Isolation and Properties of Cholesterol Ester-Storage Granules from Ovarian Tissues

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Cholesterol ester-storage granules were isolated from luteinized rat ovary and rabbit ovarian interstitial tissue by centrifugal flotation and were investigated with regard to their structure and function. Cholesterol ester, protein, phospholipid and unesterified cholesterol accounted for the dry weight of granules from luteinized rat ovary. The protein and the phospholipid were resistant to removal by washing. Substrate specificities of nucleotide phosphatase and specific radioactivities of lipid-soluble P (determined after administration of [32 P]P_i *in vivo*) were the same in granules and in a microsomal fraction from the same tissue. After administration of [32 P]P_i *in vivo*, luteinizing hormone increased the specific radioactivity of lipid-soluble P in granules, mitochondria and the microsomal fraction. Since granules did not swell in hypo-osmotic media, whereas microsomal particles did, it is suggested that adherent phospholipid and protein in granule suspensions is unlikely to result from contamination with endoplasmic reticulum. Luteinizing hormone administered *in vivo* increased the phospholipid and unesterified cholesterol contents of isolated granules relative to their cholesterol ester content, and also tended to raise their protein content. This treatment decreased the ability of isolated granules to act as a substrate for cholesterol esterase *in vitro* and increased the activity of cholesterol esterase. Cycloheximide *in vivo* also raised the unesterified cholesterol/cholesterol ester ratio of isolated granules, and when administered with luteinizing hormone acted synergistically to bring about a further increase. These results are considered compatible with evidence obtained by microscopy which suggests that granules may be surrounded by a membrane, that they arise by pinching off from the endoplasmic reticulum, and that they shrink on trophic stimulation of the tissue.

Ovaries and most other steroid-secreting endocrine tissues store large quantities of cholesterol ester, which on trophic stimulation they convert into steroids. Claesson *et al.* (1949) stated that lipid (mostly cholesterol ester) may account for up to 50% of the dry weight of the ovary of the pregnant rabbit. Most of the cholesterol ester in these tissues is localized within the cells in lipid granules (or droplets) (Deane & Seligman, 1953; Claesson, 1954). Owing to their lipid nature these structures have a low density and can conveniently be isolated, in relatively stable suspensions, from homogenates by centrifugal flotation (Claesson, 1954). By microscopy it has been shown that granules decrease in both size (Deane *et al.*, 1948; Barker, 1951; Claesson, 1954) and number (Ashworth *et al.*, 1959; Giacomelli *et al.*, 1965) on trophic stimulation of the tissue, and that they may be surrounded by a membrane (Giacomelli *et al.*, 1965; Bjersing, 1967*a,b*; Smith, 1968; Christensen & Gillim,

1969; see also, for example, the electron micrographs of Blanchette, 1966). The experiments described here were undertaken to confirm and extend these findings, by using biochemical techniques. Lipid-containing granules from luteinized (or 'superovulated') rat ovary and rabbit ovarian interstitial tissue have been used, since these tissues store large quantities of cholesterol ester and are relatively homogeneous with respect to cell type.

Some of these findings have previously been presented elsewhere (Flint & Armstrong, 1972).

Experimental

Materials

Luteinizing hormone (NIH-LH-S6; ovine) was the kind gift of the National Institutes of Health, Bethesda, Md., U.S.A. [32 P]P_i (98 Ci/mg of P) was obtained from Amersham Searle Corp., Toronto, Ont., Canada. Pregnant-mare serum gonadotrophin and human chorionic gonadotrophin were the gift of Ayerst Laboratories Inc., New York, N.Y., U.S.A., through their office in Montreal, P.Q., Canada.

