The preparation and purification of isolated rat corpus-luteum cells and their use in studying the relationship between cholesterol biosynthesis and the lutropin-stimulated formation of progesterone

Brian C. McNAMARA, Christine E. G. CRANNA,* Roger BOOTH and David A. STANSFIELD
Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee DD1 4HN,
Scotland, U.K.

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Isolated luteal cells, prepared from superovulated rat ovaries by digestion with collagenase, were subjected to density-gradient centrifugation on Percoll to give a more highly purified preparation of luteal cells than has been reported previously. The cells formed progesterone when incubated *in vitro*; lutropin stimulated this steroidogenesis. Progesterone formation was linear for at least 2 h; a minimal lutropin concentration of 1.0 ng/ml was needed for stimulation and concentrations of 3.0 and 100 ng/ml gave half-maximal and maximal responses respectively. The cells were unresponsive towards hormones other than lutropin. Exposure to lutropin raised the cellular cyclic AMP concentration, and dibutyryl cyclic AMP, but not dibutyryl cyclic GMP, was as effective in stimulating steroidogenesis as was lutropin. Aminoglutethimide, an inhibitor of cholesterol side-chain cleavage, completely blocked progesterone formation by the cells, showing cholesterol side-chain cleavage to be an obligatory step in steroidogenesis by these cells. Neither the activity of 3-hydroxy-3-methylglutaryl-CoA reductase nor the incorporation of radioactively labelled acetate or mevalonate into cholesterol by cells incubated *in vitro* were detectable unless the rats had been treated previously with 4-aminopyrazolo[3,4-*d*]pyrimidine. In cells from rats so treated, compactin was found to block almost completely the incorporation of radioactively labelled acetate, but not of mevalonate, into cholesterol, indicating that this inhibitor acts in corpus luteum in the same way as it does in other tissues. In cells from rats not treated with 4-aminopyrazolo[3,4-*d*]pyrimidine compactin had no effect on progesterone formation *in vitro*, showing cholesterol biosynthesis to be unnecessary for the rapid steroidogenic response by luteal cells to lutropin.

Lutropin stimulates the production of progesterone by the corpus luteum. The cells of this tissue have a specific receptor to which lutropin binds (Conti *et al.*, 1977; Catt *et al.*, 1979) and, by activating the membrane adenylate cyclase, consequently raises the concentration of intracellular cyclic AMP (Conti *et al.*, 1977; Sala *et al.*, 1979). Corpora lutea of both rat (Webb & Stansfield, 1974) and cow (Ling & Marsh, 1977; Hardie & Stansfield, 1977) are known to contain cyclic AMP-dependent protein phosphokinases and these were found to be stimulated in slices of bovine corpus luteum incubated with lutropin (Ling & Marsh, 1977). However, the way in which the events so far described are involved in the

stimulation of progesterone formation by lutropin is not understood because the site(s) on the pathway of progesterone formation that is stimulated in corpus luteum exposed to lutropin has not been unequivocally identified (Marsh, 1975).

During progesterone formation, the side-chain of cholesterol is removed by the mitochondrial side-chain-cleavage enzyme, giving pregnenolone, which is then converted into progesterone (Savard, 1973). But since mitochondria neither store nor synthesize cholesterol, the mechanism whereby cholesterol is provided for progesterone formation must be considered as a possible target for the action of lutropin. Corpus luteum is known to contain cholesteryl esters and a cholesteryl ester hydrolase (Coutts & Stansfield, 1968); it is able to synthesize cholesterol from acetate (Hellig & Savard, 1965) and can possibly ingest circulating lipoproteins as

* Present address: Department of Forensic Medicine and Science, University of Glasgow, Glasgow, Scotland, U.K.

several other tissues have recently been shown to do (Goldstein & Brown, 1977). The finding by Shima *et al.* (1968) that cholesterol infused into rats was used, in preference to that synthesized *de novo*, for progesterone formation supports this latter possibility.

