Biochem. J. (1973) **132**, 313–321
Printed in Great Britain

Control of Ovarian Cholesterol Ester Biosynthesis

By A. P. F. FLINT*, D. L. GRINWICH and D. T. ARMSTRONG
Departments of Physiology and of Obstetrics and Gynaecology, Health Sciences Centre, University of Western Ontario, London, Ont., Canada

(*Received* 6 *September* 1972)

1. Experimental evidence is presented for a role of progesterone and 20α-hydroxypregn-4-en-3-one as inhibitors of cholesterol ester synthetase in the acute depletion of ovarian cholesterol ester after trophic stimulation. 2. Luteinizing hormone *in vitro* decreased by 84% the rate of esterification of cholesterol with added [^{14}C]oleate by slices of rabbit ovarian interstitial tissue; this effect was mimicked by cyclic AMP (adenosine 3′:5′-cyclic monophosphate) *in vitro*, and occurred without large changes in precursor pool sizes or membrane permeability. 3. Cyclic AMP was shown to have no direct effect on cholesterol ester synthetase or cholesterol esterase in cell-free extracts of rabbit ovarian interstitial tissue, but decreased the activity of cholesterol ester synthetase (not that of cholesterol esterase) in extracts prepared from slices previously incubated with it. 4. The inhibitory effect of cyclic AMP on esterification of cholesterol with added [^{14}C]-oleate was prevented by both cycloheximide and aminoglutethimide phosphate (which also inhibited steroid synthesis in response to cyclic AMP). 5. Cyclic AMP raised the intracellular concentrations of progesterone and 20α-hydroxypregn-4-en-3-one in incubated slices by factors of 2.8 and 3.9 respectively. 6. Cycloheximide and aminoglutethimide phosphate administered *in vivo* blocked cholesterol ester depletion in response to luteinizing hormone in rats; in these ovaries cycloheximide and aminoglutethimide phosphate decreased the concentrations of progesterone and 20α-hydroxypregn-4-en-3-one and luteinizing hormone raised them. 7. Progesterone and 20α-hydroxypregn-4-en-3-one added to cell-free extracts of rabbit ovarian interstitial tissue *in vitro* (at concentrations comparable with those found in incubated slices) inhibited cholesterol ester synthetase by up to 85%. 8. The results are discussed with reference to the acute control of cholesterol ester concentrations in the ovary and adrenal cortex.

Trophic stimulation of the ovary in many mammalian species results in rapid loss of cholesterol ester from the gland (Claesson & Hillarp, 1947; Claesson *et al.*, 1953). Whether this depletion is the result of inhibited formation or of increased degradation is unknown; in view of the apparently rapid turnover of ovarian cholesterol ester (Flint & Armstrong, 1972) it appears that either effect (or both) may be responsible. Behrman and his colleagues (Behrman & Armstrong 1969; Behrman *et al.*, 1970*a*) have shown that the total activities of cholesterol esterase and cholesterol ester synthetase are increased and decreased respectively in rat ovarian tissues under conditions of decreased cholesterol ester storage (e.g. by administration of luteinizing hormone). Such chronic effects might be expected to result in a decreased concentration of cholesterol ester in the tissue. However, it appears other factors may also act to decrease the ovarian cholesterol ester concentration since incorporation of labelled long-chain fatty acid into cholesterol ester (which probably reflects the rate of esterification of cholesterol) is inhibited when steroid synthesis is stimulated *in vitro* (Armstrong, 1968; Armstrong *et al.*, 1969). The degree to which this inhibition is responsible for acute cholesterol ester depletion *in vivo*, and the mechanism by which it is brought about, are the subjects of the investigation reported here.

* Present address: Department of Obstetrics and Gynaecology, Welsh National School of Medicine, University Hospital of Wales, The Heath, Cardiff CF4 4XN, U.K.

Methods

Incubation and extraction procedures

Slices of rabbit ovarian interstitial tissue (30–400mg) were incubated in 2.5 or 5.0ml of a bicarbonate-buffered medium, pH7.4 (Krebs & Henseleit, 1932), containing additions as indicated in the text and tables, for periods up to 2h. On addition of cyclic AMP (as the free acid) to incubation media the pH was corrected (to 7.4) with NaOH. Incubations were terminated in one of three ways, as follows. For the determination of rates of steroid synthesis and incorporation of ^{14}C from [^{14}C]oleate into lipids, slices were removed from the medium and homogenized in 10ml of chloroform–methanol (2:1 v/v), the phases

of the extraction mixture then being split by addition of the incubation medium (Flint & Armstrong, 1973). For determination of intracellular concentrations of non-esterified fatty acids, long-chain fatty acyl-CoA and steroids in incubated tissue, slices were removed after incubation by sieving on to a grid or mesh, lightly blotted and plunged into liquid N_2. Frozen slices were subsequently weighed into homogenization medium, without prior thawing, and were homogenized either in water (for the assay of non-esterified fatty acid), chloroform–methanol (2:1, v/v) (for the assay of steroids) or 5% (w/v) $HClO_4$, (for the assay of long-chain acyl-CoA). For the determination of enzyme activities, slices were removed from the incubation medium and homogenized in 20–40vol. (w/v) of either 20mM-potassium phosphate buffer, pH7.0 (for assay of cholesterol esterase), or 0.1M-Tris–HCl buffer, containing GSH (5mM), EGTA [ethanedioxybis(ethylamine)tetra-acetate] (1mM) and defatted bovine plasma albumin (1mg/ml), pH7.4 (for assay of cholesterol ester synthetase).

