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The Compartmentation of Non-Esterified and Esterified Cholesterol in the Superovulated Rat Ovary

BY A. P. F. FLINT AND D. T. ARMSTRONG
Departments of Obstetrics and Gynaecology and of Physiology, University of Western Ontario, London, Ont., Canada

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1. The specific radioactivities of non-esterified and esterified cholesterol, progesterone and 20α-hydroxypregn-4-en-3-one were determined in slices of superovulated rat ovary after incubation with [1-^{14}C]acetate *in vitro* for various times. The specific radioactivities of progesterone and 20α-hydroxypregn-4-en-3-one were equal, and (during the fourth hour of incubation) exceeded those of the non-esterified cholesterol and the esterified cholesterol by factors of 2.8 and 7.6 respectively. 2. After separation of homogenates of superovulated rat ovary slices previously incubated with [^{14}C]acetate into subcellular fractions by differential centrifugation, the specific radioactivities of non-esterified cholesterol in the cytosol, mitochondria, lipid-containing storage granules and microsomal fraction were 1220, 1510, 1420 and 4020 d.p.m./μmol respectively; the corresponding values for the specific radioactivity of the esterified cholesterol were 600, 700, 730 and 760 d.p.m./μmol. The specific radioactivities of progesterone and 20α-hydroxypregn-4-en-3-one were equal in all fractions; the corresponding mean specific radioactivity of progesterone + 20α-hydroxypregn-4-en-3-one was 6150 d.p.m./μmol. 3. By using glutamate dehydrogenase and cytochrome ($a+a_3$) as mitochondrial markers, the presence of cholesterol side-chain cleavage enzyme was demonstrated in microsomal fraction free of mitochondrial contamination. 4. The specific radioactivities of ovarian non-esterified and esterified cholesterol, progesterone and 20α-hydroxypregn-4-en-3-one were determined up to 8 h after the intravenous injection of [4-^{14}C]cholesterol into superovulated rats. At all times the specific radioactivities of progesterone and 20α-hydroxypregn-4-en-3-one were equal to the specific radioactivity of non-esterified cholesterol and exceeded, by up to 3.3-fold, that of the esterified cholesterol. 5. It is concluded that non-esterified cholesterol formed from [^{14}C]acetate in the endoplasmic reticulum equilibrates slowly with non-esterified cholesterol in other subcellular fractions, and is preferentially converted into steroids. Such a mechanism presupposes the operation of a microsomal cholesterol side-chain cleavage enzyme using non-esterified cholesterol as its substrate. Unrelated evidence is presented in support of the existence of such an enzyme. The results are discussed in the light of other biochemical and electron-microscopic findings relating to the compartmentation of cholesterol in steroidogenic tissues.

Investigations into the metabolism of radioactively labelled acetate carbon in steroidogenic endocrine tissues *in vitro* have revealed that the specific radioactivities of the steroids produced exceed those of the non-esterified and esterified cholesterol* in the tissue. This was first observed in the perfused bovine adrenal gland (Hechter, Solomon, Zaffarini & Pincus, 1953) and has since been shown to occur in tissue-slice studies of the corpus luteum of the rat (Armstrong, O'Brien & Greep, 1964; Armstrong, Miller & Knudsen, 1969*b*) and the cow (Armstrong, Lee & Miller, 1970) and of the ovarian interstitial gland of the rabbit (Armstrong, Jackanicz & Keyes, 1969*a*). This phenomenon was originally thought to be due either to the existence of a steroidogenic route from acetate that did not include cholesterol as an intermediate,

* The term 'esterified cholesterol' has been used throughout to indicate the acyl esters of cholesterol. Where the specific radioactivity of esterified cholesterol is referred to, the specific radioactivity of the cholesterol moiety only of the acyl ester molecule is indicated.

or to the fact that not all the cholesterol pool was drawn upon for steroidogenesis (Hechter *et al.* 1953). Inhibitors of either cholesterol synthesis or cholesterol side-chain cleavage have since been used to eliminate the former possibility and to demonstrate the obligatory nature of cholesterol in the steroidogenic pathway (Armstrong *et al.* 1970). No attempt has been made, however, to determine the intracellular location of the steroidogenic cholesterol. The investigations reported here represent an attempt to identify this cholesterol by using the technique of centrifugal fractionation of tissue homogenates developed for the determination of the intracellular locations of enzymes, the tissue cholesterol pools being labelled by incubation of tissue slices with [1-^{14}C]acetate before fractionation. Similar methods have been used previously in investigations of the time-course of labelling of intracellular pools of lipids in the epididymal fat-pad (Angel, 1970). In addition, the ability of plasma cholesterol to gain access to the steroidogenic pool has been investigated, and the subcellular distribution of cholesterol side-chain cleavage enzyme determined.

MATERIALS

Silica gel GF_{254} was from E. Merck A.-G. through Brinkmann Instruments Inc., Westbury, N.Y., U.S.A.; metallic Zn was from the J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A., and 4-methylpentanoic acid (isocaproic acid) was from K & K Laboratories Inc., Plainview, N.Y., U.S.A. Pregnant-mare serum gonadotrophin (Gestyl) and human chorionic gonadotrophin (Pregnyl) were obtained from Organon Laboratories Ltd., Morden, Surrey, U.K., and NADH was from Sigma Chemical Co., St Louis, Mo., U.S.A. 2,5-Diphenyloxazole and 1,4-bis-(5-phenoxazol-2-yl)benzene were from Packard Instrument Co. Inc., La Grange, Ill., U.S.A. [4-^{14}C]-Cholesterol (59.2mCi/mmol), [26-^{14}C]cholesterol (60mCi/mmol) and [7α-^{3}H]progesterone (8.5Ci/mmol) were obtained from Amersham–Searle Corporation, Toronto, Ont., Canada; [1-^{14}C]acetate (2mCi/mmol) and 20α-hydroxy[1,2-^{3}H]pregn-4-en-3-one (35.1Ci/mmol) were from New England Nuclear Corp., Boston, Mass., U.S.A. The radiochemical purity of both [4-^{14}C]- and [26-^{14}C]-cholesterol was verified by t.l.c. and radioautography; [7α-^{3}H]progesterone and 20α-hydroxy[1,2-^{3}H]pregn-4-en-3-one were purified by t.l.c. before use as tracers for the estimation of recoveries during extraction and purification procedures. [1-^{14}C]Acetate was used without purification.

