bility that the secretin was not potent. Though poorly defined because of the small increments in flow, the changes in biliary clearance which followed secretin in the rat are clearly greater than those obtained in the guinea pig. Final interpretation of this result must await a more refined investigation which may reveal, for example, whether secretin enhances bicarbonate excretion in rat bile as it does in the guinea pig and other animals (8,9).

In the present experiments bile:plasma concentration ratios greater than unity were not observed. Although we are unable to explain the difference of our results from those reported for mannitol by Schanker and Hogben, their experiments differed from ours in procedural details which may have been important. In the absence of fluid replacement and control of body temperature, their animals may have been dehydrated and hypothermic, both factors which could account for the lower flow rates which they observed (Table I). At a lower rate of bile formation we would expect to find a bile:plasma concentration ratio closer to the theoretical equilibrium value of one, but it is not easy to visualize how steady-state dehydration or hypothermia could lead to values higher than unity unless they activated a mechanism for distal fluid reabsorption or unmasked an underlying reabsorptive process by selectively inhibiting distal fluid secretion.

Summary. In the rat, as in the guinea pig, dehydrocholate choleresis leads to increased excretion of mannitol and erythritol, but in the rat the amounts of the solutes appearing in bile are greater. This difference is much more pronounced for the larger solute, mannitol. For mannitol, but not for erythritol, the rate of change of clearance following dehydrocholate is also greater in the rat. Unlike its effect in the guinea pig, secretin produces only a trivial choleresis in the rat. Despite a previous report that mannitol is concentrated in rat bile, bile:plasma concentration ratios greater than unity were not observed in the present studies for either mannitol or erythritol. In both the rat and the guinea pig the extracellular fluid volume of skeletal muscle as estimated from the plasma equivalent tissue space of mannitol is twice as large for muscle from the abdominal wall as for gastrocnemius. The discrepancy is attributed to differences in connective tissue content.

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Cholesterol Precursor Pools of Progesterone in the Bovine Ovary Perfused *in vitro*.* (32381)

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Studies of cholesterol and progesterone synthesis from acetate-1-^{14}C by luteal tissue incubated *in vitro* have consistently demonstrated that the specific activity of newly synthesized cholesterol is lower than that of

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progesterone(1,2). When radioactive impurities associated with cholesterol in the digitonin-precipitable sterols (the "high counting companions" of Schwenk and Werthessen(3)) are removed by bromination, the cholesterol specific activity is reduced even further(4). Data such as these have led to the conclusion that there is a separate steroidogenic pool of cholesterol which is not equilibrated with the remaining luteal cholesterol(1,2).

In contrast with the fate of newly synthesized cholesterol in tissue incubation studies, during the course of perfusion the newly synthesized cholesterol can either be further metabolized, stored, or secreted into the ovarian venous effluent(5,6). Because the newly synthesized cholesterol and progesterone are being secreted under perfusion conditions, a different picture of the precursor-product relationships might be demonstrable in tissues after perfusion than after incubation.

In this report some of the relationships between newly synthesized cholesterol and progesterone within the ovaries at the end of a perfusion in which acetate-1-^{14}C was constantly infused are presented. Data are also presented on the relationship between the specific activity of secreted progesterone and tissue progesterone.

Methods. Perfusion. Four pairs of ovaries were obtained from cows at the slaughter house. The corpus luteum bearing (luteal) ovaries, judged by inspection to be in the early to midluteal phase of the estrous cycle, and the contralateral (follicular) ovaries of the same animals were perfused by the methods previously reported from this laboratory (5,6,7). The effluents from each type of ovary were collected separately in ice chilled containers for sequential perfusion periods.

The ovaries were infused with acetate-1-^{14}C throughout the entire perfusion, which lasted 377 minutes. During the first 60 minutes, acetate-1-^{14}C was infused at a rate of 36.0 $\times$ 10^6 dpm/min and then at a rate of 30.3 $\times$ 10^6 dpm/min during the next 94 minutes. Following this, acetate-1-^{14}C was infused for 86 minutes at a rate of 31.9 $\times$ 10^6 dpm/min along with prolactin at a concentration of 5.0 μg/ml of blood. During the remainder of the perfusion (137 minutes) acetate-1-^{14}C was infused at a rate of 20.1 $\times$ 10^6 dpm/min along with LH at a concentration of 2.5 μg/ml of blood.

The specific activity of the progesterone secreted during the final 137 minutes of the perfusion was determined on aliquots of the perfusate from each type of ovary by the method developed in this laboratory(10). In addition, 3 samples of blood were collected from the venous effluent of the luteal ovaries at 30, 15 and zero minutes before the end of the perfusion. The amount of progesterone present in the equivalent samples from the follicular ovaries was insufficient to permit precise specific activity determinations and is not reported.