Lipids were extracted from whole tissue (70–300mg) in 10ml of chloroform–methanol (2:1, v/v). The extracts were split into phases by the addition of 2ml of 0.9% (w/v) NaCl; the lower (chloroform) phase was subsequently evaporated to dryness and used for sterol and steroid analyses. Cholesterol esterase and cholesterol ester synthetase were extracted from whole tissue as described above for slices.

Assay methods

Cholesterol (unesterified and esterified), progesterone, 20α-hydroxypregn-4-en-3-one and non-esterified fatty acids were measured in lipid extracts by using standard methods for their separation, saponification and assay (Flint & Armstrong, 1973). Long-chain fatty acyl-CoA (i.e. acid-insoluble CoA thioester) was assayed fluorimetrically as free CoA in extracts from incubated tissue slices (200–400mg) with 2-oxoglutarate dehydrogenase, 2-oxoglutarate and NAD^+ (Garland *et al.*, 1965) after hydrolysis with alkali (Denton & Halperin, 1968). Recovery of palmitoyl-CoA (5–20nmol/250mg of tissue) added at the time of homogenization was 91±11% (mean±S.E.M. for five determinations).

Intra- and extra-cellular water spaces in incubated slices were determined by radioactivity assay of samples of neutralized $HClO_4$ extracts (of slices and media) after incubation of tissue in medium containing ^3H_2O and [^{14}C]sorbitol; these samples were counted for radioactivity by liquid-scintillation spectrometry in a scintillation fluid consisting of 2,5-diphenyloxazole (7.5g), 1,4-bis-(5-phenoxazol-2-yl)benzene (75mg) and naphthalene (120g) in dioxan (1 litre). Non-aqueous samples were counted for radioactivity as described by Flint & Armstrong (1971).

Cholesterol esterase activity was assayed in a supernatant fraction obtained by differential centrifugation, by using as substrate cholesterol ester-containing lipid granules simultaneously obtained by flotation, and then following release of unesterified cholesterol or non-esterified fatty acid in subsequent incubations (Flint & Armstrong, 1973). Cholesterol ester synthetase activity was assayed in whole homogenates by following the incorporation of ^{14}C from [1-^{14}C]-palmitoyl-CoA into cholesterol ester (Flint & Armstrong, 1973). Steroids were added to the cholesterol ester synthetase reaction mixture (total volume 2ml) dissolved in 25μl of ethanol; this amount of ethanol had no effect on the activity of the enzyme.

Administration of materials in vivo

Luteinizing hormone (10μg; NIH-LH-S7, ovine, gift of the National Institutes of Health, Bethesda, Md., U.S.A.) and cycloheximide (5mg) were administered intravenously in 0.4ml of 0.9% (w/v) NaCl; aminoglutethimide phosphate (30mg) was administered subcutaneously, dissolved in 0.4ml of 0.05M-acetic acid. Solutions of aminoglutethimide phosphate were prepared within 0.5h of use. In all cases, control animals received the solvents as appropriate.

Statistical treatment of results

The 2×2 factorial design experiments described in Tables 4, 5 and 6 were analysed statistically by analysis of variance; statistical differences between other means were tested by Student's *t* test.

Materials

Ovarian interstitial tissue was obtained from oestrous New Zealand White rabbits (3–4kg) which had been kept in isolation for 21 days after arrival in the laboratory. Luteinized ovaries were obtained from Wistar rats treated with pregnant-mare serum gonadotrophin (50i.u.) followed 65h later by human chorionic gonadotrophin (25i.u.) as described by Parlow (1958). They were used 5–9 days after administration of the human chorionic gonadotrophin.

^3H_2O (5mCi/ml) and [U-^{14}C]sorbitol (3mCi/mmol) were obtained from Amersham/Searle Corp., Toronto, Ont., Canada; sources and specific radioactivities of other radioactive compounds used are given in the preceding paper (Flint & Armstrong, 1973). Pregnant-mare serum gonadotrophin and human chorionic gonadotrophin were obtained from

Ayerst Laboratories Inc., New York, N.Y., U.S.A., through their office in Montreal, P.Q., Canada. 2-Oxoglutarate dehydrogenase was prepared from pig heart as described by Sanadi *et al.* (1952) and palmitoyl-CoA from palmitoyl chloride and CoA by the method of Seubert (1960). Cycloheximide was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Aminoglutethimide phosphate [2-ethyl-2-(*p*-aminophenyl)glutarimide] was from Ciba Pharmaceutical Co., Summit, N.J., U.S.A., through the kindness of Dr. J. J. Chart.

