Metabolism of Endogenous Sterol Ester by the Superovulated Rat Ovary *in vitro*

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Sterol, glyceride and phospholipid were found to account for more than 90% (w/w) of the lipid extracted from whole superovulated rat ovaries. These lipids, together with non-esterified fatty acids, were assayed in slices of the tissue after incubation for various times. Whereas the concentrations of triglyceride, diglyceride and phospholipid did not change significantly during incubation, that of sterol ester markedly decreased and those of free sterol, monoglyceride and non-esterified fatty acid increased. Evidence is presented that in this tissue (in contrast with other mammalian tissues) the main endogenous substrate for respiration is fatty acid derived from sterol ester.

The oxidation of glucose accounts for less than one-quarter of the oxygen consumption of superovulated rat ovary slices incubated *in vitro* in a bicarbonate-buffered medium containing glucose (Flint & Denton, 1969). The oxygen not accounted for in this way must be utilized by the oxidation of an endogenous substrate. In other tissues glycogen, glucose, triglyceride fatty acid or possibly amino acids derived from protein appear to be the major endogenous substrates. Initial studies showed that superovulated rat ovary contains only small amounts of glycogen (Flint & Denton, 1969) and produces neither urea nor ammonia on incubation (A. P. F. Flint, unpublished work); thus it appeared that neither glycogen nor amino acid was a significant source of endogenous substrate. The studies described below suggest that the major endogenous substrate in the superovulated rat ovary is fatty acid derived from sterol ester.

MATERIALS

Silica gels G and H were from E. Merck A.-G. through Anderman and Co. Ltd., London S.E.1. Silicic acid [100 mesh, Mallinckrodt A. R. grade; obtained through Camlab (Glass) Ltd., Cambridge] was washed before use with methanol and ether as described by Hirsch & Ahrens (1958). All solvents used were redistilled before use; diethyl ether was passed through a column of basic alumina grade 1 (M. Woelm, Eschwege, Germany) before redistillation over ferrous sulphate and storage in the dark over copper gauze. Pregnant-mare serum gonadotrophin (Gestyl) and human chorionic gonadotrophin

(Pregnyl) were obtained from Organon Laboratories Ltd., Morden, Surrey, U.K. All other chemicals were of the purest grade commercially available.

EXPERIMENTAL PROCEDURES

Incubation and extraction procedures. Superovulated rat ovaries were obtained from immature Wistar rats treated with pregnant-mare serum gonadotrophin and human chorionic gonadotrophin (Stansfield & Flint, 1967). This treatment causes ovulation and the formation of large numbers of functional corpora lutea within the ovary; the resulting tissue (the superovulated ovary) consists almost entirely of corpora lutea. There are many precedents for the use of the superovulated rat ovary as a convenient source of rat corpora lutea (see Armstrong, 1968). Ovary slices (0.3–0.4 mm thick) were incubated in either 1.5 ml (30–60 mg of tissue) or 5.0 ml (100–200 mg of tissue) of bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing glucose (1 mg/ml) gassed with O₂ + CO₂ (95:5).

Lipid from whole ovaries or slices lightly blotted to remove excess of incubation medium was extracted in 20 vol. (v/w) of chloroform-methanol (2:1, v/v) by homogenization in a Kontes grinder (Kontes Glass Co., Vineland, U.S.A.). After centrifugation the homogenate was shaken with 0.3 vol. (v/v) of 4 mM-MgCl₂. The upper phase was removed by aspiration and discarded. The lower phase, after evaporation to dryness under a stream of O₂-free N₂ at 45°C, was used either for the separation of lipid components of whole ovaries by column chromatography on silicic acid for the assay of glyceride, phospholipid and sterol in incubated slices. For the assay of non-esterified fatty acid, tissue slices (30–60 mg) plus media (1.5 ml) were extracted by homogenization together in a Kontes grinder with 6 ml of propan-2-ol-*n*-heptane-conc. H₂SO₄ (40:10:1, by vol.) as described by Dole & Meinertz (1960).

