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Ovarian Granulosa Cells Isolated with EGTA and Hypertonic Sucrose: Cellular Integrity and Function

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ABSTRACT

Chemical treatments previously shown to disrupt gap junctions were applied to rat ovaries with antral follicles prior to expressing the granulosa cells with a blunt spatula. Exposure of the ovaries to 6.8 mM EGTA [ethyleneglycol-bis-β-aminoethyl ether)-N,N'-tetracetic acid] and 0.5 M sucrose either by perfusion in situ or incubation in vitro generated monodisperse suspensions of granulosa cells with improved integrity as evaluated by several criteria.

Similar numbers of granulosa cells ($7-8 \times 10^6$ per ovary) were obtained either by direct physical expression or by pretreatment of the ovaries followed by physical expression. However, the ability of the granulosa cells to exclude trypan blue dye was consistently improved 2–3-fold by pretreatment with EGTA and hypertonic sucrose (from 25% to 40–80%). Likewise, in vitro synthetic capacities for protein, RNA and DNA were enhanced 2–6-fold, 5–10-fold and 10–20-fold, respectively. A qualitative one-to-one correspondence between protein synthesis and vital dye exclusion was demonstrable but the quantitative relationship between dye exclusion and macromolecular precursor incorporation appeared to be nonlinear. Cells obtained using the chemical pretreatments also demonstrated better survival in minimal medium for at least the first 12 h of culture. The enhancements observed could not be obtained by reversing the order of the chemical treatments or by treating granulosa cells after physical removal from ovarian follicles.

Pretreatment of ovaries with EGTA and hypertonic sucrose appears to be a reliable procedure for improving the yield of monodisperse, viable, biochemically intact granulosa cells for use in in vitro examinations of follicular physiology and function.

INTRODUCTION

Granulosa cells of the ovarian follicle are interconnected by an extensive array of gap junctions which vary in size and number with hormonal treatment (Albertini and Anderson, 1974; Albertini et al., 1975; Bjorkman, 1962; Espey and Stutts, 1972; Fletcher, 1978; Fletcher and Everett, 1973; Merk et al., 1973; Merk et al., 1972). This connecting network has presented a problem for many workers preparing granulosa cells for use in in vitro experiments. Attempts to suspend the cells in a monodisperse state result in the cells being physically torn from one another. The cells which survive such isolation may, in fact, be a subpopulation which is not necessarily typical, e.g., derived from the nearly free-floating cells near the antrum.

To alleviate this problem we have applied chemical treatments previously shown to disrupt junctional complexes of other cell types without damaging the connected cells. Modifications of methods used with liver cells (Goodenough and Gilula, 1974; Peracchia, 1977) and *Chironomus* salivary gland cells (Loewenstein et al., 1967) were applied to the ovary of the hormonally primed, immature rat. By sequentially exposing the ovaries to the specific calcium chelator, EGTA, and to hypertonic sucrose prior to squeezing the whole ovary to release granulosa cells (physical expression), we have been able to improve markedly the morphologic and functional integrity of the granulosa cells obtained. Moreover, this procedure yields essentially monodisperse cells in high yield. This paper describes the methods we have developed to obtain these cells (Campbell and Midgley, 1977; Campbell and Midgley, 1978) and our initial experiments outlining the biochemical function of these cells in comparison with cells obtained by simple physical expression.

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MATERIALS AND METHODS

Animals

Immature female rats from Holtzman Co. (Madison, WI) were stimulated to form large numbers of antral ovarian follicles by 1 of 2 regimens. Early experiments were done on rats implanted on Day 22 of age with a 1 cm × 3 mm Silastic capsule containing crystalline diethylstilbesterol. Starting on Day 25, these rats also received a total of 5 s.c. injections of 1 μg each of human follicle stimulating hormone (hFSH; LER-1577) given 12 h apart. The animals were killed on Day 27 less than 6 h after the last injection. Later experiments were done on rats which received 4 injections of estradiol-17β, i.p., coincident with 4 injections of 1.0 μg each of hFSH given s.c. The estradiol was given at 12 h intervals from Day 25 to Day 27 of age; the doses given were 0.5 mg, 0.5 mg, 1.0 mg and 2.0 mg. This "ramp" of estradiol was given to simulate the preovulatory rise seen in cycling adult rats. These animals were killed within 12 h of the last estradiol and hFSH injections. The models will be referred to in this paper as the DES-hFSH model and the E_2-ramp-hFSH model.

Chemicals and Media

Chemicals were obtained from Sigma Chemical Co., St. Louis, MO, unless otherwise indicated. Prepared media were obtained from Grand Island Biologicals, Grand Island, NY (Gibco). All media were made 10 mM in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and adjusted to pH 7.2–7.4 before use: Medium-199 plus HEPES (M-199-H), Minimal Essential Medium plus HEPES (MEM-H), Earle's Basal Salt Solution plus HEPES (EBSS-H). The medium used for EGTA treatment contained 6.8 mM EGTA and 0.2% bovine serum albumin (BSA; Fraction V) in M-199-H; this gave an EGTA concentration of 5 mM in excess of the Ca^{++} contained in M-199-H (final approximate free $[Ca^{++}]$ = 18 nM; free $[Mg^{++}]$ = 0.54 mM). Hypertonic sucrose solution contained 0.5 M sucrose, 0.2% BSA and 1.8 mM EGTA in M-199-H (final approximate free $[Ca^{++}]$ = 10 μM; free $[Mg^{++}]$ = 0.75 mM).

Cell Preparations

The best preparations were obtained by conducting all procedures, except for incubation with EGTA or hypertonic sucrose, from tissue removal to cell isolation, at room temperature (∿25°C). All cell pellets obtained after centrifugation were resuspended by gentle vortexing; no trituration was used to break up clumps of aggregated cells in any of the cell preparations.

"Expressed cells" (E) were obtained as follows: the ovary was rinsed, stripped of its bursa in isotonic media, placed into fresh M-199-H containing 1% BSA and then gently pressed with a blunt spatula to release the granulosa cells. After expression, these cells were collected by centrifugation for 5 min at 100 × g in 13 × 100 mm polypropylene tubes (2053; Falcon Plastics, Oxnard, CA) in a swinging bucket rotor. The supernatant, containing debris, was aspirated and the cells were washed with fresh media.

Cells treated with EGTA and hypertonic sucrose were obtained in 2 ways. For "Perfused cells" (P), animals anesthetized with ether were perfused through the left ventricle via the aorta with the EGTA solution (∿15°C, bubbled with 95% O_2:5% CO_2) for 15 min and then (similarly) with the sucrose solution for 5 min prior to removal of the ovaries and expression of cells into media.

