Biochem. J. (1973) **132**, 301–311
Printed in Great Britain

Activities of Enzymes Responsible for Steroid Biosynthesis and Cholesterol Ester Metabolism in Rabbit Ovarian Interstitial Tissue and Corpora Lutea

A COMPARISON OF ENZYME ACTIVITIES WITH FLOW RATES

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(*Received* 6 *September* 1972)

A method involving the use of isolated cholesterol ester-storage granules as substrate is described for the assay of cholesterol esterase in rabbit ovarian tissues. Activities of cholesterol esterase 100–200-fold higher than those previously reported in ovarian tissues were measured by using this method. In addition to that of cholesterol esterase, activities of cholesterol ester synthetase, cholesterol side-chain cleavage enzyme and 3β-hydroxy steroid dehydrogenase were determined in rabbit ovarian interstitial tissue and corpora lutea. Activities of these enzymes are in general compatible with the flows through them measured under a variety of conditions both *in vivo* and *in vitro*. It is concluded that, in the rabbit ovarian tissues investigated, these enzymes are capable of catalysing the conversions usually attributed to them.

The comparison of enzyme activities with metabolic flow rates has yielded useful information on intermediary metabolism in a variety of tissues. By using this approach it may be inferred not only whether the activity of an enzyme is sufficient to catalyse a step attributed to it, but also which enzyme in a pathway is likely to be rate-limiting. The purpose of the investigation described here was to apply this technique to the enzymes usually considered responsible for the degradation and formation of cholesterol esters [cholesterol esterase (sterol ester hydrolase, EC 3.1.1.13) and cholesterol ester synthetase (acyl-CoA–3β-hydroxy sterol acyltransferase, EC 2.3.1.–)] and the formation of progesterone and related steroids from cholesterol [cholesterol side-chain cleavage enzyme and 3β-hydroxy steroid dehydrogenase (3β-hydroxy steroid–NAD oxidoreductase, EC 1.1.1.51)], in rabbit ovarian interstitial tissue and corpora lutea.

Cholesterol esterase has been observed in the ovaries of the cow and rat (Coutts & Stansfield, 1968; Behrman & Armstrong, 1969) and the rabbit (Morin *et al.*, 1969) as well as in other steroidogenic tissues from a variety of species. It has been suggested that in the adrenal (Dailey *et al.*, 1962), mitochondria isolated from a testicular tumour (Moyle *et al.*, 1970), and testis (Bartke & Behrman, 1972), de-esterification of cholesterol ester (by cholesterol esterase) may be a pre-requisite to its conversion into steroids. The total activity of cholesterol esterase in rat ovary is influenced by both luteinizing hormone (Behrman & Armstrong, 1969) and by prolactin (Behrman *et al.*, 1970). In assaying cholesterol esterase activity difficulties have frequently been encountered in presenting the substrate to the enzyme. The most commonly used assay for cholesterol esterase activity utilizes labelled substrate and separation of labelled product from substrate by chromatography. Results obtained by this method are often difficult to relate to the total activity of the enzyme because it is not known to what extent the labelled substrate equilibrates with endogenous cholesterol ester. The method described here at least partially circumvents such difficulties by utilizing isolated cholesterol ester-containing storage granules as source of substrate; these granules are presumably the substrate for the enzyme *in vivo* and might therefore be expected to be a good substrate *in vitro*.

Cholesterol side-chain cleavage enzyme and 3β-hydroxy steroid dehydrogenase appear to be present in all steroidogenic endocrine tissues. There is little doubt, since the partial purification of the components of the mitochondrial cholesterol side-chain cleavage enzyme complex from adrenal (Simpson & Boyd, 1967; Bryson & Sweat, 1968) and ovary (Sulimovici & Boyd, 1968), that this enzyme is of key importance in the production of C_{21} steroids. However, as with cholesterol esterase, difficulties encountered with respect to isotopic dilution of substrate have hindered progress in the determination of

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total activities of cholesterol side-chain cleavage enzyme in whole homogenates; although the enzyme has frequently been assayed, activities are seldom expressed in terms that permit comparison with flow rates. 3β-Hydroxy steroid dehydrogenase is more easily assayed, by an NAD-linked spectrophotometric method, and has been measured in a variety of steroidogenic tissues including rabbit ovarian interstitial tissue (Davenport & Mallette, 1966). However, 3β-hydroxy steroid dehydrogenase has been reported on the basis of cytochemical observations to be either absent from (Rubin *et al.*, 1963) or present in low activity in (Davies *et al.*, 1966) rabbit corpora lutea.

As in liver (Mukherjee *et al.*, 1958; Goodman *et al.*, 1964), a cholesterol ester synthetase utilizing fatty acyl-CoA as acyl donor is present in adrenal cortex (Longcope & Williams, 1963) and ovary (Behrman *et al.*, 1970). No attempt appears to have been made to define optimum conditions for the assay of this enzyme with respect to substrate concentration, and as with cholesterol side-chain cleavage enzyme and cholesterol esterase, total activities have not been reported.

Determination of rates of flow of metabolites through cholesterol side-chain cleavage enzyme and 3β-hydroxy steroid dehydrogenase are made here on the basis of the rates of synthesis of progesterone and 20α-hydroxypregn-4-en-3-one *in vitro*. These rates are compared with others obtained previously *in vitro* and *in vivo*, and the assumptions made in comparing these values with enzyme activities are discussed. Rates of flow through cholesterol esterase and cholesterol ester synthetase cannot at present be measured directly because of the cyclic nature of the de-esterification/esterification process. Therefore probable rates of these flows *in vivo* have been inferred indirectly from results already available; methods for assessing the flow through these enzymes *in vitro* are discussed.

