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Synthesis of zona pellucida proteins by denuded and follicle-enclosed mouse oocytes during culture *in vitro*

(zona pellucida/oogenesis/mouse/[^{35}S]methionine incorporation/[^{3}H]fucose incorporation)

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ABSTRACT **During growth of the ovarian follicle, the mammalian oocyte becomes surrounded by an acellular coat called the zona pellucida. Whether the zona pellucida originates from the oocyte, surrounding follicle cells, or both has remained an unresolved issue. In experiments described here, denuded and follicle-enclosed mouse oocytes at various stages of growth were isolated and cultured *in vitro* in the presence of either [^{35}S]methionine or [^{3}H]fucose in order to determine the site of synthesis of the three, recently identified, zona pellucida proteins, ZP1, ZP2, and ZP3 [Bleil, J. D. & Wassarman, P. M. (1980) *Dev. Biol.*, in press]. Approximately 1.5% of the [^{35}S]methionine, and as much as 45% of the [^{3}H]fucose, that was incorporated into trichloroacetic acid-insoluble material by denuded or follicle-enclosed oocytes during a 12-hr culture period was found associated with zonae pellucidae removed from the cultured oocytes. Incorporation of [^{35}S]methionine into zona pellucida proteins was depressed to less than 1/50th when denuded oocytes were cultured in the presence of puromycin, and secretion of zona pellucida proteins by denuded oocytes was demonstrated by pulse-chase experiments. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of [^{35}S]methionine- and [^{3}H]fucose-labeled proteins present in oocytes, zonae pellucidae, and follicle cells revealed that denuded oocytes synthesize and secrete zona pellucida proteins, whereas no evidence was obtained to suggest that follicle cells synthesize these proteins. Denuded oocytes, ranging in diameter from 48 to 68 μm, incorporated both [^{35}S]methionine and [^{3}H]fucose into zona pellucida proteins during culture *in vitro*, whereas zonae pellucidae removed from fully-grown oocytes (85 μm) were not radiolabeled to a significant extent. After culture of denuded or follicle-enclosed oocytes for 12 hr, more than 95% of the [^{3}H]fucose incorporated into oocyte proteins was found in ZP1, ZP2, and ZP3, indicating that the zona pellucida proteins are the major class of proteins glycosylated during oocyte growth. These results provide biochemical evidence supporting the idea that the zona pellucida originates from the mammalian oocyte itself, rather than from the surrounding follicle cells.**

The zona pellucida is a relatively thick, translucent, acellular coat that surrounds the plasma membrane of fully grown mammalian oocytes and performs a variety of vital biological functions during early mammalian development. The zona pellucida is laid down during growth of the ovarian follicle, remains throughout preimplantation development, and is finally shed as the blastocyst readies for implantation (1–3). Several lines of evidence suggest that the zona pellucida possesses a receptor for sperm, that it acts as a barrier to further sperm penetration after fertilization, and that its presence is necessary for normal early development *in vivo* (3–5). In certain respects the zona pellucida is morphologically and functionally analogous to the vitelline envelope that surrounds oocytes of lower animal species (6, 7).

The site of origin of the zona pellucida has been the subject of considerable interest for more than a century (8–10). Despite numerous morphological, ultrastructural, histological, and autoradiographic studies, it has remained an unresolved issue as to whether the zona pellucida originates from the oocyte, follicle cells, or both (11–14).

Recently, we identified and characterized those proteins that compose the mouse oocyte's zona pellucida (15, 16). It has been known for some time, primarily on the basis of its staining properties, that the zona pellucida contains protein and carbohydrate (3, 5), and our recent work demonstrated that the zona pellucida is composed of three different protein species, which we have designated ZP1, ZP2, and ZP3. Having identified the proteins that compose the zona pellucida has permitted us to examine the site of synthesis of these proteins during follicular development. In this report we provide biochemical evidence supporting the idea that the zona pellucida originates from the oocyte itself.