To obtain "Incubated cells" (I) ovaries were removed, placed into an EGTA solution identical to that used for perfusion and punctured several times with a set of fine beading needles held together in a pincushion arrangement. They were then incubated 15 min at 37°C under 95% O_2:5% CO_2 in a test tube, centrifuged 5 min at 100 × g, resuspended in 0.5 M sucrose solution and incubated an additional 5 min. After the incubation in sucrose, the solution was diluted with 3 volumes of media without sucrose and centrifuged 5 min at 100 × g. The cells were then expressed into media. Cells were collected into the same tubes used for processing the ovaries to prevent loss of those cells released during puncturing of the ovaries.

Control preparations designated "Expressed and Incubated" (E + I) were obtained by expressing granulosa cells prior to subjecting them to the incubation procedures described above.

Vital Dye Exclusion

A primary examination of the morphology of the cells at the light microscopic level was made by vital dye exclusion. Trypan blue exclusion was measured by diluting cells which had been washed in isotonic media without protein (Simmons et al., 1976) with an equal volume of 0.2% trypan blue (Matheson, Coleman and Bell, Manufacturing Chemists, Norwood, OH) in isotonic media. After waiting 5 min or more, random fields on smeared slides were photographed with a Zeiss photomicroscope (Kodak Professional 50 or 2483 Photomicrography film). Determinations of the percentage of cells excluding dye were subsequently made from the photographs.

Time courses of dye exclusion were performed by incubating cells in test tubes in sterile M-199-H + 1% BSA at 37°C under 95% O_2:5% CO_2 and sampling at appropriate time intervals. Subsamples from these tubes were stained with trypan blue and immobilized with gelatin (see Vital Dye Exclusion and [^{35}S]-Methionine Incorporation below) prior to photography.

Macromolecular Syntheses

Protein. Incorporation of L-[^{35}S]-methionine (>500 Ci/mmole, 4–8 mCi/ml; Amersham Corporation, Arlington Heights, IL) into protein was accomplished by incubating washed cells (10^5–10^6/tube) in EBSS-H + 1% BSA with addition of 5 or 10 μl of isotope. Incubations were done with or without cycloheximide (final concentration 30 μg/ml) in 1.5 ml Eppendorf microfuge tubes at 37°C under 95% O_2:5% CO_2. Total incubation volume was 1.0 ml. At appropriate times incorporation was halted by addition of an excess (0.4 ml, 15 mg/ml) of unlabeled D,L-methionine (Sigma Grade I) at 4°C in isotonic media. Cells were immediately pelleted by a 30 second

centrifugation at 12,000 rpm in an Eppendorf Model 5412 microfuge. The supernatant was aspirated and cells were lysed with 1.0 ml 10% trichloroacetic acid (TCA; Mallinckrodt Chemical Co., St. Louis, MO) and boiled for 15 min to hydrolyze charged t-RNA (Siekevitz, 1952). The TCA solution was chilled for 30 min or more to precipitate proteins. The precipitate was collected by a 20 min centrifugation at 15,000 rpm in a Sorval SM-24 rotor and subsequently washed twice with cold 10% TCA. The washed pellet was dissolved in 0.1 N NaOH by brief sonication with the microprobe of a Bronwill Biosonik IV sonicator (75%, low power) and neutralized with an equal volume of 0.1 N HCl. Aliquots were taken for determination of protein by the method of Lowry et al. (1951) and for scintillation counting in Aquasol (New England Nuclear, Boston, MA) or ACS (Amersham-Searle).

Similar procedures were used for incorporating mixed [^{3}H]-amino acids (synthetic yeast hydrolyzate; 1 mCi/ml; Schwarz-Mann, Orangeburg, NY) into proteins; excess unlabeled amino acids were added as a 17× solution of MEM-amino acids (essential plus nonessential; Gibco).

RNA. Incorporation of [5-^{3}H]-uridine (24.9 Ci/mmole, 1 mCi/ml; Amersham-Searle) was assessed in a similar fashion. The incubations (10^5–10^6 cells/tube) were done either in M-199-H + 1% BSA or in EBSS-H + 1% BSA + MEM-vitamins (Gibco) + MEM-amino acids, both of which gave comparable results. Incubations were done with 10 μl isotope and with or without actinomycin D (1.25 μg/ml). Unlabeled uridine (0.3 ml, 250 μg/ml) was used to stop the incorporation. Cells were lysed and nucleic acids precipitated with TCA without boiling. The pellet was washed once with 10% TCA and once with ice cold ethanol. The washed pellet was incubated, after vortexing, for 45 min in 0.4 ml 0.5 N NaOH at 37°C. Following incubation, 0.12 ml of cold 50% TCA was added to bring the solution to 5% TCA. After chilling for over 30 min at 0°C, the TCA solution was cleared by centrifugation for 20 min at 15,000 rpm in an SM-24 rotor. The supernatant, containing the hydrolyzed RNA, was transferred to a second tube and lyophilized. The residue was taken up with double-distilled water and aliquots were assayed for RNA by the orcinol procedure (Ceriotti, 1955) or counted in ACS for radioactivity. Counts were corrected for quench by the external standard channel's ratio method (Laney, 1976).

DNA. Incorporation of [methyl-^{3}H]-thymidine (52.4 Ci/mmole, 1 mCi/ml; New England Nuclear) into DNA was accomplished by incubating washed cells (10^5–10^6/tube) in M-199-H + 1% BSA with 10 μl [^{3}H]-thymidine and with or without hydroxyurea (25 μg/ml). Total incubation volume was again 1.0 ml. Incorporation was halted by the addition of unlabeled thymidine (0.3 ml, 500 μg/ml in M-199-H) and by centrifugation at 12,000 rpm in the Eppendorf microfuge. After cell lysis and precipitation with 10% TCA, the pellet was washed once each with 10% TCA and with cold ethanol. The pellet was hydrolyzed as described for RNA determination but here the 5% TCA pellet was saved, dissolved in 0.1 N NaOH with sonication and neutralized with 0.1 N HCl. Aliquots were assayed for radioactivity in Aquasol or ACS or for DNA by the method of Burton (1956).