Separation of lipid components of whole ovaries by

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column chromatography on silicic acid. The chloroform-methanol extract of whole ovaries (containing about 450mg of lipid from approx. 9g of tissue) was applied in *n*-heptane to a column (2cm × 24cm) of methanol- and ether-washed (Hirsch & Ahrens, 1958) silicic acid (50g). Elution involved a series of steps and gradients in sequence as follows: (a) 250ml of *n*-heptane; (b) a linear gradient of 10% (v/v) diethyl ether in *n*-heptane (500ml) run into *n*-heptane (500ml); (c) a linear gradient of diethyl ether (500ml) run into 10% (v/v) diethyl ether in *n*-heptane (500ml); (d) a linear gradient of methanol (500ml) run into chloroform (500ml); (e) 250ml of methanol. Linear gradients were generated in open glass containers and delivered to the column in an all-glass system. Peaks of material eluted from the column were detected by weighing after evaporation to dryness *in vacuo* in a pre-weighed flask. Each fraction was then assayed for sterol, glyceride and phospholipid. The recoveries of sterol (free plus esterified), glyceride (tri- plus di- plus mono-) and of phospholipid phosphorus were 92.2, 82.5 and 94.2% respectively of the molar quantities applied to the column.

Assay procedures. Sterols were assayed by a Lieberman-Burchard reaction as follows: chloroform (1ml) and conc. H₂SO₄-acetic anhydride (1:25, v/v) (2ml) were added to each lipid sample after evaporation to dryness under a stream of O₂-free N₂ at 45°. After mixing and standing for exactly 20min in the dark at room temperature the E₆₂₀ value was measured. The readings obtained were compared with the values given by standard solutions of cholesterol. For the assay of free and esterified sterols, total lipid extracts were chromatographed on silica gel G plates developed in benzene-ethyl acetate (19:1, v/v) (Flint & Denton, 1969). Silica gel was scraped from the plates in two bands, R_F 0.05–0.5 (containing free sterol) and R_F 0.5–1.0 (containing sterol ester) and lipid was extracted three times with 3ml of chloroform-methanol (2:1, v/v). The average recovery of total sterol after t.l.c. was 97.1 ± 1.57% (mean ± s.e.m. of four values). Sterol was assayed in each fraction as described above.

Glyceride was assayed as glyceride glycerol after removal of phospholipid with silicic acid, t.l.c. and saponification (Denton & Randle, 1967). Phospholipid was determined as lipid phosphorus (Denton & Randle, 1967).

Non-esterified fatty acid was assayed by the copper-chelation method of Duncombe (1963) as modified by Itaya & Ui (1965), after separation of the propan-2-ol-*n*-heptane-conc. H₂SO₄ extracts by t.l.c. on silica gel G plates developed in *n*-hexane-diethyl ether-methanol-acetic acid (90:20:2:3, by vol.) as described by Garland & Randle (1963). Since the recovery of palmitate added to the tissue plus medium during extraction was only 87 ± 2% (mean ± s.e.m. of four values) after t.l.c., standard samples of palmitate were subjected to t.l.c. and eluted by the same methods as were applied to the experimental samples.

RESULTS AND DISCUSSION

Column chromatography of whole-ovary lipid on silicic acid. The elution pattern obtained by chromatography on silicic acid of lipid extracted from whole ovaries consisted of five major peaks that accounted for 92% (w/w) of the lipid applied to the column.

Table 1. Rates of change of concentrations of lipid components of superovulated rat ovary slices during incubation *in vitro*

Initial concentrations of lipid components of slices are expressed as $\mu\text{mol/g}$ wet wt. (means ± s.e.m.). Rates of change of concentration of the lipid components during incubation are given as $\mu\text{mol/h}$ per g wet wt. (regression rates ± s.e.). * $P < 0.05$; ** $P < 0.005$ (significance of rate of change in lipid concentration; for other rates of change $P > 0.05$). The numbers of observations are given in parentheses.