Results

Effects of luteinizing hormone and cyclic AMP on esterification of [1-^{14}C]oleate in vitro

Slices of rabbit ovarian interstitial tissue incorporated ^{14}C from [1-^{14}C]oleate into all lipid fractions investigated (Table 1). Incorporation into cholesterol derived from cholesterol ester and unesterified cholesterol probably reflects synthesis of cholesterol from [^{14}C]acetate derived from [^{14}C]oleate by β-oxidation; incorporation into other fractions is probably due primarily to incorporation as oleate. Incorporation of oleate carbon into fatty acid derived from cholesterol ester was decreased to 16% of the control value by luteinizing hormone *in vitro*, and this effect was mimicked by cyclic AMP. Neither luteinizing hormone nor cyclic AMP had any effect on incorporation into triglyceride, unesterified cholesterol or phospholipid; any inhibitory effect (not significant in the experiment cited) on incorporation into cholesterol derived from cholesterol ester probably reflects the diminished rate of esterification of cholesterol, in view of the lack of effect on incorporation of ^{14}C into unesterified cholesterol.

It appeared possible that the apparent inhibitory effects of luteinizing hormone and cyclic AMP on esterification may be caused by decreased specific radioactivities of precursors resulting from changes in the intracellular concentrations of fatty acid, or [since incorporation of fatty acid into fatty acid derived from cholesterol ester involves fatty acyl-CoA formation before esterification (Mukherjee *et al.*, 1958; Longcope & Williams, 1963; Behrman *et al.*, 1970*a*)] of long-chain fatty acyl-CoA. Changes in the permeability of the slices to fatty acid in the medium may also influence rates of uptake of labelled oleate and, hence, its esterification. To investigate these possibilities, non-esterified fatty acid and long-chain fatty acyl-CoA were assayed in slices of ovarian interstitial tissue incubated with and without cyclic AMP. Cyclic AMP affected neither extracellular or intracellular water spaces, nor the extracellular or intracellular fatty acid concentration (Table 2). Rates of production of non-esterified fatty acid by these slices (calculated from production during 2h incubations, and corrected for initial fatty acid content of the tissue) were not altered by incubation with cyclic AMP; the rate of production in control slices was 2.43±0.44μmol/h per g wet wt.; in the presence of 10mM-cyclic AMP it was 2.53±0.23μmol/h per g wet wt. (means±S.E.M. for eight determinations in each case). Long-chain fatty acyl-CoA concentrations in slices incubated for 1h were: 11.4±0.9nmol/g wet wt. in control slices and 15.0±0.5nmol/g wet wt. in slices incubated with cyclic AMP (means±S.E.M. for six determinations in each case; $P<0.005$). This increase (32%) is unlikely to be high enough to cause the decrease (84%) in incorporation of oleate carbon

Table 1. *Metabolism of [1-^{14}C]oleate in vitro by slices of rabbit ovarian interstitial tissue: effects of luteinizing hormone and cyclic AMP*

Slices of rabbit ovarian interstitial tissue were incubated for 2h in 2.5ml of bicarbonate-buffered medium containing glucose (1mg/ml), [1-^{14}C]oleate (1μCi/ml; 56.2μCi/μmol), bovine plasma albumin (0.36mg/ml) and ethanol (9.8mM); treatment flasks also contained either luteinizing hormone or cyclic AMP. After incubation slices and media were extracted together and lipid extracts separated by t.l.c. into the fractions listed; cholesterol ester was saponified. Values are $10^{-3}\times$d.p.m./g wet wt. of tissue (means±S.E.M. of three determinations). * $P<0.01$.

Additions to incubation medium	$10^{-3}\times$Incorporation of ^{14}C (d.p.m./2h per g) from [1-^{14}C]oleate into: Cholesterol derived from cholesterol ester	Fatty acid derived from cholesterol ester	Triglyceride	Unesterified cholesterol	Phospholipid
None	68±18	800±107	193±54	151±77	1474±250
Luteinizing hormone (10μg/ml)	25±17	129± 43*	148±60	228±27	1133±119
Cyclic AMP (10mM)	75±61	198± 58*	134±61	188±43	1232± 24

Table 2. *Effect of cyclic AMP in vitro on ^3H_2O and [^{14}C]sorbitol spaces and on intracellular non-esterified fatty acid concentration in interstitial tissue slices*

Slices of rabbit ovarian interstitial tissue were incubated for 1.5h in 5ml of bicarbonate-buffered medium containing glucose (1mg/ml), ^3H_2O (0.2μCi/ml) and [U-^{14}C]sorbitol (0.5mg/ml; 0.1μCi/ml) with and without cyclic AMP. After incubation, slices were removed from the medium, rapidly frozen, and homogenized in water before extraction with either $HClO_4$ or Dole's solvent (Dole & Meinertz, 1960) for determination of radioactivity or non-esterified fatty acid. Spaces were calculated from determinations of ^{3}H and ^{14}C radioactivity by the method of Randle & Smith (1958), and are expressed as μl/g wet wt. Non-esterified fatty acid was assayed in extracts of slices and media, and concentrations are expressed as μmol/ml of intracellular water or medium. Intracellular concentrations are corrected for non-esterified fatty acid content of the extracellular water. All values are means±S.E.M. of six determinations. $P>0.05$ for all differences between treatment groups.