Vital Dye Exclusion and [^{35}S]-Methionine Incorporation

Correlation of trypan blue exclusion and L-[^{35}S]-methionine incorporation was accomplished by incubating cells using the above media and conditions. At appropriate times tubes containing cells were diluted with cold, unlabeled methionine and were centrifuged 5 min at 100 × g. The pelleted cells were washed once with M-199-H. An aliquot of the cells was mixed with an equal volume of 0.2% trypan blue in M-199-H and allowed to stand for 5 min at room temperature. Then 10 μl were placed on a rinsed, chromic-acid washed slide and mixed with 10 μl of warm (40°C) 4% gelatin (pigskin; Eastman Kodak Company, Rochester, NY) in M-199-H. The mixture was spread thinly over the slide with the side of a syringe needle and allowed to gel on a dry metal pan placed on ice in a closed chamber. After about 3 min, several fields on each gelled slide were photographed with the photomicroscope and their coordinates recorded. Following photography the slides were placed on a rack in a closed container with a solution of 10% neutral formaldehyde (paraformaldehyde; Fischer Scientific, Fair Lawn, NJ) in 20% glutaraldehyde (Earnest F. Fullam, Inc., Schenectady, NY). The slides were allowed to fix in the vapor phase for several hours at room temperature and then at 4°C overnight. The fixed slides were washed for 2–3 h in 2–3 changes of cold phosphate buffered saline (PBS), pH 7.0, then placed in a humid chamber prior to manual dipping in autoradiographic emulsion. Emulsion (NTB3, Kodak) was applied and allowed to drain and dry in a vertical position for 1 h at 28°C and ∿70% humidity. The dipped slides were placed into a light tight box with a packet of desiccant. Exposure was carried out in a dry-ice cabinet (−70°C) for 48 h. Slides were developed for 6 min with Amidol developer (Kodak, 1977) at 17°C and fixed for 5 min with Kodak Fixer at 17°C. Photographs of the developed autoradiograms were taken at the same coordinates previously photographed for trypan blue exclusion. Some autoradiograms were subsequently stained with Giemsa (Matheson, Coleman and Bell) following a 30 min dehydration in absolute methanol; excess stain was removed by 2–3 rinses with PBS.

RESULTS

Vital Dye Exclusion

Vital dye exclusion has been used throughout these studies as a simple comparison of various cell preparations. Table 1 presents the findings for this parameter of cell integrity both as a function of cell preparation and as a function of the length of exposure to 6.8 mM EGTA and 0.5 M sucrose. The values given are mean ± 1 SEM for the number of independent determinations shown. The results include values from 14 experiments run over an 11 month period on both animal models.

Expression alone released only 26.5% nonstaining cells. Perfusion with hypertonic

TABLE 1. Percent of cells excluding trypan blue for various preparation protocols.[a]

	Treatment length (min)		% Cells excluding dye (mean ± SEM)	n[b]
	EGTA	Sucrose		
Expressed	0	0	26.5 ± 3.0	16
Perfused	0	2–10	39.7 ± 5.6	3
	2–10	0	52.0 ± 5.6	3
	2	2–10	62.3 ± 7.6	3
	5–10	2–10	77.1 ± 4.2	12
	10–15	1	29.1 ± 5.9	2
	15	2	33.8	1
	20	1–10	37.4 ± 1.8	4
Incubated	5–10	2–5	64.4 ± 2.4	5
	15	5	45.9 ± 3.1	16
Expressed + Incubated	15	5	17.1 ± 2.5	3

[a]The protocols for preparations followed those given in Materials and Methods with modifications as to times of chemical incubations and presence or absence of all chemical treatment steps as indicated. EGTA concentration was 6.8 mM; hypertonic sucrose was 0.5 M.

[b]The percent of cells excluding trypan blue was determined for each of n independent preparations of cells.

sucrose alone moderately enhanced the value (39.7%); EGTA alone brought about a 2-fold enhancement (52.0%). Treatment of the ovaries by the perfusion approach gave marginal increases in vital dye exclusion when sucrose treatment was shorter than 5 min (29.1%, 33.8%). Perfusion for 2 min with EGTA and 2–10 min with hypertonic sucrose enhanced dye exclusion 2.5-fold (62.3%). If the EGTA perfusion was lengthened to 5–10 min there was an even more marked improvement to ∿80%. On the other hand, perfusion with EGTA for 20 min seemed detrimental by comparison (37.4%).

Optimal times for incubation of the punctured ovaries (Incubated preparation) appear to be between 5–15 min in EGTA and ∿5 min in hypertonic sucrose (45.9%–64.4%).

The enhancement of dye exclusion shown by sequential perfusion (62.3% and 77.1%) vs that seen with either chelator or sucrose treatment alone (52.0% and 39.7%, respectively) implies a role for both treatments in the removal and dispersal of the cells. Further, the similarity of staining of the cells incubated following expression (E + I; 17.1%) to those only expressed (E; 26.5%) implies that the chemical treatments are effective only if applied prior to the removal of the cells from the ovarian follicles.

The overall comparison of Perfused vs Incubated cells indicates that optimized perfusion is the preferential preparative technique. However, there is a tendency for cellular dye exclusions to fall to or below levels seen with the incubation protocol when several animals are perfused simultaneously. Moreover, the preparation of large numbers of cells by the perfusion technique is very time consuming due to the necessity of handling animals individually. Therefore, for many biochemical studies the Incubated preparation will probably be preferred.

Macromolecular Synthesis

Although vital dye exclusion has been used as a parameter of cell integrity, it does not necessarily reflect the ability of isolated cells to perform biochemically. The granulosa cells may, for example, only function while the syncytial arrangement of the membrana granulosa is maintained. Macromolecular syntheses were compared among the different cell preparations as a means of evaluating biochemical processes since they are known to be active within the follicle at the times when these cells were isolated (Hirshfield and Midgley, 1978; Pool and Lipner, 1966; Pool and Lipner, 1969; Rao et al., 1978).

The precursor incorporation results shown in Fig. 1 are representative experiments; all incorporation experiments have been repeated at least 3 times. Each time point for these

incorporations was done with a total of $\sim 10^5 - 10^6$ cells, demonstrated to be the optimal range for the conditions and assay systems used. The specific activities of the resultant macromolecules were linear with amount of isotope used for all precursors studied.

Protein synthesis. Figure 1a depicts the time course of incorporation of [^{35}S]-methionine into protein for several of the cell preparations. The Incubated cells clearly incorporated more label by 3 h of incubation 5592 cpm/μg protein) than either the Perfused (3266 cpm/μg protein) or Expressed (2029 cpm/μg protein) cells. In all cases the incorporation could be blocked by 30 μg/ml cycloheximide. Dye exclusions by these preparations were 54%, 48% and 30% for Incubated, Perfused and Expressed cells, respectively.

Incorporation of mixed [^{3}H]-amino acids is shown in Fig. 1b. The cells used in this experiment were from the same batches shown in Fig. 1a. As anticipated from the results with methionine, treatment with EGTA and hypertonic sucrose strikingly enhanced the incorporation of mixed amino acids into protein. Here again, cycloheximide blocked incorporation.

The time courses for both methionine and mixed amino acid incorporation appear similar. Sigmoidal kinetics probably reflect an initial equilibration of intracellular pools with labeled amino acids, taking less than 10–15 min, followed by linear incorporation between 15 min and 2 h. Specific activities do not continue to rise beyond 2 h, presumably due to losses in cell viability, in vitro proteolysis and dilution of label or undetermined changes in medium conditions.