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Cycloheximide and nucleotides were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Isolation of granules and other subcellular fractions

Ovarian interstitial tissue was obtained from oestrous rabbits (New Zealand White, 3–4 kg). Lutein tissue was obtained from the heavily luteinized ovaries of immature rats treated with 50 i.u. of pregnant-mare serum gonadotrophin and subsequently with 25 i.u. of human chorionic gonadotrophin as described by Parlow (1958); these ovaries were used 5–9 days after the administration of human chorionic gonadotrophin. Lipid-containing granules were prepared as a routine from homogenates of these tissues made in 0.25 M-sucrose, pH 7.3, containing 10 mM-triethanolamine-HCl and 10 mM-MgCl₂, by flotation (Claesson, 1954) during centrifugation at 15000–25000 *g*_{av} for 20 min in a swinging-bucket head. Granules were removed from the surface of the liquid in the centrifuge tube on a dry glass rod and were suspended in the homogenization medium by shaking or with a vortex mixer; they were washed by re-suspension in the homogenization medium and repeated centrifugation and isolation, up to four times; losses at each wash were 20–30%. Unless otherwise stated granules were washed once after preparation and before use. Suspensions of granules coalesced when kept overnight. Rapid and approximate quantitative measurement of the amount of cholesterol ester in granule suspensions was possible on the basis of light-scattering; with various volumes of a single suspension (Fig. 1) or with granules isolated from the same type of ovary under similar conditions, e.g. within a single batch of identically treated animals the absorbance (measured at 580 nm) was approximately proportional to their cholesterol ester content.

Mitochondria were prepared by using the centrifugal conditions used for granules. Supernatant and microsomal fractions were prepared by centrifuging the cytosol thus obtained at 110000 *g*_{av} for 1 h.

Extraction and assay procedures

Lipids were extracted from granule suspensions, subcellular fractions and other extracts by adding samples (0.5 ml) to chloroform-methanol (2:1, v/v; 10 ml), and the phases were then split with 0.9% (w/v) NaCl (2 ml). The chloroform layer was retained and used for the assay of unesterified cholesterol, cholesterol ester and phospholipid-P (Flint & Denton, 1970a) and for counting radioactivity [by using either a Nuclear-Chicago mark I or a Nuclear-Chicago Isocap 300 β -scintillation counter in a scintillation fluid consisting of 2,5-diphenyloxazole (7.5 g) and 1,4-bis-(5-phenoxazol-2-yl)benzene (75 mg) in toluene (1 litre)]. Protein was assayed in suspensions of

Fig. 1. Light-scattering by suspensions of granules

The E_{580} was measured as a function of volume of a granule suspension. Granules prepared from 1.0 g of rabbit ovarian interstitial tissue were resuspended in 10.0 ml; the final volume in the cuvette was 2.5 ml.

granules by the method of Lowry *et al.* (1951) in a split-beam spectrophotometer, by using suitably diluted granule suspensions as blanks and bovine plasma albumin as standard; blanks were seldom more than 25% of the reading in the presence of the reagents. Lactate dehydrogenase (EC 1.1.1.27), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), NADP-malate dehydrogenase ('malic' enzyme; EC 1.1.1.40) and cholesterol ester synthetase (EC 2.3.1.-) were assayed as described previously for extracts of luteinized rat ovary (Flint & Denton, 1969, 1970b; Flint & Armstrong, 1973). Nucleotide phosphatase was assayed by following release of P_i from added nucleotide by using the assay for phosphate described by Ashcroft & Randle (1970); incubations were done in 100 mM-triethanolamine-HCl (adjusted to pH 7.4 with KOH) containing 10 mM-MgCl₂ and 1 mM-nucleotide (sodium salt). Since the nucleotide phosphatase had a K_m of 0.6 mM for ATP (A. P. F. Flint & D. T. Armstrong, unpublished work) it was not assayed at maximum activity under these conditions. The use of a higher ATP concentration was precluded by the high blanks obtained in the phosphate assay, owing to contamination of the ATP with approx. 2% of P_i . The ATPase* reaction was linear with respect to time and granule concentration. Granule Na^+ + K^+ -stimulated ATPase activity (measured in the

* Abbreviation: ATPase, adenosine triphosphatase.

presence of 15mM-Na⁺, 150mM-K⁺, 10mM-MgCl₂ and 1mM-Tris ATP) was less than 10% of the activity of the Mg²⁺-stimulated ATPase (measured in the absence of Na⁺ and K⁺). All enzymes were assayed at 37°C.