Tissue extraction. At the end of the perfusion, the follicular ovaries were weighed, minced and extracted with ethanol:acetone (1:1, v/v)(10). The corpora lutea were dissected from the residual stromal tissue, and these 2 tissues were pooled separately, weighed, minced and extracted with ethanol:acetone. The 3 extracts were evaporated to dryness under reduced pressure, and each residue was dissolved in methylene chloride. After being washed with water, each methylene chloride solution was dried over anhydrous sodium sulfate and then evaporated to dryness under N_2 and reduced pressure. Each residue was taken up in n-hexane and extracted 4 times with half volumes of 70% methanol. The n-hexane fraction and the combined methanolic extracts were evaporated to dryness under reduced pressure.

Progesterone analyses. The 70% methanolic rseidues from the stromal and follicular tissue pools were dissolved in 2 ml of n-hexane:benzene (1:1, v/v), and applied to silica gel columns (2.0 g) prepared with n-hexane (8). After washing the columns with 20 ml of 5% diethyl ether in benzene, the progesterone was eluted with 30 ml of ethyl acetate. The ethyl acetate was evaporated to dryness under reduced pressure. The residue from the 70% methanolic fraction of the corpus luteum tissue pool extract was subjected to paper chromatography in the ligroin-propylene glycol system(9). The progesterone area, delineated by U.V. absorption (253 mμ) and

reference to parallel standards, was eluted with methanol and the eluate was evaporated to dryness under N_2. From these residues, radiochemically pure progesterone was isolated and quantitated as previously described (10).

In brief in the method for the above purpose, there was a preliminary thin chromatography (TLC) on silica gel in the system methylene chloride: methanol (98:2). The eluate of the area containing progesterone was subjected to acetylation and the product was chromatographed (TLC) in the system cyclohexane:ethyl acetate (1:1). The radiochemically pure progesterone was eluted from the appropriate area and after conversion to progesterone bisdinitrophenylhydrazone (prog.-DNPH) was chromatographed in the TLC system chloroform:cyclohexane (3:1). The prog.-DNPH was eluted with chloroform and quantitated in chloroform by measuring the optical absorbancies at 350, 380 and 410 mμ. The "Allen-corrected" absorbancy was compared with that of standard prog.-DNPH.

Cholesterol analyses. The method for isolation of radiochemically pure cholesterol has been previously reported(5). By crystallizing the cholesterol dibromide from ether:acetic acid:water, the "high counting companions" (3,11) were effectively removed.

All radioactivity measurements were made by plating suitable aliquots of progesterone or cholesterol on aluminum planchets at infinite thinness, and counting in a Tracerlab automatic gas-flow window counter (efficiency 23.6% ^{14}C).

TABLE I. Progesterone and Cholesterol Concentrations in Tissues at End of Perfusion.

	Luteal ovaries		
	Corpus luteum	Stroma	Follicular ovaries
Wt (4 ovaries), g	36.5	48.4	43.0
Tissue progesterone, μg/g	44.9	2.2	.3
Tissue cholesterol, μg/g	86.3	63.2	95.8

Results. The weights of ovarian tissues and their concentrations of progesterone are presented in Table I. These values are not corrected for analytical losses. The average recovery of tissue progesterone using the methods described was 65%. Because emphasis was placed on demonstrating radiochemical purity, the cholesterol concentration within the tissues was lower than reported in the literature(12). These values are also presented in Table I.

Evidence has already been presented to show radiochemical purity of both progesterone(10) and cholesterol(5) isolated according to the methods used in this laboratory.

Table II presents data on the specific activities of cholesterol and progesterone isolated from the respective tissues and the specific activity of the progesterone secreted into the venous effluents. The average specific activity of the progesterone secreted during the total 137 minutes of the final perfusion period with LH was 725,000 dpm/μmole for the luteal ovaries and 835,000 dpm/μmole for the follicular ovaries. The specific ac-

TABLE II. Specific Activities of Secreted Progesterone, Tissue Progesterone and Tissue Cholesterol.

	Luteal ovaries		
	Corpus luteum	Stroma	Follicular ovaries
Secreted progesterone specific activity, dpm/μmole			
During entire LH infusion interval*	725,000		835,000
During final 45 min of perfusion†	409,000		—
Tissue progesterone specific activity, dpm/μmole	466,000	428,000	146,000
Tissue cholesterol specific activity, dpm/μmole	498,000	12,400	12,600

* Specific activity of the secreted progesterone was determined on an aliquot of blood obtained from the respective pooled venous effluents collected during the final 137 min of perfusion (*i.e.*, during infusion with LH).

† Specific activity of the secreted progesterone was determined on three aliquots of blood collected at 15-min intervals during the final perfusion interval. The average of these 3 values is presented.

tivities of the progesterone secreted by the luteal ovaries at 30, 15 and zero minutes before the end of the perfusion were 413,000, 387,000 and 425,000 (average 409,000 dpm/ μmole).

The specific activities of the progesterone isolated from the 3 pools of tissue were all much less than the specific activities of the progesterone secreted during the entire final 137 minutes of the perfusion. However, the average specific activity of the progesterone secreted by the luteal ovaries during the last 30 minutes of the perfusion period was only slightly less than that of the progesterone in either stroma or corpus luteum.