Note should be taken here that the ratio of the viabilities of the various preparations do not, necessarily, agree quantitatively with the ratios of their respective protein specific activities. For example, the ratio of the viabilities of the Incubated and Perfused preparations is 54%:48% = 1.125 while the ratio of their protein specific activities at 2 h of incubation is (3946 cpm/μg protein:2129 cpm/μg protein) = 1.853 for [^{35}S]-methionine or (377.1 cpm/μg protein:150.8 cpm/μg protein) = 2.501 for mixed [^{3}H]-amino acids.

DNA synthesis. The synthesis of DNA as shown by incorporation of [^{3}H]-thymidine is depicted in Figure 1c. Once more the incubation protocol clearly increased the specific activity of the macromolecular end product.

As with protein synthesis, there is a dissociation between the quantity of label incorporated and the number of cells excluding trypan blue.

RNA synthesis. Figure 1d shows the time course of incorporation of [^{3}H]-uridine into RNA. Once again the relative synthetic capacities of the various cell preparations decrease in the order of decreasing morphologic integrity: I > P > E + I = E. RNA synthesis is blocked by actinomycin D (1.25 μg/ml) in all preparations.

Direct Correlation of Trypan Blue Exclusion with Protein Synthesis

Figure 2 is a direct comparison of the ability of cells to exclude vital dye and to incorporate [^{35}S]-methionine into protein. Although these photographs were taken of Incubated cells labeled for 60 min in vitro, the comparison is representative of the results obtained for all 4 cell preparations. Identical cells were photographed in both panels. Panel 2a shows the cells after trypan blue staining and immobilization in gelatin. Panel 2b shows the same cells after trypan blue staining, immobilization in gelatin, fixation in aldehyde vapor, extensive washing in PBS to remove unincorporated [^{35}S]-methionine, autoradiography and staining with Giemsa. Under bright field conditions, the cells which exclude vital dye are refractile; they appear to be the only cells which incorporate label into protein since they are the only cells with a reduced silver grain density greater than background. The cells which do not exclude dye are nonrefractile, flattened and do not demonstrate label incorporation.

This qualitative correlation was found to be highly significant when 34 sets of matched photographic fields like those shown in Fig. 2, representing all 4 cell preparations, were examined for the number of cells excluding dye and the number of cells exhibiting more silver grains than the background. The results are plotted in Fig. 3. The correlation coefficient was 0.964 while the slope and intercept for the line described were 1.051 and −1.178, respectively. Much of the variability that was observed in this examination and presumably the reason for the nonzero intercept, was the difficulty in achieving an absolutely exact realignment of the fields during the photography of the autoradiograms; a few cells do appear in some of the dye exclusion photographs which do not appear in the photographs

of the autoradiograms, and vice versa. It should be emphasized that the correlation held for cells prepared by Incubation, Expression, Perfusion or Expression + Incubation methods and labeled for up to 150 min in vitro. The closeness of this correlation strongly implies that morphologic integrity as shown by vital dye exclusion, does, in fact, qualitatively reflect

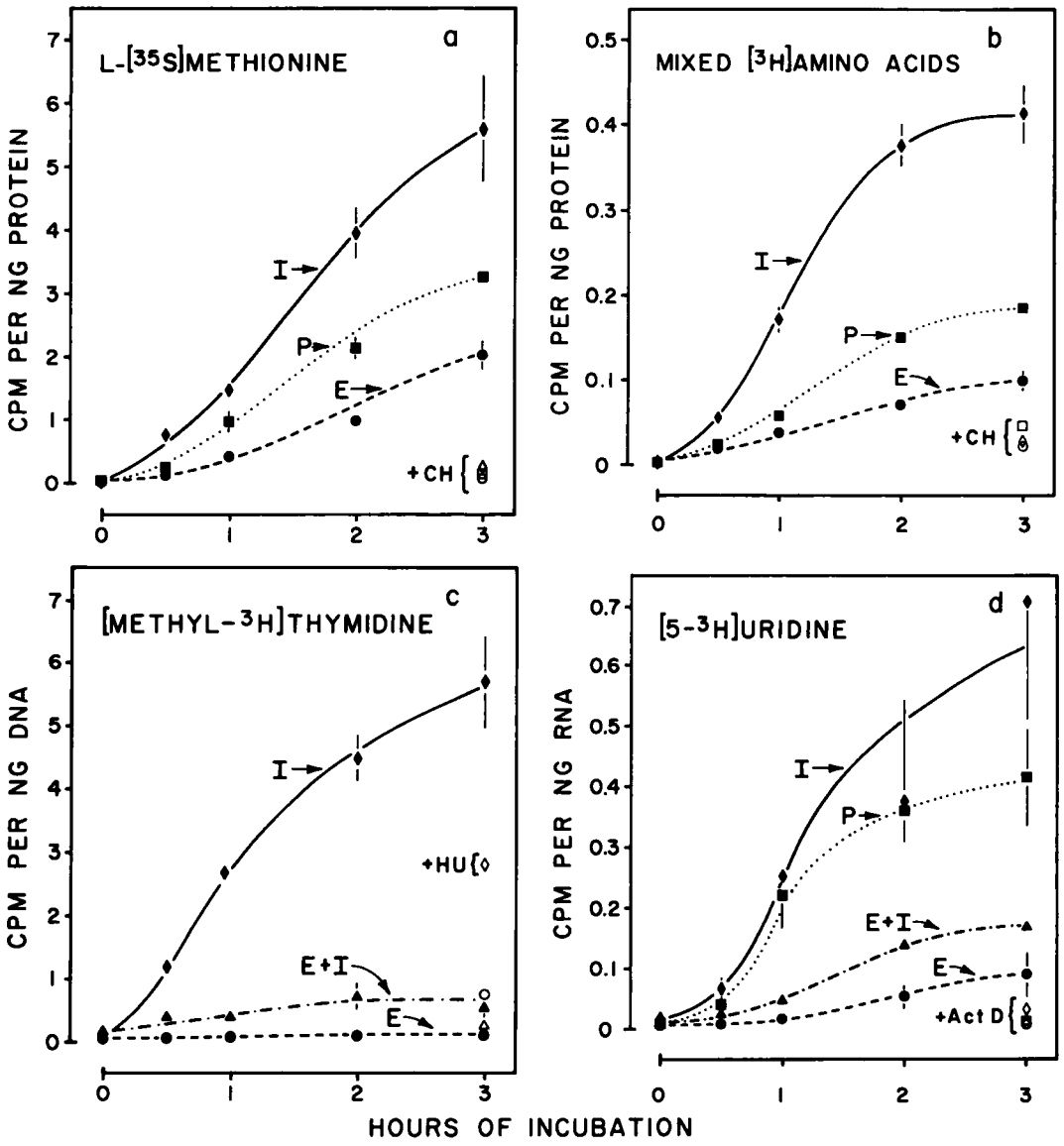


FIG. 1. Labeled precursor incorporation into macromolecules for various granulosa cell preparations.

a) Time courses of specific activity for in vitro incorporation of [^{35}S]-methionine into protein for Incubated (I), Perfused (P) and Expressed (E) cell preparations. Cycloheximide (CH) at 30 μg/ml blocks almost all incorporation when added at time zero.

b) Similar time courses for in vitro incorporation of mixed [^{3}H]-amino acids.

c) Time courses of specific activity for DNA after in vitro incorporation of [^{3}H]-thymidine into I, E and E + I cell preparations. Hydroxyurea (HU) at 25 μg/ml does not completely block synthesis in the I preparation and shows no effect in the E and E + I preparations.

d) Time courses of specific activity for in vitro incorporation of [^{3}H]-uridine into RNA. Actinomycin D (Act D) at 1.25 μg/ml totally blocks label incorporation.