In theory, therefore, cholesterol for steroidogenesis could arise by the mobilization of cellular cholesteryl esters, or of a special pool of unesterified cholesterol, by synthesis *de novo* of cholesterol or by ingestion of external cholesterol or its esters, and one or more of these processes could be the site of stimulation by lutropin.

The purpose of the work described here was to develop procedures for isolating corpus-luteum cells from superovulated rat ovaries and to characterize suspensions of these so as to assess their value in attempting to define the mechanism of cholesterol provision for steroidogenesis. In addition the possible role of cholesterol biosynthesis in lutropin-stimulated progesterone formation by the corpus luteum has been evaluated.

Experimental

Animals

Immature female Wistar rats were purchased at 26 days old from Bantin and Kingman (Aldborough, Hull, U.K.), and were superovulated (Parlow, 1958) by giving a subcutaneous injection of 50 i.u. of pregnant-mare's-serum gonadotropin, followed 66 h later by a subcutaneous injection of 25 i.u. of human choriongonadotropin. The rats were housed in a room artificially illuminated between 06:00 h and 18:00 h and in darkness between 18:00 h and 06:00 h, and allowed free access to food and water. They were killed, by a blow on the head and neck fracture, between 09:00 h and 10:30 h 5–7 days after the second of the two gonadotropin injections. Rats treated with 4-aminopyrazolo[3,4-*d*]pyrimidine were given a daily intraperitoneal injection of this compound in 0.01 M-KH₂PO₄ for 3 days before use (Balasubramaniam *et al.*, 1977; Christie *et al.*, 1979).

Chemicals

Pregnant mare's serum gonadotropin (Gestyl) and human choriongonadotropin (Pregnyl) were purchased from Organon Laboratories, Morden, Surrey, U.K. Dulbecco's Modified Eagle's (DME) medium containing Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonate] and DME '10×' medium (10 times-concentrated DME medium) were obtained from Flow Laboratories, Irvine, Scotland, U.K. Collagenase (type I), bovine serum albumin (fraction V), 4-aminopyrazolo[3,4-*d*]pyrimidine, gelatin and thimerosal were purchased

from Sigma London Chemical Co., Poole, Dorset, U.K. Ovine lutropin (DLH NIAMDD-LH-21), ovine follitropin (NIH-FSH-S9), ovine prolactin (NIH-P-S6) and bovine thyrotropin were kindly given by the National Institutes of Health, Bethesda, MD, U.S.A. Progesterone-specific antiserum, raised in rabbits to progesterone-11 α -hemisuccinyl-bovine serum albumin (deVilla *et al.*, 1972), was generously donated by Dr. G. Wilson, Forresterhill Maternity Hospital, Aberdeen, Scotland, U.K., and compactin (ML-236B) was kindly given by Dr. R. Fears, Beecham Pharmaceuticals, Animal Health Research Centre, Walton Oaks, Tadworth, Surrey, U.K. Percoll and Dextran T70 were purchased from Pharmacia, Hounslow, Middx., U.K. [1,2,6,7(n)-³H]Progesterone (sp. radioactivity 85 Ci/mmol), [U-¹⁴C]sodium acetate (sp. radioactivity 57 Ci/mol), DL-[2-¹⁴C]mevalonic acid lactone (sp. radioactivity 5–10 Ci/mol) and 3-hydroxy-3-methyl[3-¹⁴C]-glutarate (sp. radioactivity 40–60 Ci/mol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Compactin is a lactone; it and DL-[2-¹⁴C]mevalonic acid lactone were converted into their acid forms before use by being dissolved in 0.1 M-KOH and held at 37°C for 30 min. For storage, solutions of these compounds were adjusted to pH 7.5 and frozen at –20°C. Aminoglutethimide was purchased from Ciba-Geigy (UK) Pharmaceuticals, Cambridge, U.K. Dibutyryl cyclic AMP and coenzyme A were obtained from The Boehringer Corp. (London), Lewes, East Sussex, U.K., and Unisolve I was from A. and J. Beveridge, Edinburgh, Scotland, U.K. 3-Hydroxy-3-methyl[3-¹⁴C]glutaryl-CoA was prepared by methods described previously (Gregory *et al.*, 1972).