MATERIALS AND METHODS

Collection and Culture of Denuded and Follicle-Enclosed Oocytes. Ovaries were excised from 8 to 15-day-old Swiss albino mice (CD-1, Charles River Breeding Laboratories), placed into standard egg culture medium (SECM) (17), and then punctured with fine steel needles in order to obtain denuded oocytes (i.e., free of follicle cells by morphological criteria) and intact follicles ("follicle-enclosed oocytes"). Oocytes and follicles were washed throughly with SECM and transferred to 50-μl drops of culture medium in plastic dishes (Falcon). To radiolabel oocytes and follicles with [^{35}S]methionine, they were cultured in Earle's modified medium 199 prepared without unlabeled methionine (GIBCO), containing L-[^{35}S]methionine (400–800 Ci/mmol, New England Nuclear; 1 Ci = 3.7×10^{10} becquerels) at 0.5–1.0 mCi/ml, and supplemented with pyruvate (37 μg/ml). To radiolabel oocytes and follicles with L-[^{3}H]fucose, they were cultured in complete Earle's modified medium 199, containing [^{3}H]fucose (60 Ci/mmol, New England Nuclear) at 1–2 mCi/ml and supplemented with pyruvate (37 μg/ml). All cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air under paraffin oil. At the end of the culture period, denuded oocytes were harvested and washed. Follicles were torn apart with steel needles and oocytes and oocyte-free follicles were separated from each other, harvested, and washed. Oocyte diameters, exclusive of the zona pellucida, were measured with an ocular micrometer attached to an inverted microscope. Fully grown oocytes were obtained from adult mice (6–12 weeks of age) as described (18).

Removal of Zonae Pellucidae from Cultured Oocytes. Zonae pellucidae were removed from oocytes either intact, by using mouth-operated micropipettes having a bore size no greater than one-third the diameter of the oocyte, or in a soluble form by using Tyrode's acidic buffer (TAB) at pH 2.5 (19), a

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Abbreviations: SECM, standard egg culture medium; TAB, Tyrode's acidic buffer; DASA, diazonium salt of iodosulfanilic acid.

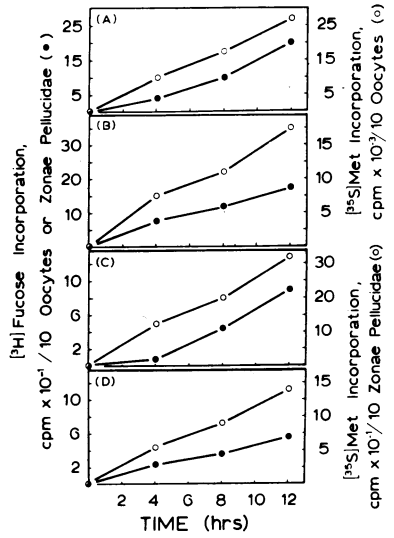

FIG. 1. Incorporation of [^{35}S]methionine and [^{3}H]fucose into oocyte and zona pellucida proteins. Denuded and follicle-enclosed oocytes (59 μm) were harvested and washed after 0, 4, 8, and 12 hr of culture in the presence of either [^{35}S]methionine (0.5 mCi/ml) or [^{3}H]fucose (2 mCi/ml). Oocytes were treated with TAB and then the zona pellucida-free oocytes and solubilized zonae pellucidae were analyzed for incorporation of radioactivity into acid-insoluble material. (*A*) Follicle-enclosed, zona pellucida-free oocytes; (*B*) denuded, zona pellucida-free oocytes; (*C*) zonae pellucidae of follicle-enclosed oocytes; (*D*) zonae pellucidae of denuded oocytes.

reagent specific for the dissolution of only the zona pellucida (16, 20). In the latter case, washed oocytes were transferred in about 0.5 μl to a 5-μl drop of TAB and, when the zonae pellucidae had dissolved (about 5 sec), the drop was collected, leaving the oocytes behind, and replaced with another 5 μl of TAB. The second drop was collected, combined with the first, and transferred to a Microfuge tube containing 10 μl of 0.25 M Tris·HCl, pH 6.8, brought to 40% (vol/vol) glycerol and 4% $NaDodSO_4$ (2× "solubilization buffer"). The zona pellucida-free oocytes were collected and placed in 10 μl of 2× solubilization buffer to which 10 μl of TAB was added. The samples were then frozen (−70°C) and thawed (60°C) three times.

