–2769.
Hartree, E. F. (1977) *Biochem. Soc. Trans. 5*, 375–394.
Holman, R. T., & Hofstetter, H. H. (1965) *J. Am. Oil Chem. Soc. 42*, 540–544.
Holzbecher, J., & Ryan, D. E. (1973) *Anal. Chim. Acta 64*, 147–150.
Inoue, M., & Wolf, D. P. (1974a) *Biol. Reprod. 10*, 512–518.
Inoue, M., & Wolf, D. P. (1974b) *Biol. Reprod. 11*, 558–565.
Inoue, M., & Wolf, D. P. (1975) *Biol. Reprod. 12*, 535–540.
Jeanloz, R. D. (1971) in *Biology of the Blastocyst* (Blandau, R. L., Ed.) pp 131–137, University of Chicago Press, Chicago.
Jilek, F., & Pavlok, A. (1975) *J. Reprod. Fertil. 42*, 377–380.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lowry, O. H., Rosebrough, M. J., Farr, A. L., & Randall, R. (1951) *J. Biol. Chem. 193*, 265–275.
Mauleon, P., & Mariana, J. C. (1977) in *Reproduction in Domestic Animals* (Cole, H. H., & Cupps, P. T., Eds.) p 190, Academic Press, New York.
McGuire, E. J., Chipowsky, S., & Roseman, S. (1972) *Methods Enzymol. 28*, 755–763.
McRorie, R. A., & Williams, W. L. (1974) *Annu. Rev. Biochem. 43*, 777–803.
Menino, A. R., Jr., & Wright, W. W., Jr. (1979) *Proc. Soc. Exp. Biol. Med. 160*, 449–452.
Nicolson, G. L., Yanagimachi, R., & Yanagimachi, H. (1975) *J. Cell Biol. 66*, 263–274.
Norberg, H. S. (1972a) *Z. Zellforsch. Mikrosk. Anat. 124*, 520–531.
Norberg, H. S. (1972b) *Z. Zellforsch. Mikrosk. Anat. 131*, 497–517.
Ochoa, S. (1955) *Methods Enzymol. 1*, 735–739.
Oikawa, T. (1978) *Gamete Res. 1*, 265–267.
Oikawa, T., & Yanagimachi, R. (1975) *J. Reprod. Fertil. 45*, 487–494.
Oikawa, T., Yanagimachi, R., & Nicolson, G. L. (1973) *Nature (London) 241*, 256–259.
Oikawa, T., Nicolson, G. L., & Yanagimachi, R. (1974) *Exp. Cell Res. 83*, 239–246.
Ownby, C. L., & Shivers, C. A. (1972) *Biol. Reprod. 6*, 310–318.
Penney, C. L. (1976) *Anal. Biochem. 75*, 201–210.
Pikô, L. (1969) in *Fertilization* (Metz, C., & Monroy, A., Eds.) Vol. 2, pp 325–403, Academic Press, New York.
Polakowski, K. L., Zaneveld, L. J. D., & Williams, W. L. (1972) *Biol. Reprod. 6*, 23–29.
Porter, W. H. (1975) *Anal. Biochem. 63*, 27–43.
Repin, U. S., & Akimova, I. M. (1976) *Biokhimiya 41*, 50–57.
Sacco, A. G., & Shivers, C. A. (1973) *Biol. Reprod. 8*, 481–490.
Sacco, A. G., & Palm, U. S. (1977) *J. Reprod. Fertil. 51*, 165–168.
Schleuning, W. D., Schiessler, H., & Fritz, H. (1973) *Hoppe-Seyler's Z. Physiol. Chem. 354*, 550–554.
Seshachar, B. R., & Bagga, S. (1963) *J. Morphol. 113*, 119–129.
Shivers, C. A., Dudkiewicz, A. B., Franklin, L. E., & Fussel, E. N. (1972) *Science 178*, 1211–1213.
Soupart, P., & Noyes, R. W. (1964) *J. Reprod. Fertil. 8*, 251–253.
Srivastava, P. N., Adams, C. E., & Hartree, E. F. (1965) *J. Reprod. Fertil. 10*, 61–67.
Stambaugh, R., & Buckley, J. (1969) *J. Reprod. Fertil. 19*, 423–432.
Stumpf, P. K., & Boardman, N. K. (1970) *J. Biol. Chem. 245*, 2579–2587.
Suszkiw, J. B., & Brecher, A. S. (1970) *Biochemistry 9*, 4008–4017.
Tadano, Y., & Yamada, K. (1978) *Histochemistry 57*, 203–215.
Terho, T. T., & Hartiala, K. (1971) *Anal. Biochem. 41*, 471–476.
Tsunoda, Y., & Chang, M. C. (1976a) *J. Exp. Zool. 195*, 409–416.
Tsunoda, Y., & Chang, M. C. (1976b) *Biol. Reprod. 14*, 354–361.
Udenfriend, S., Stein, S., Böhlen, P., Dairman, W., Leimgruber, W., & Weigele, M. (1972) *Science 178*, 871.
Warren, L. (1959) *J. Biol. Chem. 234*, 1971–1975.
Williams, C. A., & Chase, M. W. (1967) *Methods Immunol. Immunochem. 1*.
Wolf, D. P., Nishihara, T., West, D. M., Wyrick, R. E., & Hedrick, J. L. (1976) *Biochemistry 15*, 3671–3678.
Yanagimachi, R., & Nicolson, G. L. (1976) *Exp. Cell Res. 100*, 249–257.
Yanagimachi, R., Winkelhake, J., & Nicolson, G. L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2405–2408.
Yanagishita, M., Rodbard, D., & Hascall, V. C. (1979) *J. Biol. Chem. 254*, 911–920.