Spaces (μl/g of tissue)	Control slices	Slices incubated with cyclic AMP (10mM)
Total water space (TW)	871±10	894±18
Extracellular water space (EW)	542± 7	558±18
Intracellular water space (TW−EW)	329± 6	336± 3
Concentration of non-esterified fatty acid (mM)		
In medium	0.062±0.007	0.063±0.007
In slices	25.9 ±1.8	29.6 ±5.7

into fatty acid derived from cholesterol ester observed with cyclic AMP. It is unlikely therefore that the observed effects of luteinizing hormone and cyclic AMP were due to changes in pool size. The observation that neither luteinizing hormone nor cyclic AMP had an effect on incorporation of oleate carbon into triglyceride, phospholipid and unesterified cholesterol suggests there is no change in the permeability of the slices to oleate, and confirms the observed lack of effect on pool sizes.

Effect of cyclic AMP on cholesterol esterase and cholesterol ester synthetase in cell-free extracts in vitro

Since trophic stimulation is known to activate adenylate cyclase and raise the intracellular concentration of cyclic AMP in a number of steroidogenic tissues (Haynes, 1958; Marsh *et al.*, 1966; Grahame-Smith *et al.*, 1967; Dorrington & Baggett, 1969) it was considered possible that cyclic AMP might have a direct effect on cholesterol ester synthetase or cholesterol esterase. A direct stimulatory effect on cholesterol esterase could result in an apparent inhibition of esterification by causing rapid hydrolysis of recently formed cholesterol [1-^{14}C]oleate. However, addition of cyclic AMP to assay systems for cholesterol ester synthetase and cholesterol esterase activities, at a concentration comparable with those found in stimulated steroidogenic tissues, had no effect on either enzyme from rabbit ovarian interstitial tissue (Table 3).

To determine whether there was any effect on these enzymes when they were extracted from slices previously incubated with cyclic AMP, slices (30–70mg, incubated for 1h) were homogenized in 1.5ml of extraction medium and the homogenates used for enzyme assays. No effect on cholesterol esterase activity was observed after treatment of slices with cyclic AMP *in vitro*; the activity of cholesterol ester synthetase decreased from 2.66±0.26μmol/h per g wet wt. in homogenates from control slices to 1.55±0.16μmol/h per g wet wt. in homogenates from slices treated with cyclic AMP (means±S.E.M. for nine determinations in each case; $P<0.005$). It was therefore concluded that cyclic AMP has no direct effect on cholesterol esterase or cholesterol ester synthetase activities, but causes production of one (or more) inhibitor(s) of the latter enzyme when incubated with a whole-cell preparation.

The lack of a direct effect of cyclic AMP observed here on cholesterol esterase activity from rabbit ovarian interstitial tissue confirms the finding of Behrman & Armstrong (1969) on the enzyme from luteinized rat ovarian tissue.

Effects of cycloheximide and aminoglutethimide phosphate on inhibition of esterification by cyclic AMP

Aminoglutethimide phosphate (Kahnt & Neher, 1966; Camacho *et al.*, 1967; Wilroy *et al.*, 1968; Johnston *et al.*, 1968) and inhibitors of protein synthesis (Ferguson, 1963; Farese, 1964; Savard *et al.*, 1965; Gorski & Padnos, 1966; Davis & Garren, 1968) prevent trophic stimulation of steroidogenesis in a wide variety of steroid-synthesizing tissues. To

Table 3. *Effects of cyclic AMP on activities of cholesterol ester synthetase and cholesterol esterase in vitro*

Enzyme assays were performed on extracts of rabbit ovarian interstitial tissue as described in the Methods section in the presence of various concentrations of cyclic AMP, with and without theophylline. Activities are given as μmol/h per g wet wt. of tissue, as means±S.E.M. of at least three determinations. $P>0.05$ for all differences between treatment groups. —, Not determined.

Additions (μM)		Enzyme activity (μmol/h per g)	
Theophylline	Cyclic AMP	Cholesterol ester synthetase	Cholesterol esterase
0	0	0.98±0.26	—
500	0	1.11±0.19	0.92±0.14
500	10	1.39±0.49	—
500	25	0.88±0.09	1.46±0.23
500	50	1.04±0.20	1.18±0.49
500	100	1.15±0.41	1.31±0.24

Table 4. *Effects of cycloheximide and aminoglutethimide phosphate on the cyclic AMP-induced inhibition of [1-^{14}C]oleate esterification and stimulation of steroid synthesis in rabbit ovarian interstitial-tissue slices in vitro*

Slices of rabbit ovarian interstitial tissue were incubated for 2h in 2.5ml of bicarbonate-buffered medium containing glucose (1mg/ml), [1-^{14}C]oleate (1μCi/ml; 56.2μCi/μmol), bovine plasma albumin (0.36mg/ml) and ethanol (9.8mM); treatment flasks also contained cyclic AMP, cycloheximide or aminoglutethimide phosphate alone or in combination. After incubation, slices and media were homogenized together; cholesterol ester was saponified and steroids assayed after separation from other lipids in the total lipid extract by t.l.c. Values for oleate incorporation are $10^{-3}\times$d.p.m. incorporated/g wet wt. of tissue; values for steroid synthesis are nmol synthesized/g wet wt. of tissue (means±S.E.M. for four determinations in each case; both parameters are corrected for zero-time controls and are per 2h). Results of statistical analyses (by analysis of variance) are given in the text.