Determination of [^{35}S]Methionine Incorporation into Oocyte Proteins. Denuded and follicle-enclosed oocytes were harvested and washed after culture *in vitro* and treated twice with 5 μl of TAB as described above to remove zonae pellucidae. The two 5-μl washes of TAB (containing dissolved zonae pellucidae) and the zona pellucida-free oocytes were transferred to Microfuge tubes, adjusted to a final concentration of 50 mM $NaHCO_3$, pH 9.8/2% $NaDodSO_4$/0.08% bovine serum albumin in a final volume of 30 μl. Samples were frozen (−70°C) and thawed (60°C) three times, 30 μl of ice-cold 25% trichloroacetic acid was added, and the samples were kept at 0°C for 20 min. After centrifugation, the supernatants were collected and the pellets were washed with ice-cold 10% trichloroacetic acid and then dissolved in 10 μl of 0.1 M NaOH. Supernatants and dissolved pellets were subjected to liquid scintillation counting in Liquifluor (New England Nuclear) containing 0.3% BBS-3 Biosolv (Beckman).

[^{125}I]Iodosulfanilic Acid Labeling of Zonae Pellucidae. The diazonium salt of [^{125}I]iodosulfanilic acid ([^{125}I]DASA) was used to radiolabel proteins of isolated zonae pellucidae as described (16, 21). One hundred to 400 μCi of [^{125}I]iodosulfanilic acid (>1000 Ci/mmol, New England Nuclear) in propanol/H_2O (1:1, vol/vol) was dried down under N_2, taken up in 5 μl of distilled H_2O, and cooled on ice. DASA was prepared by the addition of 5 μl of 0.1 M HCl followed by 5 μl of 0.05 M $NaNO_3$. The reaction was carried out on ice for 10 min, after which the solution was neutralized with 5 μl of 0.1 M NaOH. To label isolated zonae pellucidae or intact oocytes, 4 μl of [^{125}I]DASA was added to the samples in about 1 μl of phosphate-buffered saline/0.1% polyvinylpyrrolidone-40 and, after incubation at 25°C for about 30 min, either the isolated zonae pellucidae were washed thoroughly or surface-labeled intact oocytes were washed thoroughly and zonae pellucidae were removed by TAB as described above. Radiolabeled material was stored frozen at −20°C for further use.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis of Radiolabeled Proteins. Samples in solubilization buffer were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis as described by Laemmli (22). The gels were processed for fluorography according to the procedure of Bonner and Laskey (23) and exposed to Kodak SB-5 film at −70°C. Molecular weights of radiolabeled proteins were determined by comparing their electrophoretic mobilities with those of standard proteins of known molecular weights.

RESULTS

Incorporation of [^{35}S]Methionine and [^{3}H]Fucose into Oocyte, Zona Pellucida, and Follicle Cell Proteins. To determine whether growing mouse oocytes synthesize and secrete zona pellucida proteins, denuded oocytes and follicle-enclosed oocytes isolated from juvenile mice were cultured *in vitro* in the presence of either [^{35}S]methionine or [^{3}H]fucose. After 4, 8, and 12 hr of culture, samples were harvested and washed, oocytes were removed from the follicles, zonae pellucidae were either isolated from the oocytes with micropipettes or dissolved by using TAB, and the amount of trichloroacetic acid-soluble and insoluble radiolabel associated with zona pellucida-free oocytes, zonae pellucidae, and follicle cells was determined. The kinetics of incorporation of [^{35}S]methionine and [^{3}H]fucose into oocytes and zonae pellucidae are described in Fig. 1 and a summary of incorporation data for a 12-hr culture period is presented in Table 1.

Table 1. Incorporation of [^{35}S]methionine and [^{3}H]fucose into denuded and follicle-enclosed mouse oocytes

	cpm incorporated/10 oocytes or zonae pellucidae					
	Oocyte (acid-soluble)		Oocyte (acid-insoluble)		Zonae pellucidae (acid-insoluble)	
Sample	[^{35}S]Methionine	[^{3}H]Fucose	[^{35}S]Methionine	[^{3}H]Fucose	[^{35}S]Methionine	[^{3}H]Fucose
Denuded oocytes	6,081 ± 1,432 (4,500–7,551)	488 ± 147 (328–619)	28,985 ± 9,905 (15,510–38,000)	32 ± 15 (22–50)	371 ± 192 (147–500)	14 ± 11 (6–27)
Follicle-enclosed oocytes	10,330 ± 2,054 (8,000–11,880)	894 ± 366 (532–1326)	52,090 ± 23,418 (27,800–74,500)	79 ± 31 (40–114)	1090 ± 541 (640–1690)	29 ± 20 (11–49)

Denuded and follicle-enclosed oocytes ranging from 54 to 59 μm in diameter were cultured *in vitro* for 12 hr in the presence of either 500 μCi of [^{35}S]methionine per ml or 1 mCi of [^{3}H]fucose per ml. Approximately 200 [^{3}H]fucose-labeled cells or 50 [^{35}S]methionine-labeled cells were included in each measurement; backgrounds of about 10 cpm were subtracted. Data are averages of at least four experiments ± SD, with the range of values given in parentheses.

