QUANTITATIVE METHODS FOR DETERMINING PROGESTINS IN CORPORA LUTEA, OVARIES, AND ADRENALS OF THE COW AND SOW USING PROGESTERONE-4-C\(^{14}\) AS A MEASURE OF RECOVERY OF EXTRACTED HORMONE

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SUMMARY

A quantitative method is described for the chemical assay of progesterone and \(\Delta^4\)-pregnene-20\(\beta\)-ol-3-one. The tissues were extracted with ethyl acetate, followed by vacuum distillation and partitioning of the residue between Skellysolve B – 70% methanol and benzene. The progestins were separated and further purified by paper chromatography using the Bush Skellysolve B – 95% methanol system, eluted, and rechromatographed in the same system. Progesterone-4-C\(^{14}\) (0.08 \(\mu\)g; 3,500 c.p.m.) was added at the time of initial extraction to estimate recovery of progesterone during each assay. The radiochemical purity of the compound was 93-94%. About 4% of the radioactivity remained in the tissue during initial extraction and approximately 16% was lost during each elution of the paper chromatograms. Average recovery of progesterone-4-C\(^{14}\) was 49% for 224 samples of cow tissues (standard error 0.7%) and 51% for 114 samples of sow tissues (standard error 1.1%). No \(\Delta^4\)-pregnene-20\(\beta\)-ol-3-one was detected in sow tissues.

Zarrow et al. (28) tested 17 compounds related to progesterone with the Hooker-Forbes bioassay and confirmed that the \(\Delta^4\)-ene-3-one group must be present for progestational activity. This grouping is also a convenient chemical characteristic, since all steroids having it absorb ultraviolet light at 240 m\(\mu\) when in alcoholic solution (9). Although this area of absorption is not specific for progestins, it does allow detection and quantitation after some means of isolation and separation have been used.

Chemical methods for assay of progesterone are of recent origin. Two important developments have aided recent progress in steroid analysis (21). One is the use of radioactive isotopes and the other is the use of paper chromatography. Paper chromatography, originally suggested in 1861 by Schoenbein (23), was first used for separating amino acids in 1944 (8). After Zaffaroni et al. (27) introduced the use of noneaqueous solvent systems in 1949, paper chromatography gained wide acceptance as a method for separating and purifying steroids. The Zaffaroni noneaqueous solvent systems use a high-boiling organic solvent such as formamide or propylene glycol as the stationary phase and low-

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boiling solvents as the mobile phase. Prior to development, the paper is impregnated with the nonvolatile stationary phase. Paper chromatography systems developed by Bush (4, 5) eliminated this latter step, because both phases are volatile solvents and equilibration of the paper supporting medium occurs entirely via the vapor phase of the system.

Many methods for the chemical assay of progesterone have been developed (7, 10, 12, 14, 15, 17-19, 24, 25). Most (7, 10, 12, 14, 19, 24, 25) of these employ paper chromatography for the final separation and purification of the steroid. Methods have varied, depending on the type of tissue or fluid and species of animal and objectives of the experiments. The recent identification of Δ 4-pregnene-20 β-ol-3-one (13) in cow ovaries had led to methods modified to simultaneously measure this progestin as well as progesterone. All methods are time-consuming and because of the number of steps required for separation and purification of the steroids, recoveries of progesterone added at initial extraction have generally been below 70% and quite variable. Accordingly, a method is frequently a compromise using the least number of steps for achieving minimum purification for quantitative measurement; this reduces time for assay and loss of the steroid originally extracted.

This paper will describe a procedure which estimates the loss of extractable progesterone during the remainder of the method and simultaneously allows quantitation of progesterone and Δ 4-pregnene-20 β-ol-3-one. The latter compound hereafter will be referred to as 20 β-ol.

**METHODS**

In recent years, two methods of analysis for progestins of ovarian and adrenal origin have been developed in our laboratories. The first method (12, 14) was used to isolate progestins from ovarian and adrenal tissue. The second method (25), a modification of the first, was used primarily for the study of the corpus luteum and involves the following steps: (a) Extraction of the tissue with ethyl acetate followed by filtration of the ethyl acetate-steroid-lipid mixture; (b) vacuum distillation of the filtrate and dissolving of the residue in 10 ml. of Skellysolve B, followed by extracting three times with 10-ml. volumes of 70% methanol; (c) removal of methanol under N₂ in a 45°C water bath, with extraction of the resulting aqueous phase three times with 10-ml. portions of redistilled benzene; and (e) drying the benzene extract, followed by chromatography in the Bush Skellysolve B-95% methanol system. This latter method is unsatisfactory for assay of ovarian and adrenal tissues of the bovine. The method fails to remove all of the extraneous tissue residues which exhibit absorption at 240 mµ.