Swelling of microsomal fractions and granules was investigated by measuring at 37°C the decrease in E₅₈₀ observed on adding these fractions to hypo-osmotic medium by using a Perkin-Elmer model 124 split-beam spectrophotometer equipped with water jacket and recorder.

Treatment of animals

Luteinizing hormone (10µg), cycloheximide (5mg) and [³²P]P_i (98Ci/mg of P) dissolved in 0.4ml of 0.9% (w/v) NaCl were administered to rats via the tail vein, under light ether anaesthesia.

Results

Composition of lipid-containing granules

Up to 56% of total ovarian cholesterol ester can be isolated as granules by centrifugal flotation. Values for the percentage distribution of cholesterol ester in luteinized ovaries from control rats were, for the granules, mitochondria, microsomal fraction and soluble fractions, 55.7, 30.8, 10.1 and 3.4% respectively. Lower recoveries of cholesterol ester in granule fractions were occasionally caused by incomplete removal of floating material. This was particularly marked in homogenates containing large amounts of cholesterol ester, where the lipid plug obtained was too thick to adhere to the glass rod used to remove it. More than 95% of the cholesterol ester obtainable in granules from homogenates centrifuged at 110000g_{av.} for 1h was obtained after centrifugation at 15000g_{av.} for 20min.

Assay of lipids, protein and enzymes at each stage of the serial washing procedure (Tables 1 and 2) indicated that granules were associated with phospholipid and protein that were not removed by washing. Assay of lactate dehydrogenase, glucose 6-phosphate dehydrogenase and NADP-malate dehydrogenase, which are soluble enzymes in luteinized rat ovary (Flint & Denton, 1970b), showed that the suspensions were free of soluble cytoplasmic contamination after one wash. The presence in granules of nucleotide phosphatase activity [assayed with MgATP²⁻ but also capable of hydrolysing Mg²⁺ salts of ADP, AMP, CTP, GTP and UTP (Table 3)], confirmed that they contained protein.

To determine the relative contents of phospholipid, protein and cholesterol in lipid granules a suspension prepared from luteinized rat ovary was dried to constant weight and the residue extracted with chloroform-methanol (2:1, v/v). Assuming that the cholesterol ester present was cholesterol oleate,

Table 1. Effects of serial washing on protein, phospholipid, ATPase and cytoplasmic enzymes associated with lipid-containing granules from luteinized rat ovary

After wash no.	Lipid-soluble P (ng-atoms of P/ μ mol of cholesterol ester)	Protein (μ g/ μ mol of cholesterol ester)	Activities of soluble enzymes				ATPase (nmol/min per μ mol of cholesterol ester)
			Glucose 6-phosphate dehydrogenase	Lactate dehydrogenase	'Malic' enzyme		
0	35 $\pm$ 4	65 $\pm$ 5.6	1.10	1.35	1.90	7.3	
1	37 $\pm$ 5	61 $\pm$ 19.6	—	—	—	4.4	
2	47 $\pm$ 5	61 $\pm$ 8.6	—	—	—	3.3	
3	36 $\pm$ 6	75 $\pm$ 11.8	—	—	—	4.2	
4	34 $\pm$ 3	67 $\pm$ 15.4	—	—	—	4.2	

Table 2. *Effects of serial washing on protein, phospholipid and ATPase associated with lipid-containing granules from rabbit ovarian interstitial tissue*

Granules were isolated from rabbit ovarian interstitial tissue. All values are means $\pm$ S.E.M. for four determinations; other details are as described in Table 1.