Overall, these experiments revealed that: (*i*) Zonae pellucidae removed from both denuded and follicle-enclosed oocytes (48–68 μm in diameter) contained significant amounts of radiolabel in acid-insoluble material and the amount increased continuously during culture *in vitro*. (*ii*) Approximately 1.5% of the [^{35}S]methionine and as much as 45% of the [^{3}H]fucose that was incorporated into acid-insoluble material by either denuded or follicle-enclosed oocytes during a 12-hr culture period was found associated with zonae pellucidae. (*iii*) While the presence of follicle cells did not have a significant effect on the percentage of radiolabel associated with zonae pellucidae, about 90% of the [^{35}S]methionine incorporated into acid-insoluble material of intact follicles was present in the follicle cells themselves. When oocyte-free follicles were isolated, less than 0.02% of the radiolabel was extractable with TAB. (*iv*) Although 2- to 3-fold more radiolabel was incorporated into zonae pellucidae of follicle-enclosed, as compared to denuded oocytes, the difference appeared to be due, at least in part, to about a 2-fold greater uptake of the [^{35}S]methionine and [^{3}H]fucose by follicle-enclosed oocytes. Recent work in our laboratory has shown that the size of the free methionine pool of follicle-enclosed oocytes is identical to that of denuded oocytes (unpublished results). (*v*) Less than 0.01% of the [^{35}S]methionine incorporated into acid-insoluble material by fully grown oocytes (85 μm) isolated from adult mice was found associated with zonae pellucidae.

When denuded oocytes were cultured for 12 hr in the presence of puromycin (10 μg/ml), the incorporation of [^{35}S]methionine into acid-insoluble material was reduced to about 1/10th and the amount of radiolabel associated with zonae pellucidae was reduced to less than 1/50th. The results of this experiment diminish the likelihood that [^{35}S]methionine is simply adventitiously bound to zonae pelludidae during culture of the oocytes *in vitro*.

Finally, in order to distinguish secretion of zona pellucida proteins from synthesis, denuded oocytes were cultured in the presence of [^{35}S]methionine for 8 hr ("pulse") and then for an additional 8 hr ("chase") in the absence of radiolabel and the presence of excess unlabeled methionine (30 μg/ml). At the end of each of the culture periods zonae pellucidae were removed from the oocytes with TAB and the amount of radiolabel present in acid-insoluble material was determined. During the chase period there was less than a 1% increase in the amount of [^{35}S]methionine incorporated into oocyte proteins. On the other hand, during the same period there was about a 45% increase in the amount of label associated with the zonae pellucidae, indicating that zona pellucida proteins synthesized during the pulse were secreted during the chase period.

Taken together, these results strongly suggest that zona pellucida proteins are synthesized and secreted by growing oocytes during culture *in vitro*.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis of [^{35}S]Methionine-Labeled Zonae Pellucidae. Previously, we found that when intact fully grown oocytes and isolated zonae pellucidae were radiolabeled with [^{125}I]DASA and then subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis, three major bands were observed upon autoradiography of the gel (15, 16). The positions of the bands on the gels corresponded to apparent molecular weights of 200,000, 120,000, and 83,000, and we designated the three protein species ZP1, ZP2, and ZP3, respectively. Treatment of growing oocytes (54 μm) in an identical manner has revealed that these same three proteins compose the growing oocyte's zona pellucida (Fig. 2, lanes A–C).