EXPERIMENTAL PROCEDURES

Radioactivity determination. Radioactivity was assayed by liquid-scintillation spectrometry with either a Nuclear–Chicago Mk. 1 or a Packard Tri-Carb liquid-scintillation spectrometer. The radioactivity of non-aqueous samples was counted in a scintillation fluid consisting of 2,5-diphenyloxazole (15g), 1,4-bis-(5-phenoxazol-2-yl)benzene (150mg), naphthalene (240g), xylene (1litre) and dioxan (1litre); the xylene was replaced by dioxan for the counting of aqueous samples. Counting efficiencies, which were determined by external standardization, were never less than 68%. More than 10^4 disintegrations were normally counted, although when counting low disintegration rates (<200c.p.m. above background) a total of 4×10^3 disintegrations was recorded. Counting vials with previously determined background count rates were used when counting samples with low disintegration rates.

Administration of [^{14}C]*cholesterol* in vivo. [4-^{14}C]-Cholesterol (2μCi; 59.2mCi/mmol) was injected into the tail vein in 0.2ml of 1% (w/v) Tween 80 in 0.9% (w/v) NaCl (Major, Armstrong & Greep, 1967).

Incubation, subcellular fractionation and extraction procedures

Ovaries of immature Wistar rats were luteinized by treatment with 50i.u. of pregnant-mare serum gonadotrophin and 25i.u. of human chorionic gonadotrophin, as in previous studies (Armstrong *et al.* 1964; Flint & Denton, 1970*a*). Ovary slices (0.3–0.4mm thick) were incubated in either 5.0ml (90–150mg of tissue) or 10.0ml (150–300 mg of tissue) of bicarbonate-buffered medium (Krebs & Henseleit, 1932), containing glucose (1mg/ml) and other additions as specified, and gassed with O_2+CO_2 (95:5).

For extraction of sterols and steroids after incubation, slices were homogenized in the incubation medium in Potter–Elvehjem glass homogenizers and extracted three times with 3vol. of ethyl acetate. Ethyl acetate extracts were subsequently combined for sterol and steroid assays or specific radioactivity determinations or both. Mean recoveries (%±S.E.M.) of added tracers were 96±1.4 for [^{3}H]progesterone and 94±2.5 for 20α-hydroxy[^{3}H]pregn-4-en-3-one and 104±0.7 for [^{3}H]cholesterol.

For the centrifugal separation of subcellular fractions from either whole tissue or tissue slices previously incubated with [1-^{14}C]acetate and separated from incubation media by aspiration, tissue (0.5–1.0g) was homogenized in 0.25M-sucrose containing 10mM-$MgCl_2$ and 10mM-triethanolamine–HCl, pH7.3 (5.0ml), in a hand-operated all-glass Potter–Elvehjem homogenizer at 4°C. After centrifugation for 1min at 500$g_{av.}$ to sediment unbroken cells and large debris, the homogenate was centrifuged for 20min at 10000$g_{av.}$ to yield a pellet containing predominantly mitochondria (as judged by its glutamate dehydrogenase and cytochrome contents). The resulting supernatant was further centrifuged for 1.5h at 110000$g_{av.}$ to yield a microsomal pellet and a supernatant containing the cytoplasmic components (the cytosol). The lipid-containing granules, which rose to the surface during the 10000$g_{av.}$ and 110000$g_{av.}$ centrifugations, were carefully removed with a dry glass rod, to which it was found they would adhere almost quantitatively. They were subsequently resuspended by manual agitation in the homogenization buffer. Contamination of the cytoplasmic fraction with lipid-containing granules was prevented by aspiration of material from below the level of any remaining granules. The two centrifugation steps used to prepare mitochondrial and microsomal pellets were carried out at 2°C in an MSE Superspeed 25 centrifuge with a 6×12ml swinging-bucket rotor.

Sterols and steroids were extracted from the subcellular

fractions obtained with 4×20ml of ethyl acetate. The mitochondrial and microsomal fractions were homogenized in water before extraction; the cytosol (4–5ml) and the suspension of lipid-containing granules (4–5ml) were extracted without prior dilution or homogenization. Mitochondrial and microsomal fractions to be used for enzyme assays were homogenized in 50mM-potassium phosphate buffer, pH7.3 (5ml), with a Potter–Elvehjem homogenizer.

Whole ovaries from animals treated *in vivo* with [4-^{14}C]cholesterol were extracted by homogenization with 10ml of chloroform–methanol (2:1, v/v). Lipids were extracted and removed in chloroform solution after separation into two phases by the addition of 2ml of 0.9% (w/v) NaCl. The upper (aqueous methanolic) phase was discarded.