After wash no.	Lipid-soluble P (ng-atoms of P/ μ mol of cholesterol ester)	Protein (μ g/ μ mol of cholesterol ester)	ATPase (nmol/min per μ mol of cholesterol ester)
0	38 $\pm$ 3.7	107 $\pm$ 11.3	7.6 $\pm$ 0.3
1	35 $\pm$ 4.1	86 $\pm$ 29.8	6.0 $\pm$ 1.0
2	46 $\pm$ 8.8	137 $\pm$ 28.3	8.0 $\pm$ 0.6
3	35 $\pm$ 8.8	189 $\pm$ 10.8	9.4 $\pm$ 0.3
4	41 $\pm$ 5.5	188 $\pm$ 16.7	7.6 $\pm$ 0.5

Table 3. *Relative substrate specificities of nucleotide phosphatases of granules and a microsomal fraction*

Nucleotide phosphatase activity was determined in granules and a microsomal fraction obtained from the same homogenate of luteinized rat ovary. Activities were measured in a medium containing 1 mM-nucleotide (pH 7.4) and 10 mM-MgCl₂, and are expressed as a percentage of the rate obtained with ATP $\pm$ S.E.M. (number of values are shown in parentheses).

Nucleotides tested	Nucleotide phosphatase activity (% of activity with ATP)	
	In granules	In microsomal fraction
ADP	56 $\pm$ 12 (9)	78 $\pm$ 13 (6)
AMP	116 $\pm$ 16 (8)	130 $\pm$ 16 (6)
GTP	145 $\pm$ 11 (8)	148 $\pm$ 17 (6)
UTP	125 $\pm$ 11 (8)	105 $\pm$ 9 (6)
ITP	180 $\pm$ 23 (8)	187 $\pm$ 41 (6)
CTP	59 $\pm$ 12 (7)	65 $\pm$ 19 (6)

and that the phospholipid had mol.wt. 1000, the material extracted consisted, on a weight basis, of 85.3 $\pm$ 3.9% cholesterol ester, 4.5 $\pm$ 0.2% phospholipid and 2.1 $\pm$ 0.1% unesterified cholesterol; 92 $\pm$ 4.7% of the weight could be accounted for by these lipids (means $\pm$ S.E.M. for four determinations). On the basis of the results in Table 1, protein accounts for 8.6 $\pm$ 0.4% of the weight of lipid granules. Visual examination of t.l.c. plates after separation of lipid extracts of granules showed in addition the presence of small amounts of non-esterified fatty acid and triglyceride.

It is possible that protein, phospholipid and unesterified cholesterol were associated with granules as the result of contamination by fragments of endoplasmic reticulum adhering to them during homogenization. Three techniques were used in an attempt to evaluate this possibility. Since membranous material derived from endoplasmic reticulum forming a sac round granules might be expected to enclose within it a water-accessible space, and therefore be susceptible to swelling in hypo-osmotic media,

attempts were made to cause granules to swell. Comparisons were also made between the substrate specificities of the nucleotide phosphatases from granules and endoplasmic reticulum, and of the specific radioactivities of phospholipid from granules and endoplasmic reticulum after administration of [³²P]P_i *in vivo*. All attempts to produce swelling failed, even when granules prepared and suspended in 0.25 M-sucrose were added to water. Microsomal particles were shown to swell well under these conditions. These findings confirm those made by Claesson (1954), who tested isolated granules for swelling by microscopic examination. There were no differences between the microsomal fraction and granules, with respect to other properties investigated (Tables 3 and 4).

Effects of luteinizing hormone on composition of lipid-containing granules

Administration of luteinizing hormone to rats with luteinized ovaries causes rapid depletion of ovarian

Table 4. *Effect of luteinizing hormone on incorporation of [^{32}P]P_i into lipid in various subcellular fractions in vivo*

Rats with luteinized ovaries received 48 μCi of [^{32}P]P_i with or without 10 μg of luteinizing hormone intravenously 1 h before autopsy. Ovaries were homogenized, and homogenates fractionated centrifugally, as described in the Experimental section. Homogenates and subfractions were extracted with chloroform-methanol (2:1, v/v), and extracts assayed for radioactivity and P. Values are d.p.m./ μg -atom of P (means $\pm$ S.E.M. for five determinations). $P < 0.001$ for all effects of luteinizing hormone.