The method to be described differs from the second method by: (a) The use of radioactive progesterone in trace quantities with each sample for the evaluation of losses during the procedure; (b) deletion of the equilibration step in chromatography; (c) rechromatography in the Bush Skellysolve B-95% methanol system to completely remove interfering tissue residues; and (d) correction for the contribution of the blank paper eluate to the ultraviolet absorption at
240 m\( \mu \) by a direct subtraction of the optical density of this blank rather than by the use of Reineke's method (20).

**Radioactive progesterone standard.** Absolute ethyl alcohol was used to prepare the solution of progesterone-4-C\(^{14} \) (62 \( \mu \)e/mg; purchased from California Corporation for Biochemical Research, Los Angeles, California, in 1-\( \mu \)e quantities). Approximately 1 ml. of absolute ethanol was transferred to a vial containing 1 \( \mu \)e of progesterone-4-C\(^{14} \) and allowed to stand at room temperature for 2 hr. This solution was quantitatively transferred to a 10-ml. weighed glass-stoppered flask with the addition of approximately 2.5 ml. of ethanol. The final volume of the solution was determined by weight and density. The absorption spectrum determined on a Beckman DU spectrophotometer agreed with that of progesterone. The maximum optical density corresponded to 17.7 \( \mu \)g. progesterone-4-C\(^{14} \) per microcurie of radioactivity. This is in reasonable agreement with the value of 16.1 \( \mu \)g. per microcurie calculated from the specific activity of 62 \( \mu \)e/mg. Since later samples of progesterone-4-C\(^{14} \) were all from the same lot, they were not subjected to this ultraviolet absorption measurement. One microcurie of progesterone-4-C\(^{14} \) was finally made to a volume of 100 ml. and used as a standard stock solution.

**Extraction of tissues.** All tissues were extracted three times with 50-ml. portions of ethyl acetate in a Waring Blender. After complete homogenization of the tissue during the first extraction, 500 \( \lambda \) of progesterone-4-C\(^{14} \), ca. 3,500 c.p.m. and 0.08 \( \mu \)g., was added and the extraction continued.

**Paper chromatography.** Paper chromatography in the Bush Skellysolve B-95% methanol system adequately separates progesterone and 20 \( \beta \)-ol. It was found that equilibration, the time required for saturation of the chromatogram with solvent vapors of the system, was not essential in this method. Omission of this step reduced the total time for developing the chromatograms by one-half. At the end of 3.5-4 hr. the chromatogram was removed from the tank, the concentrations of progestins located on the 3-cm. strips through use of an ultraviolet scanner, and eluted with 10 ml. of redistilled methanol.

A chromatographic standard of 50 \( \mu \)g. of pure progesterone was run with each sheet of chromatograms to aid in the location. The eluted fraction was then evaporated under a stream of nitrogen in a water bath at 45\(^\circ\) C. and rechromatographed for the same length of time. The progestins on the rechromatogram were detected and a 6-cm. section containing the steroid eluted to 5 ml. in a calibrated test tube. A paper blank of the same size was cut from an adjacent control strip, which was subjected to solvents only and treated in a similar manner.

Edgar (10) and Bush (6) found that filter paper contained material which absorbed strongly in the same region as progesterone. Therefore, if the eluted blank portion of the paper differed appreciably in size or position on the chromatogram from that containing the progesterone spot, the absorption spectrum of the latter, when corrected for the paper blank, was distorted and the absorption maximum differed from 240 m\( \mu \). Preliminary work with this method confirmed these findings.
Quantitative measurements. Quantitative measurements were based on the optical density of the samples at 230, 240, and 250 m\(\mu\). In the earlier method (12, 14) a procedure similar to that described by Reineke (20) was used to correct for the paper blank and calculate the amount of progestin present. In the present study this has been replaced by a simple calculation involving the direct subtraction of the optical density of the paper blank at 240 from the optical density of the samples at 240 m\(\mu\). The optical density of the paper blank at 240 m\(\mu\) varied somewhat from sample to sample, as noted by Reineke (20). However, the shape of the spectrum of the paper blank also varied and the variation in the ratio of densities at 230 and 240 m\(\mu\), as used in Reineke’s calculation, led to an uncertainty in the final corrected density which was appreciably larger than in the case of direct subtraction. Thus, direct subtraction of the density of the paper blank was deemed more appropriate than the use of Reineke’s method of calculation.