Preparation and incubation of isolated luteal cells

Superovulated rat ovaries were trimmed of fat and connective tissue and chopped in DME medium buffered with 20 mM-Hepes and containing 0.5% (w/v) bovine serum albumin at pH 7.2. The chopped tissue was washed three times with this medium to remove blood, then incubated at 37°C in a polypropylene tube in the above medium containing 0.5% (w/v) collagenase. Each 125 mg of chopped tissue was incubated with 1 ml of medium. The tubes were gassed with O₂/CO₂ (19:1) before being sealed with a plastic cap. At intervals of 7 min the digest was drawn in and out of a 2 ml plastic syringe to aid disaggregation and after 35 min the tube contents were filtered through a 64 μ m-mesh nylon gauze to remove undigested tissue. Cells were sedimented from the filtered suspension by centrifugation at 1000 g for 5 min at 4°C (r_{av} = 12 cm), then suspended in 1 ml of DME medium containing 20 mM-Hepes and 0.5% (w/v) bovine serum albumin at pH 7.2. The suspension was filtered through 64 μ m

nylon gauze to remove large aggregates of cells, then layered on to a 0–75% gradient of Percoll. This was formed by adding 9 ml of Percoll to 1 ml of DME '10x' medium containing 200 mM-Hepes at pH 7.2, then diluting this to 75% Percoll with DME medium containing 20 mM-Hepes (pH 7.2) and 0.5% (w/v) bovine serum albumin. The gradient was then prepared by mixing from a gradient maker 7.5 ml of the 75% Percoll and 7.5 ml of DME medium containing 20 mM-Hepes (pH 7.2) and 0.5% (w/v) bovine serum albumin. The cells were centrifuged on this gradient at 5000 *g* for 5 min ($r_{av.} = 10$ cm). Steroidogenic cells were removed from the gradient using a plastic pipette and washed three times by centrifugation at 1000 *g* for 5 min at 4°C ($r_{av.} = 12$ cm) and resuspension in DME medium containing 20 mM-Hepes and 0.5% (w/v) bovine serum albumin at pH 7.2. The final cell suspension was filtered through 64 μ m nylon gauze and 500 μ l portions of the suspension were immediately dispensed into polypropylene tubes. A sample was also transferred to a haemocytometer for cell counting. Routinely, tubes contained cells at a concentration of 10^6 – 10^7 cells/ml in a final volume of 0.6 ml. They were gassed with O₂/CO₂ (19:1), then capped and, unless otherwise stated, incubated for 2 h at 37°C with shaking at 60 oscillations/min. After incubation the tube contents were immediately sonicated and extracted as described below.

Progesterone assays

Sonicated cell suspensions were extracted with two 5 ml volumes of light petroleum (b.p. 40–60°C); freezing the aqueous phase in a solid CO₂/ethanol bath separated the two layers cleanly, allowing easy transfer of the organic layer to a clean tube. The organic solvent was evaporated under N₂ and the residue was suspended in 2.5 ml of 0.1 M-Tris/HCl buffer, pH 8.3, containing 0.2 M-NaCl, 1 g of gelatin/litre and 0.1 g of thimerosal/litre. A portion of this suspension was made up to 0.5 ml with 0.1 M-Tris/HCl buffer, pH 8.3, and 12.5 nCi of [1,2,6,7(n)-³H]progesterone, dissolved in the same buffer, was added; 500 μ l of progesterone antiserum (diluted 500–1000 fold with 0.1 M-Tris/HCl buffer, pH 8.3) was added and the mixture was incubated at 45°C for 15 min. After cooling on ice for 15 min, 0.5 ml of a suspension containing 1.25 mg of charcoal and 12.5 μ g of Dextran T70 in 0.1 M-Tris/HCl buffer, pH 8.3, was added with thorough mixing. The mixture was placed on ice for 10 min, then centrifuged at 2500 *g* for 15 min at 4°C ($r_{av.} = 23$ cm). Samples (0.5 ml) of the supernatants were added to 5 ml of Unisolve I scintillation fluid and counted for radioactivity in a Beckman LS 7000 scintillation counter. Counting efficiency, as determined by the channels ratio method (Baillie, 1960),

was 40–50%. A range of standards containing up to 16 ng of progesterone was assayed at the same time as were the cell extracts, giving a standard curve from which the progesterone content of unknowns could be calculated. This method was based on that of Morgan & Cooke (1972).