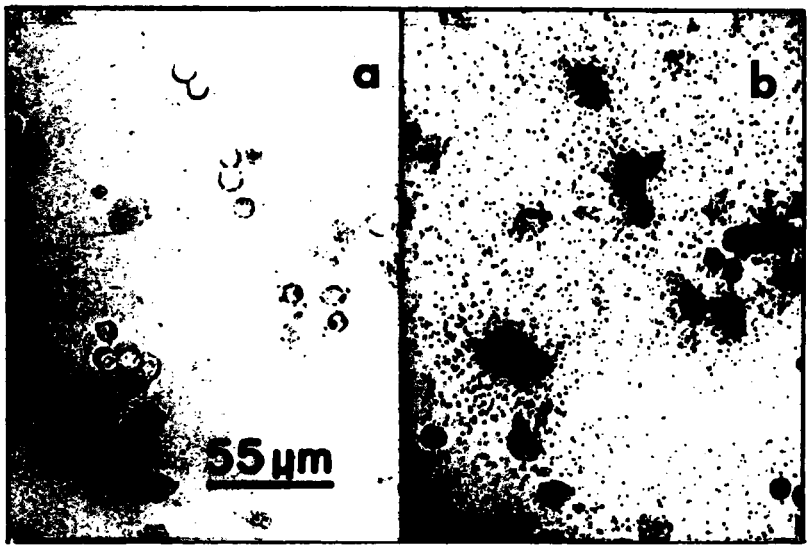


FIG. 2. Direct comparison in individual cells of trypan blue exclusion with protein biosynthesis.

a) The ability of several granulosa cells to exclude trypan blue (absence of color) and to exhibit light refractility coordinately under bright field conditions is shown. Incubated cells were labeled for 60 min in vitro with [^{35}S]-methionine, stained with 0.1% trypan blue and immobilized in gelatin. Magnification of both figures is the same; the length of the bar is 55 µm.

b) The same cells after fixation and processing for autoradiography is shown. Reduced silver grains (black) exceed background levels only for cells having incorporated [^{35}S]-methionine into protein. Only cells excluding vital dye and showing refractility exhibit evidence of protein synthesis.

Qualitatively similar comparisons were obtained for all 4 cell preparations.

the functional integrity of the cells as measured by methionine incorporation into protein.

Grain counts of the autoradiograms were not made during these studies but qualitative comparisons of the time course of the incorporation of label into the various cell preparations were made by examining low power photographs of the autoradiograms. Figure 4 shows these photographs for cells prepared by Expression, Expression + Incubation, Incubation and Perfusion methods. Areas of relatively monodispersed cells were chosen to simplify illustration, but label accumulation in cell aggregates in the various cell preparations also followed the patterns shown. Little or no accumulation of grains above background is found in cells removed at zero time, immediately after introduction into the labeling media (Fig. 4a–d). Progressively more grains are seen over cells at 30 min (Fig. 4e–h), 60 min (Fig. 4i–l) and 150 min (Fig. 4m–p) of incubation. Clearly the number of cells incorporating label in the Incubated and the Perfused preparations are larger than the number in the Expressed or Expressed + Incubated preparations. The intensity of incorporation for the 4 cell preparations, subjectively, follows the relative dye exclusion and protein specific activities shown previously.

In the presence of cycloheximide (30 µg/ml), incorporation as determined by autoradiography was partially, but not totally, suppressed at 150 min (Fig. 4q–t). There is great similarity between the intensity of radioactivity detected under the influence of cycloheximide and that found at 30 min of incubation. The residual grains probably reflect the incorporation of label that occurred prior to the completion of the cycloheximide blockade; cycloheximide and label were both added at time zero.

Cell Survival with Time

The ability of cells from the 4 preparations to survive incubation for various periods of time in fully defined media (M-199-H + 1% BSA) without serum supplementation was examined by checking the trypan blue exclusion of suspensions of cells at time intervals up to 12 h. Figure 5 demonstrates these time courses for cells obtained by Incubation (5a),

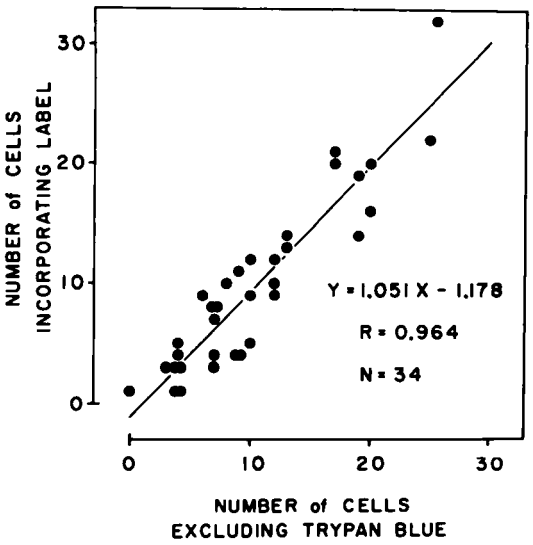


FIG. 3. Correlation of protein biosynthesis and trypan blue exclusion in individual cells.

Thirty-four pairs of photographs such as those shown in Fig. 2 were independently evaluated for the numbers of cells excluding trypan blue and the numbers of cells exhibiting more silver grains than background levels. The results of these counts were plotted and a least squares linear regression calculated. The regression line calculated was $y = 1.051x - 1.178$ with a correlation coefficient of 0.964. The photographs used were derived from all 4 cell preparations (Incubated, Perfused, Expressed and Expressed + Incubated) at labeling times of 30–150 min; this qualitative correlation did not differ among the preparations.

Expression (5b), Perfusion (5c) and Expression + Incubation (5d). Statistical comparisons between cell preparations and time intervals were made by analysis of variance (Croxton, 1959; Selby and Girling, 1965). There was an association between survival under these minimal conditions and the preparation technique used to obtain the cells; Incubation was significantly superior to other preparations ($P<0.001$).