We used NaDodSO$_4$/polyacrylamide gel electrophoresis to analyze the distribution of [^{35}S]methionine incorporated into zona pellucida proteins during culture of denuded and follicle-enclosed oocytes *in vitro*. After a 12-hr culture period, zonae pellucidae were removed from one group of oocytes (54 μm) by using mouth-operated micropipettes and from a second

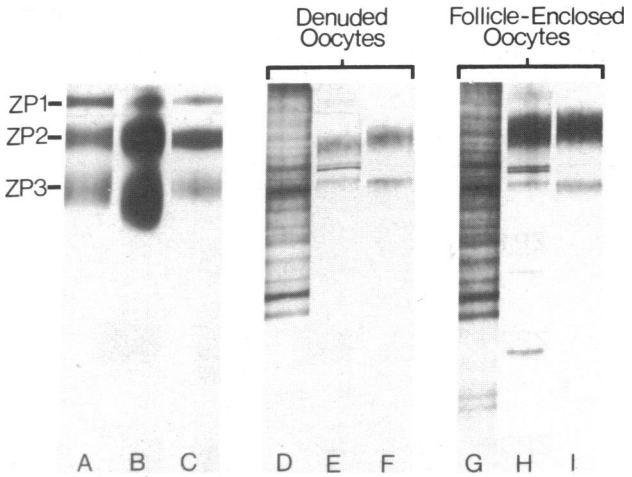

FIG. 2. Discontinuous NaDodSO$_4$/polyacrylamide gel electrophoresis of [^{125}I]DASA- and [^{35}S]methionine-labeled oocyte and zona pellucida proteins. Zonae pellucidae isolated from fully grown (85 μm) (lane A) or growing (54 μm) (lane C) oocytes by using micropipettes were radiolabeled with [^{125}I]DASA and then subjected to electrophoresis in parallel with zonae pellucidae removed from [^{125}I]DASA-labeled fully grown oocytes by using TAB (lane B). Lane B was purposely overexposed in order to demonstrate that treatment of oocytes with TAB resulted in solubilization of only zona pellucida proteins. Denuded oocytes and follicle-enclosed oocytes (54 μm) isolated from 10 day-old mice were cultured for 12 hr in the presence of [^{35}S]methionine (1 mCi/ml) and then the following samples were subjected to electrophoresis: (*i*) oocytes treated with TAB to remove zonae pellucidae (lanes D and G), (*ii*) zonae pellucidae isolated by using micropipettes (lanes E and H), and (*iii*) zonae pellucidae removed with TAB (lanes F and I). The gel was processed for and subjected to fluorography.

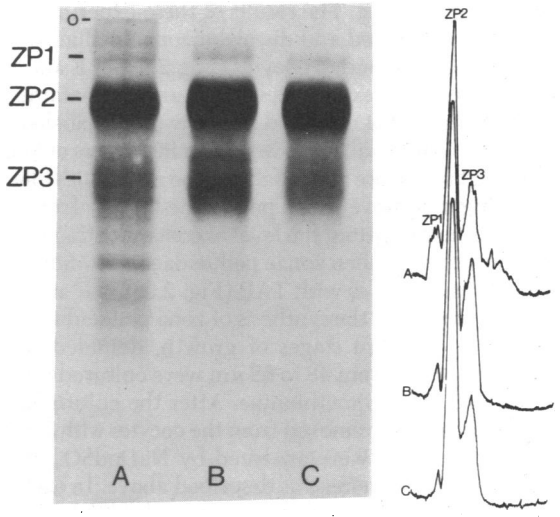

FIG. 3. Discontinuous NaDodSO$_4$/polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled zonae pellucidae removed from oocytes at various stages of growth. Denuded oocytes 48, 58, and 68 μm in diameter, isolated from 6-, 10-, and 15-day-old mice, respectively, were cultured for 12 hr in the presence of [^{35}S]methionine (1 mCi/ml). At the end of the culture period the oocytes were harvested, washed, and treated with TAB, and the solubilized zonae pellucidae were subjected to electrophoresis followed by fluorography. Shown are the fluorographs and corresponding densitometer tracings of zonae pellucidae removed from: 68-μm (lane A), 54-μm (lane B), and 48-μm (lane C) oocytes. o, Origin.