Methods

Assay methods

Lipids. Progesterone and 20α-hydroxypregn-4-en-3-one were assayed after separation by t.l.c., reduction of progesterone to 20β-hydroxypregn-4-en-3-one (with 20β-hydroxy steroid dehydrogenase and NADH), acetylation of both steroids and fluorimetric analysis in ethanolic H_2SO_4, as described by Flint & Armstrong (1971). This method was modified in that after the final t.l.c. the steroid acetates were simultaneously eluted and partitioned by mixing the silica gel from the t.l.c. plates with 2ml of water and extracting the slurry with 3×3ml of diethyl ether. Ether extracts were pooled and dried before assay and radioactivity determination for an estimate of recovery of [^{3}H]progesterone and 20α-hydroxy[^{3}H]-pregn-4-en-3-one (10–20nCi, 0.2–3pmol) added immediately after homogenization of the tissue. Overall recoveries were: for [^{3}H]progesterone, 64.1±0.8%; for 20α-hydroxy[^{3}H]pregn-4-en-3-one, 74.1±0.6% (means±S.E.M. for 30 determinations).

Non-esterified fatty acid eluted from silica gel after t.l.c. was assayed by the copper-chelation method of Duncombe (1963) as modified by Itaya & Ui (1965). Cholesterol, esterified and unesterified, was assayed by a modification of the Lieberman–Burchard reaction (Flint & Denton, 1970) after elution from silica gel.

Radioactivity. Radioactivity was assayed by liquid-scintillation spectrometry with either a Nuclear-Chicago mark 1 or a Nuclear-Chicago Isocap 300 liquid-scintillation spectrometer. Non-aqueous samples were counted for radioactivity in a scintillation fluid consisting of 2,5-diphenyloxazole (7.5g) and 1,4-bis-(5-phenoxazol-2-yl)benzene (75mg) in toluene (1 litre), and aqueous samples were counted for radioactivity as described previously (Flint & Armstrong, 1971). Counting efficiencies were determined by external standardization and were never less than 80%; more than 10^4 disintegrations were normally counted.

Separation of lipid fractions

Thin-layer chromatography. All lipid extracts were evaporated to dryness under a stream of N_2 at 45°C and the residues applied to t.l.c. plates coated with silica gel GF_{254} (0.25 or 0.5mm thick). For separation of cholesterol esters, triglycerides, non-esterified fatty acids, free cholesterol, steroids and phospholipids (in extracts of incubated slices+media, whole tissue, cholesterol esterase and cholesterol ester synthetase assay mixtures), t.l.c. plates were run in *n*-hexane–diethyl ether–acetic acid (90:10:1, by vol.), dried in air, and then re-run, in the same direction, in *n*-hexane–diethyl ether–acetic acid (75:25:2, by vol.). After drying, lipids on the plates were detected by spraying with 0.02% (w/v) dichlorofluorescein in ethanol and viewing under long-wavelength u.v. light (λ 366nm). Approximate R_F values of cholesterol esters, triglycerides, non-esterified fatty acids and free cholesterol were respectively 0.9, 0.7, 0.5 and 0.3; phospholipids remained at the origin. Progesterone and 20α-hydroxypregn-4-en-3-one (located by means of concurrently run standards observed under short-wavelength u.v. light; λ 254nm) had R_F values of 0.1–0.15.

For separation of steroids and sterols (in extracts from cholesterol side-chain cleavage enzyme and 3β-hydroxy steroid dehydrogenase assay mixtures) t.l.c. plates were run in methylene chloride–diethyl ether (5:2, v/v) and compounds were located by short-wavelength u.v. light and radioautography. Approximate R_F values were: progesterone, 0.65;

cholesterol, 0.55; pregnenolone, 0.45; and 20α-hydroxypregn-4-en-3-one, 0.30. Radioautographs were made by exposure of the t.l.c. plates to Kodak 'no-screen' medical X-ray film for 4–5 days.

Lipids were eluted from silica gel with chloroform–methanol (2:1, v/v).

Saponification. Cholesterol esters eluted from t.l.c. plates were saponified by adding 3ml of 6% (w/v) KOH in aq. 90% (v/v) ethanol to the dried material in a closed tube, flushing with N_2 and heating at 80°C for 2h. After cooling and the addition of 2ml of water, cholesterol derived from cholesterol ester was extracted with 3×2ml of *n*-hexane; fatty acid derived from cholesterol ester was extracted with 3×2ml of *n*-hexane after subsequent acidification with 10 drops of conc. HCl. By this method 99.8% of cholesteryl stearate and cholesteryl arachidonate (up to 0.5mg) were saponified. Extraction after saponification was 90–95% efficient. After saponification of cholesterol ester extracted from slices incubated with [^{14}C]oleate, the mean recovery of ^{14}C as cholesterol derived from cholesterol ester plus fatty acid derived from cholesterol ester was 92± 4.9%. Extracts in *n*-hexane of lipid fractions after saponification were evaporated under air before liquid-scintillation counting for radioactivity.

Incubation and extraction procedures

Incubations. Interstitial tissue (+follicles) and corpora lutea were separated by blunt dissection. Interstitial-tissue slices (0.3–0.4mm thick) and corpora lutea quarters were cut with a razor blade and either tissue (40–120mg) was incubated in 2.5ml of bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing glucose (1mg/ml) and other additions as indicated, gassed with O_2+CO_2 (95:5). For addition to the incubation medium [^{14}C]oleic acid (50μCi) was dissolved in ethanol (25μl) and mixed with incubation medium (usually 2ml) containing 2mg of bovine plasma albumin/ml and sufficient KOH to neutralize the acid. After mixing, the dissolved oleate was diluted to the required volume (usually 50ml) with gassed incubation medium; final oleate concentration was 18μM.

Extractions. For extraction of progesterone, 20α-hydroxypregn-4-en-3-one and cholesterol esters after incubation of tissue slices, tissue was removed from the incubation medium and homogenized in 10ml of chloroform–methanol (2:1, v/v); the chloroform–methanol extract was then split into chloroform and aq. methanolic phases by the addition of the incubation medium. The aqueous-methanolic phase was discarded, the chloroform solution evaporated and the residue used for t.l.c. By using this extraction procedure mean recoveries of added tracers were: [^{3}H]progesterone, 98±2.1%; 20α-hydroxy[1,2-^{3}H]-pregn-4-en-3-one, 95±3.9%; [^{3}H]cholesteryl oleate, 97±0.9% (means±S.E.M. for four determinations in each case).