The Incubated and the Perfused cell preparations both showed a smooth fall of ∿15–20% in trypan blue exclusion over the first 3 h of culture followed by an apparent 10–15% rise in viability at 4–6 h and a subsequent slow fall thereafter. The source of the "recovery" could not be determined in this set of experiments since only relative cell counts were done. Dissolution of some of the dead or damaged cells with a resultant decrease in total cell number and an increase in the percentage of dye excluding cells could not be ruled out without these total cell numbers.

In contrast to the Incubated and the Perfused cells, the Expressed and the Expressed + Incubated cells showed no signs of "recovery" but continued to die throughout the time course. These groups reached virtually 0% dye excluding cells by 6–12 h in culture under these minimal conditions vs 10–15% viability for Perfused cells and 25–40% viability for Incubated cells.

Purity of the Incubated Preparations

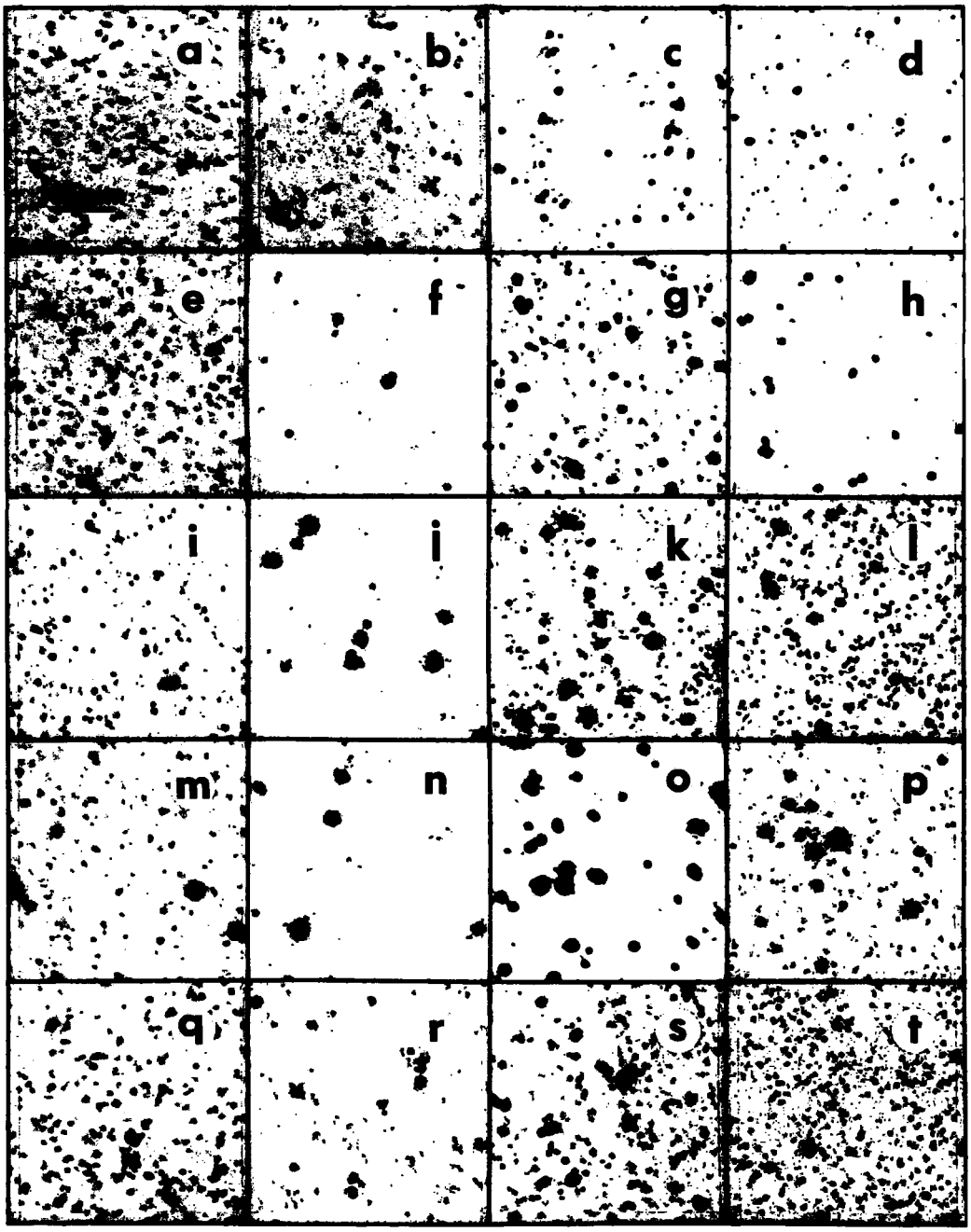
Use of chemicals to loosen junctions between ovarian granulosa cells within follicles would also be expected to loosen junctions between the thecal and interstitial cell elements occurring external to the follicular basement membrane. This might produce an artifactual increase in observed cell vitality by shifting the population of cells observed. By loosening all the cells in the ovary and consequently adding more intact nongranulosa cells to the pool of cells obtained on expression, the values of various parameters measured could be skewed

FIG. 4. Qualitative examination of the incorporation of [^{35}S]-methionine into protein as measured autoradiographically.

Granulosa cell smears taken from Expressed (a,e,i,m,q), Expressed + Incubated (b,f,j,n,r), Incubated (c,g,k,o,s) and Perfused (d,h,l,p,t) preparations at 0, 30, 60 and 150 min of incubation with tracer are shown. The cells have been stained with trypan blue, immobilized with gelatin, fixed in aldehyde vapors, autoradiographed and stained with Giemsa (see Materials and Methods). All photographs were taken at the same magnification; the length of the bar is ∿100 μm.

Cells taken at time zero, immediately after the addition of label, are shown in a–d. Cell smears made at 30, 60 and 150 min are shown in e–h, i–k and m–p, respectively. Those cells which incorporate methionine show increasing numbers of silver grains with increasing length of incubation time. The granulosa cells shown in q–t were labeled in the presence of cycloheximide at 30 μg/ml added at time zero along with the labeled methionine. The inhibitor blocked all but initial incorporation; compare to 30 min, e–h.

Incubated and Perfused cells show much heavier labeling than do Expressed or Expressed + Incubated cells throughout the time course.



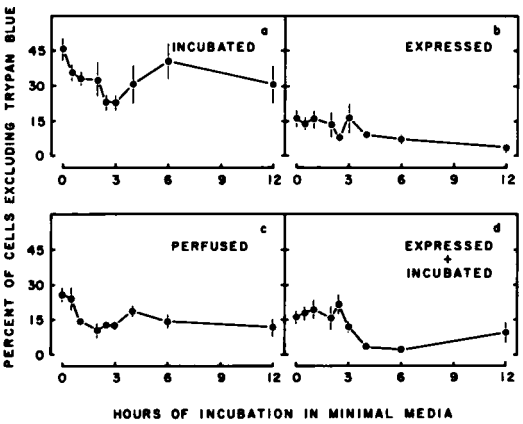


FIG. 5. Survival of granulosa cells prepared by various methods.

