A SIMPLIFIED PROCEDURE FOR THE QUANTITATIVE EXTRACTION OF PROGESTERONE FROM CORPUS LUTEUM.

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Progesterone estimations are complicated by the need to separate the non-polar lipid from the polar lipid which contains the progesterone, before this latter material is purified by chromatography. The methods at present in use are complex and do not readily lend themselves to the routine monitoring of tissue incubations. Among these methods are counter current extraction, or repeated partition in separating funnels between petroleum ether and aqueous methanol (Loy et al., 1957; Pearlman, 1954; Gospodarowicz and Legault-Demare, 1963), column chromatography (Loy et al, 1957; Duncan et al, 1960) or at the simplest storage of the lipid extract in 70% methanol at -18° to -20°C for 18 hours (Butt et al, 1951; Gospodarowicz and Legault-Demare, 1963). Often these steps are used in combination. Yields of progesterone are usually of the order of 60-65%.

The method described in this communication has the advantage of simplicity and greater speed for the worker without much technical assistance. It yields a sample of progesterone which is contaminated only with traces of fluorescent material which does not appear to interfere in any way in the ultra-violet absorption measurements which conclude the procedure. Adequate separations have been obtained even with the most heavily pigmented bovine corpora lutea.

EXPERIMENTAL AND DISCUSSION

Extraction of lipid from corpora lutea

A tissue homogenate is made to contain 500 mg. tissue in 5 ml. This suspension is extracted with 3 x 30 ml. acetone. The extracts are

combined and evaporated to aqueous volume in vacuo at temperatures not exceeding 60°C. Water (10 ml.) and ether (20 ml.) are then added and the mixture is thoroughly shaken. Centrifugation is employed to break the emulsions which tend to form during this step. The ether extract is removed and the ether extraction is twice repeated using the same volume of ether. The ethereal extracts are then evaporated to dryness in vacuo at temperatures not exceeding 60°C. The oily residue is then further dried by storage overnight in vacuo over anhydrous $CaCl_2$ or it may be dried rapidly by azeotropic distillation with benzene.

Chromatography

The whole extract is applied to the bottom left hand corner of a 20 x 20 cm. glass plate coated with a thin layer of activated Silica gel G. (Cargill, 1962). Transfer is accomplished quantitatively by repeated washings of the flask with small quantities of methylene chloride or ether. The chromatographic separation is accomplished by ascending techniques, using three solvent systems. The plate is freed from residual solvent in a cool air stream before the next solvent is applied. 6 plates can be run simultaneously using two tanks.

(a) Petroleum ether, b.p. 60-80°C

This solvent moves pigment away from the origin, the progesterone remaining stationary.

(b) Ethyl acetate/benzene

Concentrations of 10% and 15% ethyl acetate in benzene have been used with equal success. The solvent is made to migrate in the same direction as the petroleum ether, causing the progesterone to move behind a solvent front which sweeps away pigment that has tailed during the petroleum ether chromatography.

These solvent systems afford a good separation of progesterone from cholesterol, pregnenolone and 17αOH progesterone. See Table 1

Table 1

	Rf	
	10% EtOAc	15% EtOAc
Progesterone	0.14	0.32
Pregnenolone	-	0.27
Cholesterol	0.27	0.42
17α OH progesterone	-	0.14

If the plate is examined under ultra violet light at this stage of the procedure the progesterone spot can be seen within a strongly fluorescing area. This fluorescing material was also present in polar fractions obtained using the aqueous methanol freezing procedure of Butt et al (1951). Varying the concentration of ethyl acetate within the above limits has no effect upon the linearity and efficiency of the complete progesterone recovery.

(c) Ethanol/benzene

The final solvent, 10% ethanol in benzene, is run at right angles to the first two solvents. In this system progesterone moves slightly ahead of cholesterol, and its position is best defined in terms of movement of this latter compound since the Rf is variable. This is presumably caused by changes in the hydration of the silica gel during removal of solvents 1 and 2. The passage of solvent systems 1 and 2 over the plate makes progesterone move more slowly in 10% ethanol/benzene, than when 10% ethanol/benzene is the only solvent applied, and it markedly decreases the differences in Rf of progesterone and pregnenolone.