Fraction	Specific radioactivities of phospholipid-P (d.p.m./ μg -atom of P) in ovaries taken from:	
	Control animals	Animals treated with luteinizing hormone
Total homogenate	3654 $\pm$ 355	8972 $\pm$ 609
Granules	4622 $\pm$ 449	10630 $\pm$ 625
Mitochondrial fraction	3193 $\pm$ 250	7792 $\pm$ 443
Microsomal fraction	4560 $\pm$ 331	10868 $\pm$ 711

Table 5. *Effect of luteinizing hormone in vivo on the composition of lipid-containing granules from luteinized rat ovary*

Washed granules were prepared from homogenates of luteinized ovaries from animals receiving either 0.9% NaCl or luteinizing hormone (10 μg) intravenously 1 h before autopsy, and were assayed for cholesterol ester, unesterified cholesterol, phospholipid, protein and ATPase. Values are expressed per μmol of cholesterol ester (means $\pm$ S.E.M. for 15 determinations). *, $P < 0.05$; **, $P < 0.001$. Other differences, $P > 0.05$.

Component of granules	Ovaries from:	
	Control animals	Animals treated with luteinizing hormone
Phospholipid (ng-atoms of P/ μmol of cholesterol ester)	46.8 $\pm$ 2.7	75.4 $\pm$ 7.6**
Protein ($\mu\text{g}/\mu\text{mol}$ of cholesterol ester)	150 $\pm$ 20	198 $\pm$ 23
Unesterified cholesterol (nmol/ μmol of cholesterol ester)	45 $\pm$ 12	91 $\pm$ 17*
ATPase activity (nmol/min per μmol of cholesterol ester)	5.2 $\pm$ 0.7	6.1 $\pm$ 0.7

cholesterol ester (Parlow, 1961; Herbst, 1967; Armstrong, 1968). In the present study administration of 10 μg of luteinizing hormone intravenously 2 h before autopsy decreased the ovarian cholesterol ester concentration from 22.8 $\pm$ 2.6 to 14.7 $\pm$ 2.6 $\mu\text{mol/g}$ wet wt.; the concentration of unesterified cholesterol was unchanged (6.1 $\pm$ 0.4 and 6.4 $\pm$ 0.7 $\mu\text{mol/g}$ wet wt. respectively; values are means $\pm$ S.E.M. for 20 determinations). Less cholesterol ester was obtained as lipid-containing granules on isolation from the tissue after treatment with luteinizing hormone (from 8.6 $\pm$ 1.2 to 3.9 $\pm$ 1.1 $\mu\text{mol/g}$ wet wt.).

Luteinizing hormone increased the phospholipid/cholesterol ester, and unesterified cholesterol/cholesterol ester ratios in granules within 1 h of administration (Tables 5 and 6); there was also a trend towards a significant change in the protein/cholesterol ester ratio. Luteinizing hormone had no effect on granule ATPase activity (Table 5); it did increase the specific radioactivity of ovarian lipid-soluble P

(Table 4), in granules and other subcellular fractions, after administration of ^{32}P *in vivo*.

In the presence of adrenocorticotrophin, cycloheximide *in vivo* raises the unesterified cholesterol content of granules isolated from adrenal cortex (Garren *et al.*, 1971). A similar effect was observed in rat corpus luteum tissue (Table 6); both luteinizing hormone and cycloheximide *in vivo* increased the unesterified cholesterol/cholesterol ester ratio ($P < 0.01$ in each case by analysis of variance). Together they appeared to act synergistically to raise the ratio further.