Granulosa cells ($\sim 10^6$/tube were incubated in test tubes in M-199-H + 1% BSA at 37°C under 95% O_2:5% CO_2 following isolation. Individual tubes were removed at various times up to 12 h and the percent of washed cells excluding trypan blue determined. The cumulative results of 3 experiments representing 2–10 replicates/point are shown as means ± SEM.

Panels a–d represent the results for cells isolated by Incubation, Expression, Perfusion and Expression + Incubation, respectively. Analysis of variance indicates Incubation significantly enhances survival ($P<0.001$). Orthogonal contrasts further show cellular dye exclusion decreases most markedly over the first 3–4 h for all groups ($P<0.01$) and Incubated cells demonstrate a "recovery" between 3 and 6 h of culture ($P<0.005$).

in favor of the EGTA/sucrose preparations. One way to demonstrate that the increase in cell viability that has been shown for the EGTA/sucrose treated cells was not, in fact, due merely to this problem was to count cells in Incubated and in Expressed preparations. If EGTA/sucrose treatment loosened all ovarian cells equally, it would be expected that Incubated preparations would have more cells than Expressed preparations. Table 2 shows this is not the case. The table gives the representative number of cells/ovary estimated from replicated total cell counts (viable plus nonviable) made on Expressed and Incubated preparations obtained from pools of 3–11 ovaries taken 12–18 h after the last estradiol and hFSH injections given in the E_2-ramp + hFSH model. The similarity of the results for both preparations in both experiments implies that the cell populations derived by both techniques are the same. Preliminary evidence on the in vitro binding (per μg DNA) of either hFSH or human chorionic gonadotropin (hCG) indicates that there are no detectible differences in gonadotropin binding between cell preparations; this provides even stronger evidence that the cells obtained by the Incubation and the Expression methods are the same.

DISCUSSION

The presence of great numbers of large gap junctions between granulosa cells within preantral and antral ovarian follicles has been known for several years (Albertini and Anderson, 1974; Albertini et al., 1975; Bjorkman, 1962; Espey and Stutts, 1972; Fletcher, 1978; Fletcher and Everett, 1973; Merk et al., 1973; Merk et al., 1972). The techniques which have been reported for obtaining granulosa cells for examination in vitro have ignored or circumvented this junctional network by concentrating either on biological properties not requiring live cells or on properties that could be examined by using cell aggregates or cultured outgrowths of cell aggregates (Channing and Ledwitz-Rigby, 1975; Dekel and Kraicer, 1978; Gospodarowicz et al., 1977; Mueller et al., 1978). The alternative approach of disaggregating the cells prior to isolation, which has been successfully used with other cell types possessing junctional complexes, was used in the present studies. EGTA seems to be both more specific and gentler than ethylenediamino-N,N'-tetraacetic acid (EDTA) or enzymes such as trypsin (Cox et al., 1977; Culp and Black, 1972). Its use in these studies was recommended by the necessity of preserving glycoprotein hormone receptors on the granulosa cells, its successful preservation of such proteins when used to dislodge cultured Chinese hamster ovary cells (Cox et al., 1977) and its successful use in several other tissue disaggregation systems (Loewenstein et al., 1967). Hypertonic sucrose was also used because of its previously successful application in tissue disruption systems (Goodenough and Gilula, 1974; Peracchia, 1977).

We cannot yet say that the sequential exposure to EGTA and hypertonic sucrose does, in fact, open gap junctions. However, preliminary electron micrographs would seem to indicate that this is the case. Furthermore, the studies of Goodenough and Gilula (1974) on the hepatic system showed that the mode of action of these chemicals appeared to be a splitting of gap junctions. Likewise, the use of citrate (an effective chelator of calcium) by Dunbar et al. (1978) to obtain oocytes with

TABLE 2. Comparative total cell counts for Expressed and Incubated cell preparations.

	Comparative cell counts (cells/ovary; mean ± SEM)	
	E	I
Experiment 1[a]	$6.19 \pm 1.35 \times 10^6$	$7.72 \pm 1.18 \times 10^6$
Experiment 2[a]	$8.06 \pm 0.48 \times 10^6$	$7.76 \pm 0.33 \times 10^6$
	$10.60 \pm 0.11 \times 10^6$	$7.57 \pm 0.23 \times 10^6$
	$7.48 \pm 0.35 \times 10^6$	$7.20 \pm 0.38 \times 10^6$
	$7.55 \pm 0.39 \times 10^6$	$7.03 \pm 0.52 \times 10^6$
Grand mean	$8.22 \pm 0.40 \times 10^6$	$7.41 \pm 0.19 \times 10^6$

[a]Total numbers of live plus dead cells were determined for preparations generated by Expression (E) or Incubation (I) protocols. The numbers, determined on a hemocytometer, were normalized for the numbers of ovaries used (3–11/determination). All ovaries were taken 12–18 h after the last estradiol and hFSH injection in animals primed with the E_2-ramp + hFSH regimen. Means (± SEM) are given for 2–5 replicate counts done for each group of ovaries. Grand means and SEM were calculated from all the normalized individual counts made.

zona pellucida stripped of granulosa cells seems to involve disruption of the granulosa cell-oocyte junctions which occur in the zona. Further EM study will be necessary to elucidate clearly the exact mode and time course of action of the chemical treatments employed here.

The technique for incubation described yielded granulosa cells with enhanced synthetic activity (protein, DNA and RNA; Figs. 1, 4) and morphologic integrity (increased dye exclusion from 25% to ∿70%, Table 1). At this time it is impossible to tell if this is due to the rescue of a very active but fragile subpopulation normally lysed by expression or if the sparing action is a general, random one. Quantitative comparisons of synthetic activity in the syncytial membrana granulosa of the follicle are not currently available and may require autoradiographic and morphometric comparisons of intact follicles and the Incubated cell preparations.

The source of the nonlinearity in the correlation between the percentage of cells excluding vital dye and the incorporation of macromolecular precursors is not yet clear. Since label appears not to be fixed into macromolecules within stained cells, at least over the first several hours in vitro, the qualitative identity of dye exclusion and protein synthesis (Figs. 2–4) implies that the nonlinear quantitative correlation derives from something within each cell that is sufficiently intact to exclude vital dye. The existence of branched or substrate modulated metabolic pathways could easily cause such a nonlinear relationship. Alternatively, changes in the numbers of leaking or disintegrating cells could alter external precursor concentrations either to shift the specific activity of the labeled precursors or to cause changes in membrane transport or internal precursor pool sizes. Until such mechanisms are fully defined, it should not be surprising that protein, or any macromolecular synthesis, measured by the specific activity of the end product, may not be quantitatively related in a linear manner to simple membrane integrity as assessed by vital dye exclusion.