Cyclic AMP assay

Cyclic AMP was assayed as previously described (Young & Stansfield, 1977).

Cell viability

Nigrosine exclusion was used as a test of cell viability; the procedure of Kaltenbach *et al.* (1958) was followed.

Extraction of cholesterol and progesterone from cell suspensions after incubation with sodium [U-¹⁴C] acetate or DL-[2-¹⁴C]mevalonic acid

After incubation, 350 μ g of progesterone and 100 μ g of cholesterol were added to cell suspensions followed by 6 ml of 5% (w/v) KOH in aq. 77% (v/v) ethanol. The mixtures were held at 60°C for 1 h then diluted to 20 ml with water. Unsaponified lipids were extracted with two 20 ml volumes of diethyl ether. Diethyl ether extracts were evaporated to dryness under N₂ and the residues were transferred to thin-layer plates of Silica Gel GF₂₅₄. These were developed in two dimensions, the first being benzene/ethyl acetate (9:1 v/v) (Stansfield & Cargill, 1963) and the second chloroform/methanol (49:1, v/v). Progesterone spots were located under a u.v. lamp and cholesterol spots were made visible by spraying the plates with a solution containing 0.5% (w/v) rhodamine 6G in acetone. The lipids were eluted with two 5 ml volumes of diethyl ether and the eluates were transferred to scintillation vials for counting as described above.

3-Hydroxy-3-methylglutaryl-CoA reductase assay

Ovaries were homogenized in a Teflon/glass homogenizer in a medium containing 0.1 M-sucrose, 0.05 M-KCl, 0.04 M-KH₂PO₄ and 0.03 M-EDTA, pH 7.2, adding 2 ml of buffer to each 1 g of tissue. The homogenate was centrifuged at 10 000 *g* for 3 min at 4°C ($r_{av.} = 7$ cm) and the resulting supernatant centrifuged at 100 000 *g* for 1 h at 4°C ($r_{av.} = 5$ cm). The sediment was re-suspended in the homogenizing buffer and used in assays for 3-hydroxy-3-methylglutaryl-CoA reductase as previously described (Gregory *et al.*, 1972). Experiments conducted during the present study showed that this subcellular fraction contained 80% of the activity of the initial homogenate. One unit of enzyme activity generates 1 nmol of mevalonate in 1 min. Specific enzyme activity is expressed as units/mg of protein.

Presentation of results

During the course of the work, cells were found to vary from one experiment to another in the amounts of progesterone they were able to synthesize *in vitro*; each experiment was performed at least three times and each Table gives the results of three separate experiments carried out on different days. This method of presentation seems preferable to any involving the normalization of values, or the use of a ratio such as the amount of progesterone formed in the presence of lutropin divided by the amount formed in its absence; in the latter case the final value can change either because of differences in the unstimulated formation of progesterone or because of differences in the response by the cells to lutropin. The reasons for the variability of response are not known for certain, but others have made similar observations when working with cells derived from luteal tissue (Strauss *et al.*, 1978) or liver (Gibbons & Pullinger, 1979). Possibly the effects of the collagenase during digestion are responsible. Reproducibility in the dispensing of portions of cell suspensions was found to be very high. In an experiment in which a cell suspension was dispensed as described above into eight separate tubes, analysis of their progesterone contents show that the variation from the average was less than 10%. This shows that experimental error due to the dispensing of unrepresentative samples was not significant.