Enzyme associated with lipid-containing granules

Assay of cholesterol ester synthetase in washed lipid suspensions suggests that it is not associated with isolated granules. Of the total activity of the enzyme in the tissue, 95% was located in the cytosol plus microsomal fractions (Table 7). Under the assay conditions,

Table 6. *Effects of luteinizing hormone and cycloheximide in vivo on unesterified cholesterol/cholesterol ester ratios in lipid-containing granules from luteinized rat ovary*

Washed granules were isolated from luteinized ovarian tissue of rats treated with either luteinizing hormone (10 μ g) or cycloheximide (5mg) or both intravenously, 1h before autopsy. Values are expressed as nmol of unesterified cholesterol/ μ mol of cholesterol ester (means $\pm$ S.E.M. for eight determinations). Results of statistical analyses (by analysis of variance) are described in the text.

Treatment <i>in vivo</i>	Unesterified cholesterol/cholesterol ester ratio in granules (nmol/ μ mol)
None	43.3 $\pm$ 3.8
+Luteinizing hormone	70.7 $\pm$ 11.4
+Cycloheximide	60.8 $\pm$ 8.2
+Luteinizing hormone+cycloheximide	192.5 $\pm$ 15.9

Table 7. *Intracellular distribution of cholesterol ester synthetase*

Rabbit ovarian interstitial tissue was homogenized and fractionated, and cholesterol ester synthetase assayed, as described in the Experimental section. Values are means $\pm$ S.E.M. for three determinations.

Fraction	Cholesterol ester synthetase activity (μ mol/h per g)	% of total activity accounted for
Granules	0.02 $\pm$ 0.002	2.5
Mitochondrial fraction	0.04 $\pm$ 0.004	3.8
Microsomal fraction	0.22 $\pm$ 0.022	20.9
Supernatant	0.79 $\pm$ 0.077	74.1
		Total ... 101.3
Total homogenate	1.07 $\pm$ 0.067	

Table 8. *Effect of luteinizing hormone in vivo on cholesterol esterase activity*

Granules and cytosol prepared from luteinized ovaries of control rats and rats treated with luteinizing hormone (10 μ g, intravenous, 1h before autopsy) were incubated together in a 2 $\times$ 2 factorial design experiment. Cholesterol esterase activity was determined on the basis of the rate of formation of unesterified cholesterol (measured at 37°C); values are μ mol of unesterified cholesterol produced/h per g of tissue (calculated on the basis of the equivalent amount of cytosol; means $\pm$ S.E.M. for four determinations). Cholesterol ester concentrations in the incubations are given as mM (means $\pm$ S.E.M. for four values). Results of statistical analyses (by analysis of variance) are given in the text.

Cytosol from animals receiving:	Granules from animals receiving:	Rate of formation of unesterified cholesterol (μ mol/h per g)	Cholesterol ester concentration (mM)
NaCl	NaCl	1.69 $\pm$ 0.29	0.60 $\pm$ 0.02
Luteinizing hormone	Luteinizing hormone	1.96 $\pm$ 0.34	0.65 $\pm$ 0.03
NaCl	Luteinizing hormone	1.10 $\pm$ 0.16	0.62 $\pm$ 0.03
Luteinizing hormone	NaCl	2.82 $\pm$ 0.18	0.62 $\pm$ 0.03

enzyme activities in both these fractions were linear with time and enzyme concentration. Previous attempts at detecting cholesterol esterase in granules have also failed (Flint & Armstrong, 1973). The homogenization and fractionation procedures used in these experiments were similar to those employed in the experiment outlined in Table 2. As the granules

obtained in the latter were associated with a constant amount of protein, it seems unlikely that cholesterol ester synthetase could have been washed off granules during cell fractionation. Further, since all the cholesterol ester synthetase in the whole homogenate could be accounted for after fractionation it is unlikely that a cholesterol ester synthetase associated

with granules became inhibited during the fractionation process. It was concluded therefore that lipid-containing granules are not likely to bind the enzymes that catalyse their formation and degradation.