Steroid and sterol assays and specific radioactivity determinations

Progesterone and 20α-hydroxypregn-4-en-3-one. Specific radioactivities of progesterone and 20α-hydroxypregn-4-en-3-one in ethyl acetate extracts were determined, and the steroids concurrently assayed, after purification by methods involving t.l.c., enzymic reduction and acetylation, as follows. After the ethyl acetate extracts had been dried under a stream of N_2 at 45°C, the steroids were separated from sterols and other less-polar compounds by t.l.c. on silica gel G plates (0.5mm thick) run in hexane–ethyl acetate (5:2, v/v). Progesterone and 20α-hydroxypregn-4-en-3-one were then eluted together, with chloroform–methanol (2:1, v/v) and separated by t.l.c. on silica gel G plates (0.25mm thick) developed in methylene chloride–diethyl ether (5:2, v/v). After elution, separately, with chloroform–methanol (2:1, v/v), and reduction of the progesterone to 20β-hydroxypregn-4-en-3-one with 20β-hydroxy steroid dehydrogenase and NADH (Henning & Zander, 1962) both steroids were acetylated with acetic anhydride in pyridine. The steroid acetates were subsequently purified further by t.l.c. on plates of silica gel G (0.25mm thick) run in hexane–ethyl acetate (2:1, v/v) and the recovered 20α-hydroxypregn-4-en-3-one acetate and 20β-hydroxypregn-4-en-3-one acetate assayed fluorimetrically in ethanolic H_2SO_4 (Armstrong *et al.* 1969*b*) after partitioning between cyclohexane and water (equal volumes). Specific radioactivities were calculated after assays and radioactivity determinations were carried out on samples of the same final partition medium. Mean final recoveries of tracer [7-^{3}H]progesterone and 20α-hydroxy[1,2-^{3}H]pregn-4-en-3-one added before extraction of the tissue were 64±1.7% and 61±1.6% respectively (means±S.E.M. for 16 determinations in each case); with these tracers, losses occurring during extraction and purification were corrected for in each sample assayed.

Progesterone and 20α-hydroxypregn-4-en-3-one formed from [1-^{14}C]acetate and purified in this way from incubation mixtures and homogenates have been shown to be radiochemically pure (Armstrong *et al.* 1964).

Cholesterol. Specific radioactivities of non-esterified and esterified cholesterol in ethyl acetate or chloroform–methanol (2:1, v/v) extracts of superovulated rat ovary from animals previously treated with [^{14}C]cholesterol *in vivo* were determined after separation by t.l.c. on silica gel G plates (0.5mm thick) run in hexane–ethyl acetate (5:2, v/v) and subsequent saponification of both fractions (Flint & Denton, 1969). It is unneccessary to purify the cholesterol further from such tissue to achieve >95% radiochemical purity. In superovulated ovaries from animals injected with [^{14}C]cholesterol intravenously 24h previously, >98% of the ^{14}C is unsaponifiable. Since the tissue does not store large amounts of the steroids it secretes, and since the specific radioactivities of the steroids in the tissue equal the specific radioactivity of the tissue non-esterified cholesterol, error introduced by the presence of steroids in the unsaponifiable fraction of the extract is unlikely to be >3%. This is confirmed by the recrystallization results of Major *et al.* (1967), who showed that the specific radioactivity of [^{3}H]cholesterol in superovulated rat ovaries after administration of [^{3}H]cholesterol *in vivo* was not increased by purification by recrystallization as 5,6-dibromocholestanol.

In tissue slices incubated with [1-^{14}C]acetate it was necessary to purify further the cholesterol extracted, and this was done after t.l.c. and saponification as follows. After the initial t.l.c. in hexane–ethyl acetate (5:2, v/v) and saponification, the unsaponifiable fraction was plated on silica gel G plates (0.25mm thick) and brominated by the addition of 0.1–0.2ml of Br_2 in ethanol to the area of the plate containing the sterol (Major *et al.* 1967). After development in benzene–ethyl acetate (4:1, v/v) the 5,6-dibromocholestanol formed was scraped from the plates and debrominated with metallic Zn in acetic acid (Major *et al.* 1967); the resulting cholesterol was extracted into hexane and further purified by t.l.c. on silica gel G plates (0.25mm thick) run in benzene–ethyl acetate (4:1, v/v). Bromination and debromination were completed within 1 day. Cholesterol was subsequently assayed by a fluorimetric adaptation of the Lieberman–Burchard reaction (Armstrong *et al.* 1969*b*).

Determination of enzyme activities and cytochrome concentrations

Enzyme assays. Cholesterol side-chain cleavage enzyme assays were carried out by measuring the conversion of [26-^{14}C]cholesterol into 4[^{14}C]-methylpentanoic acid. Samples of mitochondria and microsomal fraction (9–14 mg of protein/ml in 50mM-potassium phosphate buffer, pH7.3) were added to a solution containing 0.05μCi of [26-^{14}C]cholesterol (60mCi/mmol, added in 0.05ml of dimethylformamide), 1mM-NADPH, 10mM-$MgCl_2$ in 50mM-potassium phosphate buffer, pH7.3. Total volume was 1ml; incubations were continued at 37°C for 2h. Incubations were terminated and the products extracted and separated by the method of Raggatt & Whitehouse (1966). This assay method does not permit the determination of absolute rates of cholesterol side-chain cleavage, since it is known neither to what extent the exogenous cholesterol added as substrate mixes with endogenous cholesterol nor how large the endogenous cholesterol pool is. This is unlikely to lead to a grossly misleading comparison of the relative cholesterol side-chain cleavage enzyme activities in mitochondria and microsomal fraction, however, since the fractions contained comparable amounts of endogenous non-esterified cholesterol, and both were disrupted by homogenization in hypo-osmotic medium before addition to the assay

system. Further, it is not possible by using this assay system to distinguish between the C-20,22 and C-17,20 cleavages of cholesterol; however, the cleavage of cholesterol at C-17,20 [which has been reported in testis by Jungmann (1968*a*,*b*)] is unlikely to occur to any large extent in the superovulated rat ovary, since progesterone and 20α-hydroxypregn-4-en-3-one are quantitatively the most important steroids produced by the tissue (Channing & Villee, 1966).

Glutamate dehydrogenase (EC 1.4.1.2) was assayed as described by Flint & Denton (1970*b*).