Results and discussion

The tissue used in this work was the super-ovulated rat ovary, which, although not the corpus luteum of a naturally cycling or pregnant animal, is known to consist predominantly of luteinized tissue and is generally accepted and used as a convenient source of luteal cells (Channing & Vilee, 1966).

By sonicating cell suspensions before assaying for progesterone, as described in the Experimental section, no distinction is made between progesterone synthesized by the cells then secreted into the medium, and progesterone synthesized but retained within the cell. As a measure of the total amount of progesterone synthesized this procedure seems preferable to that used by Christie *et al.* (1979), in which the cells were sedimented after incubation and the medium alone analysed for progesterone; this method requires the assumption that all of the progesterone synthesized is secreted, and could be subject to errors caused by leakage of progesterone from aging cells as the incubation proceeds.

Density-gradient centrifugation separated four bands of cells (Fig. 1); of these only band 4 (Fig. 1) contained progesterone and was able to synthesize progesterone when incubated *in vitro* with lutropin. The nature of the other cells is not clear; those at the

Fig. 1. Separation of isolated rat corpus-luteum cells by density-gradient centrifugation on Percoll

Chopped ovaries were digested with collagenase and the crude cell preparation was centrifuged on a gradient of Percoll as described in the Experimental section.

base of the tube were red and presumably erythrocytes, but the other bands of cells were white. They were found to be non-steroidogenic and their removal produced an increase in the specific progesterone-synthesizing ability of the steroidogenic cells of band 4 (Fig. 1), as shown in Table 1. These non-steroidogenic cell bands could also have contained dead or damaged cells, since their removal was also found to increase from 70 to 90% the ability of the final cell suspension to exclude nigrosine. This dye is taken up by damaged cells (Kaltenbach *et al.*, 1958).

Fig. 2 shows the dose-response characteristics of the isolated cells to lutropin; they responded to lutropin concentrations greater than 1.0 ng/ml, with maximal and half-maximal responses at 100 and 3.0 ng/ml respectively. These lutropin concentrations are greater than those reported by Conti *et al.* (1977), who found a lutropin concentration of between 0.001 and 0.01 ng/ml sufficient to give measurable stimulation and one of 0.1 ng/ml sufficient to elicit a maximal response, but 3-isobutyl-1-methylxanthine was present in their incubations and this, as an inhibitor of cyclic nucleotide phosphodiesterase, might be expected to potentiate the effect of lutropin. The lutropin concentrations shown in Fig. 2 are more nearly comparable with those of Thomas *et al.* (1978), who also omitted an inhibitor of cyclic nucleotide phosphodiesterase. The possible variation in potency of lutropin from different sources provides another reason why dose-response curves might be expected to vary from one laboratory to another.

Fig. 3 shows a time course of the steroidogenic response by isolated cells incubated *in vitro*; when

Table 1. *Effect of Percoll-density-gradient centrifugation on progesterone biosynthesis by isolated luteal cells*
Methods for preparing and incubating the cells, conducting the density-gradient centrifugation and assaying for progesterone are described in the Experimental section. Cells 'after centrifugation' refers to cells taken from band 4 (Fig. 1).

Experiment	Cells	Progesterone (ng/10 ⁶ cells)		
		Before incubation	After incubation for 2 h	
			Without lutropin	With lutropin (100ng/ml)
1	Before centrifugation	0.9	14.5	292.5
	After centrifugation	1.1	54.2	376.7
2	Before centrifugation	2.6	6.2	54.7
	After centrifugation	2.2	16.5	197.1
3	Before centrifugation	0.8	2.6	12.1
	After centrifugation	1.3	4.1	34.2

Fig. 2. *Dose-response relationship of isolated rat corpus-luteum cells to lutropin*

Cells were prepared and incubated as described in the Experimental section, except that the concentration of lutropin was varied as shown in the Figure. Progesterone initially present in the cells was subtracted from that found after incubation to give the amount of progesterone formed during incubation.

stimulated by added lutropin (100ng/ml), progesterone formation was linear for 2h with a slight fall from linearity thereafter up to 4h, and when not stimulated, steroidogenesis was linear for about 4h.