Experiment no.	Additions to incubation medium	$10^{-3}\times$Incorporation of ^{14}C (d.p.m./2h per g) from [^{14}C]oleate into fatty acid derived from cholesterol ester	Steroid synthesis (nmol/2h per g)	
			Progesterone	20α-Hydroxypregn-4-en-3-one
1	None	940± 84	2± 4	96± 10
	Cyclic AMP (10mM)	152± 36	124±16	1104±132
	Cycloheximide (0.5mg/ml)	868±116	20±10	44± 8
	Cyclic AMP (10mM)+cycloheximide (0.5mg/ml)	881± 80	2± 2	10± 2
2	None	247± 42	86±30	132± 8
	Cyclic AMP (10mM)	92± 9	240±34	556± 48
	Aminoglutethimide phosphate (0.2mg/ml)	267± 70	50±18	8± 2
	Cyclic AMP (10mM)+aminoglutethimide phosphate (0.2mg/ml)	231± 6	30± 8	10± 10

determine whether cyclic AMP inhibits esterification when its effect on steroid synthesis is blocked, rabbit ovarian interstitial-tissue slices were incubated with and without cyclic AMP and these inhibitors (Table 4).

Addition of cyclic AMP to the incubation medium resulted in increased rates of synthesis of both progesterone and 20α-hydroxypregn-4-en-3-one ($P<0.02$ in all cases) and inhibited by up to 84% the incorporation of oleate carbon into fatty acid derived from cholesterol ester ($P<0.02$). Both these effects of cyclic AMP were prevented by either cycloheximide or aminoglutethimide phosphate *in vitro*. These compounds not only prevented an increase in steroid

synthesis in response to cyclic AMP, but also decreased basal rates of synthesis of total steroid (i.e. progesterone+20α-hydroxypregn-4-en-3-one) ($P<0.01$, in both cases). This indicated that esterification was prevented as a result of the production of an inhibitor, presumably produced later in the chain of events initiated by cyclic AMP than the sites of action of either cycloheximide or aminoglutethimide phosphate: if it were produced before one of these sites the effect on esterification would not be expected to be blocked by both compounds. Since aminoglutethimide acts late in this sequence [it is reported to be a specific inhibitor of cholesterol side-chain cleavage enzyme (Kahnt & Neher, 1966; Dexter *et al.*, 1967*b*)], this suggests that a steroid may be the inhibitory substance.

Concentrations of progesterone and 20α-hydroxypregn-4-en-3-one in slices incubated with and without cyclic AMP

Intracellular concentrations of progesterone and 20α-hydroxypregn-4-en-3-one in rabbit ovarian interstitial-tissue slices increased on incubation with cyclic AMP. Progesterone and 20α-hydroxypregn-4-en-3-one concentrations were: in control slices, 30±5.4 and 84±11.9nmol/g wet wt. of slices respectively; in slices incubated with 10mM-cyclic AMP they were 56±7.5 and 216±15.5nmol/g wet wt. respectively (means±S.E.M. of five determinations; values corrected for steroid content of the medium comprising the extracellular space on the basis of the values reported in Table 2; slices were incubated for 1h).

Inhibition by progesterone and 20α-hydroxypregn-4-en-3-one of cholesterol ester synthetase activity in cell-free extracts

Fig. 1 shows the effects of progesterone and 20α-hydroxypregn-4-en-3-one *in vitro* on cholesterol ester synthetase activity in a whole homogenate of rabbit ovarian interstitial tissue. Enzyme activity was decreased to approx. 20% of the uninhibited value in the presence of 50μM-progesterone or 20α-hydroxypregn-4-en-3-one. This steroid concentration is considerably lower than that found in incubated slices; assuming the steroids are uniformly distributed throughout the total water space in tissue slices (see Table 2) the values given above for progesterone and 20α-hydroxypregn-4-en-3-one contents of rabbit ovarian tissue slices *in vitro* give values of 34 and 64μM for progesterone and 20α-hydroxypregn-4-en-3-one in control slices and of 95 and 245μM respectively in slices incubated with 10mM-cyclic AMP. The actual steroid concentrations are likely to be considerably higher in some parts of the cell, in view of the limited solubility of these compounds.