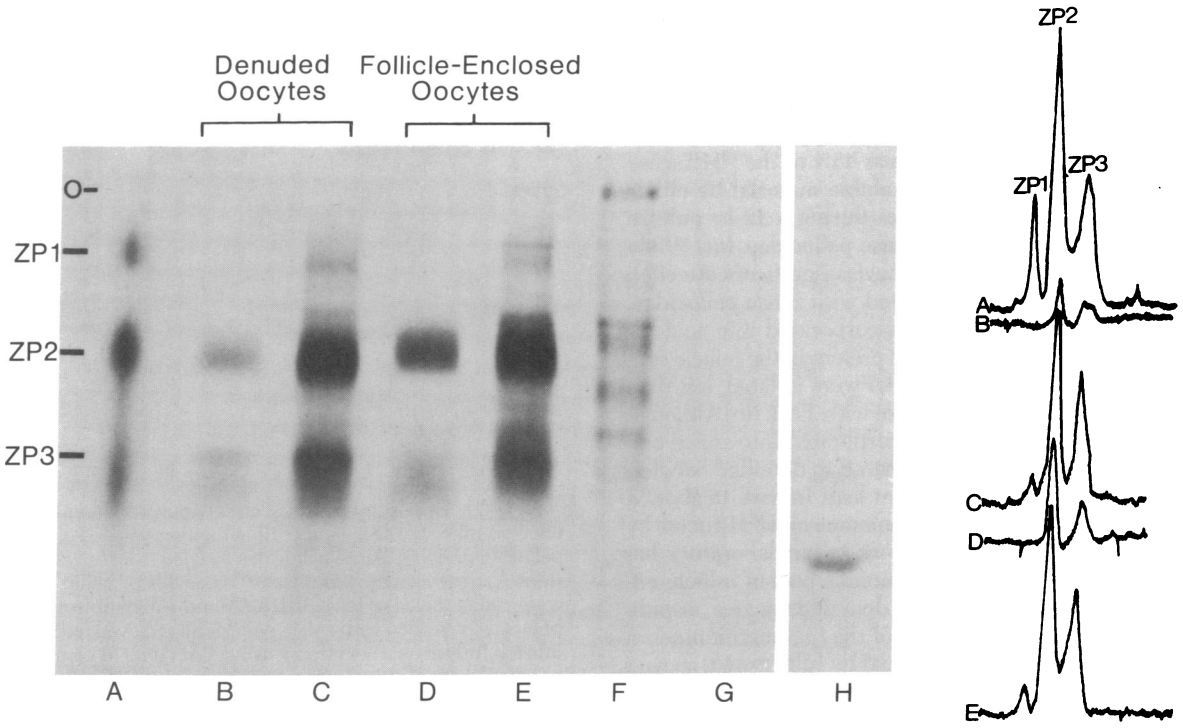

FIG. 4. Discontinuous NaDodSO$_4$/polyacrylamide gel electrophoresis of [^{3}H]fucose-labeled zonae pellucidae. Denuded and follicle-enclosed oocytes (54 μm) isolated from 10-day-old mice were cultured for 12 hr in two separate 50-μl drops of medium containing [^{3}H]fucose (1 mCi/ml). After the culture period, 200 denuded oocytes and 200 oocytes isolated from follicles were treated with TAB and the zona pellucida-free oocytes (lanes C and E) and solubilized zonae pellucidae (lanes B and D) were subjected to electrophoresis in parallel with 40 oocyte-free follicles (lane F), material released from 40 oocyte-free follicles by TAB (lane G), and 10 μl of medium in which follicles had been cultured (lane H). [^{125}I]-DASA-labeled zonae pellucidae were included as standards (lane A). Shown is a photograph of the fluorogram and the corresponding densitometer tracings of lanes A–E. o, Origin.

group by using TAB. Three samples were then subjected to electrophoresis followed by fluorography; (*i*) oocytes from which zonae pellucidae had been removed with TAB, (*ii*) zonae pellucidae dissolved with TAB, and (*iii*) zonae pellucidae isolated by micropipetting. The results of these experiments are shown in Fig. 2. Isolated and dissolved zonae pellucidae, obtained from either denuded oocytes (Fig. 2, lanes E and F) or follicle-enclosed oocytes (Fig. 2, lanes H and I), contained radiolabeled proteins that migrated similarly to ^{125}I-labeled ZP1, ZP2, and ZP3. Additional bands were seen in samples of isolated zonae pellucidae, but are attributable to the difficulties inherent in attempting to remove zonae pellucidae cleanly from small oocytes with micropipettes; the level of contaminating proteins was greatly reduced when zonae pellucidae were obtained by treatment of the oocytes with TAB (Fig. 2, lanes F and I).