The results summarized in Table 2 show that, of the hormones tested, only lutropin was able to stimulate progesterone formation by the cells, so that

Fig. 3. *Time course of the response by isolated rat corpus-luteum cells to lutropin*

Cells were prepared and incubated as described in the text in the Experimental section, except that the incubation time was varied as shown in the Figure. Symbols: ●, incubation containing added lutropin (100ng/ml); ○, no lutropin present. Progesterone initially present in the cells was subtracted from that found after incubation to give the amount of progesterone formed during incubation.

these are clearly responding specifically to their tropic hormone.

Evidence for the role of cyclic AMP in mediating the response to lutropin is given in Table 3, which indicates that, in the presence of 3-isobutyl-1-methylxanthine, the cells are stimulated to produce cyclic AMP when incubated with lutropin. Further evidence is given in Table 4, which shows that dibutyryl cyclic AMP can stimulate progesterone

Table 2. *Specificity of response by isolated luteal cells to tropic hormones*

Cells were prepared and incubated as described in the Experimental section, except that various hormones were used as indicated in the Table. Each was added to give a final concentration of 100ng/ml during incubation. Progesterone was assayed as described in the Experimental section.

Experiment	Progesterone (ng/10 ⁶ cells)					
	Before incubation	After incubation for 2 h				
		Without hormone	Follitropin	Thyrotropin	Prolactin	Lutropin
1	3.7	105.3	119.2	114.8	118.3	375.5
2	3.0	125.3	137.8	125.3	120.8	228.9
3	0.8	98.2	88.6	80.1	98.2	177.2

Table 3. *Effect of lutropin on cyclic AMP formation by isolated luteal cells*

Cells were prepared and incubated as described in the Experimental section, except that 1-methyl-3-isobutyl-xanthine (0.125 mM) was added to all incubations. Progesterone and cyclic AMP were assayed as described in the Experimental section.

Experiment	Progesterone (ng/10 ⁶ cells)			Cyclic AMP (pmol/10 ⁶ cells)		
	Before incubation	After incubation for 2 h		Before incubation	After incubation for 2 h	
		Without lutropin	With lutropin (100ng/ml)		Without lutropin	With lutropin (100ng/ml)
1	0.7	12.9	42.2	5.3	7.7	51.3
2	1.2	14.4	24.9	1.6	2.2	43.9
3	1.3	79.5	157.6	6.6	8.9	54.9

Table 4. *Effect of lutropin and dibutyryl cyclic AMP on progesterone formation by isolated luteal cells*

Cells were prepared and incubated as described in the Experimental section. When present, the concentration of lutropin was 100ng/ml, and that of dibutyryl cyclic AMP was 2mM. Progesterone was assayed as described in the Experimental section. Abbreviations used: LH, lutropin, diBcAMP, dibutyryl cyclic AMP. — and + indicate respectively the absence of a compound from and the presence of a compound in the incubation.

Experiment	Progesterone (ng/10 ⁶ cells)				
	Before incubation	After incubation for 2 h			
		—LH —diBcAMP	+LH —diBcAMP	—LH +diBcAMP	+LH +diBcAMP
1	2.1	10.6	73.9	78.2	73.5
2	1.2	6.7	15.4	14.1	13.5
3	1.3	44.6	177.4	167.3	204.8

formation by these cells to the same extent as can lutropin. Addition of both dibutyryl cyclic AMP and lutropin to the cells did not increase the amount of progesterone formed over that produced in the presence of either stimulator alone. In a series of similar experiments, dibutyryl cyclic GMP was found to be incapable of stimulating progesterone formation (results not shown). In addition to supporting the view that lutropin acts via cyclic AMP, this result also shows that the steroidogenic

response to lutropin is not limited through the loss of, or damage to, cellular receptors for this hormone during cell isolation.

The results described so far show that the cells behave *in vitro* in a manner to be expected from their known properties *in vivo* and therefore provide evidence that the preparation is suitable for studying the mode of action of lutropin.