Consistent with this conclusion is the finding that stimulation of cholesterol ester hydrolysis probably involves activation of a component of the cytosol rather than of the granules (Table 8). Lipid-containing granules and cytosol prepared from the luteinized ovaries of control and luteinizing hormone-treated rats were incubated together in a 2×2 factorial design experiment. As reported by Behrman & Armstrong (1969), administration of luteinizing hormone 1 h before autopsy resulted in an increase in the activity of cholesterol esterase in the cytosol ($P < 0.01$). Luteinizing hormone simultaneously decreased the proficiency of the granules as a substrate for cholesterol esterase ($P < 0.05$).

Discussion

Many of the observations described above are consistent with available microscopic evidence relating to the structure, formation and function of cholesterol ester-storage granules. The presence of a nucleotide phosphatase in granules suggests that at least part of the protein they contain is accessible to the aqueous environment and not hidden within the lipid matrix. The demonstration that the granules are also associated with phospholipid is consistent with microscopic evidence suggesting that they may be surrounded by a membrane. It seems probable that a high proportion of the protein and phospholipid associated with granules may be in the form of a membrane; if this is so, the observed increases in phospholipid/cholesterol ester and unesterified cholesterol/cholesterol ester ratios in granules isolated from ovaries of luteinizing hormone-treated animals are consistent with their shrinkage. The fact that treatment with luteinizing hormone decreases the amount of ovarian cholesterol ester that can be isolated from ovaries in the form of granules also agrees with the proposed function of these organelles. These observations confirm Claesson's (1954) suggestion that the material isolated from ovarian homogenates by centrifugal flotation consists of the lipid-containing granules observed in sections of tissue under the microscope.

Similarities between the substrate specificities of the nucleotide phosphatases of granules and the microsomal fraction (Table 3), and between the specific radioactivities of lipid-soluble P extracted from the fractions after administration of [^{32}P]P_i *in vivo* (Table 4), suggest that a relationship may exist between the phospholipid and protein of granules and endoplasmic reticulum. Possible microsomal contamination of suspensions of granules appears to be ruled out by the observation that isolated granules could

not be made to swell in hypo-osmotic media. This could be interpreted as confirming the microscopic evidence of Bjersing (1967a), which indicates that lipid-containing granules arise by intraluminal deposition of cholesterol ester in the tubules of the endoplasmic reticulum. [See also the suggestion by van Lennep & Madden (1965) that lipid may accumulate in vacuoles within the endoplasmic reticulum during luteolysis.] Such a mechanism for the formation of granules is consistent with the relative lack of cholesterol ester synthetase in them, and with the presence of at least some of this enzyme in microsomal fractions (Table 7). If the endoplasmic reticulum is the source of the phospholipid and protein associated with granules, the findings described in Tables 3 and 4 suggest that, with respect to incorporation of ^{32}P into phospholipid and nucleotide phosphatase specificity, the membranes may retain their properties after becoming constituents of granules.

After administration of [^{32}P]P_i *in vivo*, the specific radioactivity of phospholipid-P in the microsomal fraction was higher than that in the mitochondrial fraction ($P < 0.01$ in both control and luteinizing hormone-treated ovaries; Table 4). This is consistent with the observations of McMurray & Dawson (1969), who showed that [^{32}P]P_i is incorporated into phospholipid in the endoplasmic reticulum of liver and thereafter transported to other sites inside the cell.

The finding that luteinizing hormone increased the incorporation of ^{32}P into ovarian phospholipid *in vivo* (Table 4) is in agreement with its effect on phospholipid in the testis (Yokoe & Hall, 1970). The mechanism by which this effect is brought about, and its consequences, are not understood. Luteinizing hormone *in vitro* does not have this effect, although it does stimulate steroid synthesis, in slices of bovine corpus luteum (Scott *et al.*, 1968).