For extraction of non-esterified fatty acids produced during incubation, tissue slices were homogenized in the incubation medium in a Kontes grinder (Kontes Glass Co., Vineland, N.J., U.S.A.) and the homogenates were extracted with 10ml of propan-2-ol–*n*-heptane–0.5M-H_2SO_4 (40:10:1, by vol.) as described by Dole & Meinertz (1960).

For extraction of lipids from non-incubated tissue, or from enzyme-assay reaction mixtures, samples of tissue (25–200mg) or homogenate (0.1–0.5ml) were added to 10ml of chloroform–methanol (2:1, v/v). After mixing or homogenization, as appropriate, the phases were split by the addition of 2ml of 0.9% (w/v) NaCl, and the aqueous-methanolic phase was discarded.

Enzyme assays

Ovaries from pseudopregnant rabbits were separated by blunt dissection into interstitial tissue + follicles and corpora lutea. Both tissues were homogenized by using a hand-operated all-glass Potter–Elvehjem homogenizer, either in 20mM-potassium phosphate buffer, pH7.0 (for the assay of 3β-hydroxy steroid dehydrogenase, cholesterol esterase and cholesterol side-chain cleavage enzyme), or in 0.1M-Tris–HCl buffer, containing GSH (5mM), EGTA [ethanedioxybis(ethylamine)tetra-acetate] (1mM) and defatted bovine plasma albumin (1mg/ml), pH7.4 (for the assay of cholesterol ester synthetase). All manipulations were done at 4°C and all enzyme assays at 37°C. Cholesterol ester synthetase and cholesterol side-chain cleavage enzyme activities were assayed in the crude homogenate; 3β-hydroxy steroid dehydrogenase and cholesterol esterase activities were assayed in supernatant fractions prepared by centrifugation at $10000g_{av.}$ for 20min in the swinging-bucket (SW-40) rotor of a Beckman model L2-65B centrifuge. After centrifugation the supernatant fractions were defatted by removal of material floating on the surface of the solution by using a dry glass rod. This material consisted largely of cholesterol ester-containing lipid granules and was used as substrate in the cholesterol esterase assay. Assay of cholesterol esterase activity in supernatant fractions is justified on the grounds that the enzyme is soluble in rat ovarian tissue (Behrman & Armstrong, 1969) and in liver (Deykin & Goodman, 1962). Determination of 3β-hydroxy steroid dehydrogenase activity in the pellet obtained after centrifugation indicated that more than 95% of the enzyme was in the supernatant fraction obtained after centrifugation at $10000g_{av.}$ for 20min.

Cholesterol esterase (*EC* 3.1.1.13). Cholesterol esterase activity was assayed by determining the rate of formation of cholesterol or non-esterified fatty

acid from a suspension of cholesterol ester-containing lipid granules by a supernatant fraction. Supernatant fractions (containing the enzyme) and lipid granules (the substrate), usually obtained from the same homogenate (containing 1g of tissue/7.5 or 10.0ml of buffer), were prepared by centrifugation as described above. Lipid granules removed from the surface of the supernatant were resuspended in 0.1M-triethanolamine–HCl buffer, pH7.5. Supernatant fractions were removed from above the crude mitochondrial pellet by aspiration. Granule suspensions (1ml) and supernatants (1ml) were incubated together for up to 4h, samples (0.5ml) of the incubation mixture being removed and extracted at various times throughout the incubation period. In a typical experiment lipid granules from 1.9g wet wt. of interstitial tissue were sufficient to provide a final cholesterol ester concentration of approx. 2mM in 25 incubation tubes. During the assays free cholesterol and non-esterified fatty acid were produced at equal rates on a mol:mol basis; non-esterified fatty acid production rates as percentages of free cholesterol production rates were 112±32 for interstitial tissue and 128±28 for corpora lutea (means±S.E.M. for nine determinations in each case). Hydrolysis of cholesterol esters depended on the presence of both enzyme and substrate (Fig. 1) and was linear with time and enzyme concentration for interstitial tissue (Fig. 2) and for corpora lutea (results not shown). There was no change in rate of hydrolysis with cholesterol ester concentrations between 0.46 and 2.50mM. Granules from interstitial tissue were as good a substrate as

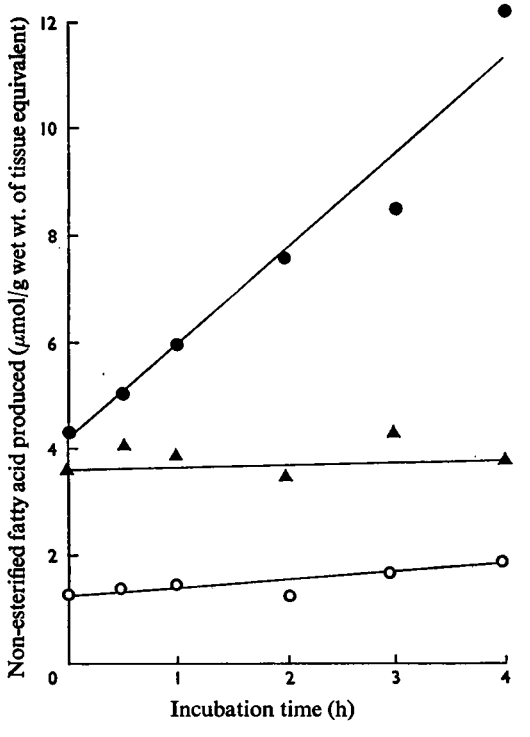

Fig. 1. *Dependence of cholesterol esterase activity on the presence of enzyme and substrate*

Incubations contained: ○, granules alone; ▲, supernatant alone; ●, granules and supernatant. Cholesterol ester concentration due to granules was 1.92mM; that due to supernatant was 0.09mM. Non-esterified fatty acid production is expressed in terms of supernatant obtained from homogenate containing 1g of interstitial tissue. For further details see the Methods section.