Cytochrome assays. Cytochromes ($a+a_3$) and *P*-450 were assayed by the methods of Cammer & Estabrook (1967) and Omura & Sato (1964) respectively. The extinction coefficients used were as determined by these authors.

Protein. Assays of protein in homogenates of subcellular fractions were carried out by the method of Lowry, Rosebrough, Farr & Randall (1951), with a solution of bovine serum albumin (fraction V) as standard.

Statistical analyses. Statistical analyses were made by Student's *t* test.

RESULTS AND DISCUSSION

Specific radioactivities of sterols and steroids formed in vitro from [^{14}C]acetate. Slices of superovulated rat ovary were incubated for various times in a bicarbonate-buffered medium containing glucose (1mg/ml) and 1mM-[^{14}C]acetate (2μCi/ml). Under these conditions progesterone and 20α-hydroxypregn-4-en-3-one were synthesized at constant rates (Fig. 1), the rate of synthesis of progesterone exceeding that of 20α-hydroxypregn-4-en-3-one by a factor of 1.7 ± 0.3 (mean$\pm$S.E.M. calculated from the rates of synthesis of each steroid).

The specific radioactivities of the progesterone and 20α-hydroxypregn-4-en-3-one synthesized during these incubations increased with time, reaching a maximum in the third hour; the mean values ($\pm$S.E.M.) for these specific radioactivities during the fourth hour of incubation were: progesterone, 6576 ± 1099d.p.m./μmol (4 determinations); 20α-hydroxypregn-4-en-3-one, 6626 ± 678 d.p.m./μmol (4 determinations). The specific radioactivities, determined after bromination and debromination, of non-esterified cholesterol in the tissue slices after incubation for 3h were 1986 ± 363 d.p.m./μmol (4) and 812 ± 177d.p.m./μmol (4) respectively; during the fourth hour of incubation these values increased to 2679 ± 437d.p.m./μmol (4) and 917 ± 204d.p.m./μmol (4) respectively. Thus during the fourth hour of incubation the mean specific radioactivity of the progesterone+20α-hydroxypregn-4-en-3-one synthesized exceeded the specific radioactivities of non-esterified and esterified cholesterol in the tissue by factors of 2.8–2.9 and 7.6–7.7 respectively. Similar observations have been made previously with a number of steroidogenic endocrine tissues (see the introduction for references).

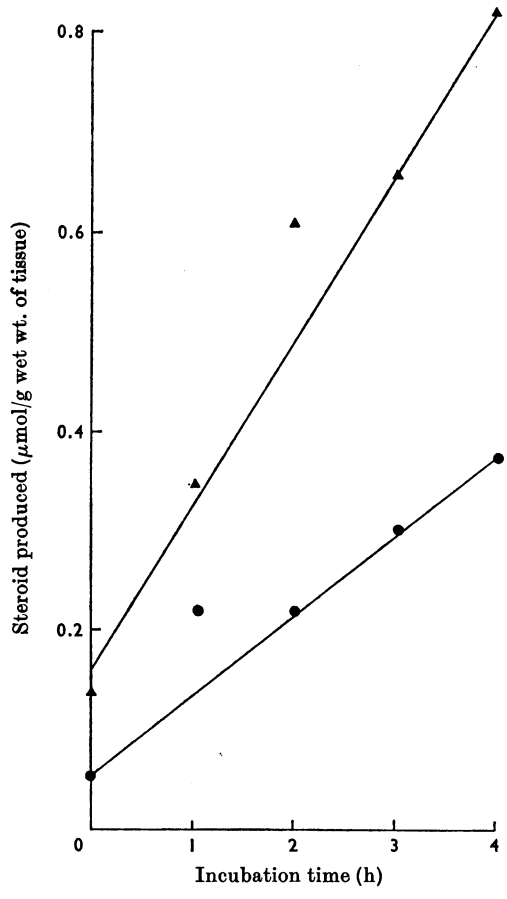


Fig. 1. Effect of incubation time on synthesis of progesterone (▲) and 20α-hydroxypregn-4-en-3-one (●) by slices of superovulated rat ovary incubated *in vitro*. Slices were incubated in 5ml of bicarbonate-buffered medium containing glucose (1mg/ml) for various times. Steroids were extracted with ethyl acetate from slices+media after homogenization together, and were subsequently assayed fluorimetrically after purification by t.l.c., enzymic reduction of progesterone to 20β-hydroxypregn-4-en-3-one and acetylation. Losses during extraction and purification were established with the aid of [7α-^{3}H]progesterone and 20α-hydroxy[1,2-^{3}H]pregn-4-en-3-one added to the homogenate of slices+medium before extraction.

To account for these differences between the steroid and cholesterol specific radioactivities, tissue slices incubated as described above with [1-^{14}C]-acetate for 3h were homogenized in buffered 0.25M-sucrose and separated by differential centrifugation into four subcellular fractions. Both non-esterified and esterified cholesterol were present in all the fractions prepared; their specific radioactivities are shown in Table 1. The specific radioactivity of

Table 1. *Specific radioactivities of non-esterified and esterified cholesterol in subcellular fractions prepared from slices of superovulated rat ovary incubated with* [1-^{14}C]*acetate*

Slices were incubated for 3h in 10ml of bicarbonate-buffered medium containing glucose (1mg/ml) and 1mM-[1-^{14}C]acetate (2μCi/ml). After incubation the slices were removed from the incubation medium and homogenized in buffered 0.25M-sucrose containing 10mM-$MgCl_2$ before centrifugal separation into subcellular fractions as described in the Experimental Procedures section. Specific radioactivities of non-esterified and esterified cholesterol in each fraction were subsequently determined after saponification, bromination and debromination; values are expressed as d.p.m./μmol of cholesterol (mean±S.E.M. of six determinations in each case).