Table 2

Rf in 10% ethanol/benzene				Rc values		
*Prog.	Chol.	Preg.	17OH prog.	Prog.	Preg.	17OH prog.
0.425	0.396	-	-	1.08	-	-
0.49	0.43	-	-	1.14	-	-
0.53	0.47	-	-	1.13	-	-
0.63	0.55	-	-	1.15	-	-
0.44	0.4	0.39	0.39	1.10	0.97	0.97

* Prog. = Progesterone Preg. = Pregnenolone

Chol. = Cholesterol 17OH Prog. = 17α hydroxy progesterone

When the plate is examined under ultra-violet light progesterone is seen to be ahead of pigmented material, and situated within a slightly fluorescing background. The fluorescence does not however, seem to influence the final U.V. absorptiometry, and is probably only present in trace amount. It cannot be detected when the progesterone spot is subjected to gas/liquid chromatography and is barely detected when the plate is heated after spraying with ceric sulphate in 15% H_2SO_4. The fluorescent background gives no colour with phosphomolybdic acid.

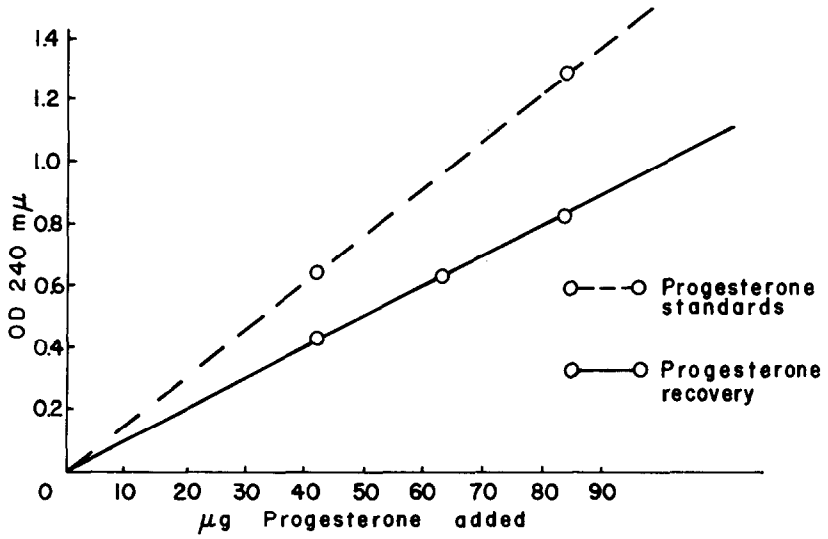
Estimation of progesterone

When the chromatographic separation is complete the chromatoplate is freed from solvent in a cool air stream. The progesterone spot is located by viewing under ultra-violet light. (It is found that the chromatography has the effect of concentrating the progesterone spot into an area much smaller than the application zone, which is, of necessity, fairly large.) The progesterone-containing silica gel is scraped off the plate into a weighed centrifuge tube and the tube weighed again. 4 ml. methylene chloride is added and the tube is immediately capped with aluminium foil and then oversealed with parafilm. After swirling the tubes with a "Vortex" mixer they are allowed to stand for at least two hours before the silica gel is centrifuged down. The supernatant is carefully decanted into capped spectrophotometer cells and the absorption of the peak at 240 mμ is measured. This is compared with the absorption of solutions of progesterone in methanol added to methylene chloride. (Concentrations of up to 84 μg progesterone in 84 μl of MeOH are diluted to 4 ml. with $MeCl_2$). A "blank" is prepared by scraping a portion of silica gel from an area of the plate which is free from any absorbing or fluorescing material, and treating it in an identical manner.

Recovery of added progesterone

Progesterone, dissolved in MeOH at concentrations of 1 μg./1 μl. added to corpus luteum homogenates in amounts between 20 and 84 μg. is recovered quantitatively with yields of up to 67%. Figure 1 is a

typical recovery plot. The mean recovery over 5 series of extractions is 62%



The recoveries are based on the absorbances at 240 mμ after correction for the silica gel background absorption and for endogenous progesterone present in an unsupplemented extract.

The method has been used successfully to demonstrate the synthesis of progesterone from pregnenolone in incubated homogenates of bovine corpus luteum.

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