In order to evaluate the synthesis of zona pellucida proteins by oocytes at different stages of growth, denuded oocytes ranging in diameter from 48 to 68 μm were cultured for 12 hr in the presence of [^{35}S]methionine. After the culture period, zonae pellucidae were removed from the oocytes with TAB and radiolabeled proteins were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis as described above. In each case, the solubilized zonae pellucidae contained [^{35}S]methionine-labeled proteins that migrated similarly to ZP1, ZP2, and ZP3 (Fig. 3); identical results were obtained with [^{3}H]fucose-labeled oocytes.

NaDodSO$_4$/Polyacrylamide Gel Elecrophoresis of [^{3}H]-Fucose-Labeled Zonae Pellucidae. Experiments identical to those described above were carried out with denuded and follicle-enclosed oocytes and [^{3}H]fucose as the radioactively labeled precursor. After a 12-hr culture period, radiolabeled zonae pellucidae, as well as oocytes freed of zonae pellucidae with TAB, were subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis. Despite relatively low cpm per zona pellucida, both ZP2 and ZP3 were detected in zonae pellucidae removed from [^{35}S]fucose-labeled denuded and follicle-enclosed oocytes (Fig. 4, lanes B and D). Furthermore, when zona pellucida-free oocytes were subjected to electrophoresis, as much as 95% of the [^{3}H]fucose incorporated into oocyte proteins was found in ZP1, ZP2, and ZP3 (Fig. 4, lanes C and E). The latter observation suggests that zona pellucida proteins are the major class of proteins glycosylated during oocyte growth. On the other hand, oocyte-free follicles did not contain any major radiolabeled proteins that migrated with zona pellucida proteins (Fig. 4, lanes F and G), and radiolabeled zona pellucida proteins were not present in culture medium from which whole follicles were removed (Fig. 4, lane H). Therefore, we have been unable to detect synthesis of zona pellucida proteins by follicle cells themselves.

DISCUSSION

The site of origin of the zona pellucida has remained uncertain despite numerous efforts to resolve this issue. According to some investigators the zona pellucida originates from the oocyte (13, 14), whereas others feel that it originates from the follicle cells (24, 25) or from both the oocyte and the follicle cells (26, 27). The conclusions of these investigators are based solely upon morphological and autoradiographic observations. For example, the finding that autoradiographs of ovarian thick sections from mice injected with [^{3}H]fucose showed a progression of grains from the inside to the outside of the zona pellucida as a function of time after injection has been taken as evidence that the zona pellucida originates from the oocyte (14).

In this report we used biochemical criteria to assess the site of origin of the zona pellucida. We found that denuded oocytes, at various stages of growth, synthesize and secrete zona pellucida proteins during culture *in vitro*. Although the presence of follicle cells increases the amount of radiolabel incorporated into zona pellucida proteins, it appears to be a consequence of greater uptake of the radioactively labeled precursors by follicle-enclosed oocytes than by denuded oocytes; a similar finding has been reported in the case of [^{3}H]uridine incorporation into RNA in mouse oocytes (28). In general, the same percentage of radiolabel taken up by denuded or follicle-enclosed oocytes was found incorporated into zona pellucida proteins. Although no evidence was obtained to suggest that follicle cells also synthesize zona pellucida proteins, this possibility cannot be completely excluded. It is clear from the results reported here, however, that the oocyte itself participates in the assembly of the zona pellucida. We do not know as yet whether the rates of synthesis of zona pellucida proteins by the oocyte during its growth period can account for the 4.8 ng of protein found in the zona pellucida of fully grown mouse oocytes (29).

The findings reported here are also of interest in connection with certain of the marked changes in ultrastructure that the mouse oocyte undergoes during its growth phase (30). In particular, the Golgi complex, which consists solely of lamellae arranged in a parallel fashion during the initial stages of growth, becomes a large conglomerate of vesicles, vacuoles, granules, and lamellae during the middle and late stages of oocyte growth. Such ultrastructural changes have been attributed to an increasing activity of the Golgi complex through its participation in the concentration and exocytosis of secretory proteins (31, 32). It is tempting to suggest that much of this activity is associated with the packaging and secretion of zona pellucida proteins during growth of the mouse oocyte.

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