To establish that cholesterol is an obligatory precursor of progesterone formed by the cells *in*

vitro, the effect on progesterone formation of aminogluthethimide, a specific inhibitor of the cytochrome *P*-450-dependent cholesterol-side-chain-cleavage system (Kowal, 1969), was tested. The results in Table 5 show that this inhibitor abolished progesterone formation by the cells irrespective of whether or not lutropin was present during incubation. Evidently cholesterol side-chain cleavage is essential if progesterone is to be formed, showing that the cells contain no pool of pregnenolone awaiting conversion into progesterone.

Since cholesterol is an obligatory precursor of progesterone, the mechanism for the provision of cholesterol is clearly a valid subject for consideration as a possible target for lutropin. Further experiments were designed to test the possibility that cellular cholesterol biosynthesis might constitute this mechanism.

The fungal product compactin is a known competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase (Endo *et al.*, 1976) and therefore of cholesterol biosynthesis in various tissues (Brown *et al.*, 1978; Goldstein *et al.*, 1979), but it does not appear to have been tested previously in the corpus luteum. To test its effectiveness, isolated luteal cells were incubated with radioactively labelled acetate or mevalonate, but no incorporation of radioactivity into cholesterol was detectable. Measurement of 3-hydroxy-3-methylglutaryl-CoA reductase activity also showed this enzyme to be undetectable under experimental conditions in which a specific activity of 0.02 would have been measurable. These observations suggest that cholesterol biosynthesis must proceed at a low rate in, or be absent from, the rat corpus-luteum cell. However, the absolute rate of cholesterol biosynthesis cannot be estimated from such isotope-incorporation data; the specific radioactivity of the precursor cannot be known accurately because of its dilution with endogenous material, so that the possibility that some cholesterol biosynthesis does take place cannot be discounted. To test the inhibitor, rats were treated with 4-

aminopyrazolo[3,4-*d*]pyrimidine as described in the Experimental section. This drug is known to lower the plasma cholesterol concentration and lead to a greatly enhanced rate of cholesterol biosynthesis in many tissues (Andersen & Dietschy, 1976, 1977; Balasubramaniam *et al.*, 1976). Table 6 shows that this is true for the rat corpus luteum; acetate or mevalonate were readily incorporated into cholesterol. Measurements also showed that 3-hydroxy-3-methylglutaryl-CoA reductase activity was detectable; tissue from eight rats assayed separately gave specific enzyme activities ranging from 0.10 to 0.85 with an average of 0.36. The results in this Table also show that compactin decreased the incorporation of acetate, but not of mevalonate, into cholesterol. Inhibition in the former case was over 90%. In spite of this, compactin was found to have no effect on the formation of progesterone by the isolated cells (Table 7). The conclusion that cholesterol biosynthesis is not involved in the lutropin-stimulated formation of progesterone agrees with and strengthens that of Armstrong *et al.* (1970), since compactin is a superior inhibitor to compound AY 9944 used by Armstrong *et al.* (1970). This is because compound AY 9944 acts at a late stage in cholesterol biosynthesis and therefore allows the formation of sterols earlier in the biosynthetic pathway than cholesterol. The terminal pathways of cholesterol biosynthesis in the corpus luteum are still uncertain, and the cholesterol-side-chain-cleavage enzyme is known to have a fairly broad degree of specificity (Arthur *et al.*, 1976), so that the possibility of compound AY 9944 allowing the formation of a sterol capable of being converted into pregnenolone cannot be ignored. Compactin, which acts early in the pathway of cholesterol biosynthesis, does not allow the formation of the sterol ring structure and so does not suffer from this drawback.