Effects of cycloheximide and aminoglutethimide phosphate on luteinizing hormone-induced depletion of cholesterol ester in vivo

It was concluded from the experiments with rabbit ovarian interstitial tissue (Tables 1 and 2) that the incorporation of oleate carbon into fatty acid derived from cholesterol ester *in vitro* is a reliable index of the rate of esterification of cholesterol. Since cholesterol esters appear to be in a state of rapid turnover in ovarian tissue *in vivo* (Flint & Armstrong, 1972), it seems likely that the inhibition of esterification observed with luteinizing hormone or cyclic

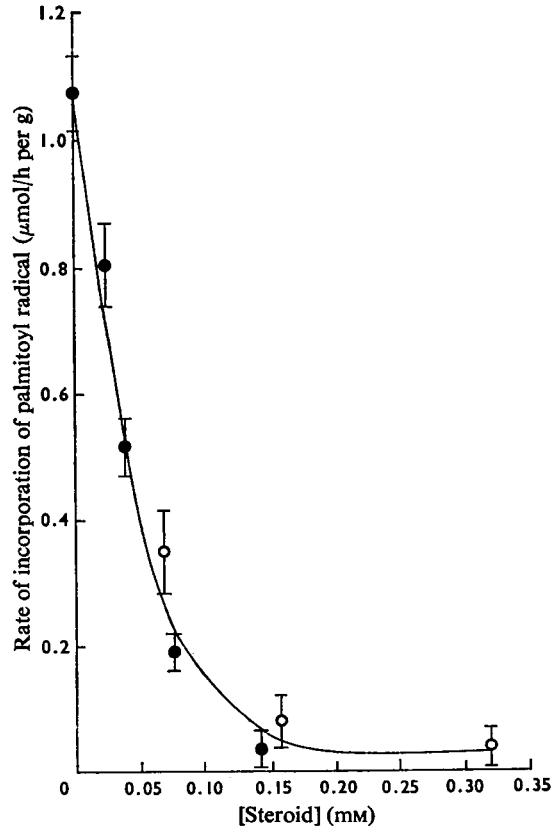

Fig. 1. *Effects of progesterone and 20α-hydroxypregn-4-en-3-one on cholesterol ester synthetase activity in extracts of rabbit ovarian interstitial tissue*

Cholesterol ester synthetase activity was assayed by following incorporation of ^{14}C from [1-^{14}C]palmitoyl-CoA into cholesterol ester in the presence of various concentrations of: ●, progesterone; ○, 20α-hydroxypregn-4-en-3-one. Values are means of at least three determinations; vertical bars indicate S.E.M.

Table 5. *Effects of luteinizing hormone and cycloheximide in vivo on cholesterol contents of luteinized rat ovaries*

Rats with luteinized ovaries were given luteinizing hormone (10μg) or cycloheximide (5mg) or both in 0.4ml of 0.9% (w/v) NaCl intravenously 2h before autopsy. Ovaries were removed at autopsy and extracted in chloroform–methanol (2:1, v/v). Cholesterol ester and unesterified cholesterol were assayed after separation by t.l.c. Values are μmol/g wet wt. of tissue (means±S.E.M. for four determinations). Results of statistical analyses (by analysis of variance) are given in the text.

	Ovarian cholesterol content (μmol/g)	
Treatment	Unesterified	Esterified
None	13.5±0.9	27.1±1.8
Luteinizing hormone	11.5±0.2	9.7±1.6
Cycloheximide	20.3±2.3	28.2±3.1
Luteinizing hormone+cycloheximide	18.9±0.7	27.2±3.1

Table 6. *Effects of luteinizing hormone and aminoglutethimide phosphate in vivo on cholesterol and steroid contents of luteinized rat ovaries*

Rats with luteinized ovaries were given luteinizing hormone (10μg, intravenously, 2h before autopsy) or aminoglutethimide phosphate (30mg, subcutaneously, 5.5h before autopsy) or both. Cholesterol and steroids were extracted from ovaries at autopsy and assayed after separation by t.l.c. Values for cholesterol are given as μmol/g wet wt., and those for steroids as nmol/g wet wt. (means±S.E.M. for eight determinations). Results of statistical analyses (by analysis of variance) are given in the text.

	Ovarian cholesterol contents (μmol/g wet wt.)		Ovarian steroid contents (nmol/g wet wt.)	
Treatment	Unesterified	Esterified	Progesterone	20α-Hydroxypregn-4-en-3-one
None	8.1±0.4	22.4±3.0	28.2±9.3	6.7±2.6
Luteinizing hormone	8.7±0.5	10.1±1.9	77.2±7.8	20.8±3.4
Aminoglutethimide phosphate	10.4±1.1	35.6±5.1	5.4±1.0	3.8±1.0
Luteinizing hormone+aminoglutethimide phosphate	14.4±0.7	37.4±3.0	9.4±1.7	4.8±0.8

AMP *in vitro* may be responsible at least in part for the depletion of cholesterol ester observed in response to luteinizing hormone *in vivo*. As this effect *in vitro* appeared to be mediated via increased concentrations of steroids, it was decided to examine the effects of cycloheximide and aminoglutethimide phosphate on cholesterol ester depletion *in vivo*. Luteinized rat ovaries were used in this investigation because more uniform responses, in terms of cholesterol ester depletion, can be obtained with luteinizing hormone in this tissue preparation (A. P. F. Flint & D. T. Armstrong, unpublished work).

Table 5 shows the effect of cycloheximide on the disappearance of ovarian cholesterol ester in response to luteinizing hormone. Luteinizing hormone decreased the cholesterol ester concentration to 36% of the control value ($P<0.001$) within 2h of administration; this effect was blocked by cycloheximide. Cycloheximide had no effect on cholesterol ester concentration, but raised that of unesterified cholesterol ($P<0.01$).