The extremity of differences between thymidine incorporation into DNA in the Incubated preparation as opposed to the Expressed preparation (4476 cpm/μg DNA vs 95 cpm/μg DNA at 2 h of incubation) raised the possibility that the EGTA/sucrose isolation protocol might have stimulated DNA synthesis in vitro. The following three observations argue that the observed improvements in thymidine incorporation seen in the Incubated cells were not artifacts produced by such a mechanism.

First, in vivo incorporation of [^{3}H]-thymidine into granulosa cells (Hirshfield and Midgley, 1978; Rao et al., 1978) shows these cells to be actively synthesizing DNA. This implies that the Incubated preparation most accurately reflects the biochemical activity occurring in vivo (Fig. 1c).

Second, when Expressed cells were subjected to EGTA and sucrose following isolation (E + I) and were then allowed to incorporate [^{3}H]-thymidine, only a comparatively modest enhancement of the specific activity of the isolated DNA occurred with respect to that of

Expressed cells (714 cpm/μg DNA vs 95 cpm/μg DNA at 2 h of incubation). This implies that the chemical treatment protocol by itself could not markedly activate DNA synthesis in granulosa cells previously removed from the follicle; if the chemicals stimulated massive synthesis they had to do so prior to removal of the cells from the follicle.

Third, EGTA/sucrose treatment did not act to increase DNA specific activities by merely washing out or blocking the action of nucleotidases normally found in the cell preparations. Degradation of DNA and RNA in dead cells by treatment of the cell preparations with a mixture of RNAse (50 μg/ml; Sigma Type XII-A) and DNAse (50 μg/ml; Sigma DN-EP) at 37°C for 20 min, followed by removal of the resulting nucleotides prior to incorporation of label (results not shown), did not change the relative rates of thymidine incorporation of the cell preparations examined (Fig. 1c). This protocol increased the specific activity of the DNA in the Incubated cell preparation 20–25% at 2 and 3 h of incubation; Expressed + Incubated cells demonstrated no change in specific activities while Expressed cells showed a modest increase in incorporation to the level seen with E + I cells. Thus, no major movements of the DNA specific activities of the E and E + I preparations toward those seen in the I preparation were observed even when sources of label dilution were removed.

This study may contain the first qualitative one-to-one mapping of cellular vital dye exclusion to protein precursor incorporation (Figs. 2, 3). This supports previous work indicating that trypan blue exclusion reflects cell viability or functional integrity, which, presumably, includes protein synthesis (Paul, 1975; Sawicki et al., 1967). The comparison presented in this paper does not depend on a statistical comparison of separate groups of cells. Rather, it compares directly the 2 parameters within the same individual cells and provides what would seem to be the strongest kind of support for concluding that vital dye exclusion is indeed a necessary and sufficient condition, during at least the initial hours in vitro, to indicate that cells can synthesize protein under physiologic conditions. This one to one correlation implies that simple vital dye exclusion is a reliable reflection of functional integrity and can be used to predict the qualitative results of acute functional comparisons of various cell preparations. This relationship between dye exclusion and protein synthesis and the technique of correlating the two by immobilizing trypan blue stained cells in gelatin becomes particularly important in cell preparations, such as granulosa cells, where >90% viability is rarely achieved and <50% is common. Without prior enrichment of such preparations for live (or dead) cells the ability to distinguish which cells are responsible for substrate uptake, product synthesis or ligand binding becomes a matter of probability and has a large possibility of error for individual cells or subgroups of cells. Such analyses, as were applied here, could be used to follow cellular enrichment protocols and could be adapted, by adjustment of amount and type of label, cell fixation and autoradiographic exposure times, to allow morphometric analysis of any of a large number of cellular processes or cellular components.

The similarity of the numbers (Table 2) and binding characteristics of cells obtained by Incubation or Expression indicates that the two methods generate equivalent cell populations. However, in defined media the ability of the cells to survive depends on the method by which they were prepared (Fig. 5). This implies that even those cells which are not markedly damaged by simple physical expression eventually succumb to incipient damage or to the larger amounts of proteolytic enzymes inherently present in those preparations with more dead cells. By whatever mechanism the difference in the preparations arises, the greater ability of the Incubated cell preparations to survive, even in minimal media, argues in favor of their use for obtaining granulosa cells for culture, particularly for experiments involving cell clones requiring nonaggregated cells.

The Incubated and the Perfused preparations seem to possess many of the biochemical functions of the cells of the intact tissue. Furthermore, they allow the application of separation and analytical techniques which have been developed for use with other monocellular dispersions of live cells derived either from tissues or from cultures. For example, lysis and subfractionation of granulosa cells to yield clean subfractions has previously been difficult because of the presence of cellular aggregates and the large nuclear to cytoplasmic ratio of the cells. If these cells are obtained intact in monodisperse suspension, however, separation, lysis and subfractionation techniques successfully used on lymphocytes (Jett et al., 1977) or

thymocytes (Allfrey, 1974; Monneron and d'Alayer, 1978) should be applicable. By using the Incubation procedure in conjunction with techniques for examining cells derived largely from the cumulus vs those from the membrana granulosa (Haney and Schomberg, 1978) it should also be possible to begin to distinguish the different properties of these 2 cellular subpopulations.

The purpose of the intercellular communication system in the follicle as it relates qualitatively and quantitatively to macromolecular synthesis, or to synchrony and differentiation is an aspect of follicular physiology which now seems to be approachable with highly viable, disaggregated cells. A part of any difference in function of granulosa cells in the follicle vs disaggregated intact cells might be attributable to intercellular junctions. If junctions could be caused to re-form in vitro, the effects of interruption of the intercellular junctional complex could be studied by comparing intact follicular responses to those of disaggregated cells and of cell aggregates derived from disaggregated cells.

Modifications of the techniques presented are of course possible and may well be advisable if a different system or species is being studied. Obvious variations for use in the rat system would be further alterations in the times or temperatures of incubation, the concentration of the EGTA and/or sucrose and the presence of enzymes like DNAse or absence of ions like Mg^{++}. In the case of ovaries from larger species, it may be advisable to bisect individual follicles prior to incubation in order to optimize the permeation of the chelator and sucrose into the antral space. Alternatively, injection of the solutions into the antral cavity might accomplish the desired effects of disaggregation prior to cellular removal.

In summary, a procedure for obtaining monodisperse granulosa cells with reasonable viability and synthetic capacity has been described. The resultant cells constitute a preparation on which experiments can be based to explore the physiology of the granulosa cell and its role within the intact ovarian follicle.

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