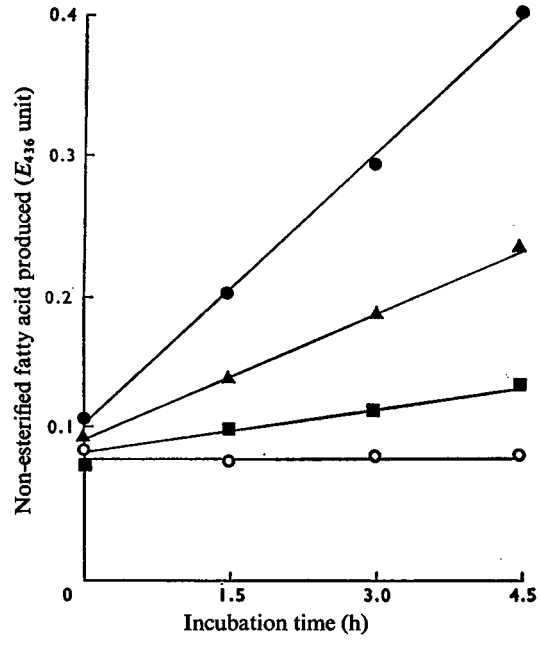

Fig. 2. *Assay of cholesterol esterase activity: dependence of non-esterified fatty acid production on incubation time and enzyme concentration*

Supernatant fraction prepared from a homogenate containing 3.24g of interstitial tissue/24.3ml of medium was incubated with lipid granules from the same homogenate; the final cholesterol ester concentration was 1.69±0.046mM (16). Supernatant contents of incubation tubes were as follows: ●, 1.0ml; ▲, 0.5ml; ■, 0.25ml; ○, 0.5ml (boiled for 5min before incubation). Non-esterified fatty acid contents were determined at the times indicated after extraction and t.l.c.

Table 1. *Hydrolysis of lipid-granule cholesterol ester by supernatant fractions from interstitial tissue and corpora lutea: effect of changing source of substrate*

Lipid granules and supernatants were prepared from 13ml of whole homogenates (1g of tissue/7.5ml of medium, in each case) of interstitial tissue and corpora lutea from the ovaries of the same pseudopregnant rabbits. After centrifugation, lipid granules from interstitial tissue and corpora lutea were suspended in 22ml and 6.5ml of medium respectively, and 1ml of each suspension was incubated with 1ml of either supernatant. Cholesterol ester concentrations were determined after incubation; rates of hydrolysis were determined from free cholesterol production. Values are means±S.E.M. for six determinations.

Source of		Rate of hydrolysis of cholesterol ester (μmol/h per g wet wt. of tissue)	Concn. of cholesterol ester (mM)
Substrate	Enzyme		
Interstitial tissue	Interstitial tissue	2.46±0.39	1.88±0.06
Interstitial tissue	Corpora lutea	1.10±0.24	2.07±0.06
Corpora lutea	Interstitial tissue	2.33±0.33	1.85±0.13
Corpora lutea	Corpora lutea	1.42±0.20	1.78±0.03

those from corpora lutea for the enzyme from either tissue (Table 1) and were used for the assay of the enzyme from both sources when sufficient granules were otherwise unavailable.

3β-Hydroxy steroid dehydrogenase (*EC* 1.1.1.51). Assays were performed spectrophotometrically by following the reduction of NAD^+ in the presence of pregnenolone by a modification of the method of Kuhn & Briley (1970). The total volume of reaction mixture was 2.8ml, and the pregnenolone concentration was 35μM. Rates were linear with time and enzyme concentration (up to 0.15ml of $10000g_{av.}$ supernatant) and there was no change in absorption when enzyme, pregnenolone or NAD^+ were omitted. Davenport & Mallette (1966) have reported that a 1:1 molar stoicheiometry exists between the oxidation of pregnenolone and the reduction of acetylpyridine nucleotide. Rates obtained by this method were corrected by Kuhn & Briley (1970) to pH7.4 (from pH9.4, at which the assay is performed) by using an experimentally determined factor of 0.61. Rates measured by us were not corrected in this way, since there appears to be no well-defined pH optimum for the enzyme from rabbit ovarian interstitial tissue (Davenport & Mallette, 1966), the activity increasing between pH8.6 and 10.0; rates are therefore given as obtained at pH9.4. Addition of [^{14}C]pregnenolone (165000c.p.m.) to cuvettes containing extracts of either interstitial tissue or corpora lutea, followed by extraction, t.l.c. and radioautography (as described above), showed that during the course of the reaction 90.5% of the pregnenolone oxidized was recoverable as progesterone.

Cholesterol ester synthetase (*EC* 2.3.1.–). Cholesterol ester formation was assayed by determining the rate of incorporation of ^{14}C from [1-^{14}C]palmitoyl-CoA into cholesterol ester by a modification of the method of Behrman *et al.* (1970). Whole homogenates (1ml) of interstitial tissue (1g wet wt. of tissue/10ml of medium) or corpora lutea (1g wet wt. of tissue/40ml of medium) were incubated for up to 3min with [1-^{14}C]palmitoyl-CoA (40μM, 12.5μCi/μmol) in a total volume of 2ml of Tris–HCl buffer (0.1M), pH7.4, containing GSH (5mM), EGTA (1mM) and defatted bovine plasma albumin (1mg/ml). Palmitoyl-CoA was added in less than 50μl of aqueous solution. After incubation for the desired length of time, 0.5ml samples of the reaction mixture were removed and extracted as described above. Cholesterol esters were separated from other lipids by t.l.c., and the amount of ^{14}C incorporated was determined. Incorporation rates were linear with time and enzyme concentration (Fig. 3) and were not dependent on addition of cholesterol. An optimum concentration of palmitoyl-CoA (40μM) was used (Fig. 4). Since enzyme activity was calculated from the specific radioactivity of added palmitoyl-CoA it was necessary to ensure that added [^{14}C]palmitoyl-CoA was not diluted by endogenous long-chain acyl-CoA; this was achieved by preincubating the tissue homogenate at 37°C for 5min before addition to the incubation mixture; during this time more than 80% of the long-chain acyl-CoA was hydrolysed by endogenous deacylase, as judged by the rate of hydrolysis of added [^{14}C]palmitoyl-CoA [assuming a tissue concentration of long-chain acyl-CoA of 13nmol/g wet wt.; concentration in rabbit ovarian interstitial tissue slices is 11–15nmol/g wet wt. (Flint *et al.*, 1973)]. Long-chain acyl-CoA remaining after this procedure would be expected to cause less than 1% error in the calculated [^{14}C]palmitoyl-CoA specific radioactivity.