Lipid component	Initial concn. ($\mu\text{mol/g}$)	Rate of change in concentration during incubation ($\mu\text{mol/h}$ per g)
Total sterol	58.8 ± 1.79 (4)	-3.02 ± 1.30* (20)
Sterol ester	51.8 ± 2.68 (4)	-6.18 ± 1.69** (20)
Free sterol	6.9 ± 0.22 (4)	+1.58 ± 0.15** (20)
Triglyceride	5.9 ± 0.43 (6)	-0.34 ± 0.21 (13)
Diglyceride	0.9 ± 0.06 (6)	+0.05 ± 0.03 (13)
Monoglyceride	0.2 ± 0.03 (6)	+0.04 ± 0.01* (12)
Phospholipid	29.4 ± 0.70 (3)	-0.41 ± 0.64 (11)
Non-esterified fatty acid	0.7 ± 0.12 (7)	+4.07 ± 0.27** (21)

Fig. 1. Sterol concentrations in slices of superovulated rat ovary *in vitro*. Slices were incubated in 5.0ml of bicarbonate-buffered medium containing glucose (1 mg/ml). Sterols were extracted from slices after incubation and separated by t.l.c. before assay. ●, Total sterol; ▲, sterol ester; ○, free sterol. Vertical bars indicate s.e.m.

These were shown by assay to be sterol ester, triglyceride, free sterol and phospholipid (two peaks) in order of elution. The small amounts of di- and monoglyceride eluted were detected by assay only. The total yields (expressed per g wet wt. of tissue extrac-

Fig. 2. Non-esterified fatty acid production by slices of superovulated rat ovary *in vitro*. Slices were incubated in bicarbonate-buffered medium containing glucose (1mg/ml) for various times. Non-esterified fatty acids in slices plus medium were extracted and separated from phospholipids by t.l.c. before assay. Vertical bars indicate S.E.M.

Table 2. Effect of incubation on the sterol and fatty acid content of the sterol ester fraction from superovulated rat ovary slices

Slices were incubated for 3 h in 5 ml of medium containing glucose (1mg/ml). Sterol ester was extracted before and after incubation and separated from triglyceride and non-esterified fatty acid by t.l.c. on silica gel G plates (0.5mm thick) developed in *n*-hexane-diethyl ether-methanol-acetic acid (90:20:2:3, by vol.). The sterol ester fraction, contaminated with less than 5% of the total glyceride fraction and with less than 2% of the total non-esterified fatty acid, was eluted with 2 × 5 ml of chloroform-methanol (2:1, v/v). After saponification sterol and fatty acid were assayed in the sterol ester fraction. Results are expressed as μmol/g wet wt. of slices extracted (means ± S.E.M. for four values in each case).

Slices	Slice content of sterol ester sterol	Slice content of sterol ester fatty acid	Fatty acid/sterol ratio
Non-incubated	50.7 ± 11.6	51.9 ± 8.9	1.1 ± 0.1
Incubated (3h)	37.0 ± 3.4	33.9 ± 1.4	0.9 ± 0.1

ted) were as follows: sterol ester, 47.3 μmol; free sterol, 8.5 μmol; triglyceride, 5.8 μmol; phospholipid, 30.4 μg-atoms of lipid phosphorus in two peaks (14.1 and 16.3 μg-atoms). Similarly high concentrations of sterol ester have been observed in other ovarian preparations (Herbst, 1967; Armstrong, 1968).

Changes in lipid concentrations in ovary slices during incubation. To identify the endogenous substrate utilized during incubation *in vitro*, measurements were made of the major lipid components in tissue slices incubated for various periods up to 3 or 4 h. The initial concentrations of the lipids found in tissue slices (Table 1) agreed closely with those found in the whole ovary. The rates of change (±S.E.) of the concentrations of the lipids were calculated by regression analysis (Draper & Smith, 1966) (Table 1). The observed concentrations of free and esterified sterols and of non-esterified fatty acids are shown in Figs. 1 and 2 respectively.

During incubation there was no significant change in the concentrations of triglyceride, diglyceride or phospholipid phosphorus. There was, however, a significant decrease in the concentration of sterol ester, at a rate approximately equal to 12% of the initial concentration/h of incubation. With this disappearance of sterol ester both the concentration of free sterol and that of non-esterified fatty acid increased significantly during incubation. There was also a small but significant increase in the concentration of monoglyceride.