In a recent study, Christie *et al.* (1979) found that isolated luteal cells from rats treated with 4-aminopyrazolo-[3,4-*d*]pyrimidine did not synthesize as

Table 5. *Effect of aminogluthethimide on progesterone formation by isolated luteal cells*

Cells were prepared and incubated as described in the Experimental section, except that aminogluthethimide was added to some incubations. When present, the lutropin concentration was 100 ng/ml and the aminogluthethimide concentrations 10 μ M during incubation. Progesterone was assayed as described in the Experimental section. Abbreviations used: LH, lutropin; AG, aminogluthethimide.

Experiment	Progesterone (ng/10 ⁶ cells)				
	Before incubation	After incubation for 2 h			
		-LH -AG	+LH -AG	-LH +AG	+LH +AG
1	1.8	16.0	30.0	1.5	1.5
2	1.3	10.2	45.3	1.3	1.9
3	1.4	4.5	14.9	1.3	1.6

Table 6. *Effect of compactin on the incorporation of [U-¹⁴C]acetate and [2-¹⁴C]mevalonate into cholesterol by isolated luteal cells prepared from rats treated with 4-aminopyrazolo[3,4-*d*]pyrimidine*

Rats were treated with 4-aminopyrazolo[3,4-*d*]pyrimidine as described in the Experimental section, where the methods for preparing and incubating the cells are also given. Where present 20 μ Ci of [U-¹⁴C]acetate was added to each incubation, and 5 μ Ci of [2-¹⁴C]mevalonate. Compactin was added to a final concentration of 2.6 μ M. Controls were heated at 60°C for 5 min before being incubated. After incubation, cholesterol was isolated as described in the Experimental section.

		10 ⁻² × Radioactivity in cell cholesterol after incubation with [U- ¹⁴ C]acetate (d.p.m./10 ⁶ cells per 2 h)		
(A)	Experiment	Control	—Compactin	+Compactin
	1	1.6	370	38
	2	4.8	930	26
	3	3.5	220	17
		Radioactivity in cell cholesterol after incubation with [2- ¹⁴ C]mevalonate (d.p.m./10 ⁶ cells per 2 h)		
(B)	Experiment	Control	—Compactin	+Compactin
	1	260	1900	1800
	2	890	3200	4400
	3	380	3300	3600

Table 7. *Effect of compactin on the stimulation by lutropin of progesterone formation by isolated luteal cells*
Cells were prepared and incubated as described in the Experimental section. When present, the lutropin concentration was 100 ng/ml during incubation. When present, the compactin concentration was 2.6 μ M. After incubation, progesterone was assayed as described in the Experimental section. Abbreviation used; LH, lutropin.

		Progesterone (ng/10 ⁶ cells)			
		After incubation for 2 h			
Experiment	Before incubation	—LH	+LH	—LH	+LH
		—compactin	—compactin	+compactin	+compactin
1	1.3	22.1	197.8	35.4	192.0
2	1.3	14.6	190.2	11.8	217.4
3	0.8	74.9	136.5	83.8	134.0

much progesterone as did normal cells and concluded that blood cholesterol constitutes the primary source of cholesterol for steroid production. However, 4-aminopyrazolo[3,4-*d*]pyrimidine is known to inhibit lipoprotein production by the liver; since the action of lutropin requires protein synthesis *de novo* impairment of this might be responsible for the poor steroidogenic performance by the cells. Alternatively, the lowering of cholesteryl ester concentrations caused by 4-aminopyrazolo[3,4-*d*]pyrimidine might lead to cellular defects, unidentified at present, causing inhibition of steroidogenesis. The observed low rate of cholesterol biosynthesis is consistent with the possibility that the rat corpus luteum might rely on circulating sterol for its cholesterol requirements, but it is not clear how this uptake of cholesterol could be related to the rapid

steroidogenic response by the cells to lutropin, with which the present studies have been concerned. The evidence given in the present paper clearly demonstrates a mechanism whereby lutropin can stimulate progesterone synthesis by cells in which cholesterol biosynthesis is blocked and, since no cholesterol was added to the medium, which are deprived of external cholesterol. Under these conditions the cells must be using cholesterol, or its esters, already present in the cell as substrate for progesterone formation and therefore lutropin might act to promote the mobilization of this cellular sterol.

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