Initial attempts to assay cholesterol ester synthetase activity in extracts of corpora lutea by the method described here for interstitial tissue (i.e. with a tissue extract containing 1g of tissue/10ml of

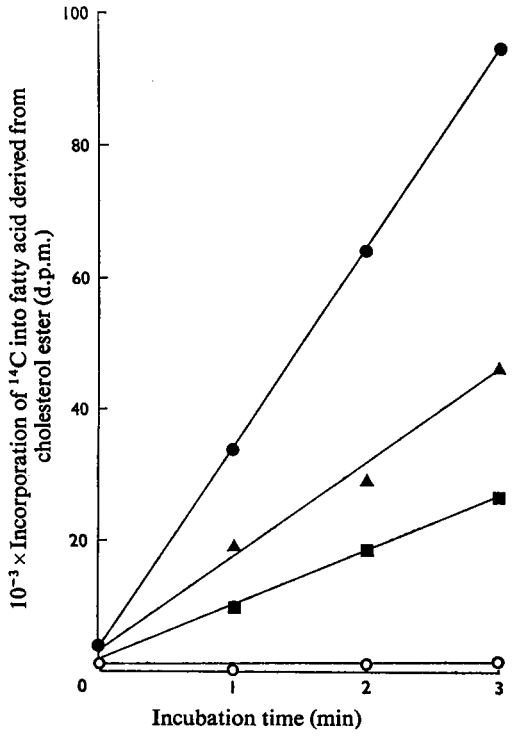

Fig. 3. *Assay of cholesterol ester synthetase activity: dependence on time and enzyme concentration of incorporation of ^{14}C from [1-^{14}C]palmitoyl-CoA into cholesterol ester*

Whole homogenate of interstitial tissue containing 2.08g of tissue/15.0ml of medium was incubated with [1-^{14}C]palmitoyl-CoA (40μM, 12.5μCi/μmol) for various times. Incorporation of ^{14}C was determined after extraction and t.l.c. Homogenate contents of incubations were: ●, 1.0ml; ▲, 0.5ml; ■, 0.25ml; ○, 0.5ml of boiled homogenate.

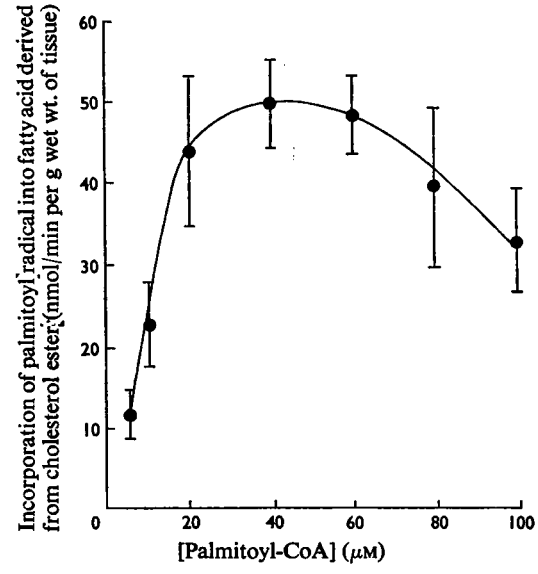

Fig. 4. *Assay of cholesterol ester synthetase activity: effect of palmitoyl-CoA concentration on incorporation of ^{14}C from [1-^{14}C]palmitoyl-CoA into cholesterol ester by whole homogenate of interstitial tissue*

Specific radioactivities of [^{14}C]palmitoyl-CoA were calculated on the basis of the quantities of labelled and unlabelled compound added. Values are expressed as nmol of palmitoyl radical incorporated/min per g wet wt. of tissue, as means±S.E.M. (shown as vertical bars) for three determinations at each point.

extraction medium) indicated that the assay was not linear with respect to enzyme concentration under these conditions, suggesting the presence of an inhibitor of the enzyme in extracts of corpora lutea.

Cholesterol side-chain cleavage enzyme. Assays were made of this enzyme activity in whole homogenates by determining the rate of conversion of added [4-^{14}C]cholesterol into steroids in the presence of NADPH. Whole homogenates (1ml) were incubated with 2.5mM-NADPH, 20mM-$MgCl_2$, 1mM-EGTA, defatted bovine plasma albumin (1mg/ml) and [4-^{14}C]cholesterol (0.1μCi, added in 50μl of dimethylformamide) in a total volume of 2ml for up to 1.0h. Samples (0.5ml) of reaction mixture were extracted, and steroids and sterols separated by t.l.c. and located by radioautography as described below. Compounds formed during incubation, and unchanged cholesterol, were eluted and assayed for radioactivity. The mass of cholesterol eluted was also determined, allowing calculation of its specific radioactivity in the reaction mixture; this value was used to calculate the reaction rate in terms of mass of cholesterol converted per unit time. The specific radioactivity of the progesterone produced during the incubations (determined as described below) was close to that of the cholesterol extracted after incubation; thus it appears that added [^{14}C]cholesterol equilibrated fully with endogenous cholesterol in the tissue extract. Mean specific radioactivity of progesterone as a percentage (±S.E.M.) of the specific radioactivity of cholesterol was: for interstitial tissue, 116±18 (12); for corpora lutea, 98±26 (9). Production of steroid during the incubation exceeded by more than 10-fold the progesterone content of the homogenate before incubation; therefore these specific radioactivities are unlikely to be greatly