Subcellular fraction	Specific radioactivity of cholesterol (d.p.m./μmol) Non-esterified	Esterified
Lipid granules	1420±195	730±100
Cytosol	1220±374	600± 91
Microsomal fraction	4020±443	760± 74
Mitochondria	1510±254	700±134

the microsomal non-esterified cholesterol exceeded the specific radioactivities of non-esterified cholesterol in the other fractions (which were equal) by a factor of 2.9. No differences were detected between the specific radioactivities of esterified cholesterol in the subcellular fractions prepared. The mean specific radioactivities of the progesterone and 20α-hydroxypregn-4-en-3-one in each subcellular fraction (not shown in Table 1) were 5855±734 d.p.m./μmol (20 determinations) and 6454±747 d.p.m./μmol (20 determinations) respectively; no differences in specific radioactivities were noted between fractions. Thus the specific radioactivities of progesterone and 20α-hydroxypregn-4-en-3-one in the slices at the end of the incubation with [1-^{14}C]acetate were significantly higher ($P<0.001$) than the specific radioactivities of both non-esterified and esterified cholesterol in the mitochondria, cytosol and lipid-containing granules. There was, however, little difference ($P>0.025$) between the mean specific radioactivity of the progesterone+20α-hydroxypregn-4-en-3-one (which was 6154±707 d.p.m./μmol) and the specific radioactivity of microsomal non-esterified cholesterol (4021±443 d.p.m./μmol). That the specific radioactivity of microsomal non-esterified cholesterol is higher than that of the cholesterol in other subcellular components is not unexpected, since the enzymes responsible for the conversion of squalene into cholesterol have been reported to be located exclusively in the endoplasmic reticulum.

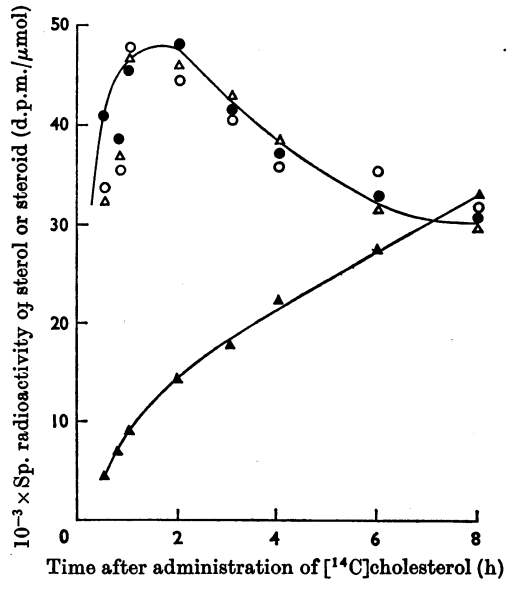


Fig. 2. Effect of time after administration on the specific radioactivities of non-esterified cholesterol (○), esterified cholesterol (▲), progesterone (△) and 20α-hydroxypregn-4-en-3-one (●) in the ovaries of superovulated rats after intravenous injection of [4-^{14}C]cholesterol. Ovaries were removed after the administration of 2μCi of cholesterol and lipids extracted in chloroform–methanol (2:1, v/v) by homogenization. Specific radioactivities of non-esterified cholesterol were determined in each ovary; specific radioactivities of progesterone and 20α-hydroxypregn-4-en-3-one were determined in pooled fractions extracted from each pair of ovaries. Thus each value for the specific radioactivity of non-esterified cholesterol represents the mean of six or more determinations; each value for the specific radioactivity of progesterone or 20α-hydroxypregn-4-en-3-one is the mean of three or more determinations.

The difference observed here between the specific radioactivities of non-esterified cholesterol in microsomal and other subcellular fractions is not in agreement with the findings of Ichii & Kobayashi (1966), which indicate that in adrenal-cortex slices incubated with [^{14}C]acetate and subsequently fractionated by differential centrifugation the mitochondrial sterol has a higher specific radioactivity than that in other fractions. However, these authors neither purified the sterol extracted nor determined the specific radioactivity of that in microsomal fraction. It is therefore possible that they were dealing with a mixture of sterols, and that microsomal material contaminated their mitochondrial fractions.

Specific radioactivities of steroids and sterols after administration of [^{14}C]*cholesterol* in vivo. Since the

specific radioactivities of the steroids in subcellular fractions prepared from ovary slices incubated with [^{14}C]acetate *in vitro* approximated to those of the microsomal cholesterol, it was considered probable that these compounds are formed from non-esterified cholesterol rather than from esterified cholesterol *in vivo*. Further evidence for this relationship was derived from an examination of the specific radioactivities of the whole-tissue progesterone, 20α-hydroxypregn-4-en-3-one, non-esterified cholesterol and esterified cholesterol after the administration of [4-^{14}C]cholesterol *in vivo* (Fig. 2). It was found that, at all times up to 8h after the administration of [4-^{14}C]cholesterol, the specific radioactivity of the non-esterified cholesterol in the tissue exceeded that of the esterified cholesterol; the difference reached a maximum after 2h, when the specific radioactivity of the non-esterified cholesterol (then 46.0$\pm$7.2d.p.m./μmol) exceeded that of esterified cholesterol (13.8$\pm$1.5d.p.m./μmol) by a factor of 3.3. The mean specific radioactivity of the progesterone+20α-hydroxypregn-4-en-3-one in the tissue at this time was 48.3$\pm$11.2d.p.m./μmol. At all times selected, the specific radioactivities of both progesterone and 20α-hydroxypregn-4-en-3-one extracted from the tissue were the same as the specific radioactivity of the non-esterified cholesterol, thus verifying *in vivo* the precursor–product relationship predicted from the observations made *in vitro*.