Luteinizing hormone and cycloheximide *in vivo* appeared to act synergistically to raise the unesterified cholesterol/cholesterol ester ratio in isolated granules (Table 6). This suggests they may be acting in different ways to produce the same effect. Possible explanations are that cholesterol esterase may be activated by luteinizing hormone through a process not requiring protein synthesis, and that cycloheximide may prevent unesterified cholesterol from leaving the granules. Clarification of these findings must await further work on the mechanism of de-esterification of cholesterol, and its relation to steroid synthesis.

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References

Armstrong, D. T. (1968) *Recent Progr. Horm. Res.* **24**, 255-319

- Ashcroft, S. J. H. & Randle, P. J. (1970) *Biochem. J.* **119**, 5-15
- Ashworth, C. T., Race, G. J. & Mollenhauer, H. H. (1959) *Amer. J. Pathol.* **35**, 425-437
- Barker, W. L. (1951) *Endocrinology* **48**, 772-785
- Behrman, H. R. & Armstrong, D. T. (1969) *Endocrinology* **85**, 474-480
- Bjersing, L. (1967a) *Z. Zellforsch. Mikrosk. Anat.* **82**, 173-186
- Bjersing, L. (1967b) *Z. Zellforsch. Mikrosk. Anat.* **82**, 187-211
- Blanchette, E. J. (1966) *J. Cell Biol.* **31**, 517-542
- Christensen, A. K. & Gillim, S. W. (1969) in *The Gonads* (McKerns, K. W., ed.), pp. 415-488, Appleton-Century-Crofts, New York
- Claesson, L. (1954) *Acta Physiol. Scand.* **31**, Suppl. **113**, 53-78
- Claesson, L., Diczfaluzy, E., Hillarp, N.-Å. & Högberg, B. (1949) *Acta Physiol. Scand.* **16**, 183-200
- Deane, H. W. & Seligman, A. M. (1953) *Vitam. Horm. New York* **11**, 173-204
- Deane, H. W., Shaw, J. H. & Greep, R. O. (1948) *Endocrinology* **43**, 133-153
- Flint, A. P. F. & Armstrong, D. T. (1972) in *Gonadotrophins* (Saxena, B. B., Beling, C. G. & Gandy, H. M., eds.), pp. 269-286, John Wiley & Sons, New York
- Flint, A. P. F. & Armstrong, D. T. (1973) *Biochem. J.* **132**, 301-311
- Flint, A. P. F. & Denton, R. M. (1969) *Biochem. J.* **112**, 243-254
- Flint, A. P. F. & Denton, R. M. (1970a) *Biochem. J.* **116**, 79-82
- Flint, A. P. F. & Denton, R. M. (1970b) *Biochem. J.* **117**, 73-83
- Garren, L. D., Gill, G. N., Masui, H. & Walton, G. M. (1971) *Recent Progr. Horm. Res.* **27**, 433-478
- Giacomelli, F., Wiener, J. & Spiro, D. (1965) *J. Cell Biol.* **26**, 499-521
- Herbst, A. L. (1967) *Endocrinology* **81**, 54-60
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- McMurray, W. C. & Dawson, R. M. C. (1969) *Biochem. J.* **112**, 91-108
- Parlow, A. F. (1958) *Fed. Proc. Amer. Soc. Exp. Biol.* **17**, 402
- Parlow, A. F. (1961) *Fed. Proc. Amer. Soc. Exp. Biol.* **20**, 187
- Scott, T. W., Hansel, W. & Donaldson, L. E. (1968) *Biochem. J.* **108**, 317-323
- Smith, A. D. (1968) *Biochem. J.* **109**, 17P-19P
- van Lennep, E. W. & Madden, L. M. (1965) *Z. Zellforsch. Mikrosk. Anat.* **66**, 365-380
- Yokoe, Y. & Hall, P. F. (1970) *Endocrinology* **86**, 1257-1263