The decrease in sterol ester concentration during incubation was considerably greater than the rise in concentration of free sterol; this is reflected in the

decrease in concentration of total sterol. The possibility was considered that this decrease might result from the conversion (without de-esterification) of sterol ester into a form unable to give the Lieberman-Burchard reaction. Sterol ester from non-incubated slices and from slices incubated for 3 h was therefore separated from triglyceride by t.l.c. and, after saponification, was assayed for sterol (by the Lieberman-Burchard reaction) and for fatty acid. There was close agreement between the amounts of sterol and the amounts of fatty acid released on saponification of the sterol ester fraction from both non-incubated slices and those incubated for 3 h (Table 2). These observations are consistent with a real loss of sterol ester during incubation and suggest that the disappearance of total sterol is the result of the metabolism of free sterol to products that do not give the Lieberman-Burchard reaction. The output of progestins can account for a small fraction of this apparent sterol loss, but the major metabolite has not been identified. A similar unaccountable disappearance of sterol has been observed in superovulated rabbit ovary after treatment with luteinizing hormone (Solod, Armstrong & Greep, 1966).

The phospholipid concentrations shown in Table 1 are based on the assay of phospholipid phosphorus and not phospholipid fatty acid; it is possible therefore that some fatty acid for oxidation could be derived from the phospholipids if appreciable amounts of lysophospholipids were formed from them during incubation. The values presented in Table 3, however, suggest that this does not occur. Phospholipids from non-incubated slices and from slices incubated for 3 h were separated by t.l.c. as

Table 3. *Effect of incubation on the content of the phospholipid fraction from superovulated rat ovary slices*

Slices were incubated for 3 h in 5 ml of medium containing glucose (1 mg/ml). Phospholipids were extracted before and after incubation and separated by t.l.c. on 0.5 mm-thick layers of silica gel H (suspended in 1.0 mm- Na_2CO_3 before spreading) developed in chloroform-methanol-acetic acid-water (25:15:4:2, by vol.) (Skipski, Peterson, & Barclay, 1964). After development the plates were dried and spots made visible in a tank of I_2 vapour. Phospholipids were eluted for subsequent phosphorus determinations (Denton & Randle, 1967) in 2×5 ml of methanol and evaporated to dryness under a stream of O_2 -free N_2 at 45°C . Results are expressed as μg -atoms of lipid phosphorus/g wet wt. of slices extracted (means $\pm$ s.e.m. for three determinations in each case).

Slices	Mean R_F of fraction:	Phosphorus content of fractions eluted from t.l.c. plates			Total lipid content
		0.15	0.35	0.9	
Non-incubated		2.1 ± 0.28	10.9 ± 0.40	11.4 ± 0.33	24.4 ± 0.29
Incubated (3h)		2.2 ± 0.17	10.6 ± 0.93	10.9 ± 1.01	23.7 ± 0.69
Incubated					
Non-incubated	ratio	1.0 ± 0.16	1.0 ± 0.09	1.0 ± 0.09	1.0 ± 0.03

described in Table 3. Three fractions were present with R_F values approx. 0.9, 0.35 and 0.15 (compared with R_F values approx. 0.9, 0.3 and 0.1 for phosphatidylethanolamine, phosphatidylcholine and lyso-phosphatidylcholine respectively). Elution and assay of these fractions showed that no change in either the relative or absolute amounts of these fractions occurred during incubation.

The decrease in the sterol ester concentration during incubation is presumably the result of de-esterification by the sterol ester hydrolase found in superovulated rat ovary by Coutts & Stansfield (1968). The release of fatty acid that would accompany the metabolism of sterol ester in this way is sufficient to account for the observed increases in concentrations of both non-esterified and mono-glyceride fatty acid and leave an excess of $2.07 \mu\text{mol}$ of fatty acid/h per g wet wt. of slices. The oxidation of this excess would require $48.5 \mu\text{mol}$ of oxygen/h per g (assuming the fatty acid to be palmitate) or $53.8 \mu\text{mol}$ of oxygen/h per g (assuming it to be stearate). This accounts for between 50 and 60% of the oxygen uptake of slices incubated under the conditions used in this study; a further 20% is accounted for by the oxidation of glucose (Flint & Denton, 1969).

The results described above show that sterol esters (which are found in high concentrations in ovarian preparations) are metabolized at an appreciable rate during incubation of superovulated rat ovary slices *in vitro*. Moreover, fatty acids released by this metabolism appear to be the main endogenous fuel for respiration in this tissue. This is in distinct

contrast with other mammalian tissues and presumably is a reflection of the important role of sterol esters in the metabolism of a tissue that is principally involved with the synthesis, storage and release of steroids.

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