These findings confirm those made previously by Daily, Swell & Treadwell (1962) and Raggatt & Whitehouse (1966), indicating that non-esterified cholesterol rather than esterified cholesterol was the substrate for side-chain cleavage in the adrenal cortex. The former authors based their proposal on the rates of steroid formation from labelled non-esterified or esterified cholesterol added separately to homogenates. The conclusions of Raggatt & Whitehouse (1966) were based on an examination of the substrate specificity of a partially purified preparation of cholesterol side-chain cleavage enzyme.

Detection of cholesterol side-chain cleavage enzyme in mitochondria and microsomal fraction. The observation that the specific radioactivities of progesterone and 20α-hydroxypregn-4-en-3-one approximate to that of microsomal non-esterified cholesterol in ovary slices incubated with [1-^{14}C]-acetate indicates that under these conditions cholesterol utilized for steroidogenesis does not equilibrate with cholesterol in other pools before side-chain cleavage. This suggests the presence of cholesterol side-chain cleavage enzyme in the microsomal fraction, in addition to its generally accepted location in mitochondria. Experiments designed to test this possibility are summarized in Fig. 3, showing the results of cholesterol side-chain cleavage enzyme assays carried out on mitochondria and microsomal fractions at different concentrations of Mg^{2+}. Whereas the activity in the microsomal fraction was not influenced by [Mg^{2+}], that in the mitochondria was increased up to 9.5-fold by Mg^{2+}, reaching a maximum activity at 10mM-Mg^{2+}. Stimulation of cholesterol side-chain cleavage enzyme by Mg^{2+} has also been observed with the enzyme from rat testis mitochondria (Drosdowsky, Menon, Forchielli & Dorfman, 1965). This is not indisputable evidence, however, for the existence of cholesterol side-chain cleavage enzyme in two separable subcellular compartments: it is possible that the activity sedimenting with the microsomal fraction was due to submitochondrial particles, and that the apparent difference in response to Mg^{2+} was due to differences in permeability characteristics, which might be expected between submitochondrial particles and unbroken mitochondria. The mitochondrial and microsomal fractions were therefore assayed for the typically mitochondrial components, cytochrome ($a+a_3$) and glutamate dehydrogenase (Table 2). Two mitochondrial markers of different intramitochondrial distribution were chosen to increase the chance of detecting contamination of the microsomal fraction by particles of broken mitochondria. Amounts of glutamate dehydro-

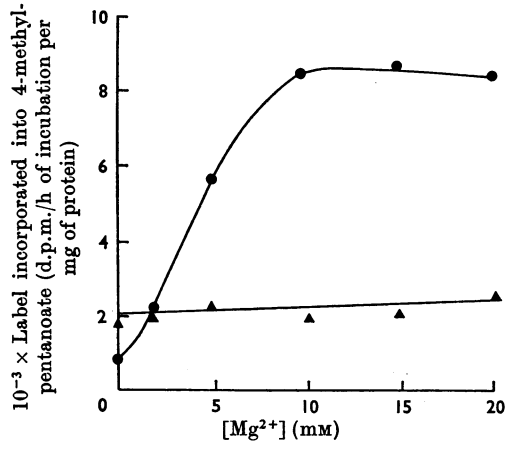


Fig. 3. Effect of Mg^{2+} concentration on the activity of cholesterol side-chain cleavage enzyme in mitochondria (●) and microsomal fractions (▲) from superovulated rat ovary. Mitochondria and microsomal fractions were prepared from whole ovaries by homogenization in buffered 0.25M-sucrose and differential centrifugation as described in the Experimental Procedures section. The activity of cholesterol side-chain cleavage enzyme was subsequently determined in each fraction by incubation with [26-^{14}C]cholesterol and isolation of the product, 4[^{14}C]-methylpentanoate. Each value is the mean of three determinations; non-incubated control values have been subtracted.

Table 2. *Enzymes and cytochromes in mitochondria and microsomal fractions from superovulated rat ovary*

Mitochondria and microsomes were prepared from whole ovaries by homogenization and centrifugation, and enzymes and cytochromes assayed, as described in the Experimental Procedures section. Both subcellular fractions contained 9–14mg of protein/ml. Values are expressed as means±S.E.M. for the numbers of determinations in parentheses. Units are specified in each case. 1 unit of enzyme activity converts 1 μmol of substrate/min (measured at 25°C). Values for cholesterol side-chain cleavage enzyme activity have been corrected for activity in non-incubated controls containing enzyme denatured by boiling, as described by Raggatt & Whitehouse (1966).

	Activity		Ratio Activity in microsomal fraction / Activity in mitochondria
	In mitochondria	In microsomal fraction	
Cholesterol side-chain cleavage enzyme (d.p.m./h per mg of protein)	8464±888 (9)	2134±649 (9)	0.25±0.05
Cytochrome *P*-450 (nmol/g of protein)	290± 10 (6)	127± 13 (5)	0.44±0.03
Cytochrome ($a+a_3$) (nmol/g of protein)	349± 27 (6)	<46 (5)	<0.13
Glutamate dehydrogenase (units/g of protein)	56± 6 (4)	<0.24 (4)	<0.0043

Table 3. *Effect of removal of possible contamination of microsomal fractions by light mitochondria on cholesterol side-chain cleavage enzyme activity in microsomal and mitochondrial fractions*

Superovulated rat ovaries were homogenized in 0.25M-sucrose containing additives as described in the Experimental Procedures section. After an initial centrifugation for 1 min at 500$g_{av.}$ the homogenate was divided into three portions and mitochondria were sedimented by centrifugation at 10000, 30000 or 50000$g_{av.}$ for 20 min. The three supernatants thus obtained were then used to prepare microsomal fractions by further centrifugation at 110000 $g_{av.}$ for 90min. Cholesterol side-chain cleavage enzyme and protein were assayed in the mitochondrial and microsomal pellets. Cholesterol side-chain cleavage enzyme activity is expressed as the mean±S.E.M. of four determinations; non-incubated control values have been subtracted as in Table 2. Values for protein contents are means of two determinations.