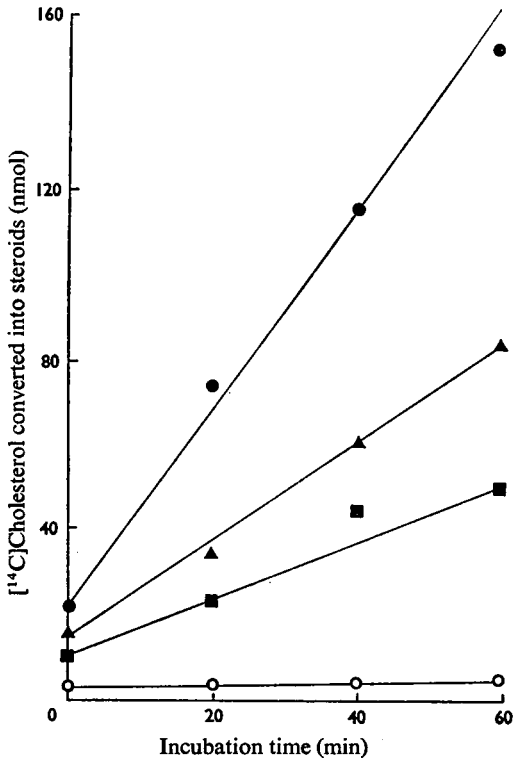

Fig. 5. *Assay of cholesterol side-chain cleavage enzyme activity: dependence of incorporation of ^{14}C from [4-^{14}C]cholesterol into pregnenolone plus progesterone plus 20α-hydroxypregn-4-en-3-one on time and enzyme concentration*

Homogenate of interstitial tissue containing 1.0g wet wt. of tissue/10ml of medium was incubated with [4-^{14}C]cholesterol (0.1μCi, 58.0mCi/mmol, added in 50μl of dimethylformamide), NADPH, bovine plasma albumin, $MgCl_2$ and EGTA. Incorporation of ^{14}C into steroids was determined after extraction, t.l.c. and radioautography, and was converted into terms of mass of cholesterol side-chain cleaved by using an experimentally determined value (which varies from one experiment to another) for the specific radioactivity of cholesterol in the incubation. Homogenate contents of incubations were: ●, 1.0ml; ▲, 0.5ml; ■, 0.25ml; ○, 0.5ml of boiled homogenate.

affected by dilution (with endogenous unlabelled steroid) of steroid formed during the incubation. Reaction rates were linear with time and enzyme concentration (Fig. 5) when plotted in terms of mass of cholesterol converted/mass of tissue equivalent. This is a further indication that equilibration occurred between labelled and endogenous substrates.

Expression of results

For enzyme activities all results are expressed as mass of substrate converted/g wet wt. of tissue. Where supernatant fractions were used for enzyme assays the activity is corrected for dilution involved in their preparation. Differences between means were analysed statistically by Student's *t* test.

Materials

Animals. Ovaries were obtained at autopsy from New Zealand White rabbits (3–4kg) on day 10 of a pseudopregnancy induced by the intravenous administration of 50μg of bovine luteinizing hormone (NIH-LH-B6 or 7). At this stage of the pseudopregnancy (which normally lasts for 16–18 days) corpora lutea are at their maximum size (16–20mg each, five to eight per ovary) and secretory activity. Animals were killed by intravenous administration of 2–3ml of Euthansol (sodium pentobarbital, 3g/ml; W. E. Saunders Ltd., London, Ont., Canada).

Chemicals. [7α-^3H]Progesterone (8.5Ci/mmol), [4-^{14}C]pregnenolone (56.4mCi/mmol), [4-^{14}C]cholesterol (58.0mCi/mmol) and [7α-^3H]cholesterol (12.3Ci/mmol) were from Amersham Searle Corp., Toronto, Ont., Canada; [1-^{14}C]palmitoyl-CoA (54.9mCi/mmol), [1-^{14}C]oleic acid (56.2mCi/mmol) and 20α-hydroxy[1,2-^3H]pregn-4-en-3-one (50.3Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass., U.S.A. Labelled progesterone, 20α-hydroxypregn-4-en-3-one, pregnenolone and cholesterol were purified by t.l.c. before use. All radioactive compounds (with the exception of palmitoyl-CoA) were used without prior decrease of specific radioactivity. [7α-^3H]Cholesteryl oleate (12.3mCi/mmol) was prepared from [7α-^3H]cholesterol and oleoyl chloride (Eastman–Kodak Co., Rochester, N.Y., U.S.A.) by the method of Deykin & Goodman (1962) and the reaction products were separated by t.l.c. Unlabelled palmitoyl-CoA was prepared from CoA and palmitoyl chloride (Eastman–Kodak Co.) by the method of Seubert (1960). Bovine plasma albumin (fraction V, from Armour Pharmaceutical Co., Chicago, Ill., U.S.A.) was defatted with charcoal as described by Chen (1967). Silica gel GF_{254} was obtained from E. Merck A.-G. through Brinkmann Instruments Inc., Westbury, N.Y., U.S.A. Luteinizing hormone was a gift from the National Institutes of Health, Bethesda, Md., U.S.A.

Results and Discussion

Sterol and steroid contents of rabbit ovarian interstitial tissue and corpora lutea, and rates of synthesis of progesterone and 20α-hydroxypregn-4-en-3-one in these tissues *in vitro*, are given in Table 2. In confirmation of the findings of Hilliard *et al.* (1968) and

Table 2. *Sterol and steroid contents of, and rates of steroid production by, ovarian interstitial tissue and corpora lutea from pseudopregnant rabbits*

Cholesterol ester, unesterified cholesterol, progesterone and 20α-hydroxypregn-4-en-3-one were assayed in extracts of tissues obtained at autopsy. Rates of synthesis of progesterone and 20α-hydroxypregn-4-en-3-one *in vitro* were determined after slicing and incubating the tissues, and are corrected for steroids in the tissue before incubation. All values are given as means±S.E.M. for the number of determinations in parentheses; *, $P<0.02$; **, $P<0.005$, for differences between tissues.