The specific activity of tissue cholesterol was higher than that of tissue progesterone only within the corpus luteum.

Discussion. During the perfusion of ovaries with acetate-1-^{14}C, newly synthesized cholesterol-^{14}C and progesterone-^{14}C may be either further metabolized or secreted into the ovarian venous effluent.

In the follicular ovaries and in the residual stromal tissue of the luteal ovaries, the specific activity of the isolated cholesterol is only 1/40th that of the cholesterol in the luteal tissue at the end of the perfusion. It would appear that under the conditions of this experiment the principal site of new cholesterol synthesis was the corpus luteum.

The specific activity of the cholesterol in the luteal tissue at the end of the experiment is, if anything, higher than the specific activity of either the luteal tissue progesterone or of the progesterone secreted into the ovarian venous effluent. This is in contrast to results of incubations with corpus luteum slices where the reverse has been reported (1,2).

If the turnover rate is defined as being the rate at which all the substance present is replaced in the tissue, then on the basis of the specific activities, the turnover rate of luteal cholesterol was greater than or equal to that of progesterone, and was less than 377 minutes, which was the total duration of acetate-1-^{14}C infusion.

Concomitantly, there was a large pool of less rapidly turning-over cholesterol in the stromal tissue and in the follicular ovaries. LH is known to induce cholesterol depletion in both rat(13) and rabbit(14) ovaries, and it may be that this contributed to the rapid turnover of luteal cholesterol.

The specific activity of the progesterone secreted by the luteal ovaries during the final 30 minutes of the perfusion was slightly lower than the specific activity of the luteal cholesterol. This would suggest that there is a source of cold cholesterol contributing to the secreted progesterone.

The possible sources of cold cholesterol are either the stroma or the blood. Preliminary experiments already conducted in our laboratory indicate that blood cholesterol labeled with cholesterol-7α-^{3}H contributes significantly to progesterone secreted by bovine luteal ovaries perfused *in vitro*§.

Summary. Bovine ovaries were perfused *in vitro* with acetate-1-^{14}C throughout an experiment which lasted 377 minutes. Luteal ovaries and the contralateral follicular ovaries were sequentially infused with prolactin, and then with LH. At the end of the perfusion, the specific activity of luteal cholesterol-^{14}C was slightly greater than the specific activity of either the luteal tissue progesterone-^{14}C or the secreted progesterone-^{14}C. The specific activity of the cholesterol-^{14}C isolated from the residual stromal tissue and from the follicular ovaries was much lower than that observed in the corpus luteum.

§ Unpublished observations.

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Probenecid Binding by Renal Cortical Slices And Homogenates.* (32382)

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Previous studies from this laboratory have implicated tissue binding as a mechanism for probenecid accumulation by isolated rabbit renal tissue(1,2). This information is relevant to studies of organic acid transport since probenecid is often used as a specific inhibitor of those transport processes. In fact, the ability of probenecid to reduce the secretion of a substance has been taken as experimental evidence that the substance in question was handled by the renal organic acid transport system(3).

Although under *in vivo* conditions there are clearance data to support renal secretion of probenecid(4), unequivocal evidence for carrier mediated transport of this acid by isolated tissues is not available. For example, although bromcresol green does reduce probenecid accumulation by renal cortex slices, p-aminohippurate (PAH) does not(1). In addition various alterations in experimental conditions which influence the transport of PAH do not affect probenecid uptake or run-out(2). For example, several substances that produce biphasic affects on PAH efflux have no effect on probenecid efflux.

Because of these discrepancies the following experiments were undertaken. It was hoped that the use of a standard equilibrium analysis as well as the homogenate studies would give direct evidence concerning the importance of tissue binding in the probenecid transport process.

Methods. Probenecid uptake was determined by procedures reported previously(1). Free-hand cortical slices (less than 0.5 mm thick) were prepared from freshly isolated kidneys and were stored in cold Krebs-Ringer phosphate solution until used. Slices (100-300 mg) were incubated in 3.0 ml of a modified Krebs-Ringer phosphate solution for 60 minutes. This time period has been shown in earlier studies(1) to be adequate for attainment of the steady state. The incubation medium contained 40 mM potassium and 10 mM sodium acetate in addition to the usual constituents. Probenecid was added to this medium to give the various final concentrations employed in this study. All incubations were carried out in a Dubnoff incubator at a shaker speed of 95 cycles/minute, a temperature of 25°, and with 100% oxygen as the gas phase.

After incubation the tissues were blotted, weighed, and homogenized in distilled water with a Potter-Elvehjem homogenizer. Protein precipitation was accomplished with zinc sulfate and sodium hydroxide. After centrifugation an aliquot of the supernatant solution was acidified and extracted for probenecid according to a modification of the method of Dayton *et al*(1,5). This involved shaking of the acidified sample with ethylene dichloride, washing of the organic phase 3 times with a citrate-phosphate buffer and finally shaking the ethylene dichloride with sodium hydroxide. The probenecid determin-

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