Administration of aminoglutethimide phosphate also blocked cholesterol ester depletion in response to luteinizing hormone (Table 6). Concentrations of both cholesterol ester and unesterified cholesterol were raised in response to aminoglutethimide phosphate ($P<0.01$ for the effects on both unesterified and esterified cholesterol). In this experiment total tissue concentrations of progesterone and 20α-hydroxypregn-4-en-3-one were increased by luteinizing hormone ($P<0.005$ and $P<0.01$ respectively) and decreased by aminoglutethimide phosphate ($P<0.01$ in each case).

Discussion

Two points seem of importance in assessing the results presented. First, the interpretation offered below depends heavily on the assumption that cholesterol esters are in a continual state of rapid turnover, and that cholesterol ester depletion caused *in vivo* by luteinizing hormone may therefore result from

either inhibition of esterification or activation of de-esterification. Evidence in support of this assumption has been obtained in rat ovarian tissue after intravenous administration of [^{14}C]cholesterol, when the specific radioactivity of the cholesterol ester in the tissue reaches that of the unesterified cholesterol within 8h of administration of labelled material (Flint & Armstrong, 1971, 1972). Secondly, the results obtained *in vitro* can be interpreted as indicating control of esterification only to the extent that rates of incorporation of oleate carbon into cholesterol ester are an indication of the relative rates of esterification of cholesterol. In the absence of large changes in membrane permeability or concentrations of metabolites between oleate and cholesterol ester (Tables 1 and 2), alterations in rates of incorporation of oleate probably reflect alterations in rates of cholesterol esterification.

In the light of these assumptions the results of the present investigation seem to indicate the following. The inhibition of esterification of cholesterol by luteinizing hormone *in vitro* can be mimicked by cyclic AMP, and the inhibitory effect of cyclic AMP is prevented by inhibition of the synthesis of steroids from cholesterol. Inhibitors of steroid synthesis block the luteinizing hormone-induced cholesterol ester depletion *in vivo*, possibly by preventing inhibition of cholesterol ester synthetase. That these effects of trophic stimulation may be due to increased intracellular concentrations of steroids is indicated by the inhibitory effects of progesterone and 20α-hydroxypregn-4-en-3-one on cholesterol ester synthetase in cell-free extracts. Effects of other steroids have not been ruled out.

The conclusion that cholesterol esterification is controlled by steroid concentration is strengthened by the observation, made here and previously by Behrman *et al.* (1970*b*) and Wilks *et al.* (1970), that aminoglutethimide phosphate raises the ovarian cholesterol ester concentration. In the light of the present findings, this effect can be attributed to a decrease in the concentration of steroids in the tissue, resulting in an increased activity of cholesterol ester synthetase by de-inhibition. In contrast to the effect of aminoglutethimide phosphate on cholesterol ester concentrations *in vivo*, the administration of cycloheximide did not increase cholesterol ester storage. This may be because the animals were in contact with aminoglutethimide phosphate for longer than they were with cycloheximide (5.5h as opposed to 2h).

In the present study aminoglutethimide phosphate was found to block cholesterol ester depletion in response to luteinizing hormone *in vivo*. In similar experiments (conducted with a different strain of rat) Behrman *et al.* (1970*b*) found that it did not. A possible explanation for this difference in response is that in the latter experiments the effect of aminoglutethimide phosphate (given 5.5h previously) may have been wearing off, allowing an increase in steroid concentrations in response to luteinizing hormone. In our hands, the rats began recovering from the apparent sedative effect of aminoglutethimide phosphate 4–5h after its administration. The inhibitory effect of aminoglutethimide phosphate on cholesterol ester depletion described here when luteinizing hormone is administered 2h before autopsy has been confirmed when luteinizing hormone is given either 0.5h or 4h before autopsy, with aminoglutethimide phosphate administered 5.5h before autopsy in all regimens (A. P. F. Flint & D. T. Armstrong, unpublished work), and has also been observed in both intact and hypophysectomized immature rats by Zarrow & Clark (1969).

Some of the experiments described here have also been performed with adrenal cortex. The adrenocorticotrophin-induced depletion of cholesterol ester *in vivo* (Popják, 1944; Long, 1947) is blocked by cycloheximide (Davis & Garren, 1968) and aminoglutethimide phosphate (Dexter *et al.*, 1967*a*). Aminoglutethimide phosphate also causes rapid accumulation of cholesterol in the adrenal cortex and prevents the increase in steroid concentration in the gland normally associated with the administration of adrenocorticotrophin (Dexter *et al.*, 1967*b*). Studies with adrenal cortex *in vivo* therefore support those reported here with ovary, and suggest that a similar mechanism may operate to control cholesterol ester synthetase in other steroidogenic tissues.

The findings presented here can be interpreted as suggesting a role for steroids in the acute control of cholesterol ester metabolism. The chronic control of esterification and de-esterification of ovarian cholesterol appears to be through effects of luteinizing hormone and prolactin on the total activities of cholesterol esterase and cholesterol ester synthetase (Behrman & Armstrong, 1969; Behrman *et al.*, 1970*a*).