Tissue	Sterol contents (μmol/g wet wt.)		Steroid contents (nmol/g wet wt.)		Rates of steroid synthesis *in vitro* (nmol/h per g)	
	Cholesterol ester	Unesterified cholesterol	Progesterone	20α-Hydroxypregn-4-en-3-one	Progesterone	20α-Hydroxypregn-4-en-3-one
Interstitial tissue	53.5±1.8 (4)	5.9±0.6 (4)	5.5±0.9 (10)	51.3±6.8 (10)	27.6±5.4 (5)	92.2±4.2 (5)
Corpora lutea	19.6±0.5 (4)**	7.0±0.5 (4)	50.2±4.5 (6)**	20.3±3.6 (6)*	146.0±13.6 (5)**	29.8±1.3 (5)**

Wilks *et al.* (1970), the cholesterol ester content of interstitial tissue was higher than that of corpora lutea. The 20α-hydroxypregn-4-en-3-one/progesterone concentration ratio was higher in interstitial tissue than in corpora lutea, and interstitial tissue produced more 20α-hydroxypregn-4-en-3-one than did corpora lutea during incubation *in vitro*, presumably as a result of its higher 20α-hydroxy steroid dehydrogenase activity (Strauss *et al.*, 1972).

Rates of flow through cholesterol side-chain cleavage enzyme and 3β-hydroxy steroid dehydrogenase

Rates of synthesis of progesterone plus 20α-hydroxypregn-4-en-3-one by tissue slices *in vitro* were: 119.8±7.1 and 175.8±14.2 nmol/h per g wet wt. of tissue, for interstitial tissue and corpora lutea respectively (means±S.E.M. calculated from the results in Table 2; P for the difference between means was less than 0.01). These rates of production of progesterone and 20α-hydroxypregn-4-en-3-one are comparable with rates observed with unstimulated whole ovary *in vivo* (Solod *et al.*, 1966; Hilliard *et al.*, 1969) and with rates found previously *in vitro* (Dorrington & Kilpatrick, 1966). Rates of synthesis of progesterone plus 20α-hydroxypregn-4-en-3-one presumably reflect minimum flow rates through cholesterol side-chain cleavage enzyme and 3β-hydroxy steroid dehydrogenase. Actual flow rates may be higher as a result of further metabolism of these steroids to products not measured, or loss from the tissue of pregnenolone.

Rates of flow through cholesterol ester synthetase and cholesterol esterase

Rates of esterification and de-esterification of cholesterol, i.e. flow rates through cholesterol ester synthetase and cholesterol esterase respectively, cannot be measured directly with methods at present available. Indirect methods have therefore been used in this study.

Minimum rates of flow through cholesterol esterase *in vivo* can be inferred from rates of depletion of cholesterol ester after stimulation by gonadotrophin. Results of Claesson *et al.* (1953) indicate that such depletion occurs at a rate of 9.88 μmol of cholesterol ester/h per g wet wt. of tissue (range 7.32–15.50) in rabbit ovarian interstitial tissue. Rates of flow through cholesterol ester synthetase *in vivo* can be inferred from rates of re-accumulation of cholesterol ester after depletion. Information of this kind for rabbit ovarian interstitial tissue appears to be unavailable: typical rates obtained with rat ovarian luteal tissue are (in μmol of cholesterol ester formed/h per g wet wt. of tissue) 4.0–6.5 (Herbst, 1967) and 2.1 (Armstrong, 1968). This approach to the determination of flow rates has the disadvantages that (*a*) the

tissue is stimulated hormonally, and rates obtained in this way may not be comparable with enzyme activities measured in unstimulated tissue, (*b*) the actual rates are likely to exceed these minimum rates to the extent of the additional flow through the synthetase in the case of de-esterification and the esterase in the case of esterification, and (*c*) luteinizing hormone causes lysis of corpora lutea in rabbits, during which process they do not lose (but rather gain, on a weight basis) cholesterol ester (Foster, 1938; Stormshak & Casida, 1964; Hilliard *et al.*, 1968). The method cannot therefore be applied to corpora lutea in the rabbit. A second approach to the problem of the measurement of rates of esterification and de-esterification *in vivo* has been made through the analysis of specific radioactivities of ovarian unesterified and esterified cholesterol at various times after the administration of a pulse dose of [^{14}C]cholesterol (Flint & Armstrong, 1972). This method has the advantage that it can be applied to the steady-state situation, but is more suited to application to small animals (e.g. the rat) for reasons of economy and less animal variability. Rates calculated in this way from results obtained with unstimulated rat lutein tissue (8.1 μmol/h per g wet wt.; Flint & Armstrong, 1972) are comparable with those inferred from the rate of depletion of cholesterol ester in rabbit ovarian interstitial tissue.

Two approximate methods are available for assessing these flows *in vitro*. An indication of the minimum rate of de-esterification in incubated tissue slices can be obtained from the rate of production of non-esterified fatty acid *in vitro*; this rate was found to be related to the rate of cholesterol ester disappearance and to be unaffected by any hydrolysis of glycerides in rat lutein tissue (Flint & Denton, 1970). With rabbit ovarian interstitial tissue and corpora lutea *in vitro*, rates of non-esterified fatty acid production are 1.9 ± 0.31 and 1.1 ± 0.13 μmol/h per g wet wt. respectively (means $\pm$ S.E.M. for four determinations, measured as described in the Methods section, and corrected for fatty acid content of non-incubated tissue; $P>0.05$). These rates are of the same order of magnitude as those inferred from observations made *in vivo* and discussed above. Actual rates of de-esterification are likely to exceed those indicated by fatty acid-output results to the extent of the re-esterification of fatty acid liberated on de-esterification. Rates of esterification of cholesterol *in vitro* may not equal rates of de-esterification, since in rat lutein tissue incubated *in vitro*, the cholesterol ester content of tissue slices has been observed to fall (Flint & Denton, 1970); rates of esterification do not equal rates of de-esterification if a steady state does not exist with respect to cholesterol ester concentration.