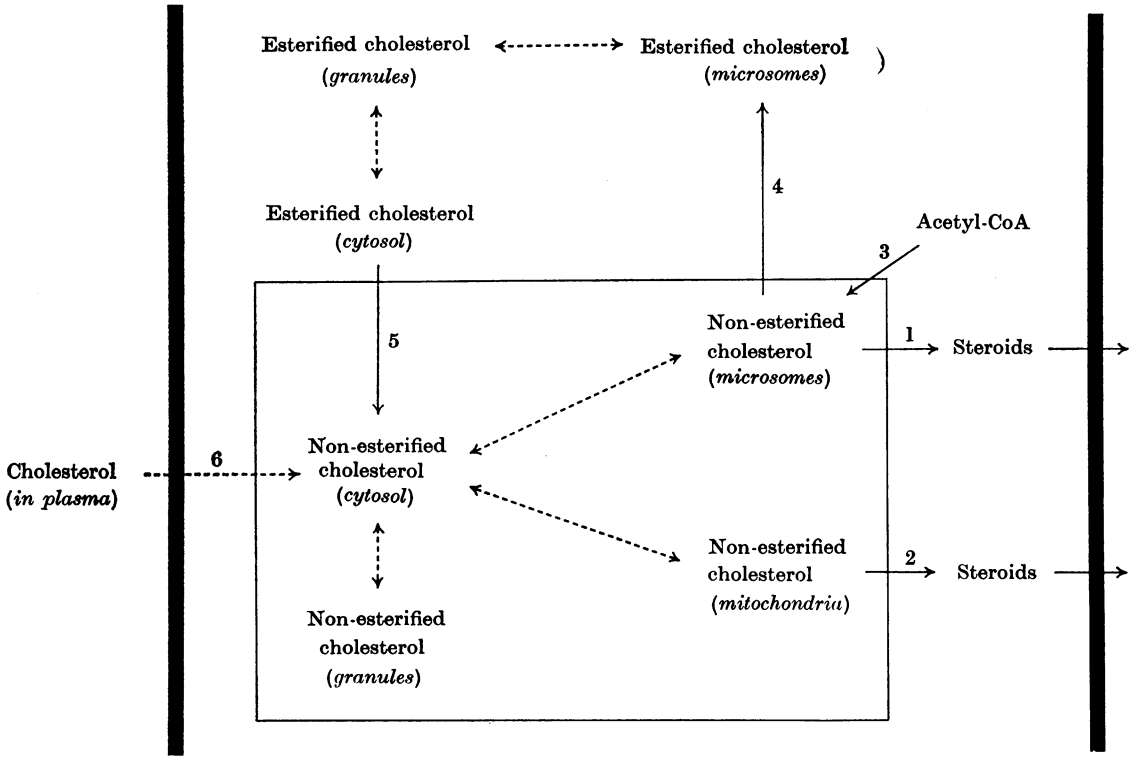
Fraction ($g_{av.}$)	Wt. of protein in pellet (mg)	Cholesterol side-chain cleavage enzyme activity (d.p.m. incorporated into 4-methylpentanoate/h per mg of protein)
Mitochondrial (10000–500)	24.5	5119±701
Mitochondrial (30000–500)	25.2	5341±383
Mitochondrial (50000–500)	27.5	4085±218
Microsomal (110000–10000)	4.1	2606±384
Microsomal (110000–30000)	3.7	4760±471
Microsomal (110000–50000)	2.5	3185±548

genase and cytochrome ($a+a_3$) present in microsomal preparations were found to be below the lower limit of reliability of the assays used, although suspensions of comparable protein content were used. Expressed as (enzyme content of microsomes)/(enzyme content of mitochondria) ratios the results clearly indicate a higher concentration of cholesterol side-chain cleavage enzyme and cytochrome *P*-450 than of mitochondrial markers in microsomal fractions.

To preclude the possibility that the cholesterol side-chain cleavage enzyme activity present in microsomes was due to the presence of light mitochondria not sedimented by centrifugation at 10000$g_{av.}$ for 20min, an experiment was undertaken in which mitochondria were sedimented from three samples of the same homogenate by centrifugation for 20min at 10000, 30000 and 50000$g_{av.}$ (Table 3). The three supernatants from these centrifugation steps were then further centrifuged at 110000$g_{av.}$ for 90min to sediment the microsomal fractions they contained. Assays of cholesterol side-chain cleavage enzyme and protein in each of the three microsomal pellets showed no decrease in the specific activity of the enzyme as mitochondria were sedimented at higher speeds. Nor was there any increase in the specific activity in the corresponding mitochondrial fractions. There was, however, a marked removal of protein from microsomal fractions into mitochondria as the latter were sedimented at increasing speeds.

It was concluded that the microsomal fraction was not contaminated significantly by mitochondrial particles. The detection in microsomal preparations of cytochrome *P*-450, a component of the cholesterol side-chain cleavage enzyme complex (Koritz, 1966; Simpson & Boyd, 1966), was regarded as further evidence for the presence of side-chain cleavage activity in microsomes.

General aspects of the compartmentation of cholesterol in subcellular organelles. The existence of a cholesterol side-chain cleavage enzyme in both



Scheme 1. Proposed inter-relationships between cholesterol pools in superovulated rat ovary. The non-esterified cholesterol pools are shown surrounded by a thin line; cell membranes are represented by thick lines. Solid arrows indicate chemical transformations; broken arrows indicate equilibration steps or transfers between pools. The following numbered steps have been demonstrated in either the superovulated rat ovary or another steroidogenic tissue: 1, microsomal cholesterol side-chain cleavage; 2, mitochondrial cholesterol side-chain cleavage; 3, incorporation of acetate carbon atoms into cholesterol; 4, microsomal esterification of non-esterified cholesterol; 5, de-esterification of esterified cholesterol by a soluble esterase; 6, uptake of cholesterol from plasma.

mitochondria and microsomal fractions raises the possibility of two discrete and totally compartmentalized steroidogenic pathways from cholesterol. Δ^5 3β-Hydroxy steroid dehydrogenase is also present in both mitochondria and microsomal fractions in the superovulated rat ovary (Sulimovici & Boyd, 1969), thus completing the pathway from cholesterol to progesterone in both compartments.