Thus the fall in cholesterol ester concentration observed in luteal elements of the ovary after hypophysectomy (King *et al.*, 1949; Armstrong, 1968), and the stimulation of storage by prolactin and of depletion by luteinizing hormone (Claesson & Hillarp, 1947; King *et al.*, 1949), can be accounted for by the effects of hypophysectomy and replacement therapy on the activities of these enzymes. The acute control of cholesterol esterification suggested by the results presented here neither limits the importance or validity of chronic effects such as these, nor overrules a possible role for an activation of cholesterol esterase in the depletion mechanism.

This work was supported by grants from the U.S. National Institutes of Health (Contract no. NIH-69-2165), the Canadian Medical Research Council (Grant no. MT-3392) and the Ford Foundation (Grant no. 710-0303).

References

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

Armstrong, D. T., Jackanicz, T. M. & Keyes, P. L. (1969) in *The Gonads* (McKerns, K. W., ed.), pp. 3–21, Appleton–Century–Crofts, New York

Behrman, H. R. & Armstrong, D. T. (1969) *Endocrinology* **85**, 474–480

Behrman, H. R., Orczyk, G. P., Macdonald, G. J. & Greep, R. O. (1970*a*) *Endocrinology* **87**, 1251–1256

Behrman, H. R., Armstrong, D. T. & Greep, R. O. (1970*b*) *Can. J. Biochem.* **48**, 881–884

Camacho, A. M., Cash, R., Brough, A. J. & Wilroy, S. R. (1967) *J. Amer. Med. Ass.* **202**, 20–26

Claesson, L. & Hillarp, N.-Å. (1947) *Acta Physiol. Scand.* **14**, 102–119

Claesson, L., Hillarp, N.-Å. & Högberg, B. (1953) *Acta Physiol. Scand.* **29**, 329–339

Davis, W. W. & Garren, L. D. (1968) *J. Biol. Chem.* **243**, 5153–5157

Denton, R. M. & Halperin, M. L. (1968) *Biochem. J.* **110**, 27–38

Dexter, R. N., Fishman, L. M., Ney, R. L. & Liddle, G. W. (1967*a*) *Endocrinology* **81**, 1185–1187

Dexter, R. N., Fishman, L. M., Ney, R. L. & Liddle, G. W. (1967*b*) *J. Clin. Endocrinol. Metab.* **27**, 473–480

Dole, V. P. & Meinertz, H. (1960) *J. Biol. Chem.* 2595–2599

Dorrington, J. H. & Baggett, B. (1969) *Endocrinology* **84**, 989–996

Farese, R. V. (1964) *Biochim. Biophys. Acta* **87**, 699–701

Ferguson, J. J. (1963) *J. Biol. Chem.* **238**, 2754–2759

Flint, A. P. F. & Armstrong, D. T. (1971) *Biochem. J.* **123**, 143–152

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 and Sons, New York

Flint, A. P. F. & Armstrong, D. T. (1973) *Biochem. J.* **132**, 301–311

Garland, P. B., Shepherd, D. & Yates, D. W. (1965) *Biochem. J.* **97**, 587–594

Gorski, J. & Padnos, D. (1966) *Arch. Biochem. Biophys.* **113**, 100–106

Grahame-Smith, D. G., Butcher, R. W., Ney, R. L. & Sutherland, E. W. (1967) *J. Biol. Chem.* **242**, 5535–5541

Haynes, R. C. (1958) *J. Biol. Chem.* **233**, 1220–1222

Johnston, G. G., Krisle, J. R. & Troop, R. C. (1968) *Proc. Soc. Exp. Biol. Med.* **129**, 20–23

Kahnt, F. W. & Neher, R. (1966) *Helv. Chim. Acta* **49**, 725–732

King, C. T. G., Macauley, M., Hisaw, F. L. & Dawson, A. B. (1949) *Anat. Rec.* **105**, 562–563

Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66

Long, C. N. H. (1947) *Recent Progr. Horm. Res.* **1**, 99–122

Longcope, C. & Williams, R. H. (1963) *Endocrinology* **72**, 735–741

Marsh, J. M., Butcher, R. W., Savard, K. & Sutherland, E. W. (1966) *J. Biol. Chem.* **241**, 5436–5440

Mukherjee, S., Kunitake, G. & Alfin-Slater, R. B. (1958) *J. Biol. Chem.* **230**, 91–96

Parlow, A. F. (1958) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **17**, 402

Popják, G. (1944) *J. Pathol. Bacteriol.* **56**, 485–496

Randle, P. J. & Smith, G. H. (1958) *Biochem. J.* **70**, 501–508

Sanadi, D. R., Littlefield, J. W. & Bock, R. M. (1952) *J. Biol. Chem.* **197**, 851–862

Savard, K., Marsh, J. M. & Rice, B. F. (1965) *Recent Progr. Horm. Res.* **21**, 285–365

Seubert, W. (1960) *Biochem. Prep.* **7**, 80–83

Wilks, J. W., Fuller, G. B. & Hansel, W. (1970) *Endocrinology* **87**, 581–587

Wilroy, R. S., Camacho, A. M., Trouy, R. L. & Hagan, A. A. (1968) *Endocrinology* **83**, 56–60

Zarrow, M. X. & Clark, J. H. (1969) *Endocrinology* **84**, 340–346