Absolute rates of esterification of cholesterol cannot be measured at present *in vitro*; relative rates may, however, be indicated by rates of incorporation by tissue slices of [^{14}C]oleate added to incubation medium into fatty acid derived from cholesterol ester. Such rates are influenced by dilution of the labelled material with fatty acid produced during the incubation. Fatty acids are esterified with CoA before incorporation into cholesterol ester (Mukherjee *et al.*, 1958; Longcope & Williams, 1963; Behrman *et al.*, 1970) and these rates may also be influenced by isotopic dilution during mixing with endogenous fatty acyl-CoA. In practice such dilution is likely to be minimal, however, as there appears to be rapid and continuous formation and hydrolysis of fatty acyl-CoA, and isotopic equilibrium will probably be reached rapidly between non-esterified fatty acid and fatty acyl-CoA. Further, the concentrations of fatty acyl-CoA in ovarian tissues are low (11–15 nmol/g wet wt., Flint *et al.*, 1973). Given similar rates of non-esterified fatty acid release and similar intracellular concentrations of fatty acids, comparison of incorporation rates is likely to indicate relative rates of esterification of cholesterol. For rabbit ovarian interstitial tissue and corpora lutea these rates, measured as described in the Methods section, were 733000 ± 99000 and 72000 ± 3000 d.p.m./h per g wet wt. of tissue respectively (means $\pm$ S.E.M. for five determinations in each case; $P<0.001$).

On the basis of the results obtained by the methods described above, with both rabbit ovarian tissues and others, flow rates through cholesterol ester synthetase and cholesterol esterase *in vivo* appear to be in the region of 6–8 μmol/h per g wet wt. of tissue. The rate of de-esterification of cholesterol ester measured *in vitro* seems to be at least 1–2 μmol/h per g wet wt. The flow rate through cholesterol ester synthetase *in vitro* is not certain, but probably does not exceed that through cholesterol esterase.

Activities of cholesterol side-chain cleavage enzyme, 3β-hydroxy steroid dehydrogenase, cholesterol esterase and cholesterol ester synthetase

Total activities of these enzymes in rabbit ovarian interstitial tissue and corpora lutea are listed in Table 3. With the exception of cholesterol side-chain cleavage enzyme, activities of these enzymes were higher in interstitial tissue than in corpora lutea.

Activities of cholesterol side-chain cleavage enzyme and 3β-hydroxy steroid dehydrogenase are 9.6–17- and 132–260-fold greater respectively than the basal rates of production of progesterone plus 20α-hydroxypregn-4-en-3-one *in vitro* (Table 2) and *in vivo* (Solod *et al.*, 1966; Hilliard *et al.*, 1969), and the measured activities are, subject to the conditions outlined above, sufficient to catalyse the flows under these conditions. However, after stimulation of interstitial tissue at coitus or by administration of luteinizing hormone, rates of synthesis of progesterone plus

Table 3. *Activities of cholesterol ester synthetase, cholesterol esterase, cholesterol side-chain cleavage enzyme and 3β-hydroxy steroid dehydrogenase in ovarian interstitial tissue and corpora lutea from pseudopregnant rabbits*

Activities were assayed in extracts of tissue obtained at autopsy and are expressed as μmol of substrate converted/h per g wet wt. of tissue, as means±S.E.M. for the number of determinations in parentheses. Assays were carried out at 37°C. *, $P<0.02$; **, $P<0.005$, for differences between tissues; other differences, $P>0.05$.

	Enzyme activities (μmol/h per g wet wt.)			
Tissue	Cholesterol ester synthetase	Cholesterol esterase	Cholesterol side-chain cleavage enzyme	3β-Hydroxy steroid dehydrogenase
Interstitial tissue	1.63±0.15 (6)	2.13±0.24 (12)	2.03±0.42 (9)	31.2±1.62 (9)
Corpora lutea	0.98±0.15 (6)*	1.26±0.16 (12)**	1.68±0.29 (9)	23.3±2.22 (9)*

20α-hydroxypregn-4-en-3-one approach 2.5 μmol/h per g wet wt. of tissue *in vivo* (Hilliard *et al.*, 1969) and *in vitro* (Solod *et al.*, 1966). Under these conditions flow rates through cholesterol side-chain cleavage enzyme may be close to or slightly exceed the activities reported here for interstitial tissue. Luteinizing hormone does not greatly increase rates of steroid synthesis in rabbit corpora lutea *in vitro* (Dorrington & Kilpatrick, 1966) or *in vivo* (where it causes luteolysis); cholesterol side-chain cleavage enzyme activity in the rabbit corpus luteum appears therefore to be sufficient to catalyse observed flows under all conditions.

In view of the suggested absence (Rubin *et al.*, 1963) or low activity (Davies *et al.*, 1966) of 3β-hydroxy steroid dehydrogenase in rabbit corpora lutea, the demonstration of a relatively high activity of the enzyme in the tissue by a chemical assay is noteworthy. Although the activity in corpora lutea is lower than that in interstitial tissue there can be little doubt that it exceeds the flow through this step, possibly by a factor as high as 130.

Inferred rates of de-esterification and esterification of cholesterol *in vivo*, although higher than the total activities of cholesterol esterase and cholesterol ester synthetase, are of the same order of magnitude as these activities. Minimum rates of de-esterification *in vitro*, as indicated by fatty acid release rates, are below these activities. It appears probable, therefore, bearing in mind the limitations of the assays for these enzymes, that cholesterol ester synthetase and cholesterol esterase catalyse the conversions usually attributed to them.

The suggestion that the concentration of cholesterol ester in rat ovary may be determined largely by the total activities of cholesterol ester synthetase and cholesterol esterase (Behrman *et al.*, 1970) seems unlikely to account for the differences in concentrations of cholesterol ester between rabbit ovarian interstitial tissue and corpus luteum (Table 2), since both activities are lower in corpora lutea than in interstitial tissue. It is possible therefore that the total activities of these enzymes are modified in the whole cell. The relatively low rate of esterification of [^{14}C]-oleate observed *in vitro* with corpora lutea, and the apparent inhibitor of cholesterol ester synthetase found in extracts of corpora lutea (see the Methods section) suggest that cholesterol ester synthetase in the corpus luteum may be partially inhibited. This inhibition may be the cause of the different concentrations of cholesterol ester in the two tissues. That either cholesterol esterase or cholesterol ester synthetase may be controlled by effectors in the whole cell is perhaps not unlikely in view of the importance of these enzymes in tissues storing and mobilizing large quantities of cholesterol ester.

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).

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