The findings described above, and those of other authors relating to the compartmentation of cholesterol, can be summarized schematically (Scheme 1). In this scheme the total non-esterified cholesterol pool is shown with three inputs and two outlets. The inputs are: (*a*) uptake from plasma by transport across the cell membrane, as can be seen to occur after administration of labelled cholesterol *in vivo*; (*b*) synthesis from acetate; (*c*) release from esterified cholesterol by hydrolysis. The input of non-esterified cholesterol from plasma is shown entering the cytosol pool, since the cytosol is generally assumed to border the cell membrane; that from acetate is depicted entering the microsomal compartment, as a result of the findings described in the present paper, and that from esterified cholesterol stored in lipid-containing granules is shown entering the cytosol pool, since the cholesterol esterase (EC 3.1.1.13) responsible for the hydrolysis is a soluble enzyme (Behrman & Armstrong, 1969). Outlets from the non-esterified cholesterol compartment are depicted occurring: (*a*) to steroids from both mitochondrial and microsomal cholesterol, as a result of the findings described in the present paper, and (*b*) from the microsomal pool to esterified cholesterol, since the cholesterol ester synthetase responsible for this conversion is microsomal in the superovulated rat ovary (Behrman, Orczyk, Macdonald & Greep, 1970) and the adrenal cortex (Longcope & Williams, 1962; Shyamala, Lossow & Chaikoff, 1966). Equilibration between subcellular non-esterified

Table 4. *Specific radioactivities of non-esterified and esterified cholesterol in, and of progesterone and 20α-hydroxypregn-4-en-3-one formed by, slices of superovulated rat ovaries taken from rats injected with [4-^{14}C]cholesterol intravenously 24h previously*

Ovaries were taken from superovulated rats injected with 2 μCi of [4-^{14}C]cholesterol 24h previously via the tail vein; ovary slices were subsequently incubated for 3h in a bicarbonate-buffered medium containing glucose (1mg/ml). Non-incubated and incubated slices were extracted with ethyl acetate after homogenization in the incubation medium, and the specific radioactivities of the non-esterified and esterified cholesterol, progesterone and 20α-hydroxypregn-4-en-3-one determined as described in the Experimental Procedures section. Values (d.p.m./μmol) are expressed as percentages (means±S.E.M. for the number of determinations in parentheses) relative to the specific radioactivity of the esterified cholesterol in non-incubated slices, defined for the purposes of the calculation as 100%. n.d., Not determined. The lowest value for the specific radioactivity of esterified cholesterol encountered in this experiment was 3.9×10^3 d.p.m./μmol.

Sterol or steroid	Sp. radioactivities (relative to that of cholesterol ester) (d.p.m./μmol)	
	Non-incubated slices	Incubated slices
Esterified cholesterol	100 (defined) (9)	100± 3.8 (9)
Non-esterified cholesterol	98±4.0 (9)	102±10.3 (9)
Progesterone	n.d.	100± 2.0 (6)
20α-Hydroxy-pregn-4-en-3-one	n.d.	106± 4.8 (6)

cholesterol pools is tentatively depicted as occurring between the cytosol and the microsomes, mitochondria and lipid-containing granules. Direct equilibration between particulate cell components is unlikely to be as rapid as that between the cytosol and the particles, since the former must rely on contact, the latter presumably being achieved by diffusion. Experimental evidence is provided by a comparison of the observed rates of equilibration of non-esterified cholesterol derived from plasma cholesterol on the one hand with that generated endogenously on the other. In the former case equilibration is rapid and the specific radioactivities of steroids produced correspond closely to those of the whole-tissue non-esterified cholesterol within 0.5h after administration of the label (Fig. 2); in the latter, non-esterified cholesterol formed in the endoplasmic reticulum does not equilibrate immediately with that in the other subcellular organelles (Table 1). That equilibration between non-esterified and esterified cholesterol compartments and steroids in the tissue reaches completion is indicated by the experimental data presented in Table 4. Slices from the ovaries of rats injected with [4-^{14}C]cholesterol intravenously 24h previously were incubated for 3h and the specific radioactivities of non-esterified and esterified cholesterol in the tissue slices and of progesterone and 20α-hydroxypregn-4-en-3-one released into the medium were determined. The specific radioactivities of sterols before and after incubation and of steroids produced during incubation were identical, indicating that equilibration between non-esterified and esterified cholesterol pools and pools of steroid is completed during exposure to [^{14}C]cholesterol for 24h.

Since (*a*) cholesterol ester synthetase is microsomal, (*b*) esterified cholesterol is stored by steroidogenic tissues in lipid-containing granules or droplets and (*c*) the action of the microsomal cholesterol ester synthetase is likely to lead to microsomal cholesterol ester, it is evident, on biochemical grounds, that esterified cholesterol is transferred from the endoplasmic reticulum to storage granules. Electron-microscopic examination of porcine luteal cells has led Bjersing (1967) to the conclusion that the lipid-containing storage granules are formed from the endoplasmic reticulum by a pinching-off process, in a manner apparently analogous to the formation of storage granules in other secretory tissues. The fact that lipid-containing storage granules are apparently surrounded by a lipoprotein layer or membrane (Smith, 1968; Christensen & Gillim, 1969) further underlines similarities between these tissues and other secretory organs where the secretory products are stored in granules generated from endoplasmic reticulum, and relatively minor chemical alterations of the storage material occur before secretion.

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