Optical density readings were made on a Beckman DU spectrophotometer, using silica cells of 1-cm. light path. The quantity of progestin in the sample was calculated from the following formula:

\[
\frac{(D - PB)}{O.D. \times F} = \mu g. \text{ of progestin in sample, where,}
\]

- \(D = \text{optical density reading of sample at 240 m\(\mu\),}\)
- \(PB = \text{optical density of paper chromatogram blank at 240 m\(\mu\),}\)
- \(O.D. = \text{optical density for 1 \(\mu g\). of progesterone in 5 ml. as determined at a concentration of 50 \(\mu g\). per 5 ml. of solvent, and}\)
- \(F = \text{fraction of progesterone-4-C}^{14} \text{ recovered.}\)

After the spectrophotometric measurements, 4 of the 5 ml. of each progesterone solution were transferred to individual aluminum planchets pretreated as described below. Each planchet was covered with a single-thickness disc of lens paper to provide uniform distribution, and counted to a total of ten thousand counts. The percentage recovery of progesterone-4-C\(^{14}\) was calculated and this value was used to correct the quantity of progesterone in the final eluate for losses sustained during the experimental procedure. Because 20 \(\beta\-ol\) exhibits properties similar to progesterone, the percentage recovery of progesterone-4-C\(^{14}\) was also used to correct for losses of 20 \(\beta\-ol\).

Preparation of planchets. All planchets used in this study were pretreated in a manner similar to that described by Pearce et al. (16). This procedure consisted of (a) washing in petroleum ether at 40° C. for 45 min., followed by washing in acetone at 40° C. for 45 min., (b) rewashing the planchets at low heat in distilled water to which a mild detergent had been added and rinsing with distilled water. It is important in this procedure to prevent planchets from adhering to each other. Washing was considered satisfactory when a drop of ethanol dispersed uniformly over the surface of the dried planchet.
One or two drops of a solution of carbon tetrachloride containing a small amount of silicone grease were applied, via an eye dropper, to the inner surface of the rim of each planchet. A thin film of silicone grease remained on this surface to prevent the sample from spreading beyond the confines of the planchet.

Sources of tissues. Ovaries and adrenals were removed within 30 min. after slaughter of sows and cows. The corpora lutea were separated from the ovaries and the tissues from individual animals were placed into sample bottles and immediately frozen with solid CO₂ and stored at -25 °C. until prepared for final analysis.

Purity determination of progesterone-4-C¹⁴. Berliner and Salhanick (2), as well as Savard (22), have reported that when employing C¹⁴-labeled steroids in paper chromatography, measurable amounts of radioactivity remain at the origin. It was, therefore, necessary to determine whether these findings apply to progesterone-4-C¹⁴. Four- and ten-milliliter portions of the standard isotope solution were concentrated and applied with 50 μg. of standard progesterone to 2-cm. strips of Whatman No. 1 chromatography paper. The chromatograms were developed in the Bush Skellysolve B–95% methanol system for 3.5 to 4 hr. Small beakers were centered under each strip to collect any solvent. A 6-cm. section containing the standard progesterone and progesterone-4-C¹⁴, as detected through use of an ultraviolet scanner, was marked, and the entire strips beginning 1 cm. above the origin were cut into 2-cm. sections, with the exception of the marked area, which was cut into 1-cm. sections. These sections were placed on aluminum planchets and counted. Any solvent collected in the beakers from the strips was also plated and counted.

Table 1 shows that 7.92% of the activity remained near the origin on the first chromatogram (4-ml. sample) and 3.2% between this area and the progesterone spot. This appears to be partly impurity, since rechromatography of the progesterone area of the 10-ml. fraction containing the progesterone-4-C¹⁴ revealed very little activity on these two areas (0.57%).

### Table 1

<table>
<thead>
<tr>
<th>Location Description</th>
<th>Distance in cm.</th>
<th>Percent of Total</th>
<th>Location Description</th>
<th>Distance in cm.</th>
<th>Percent of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vicinity of origin</td>
<td>10</td>
<td>7.92</td>
<td>Area from origin</td>
<td>20</td>
<td>0.57</td>
</tr>
<tr>
<td>Area between origin</td>
<td>20</td>
<td>3.20</td>
<td>Detected origin</td>
<td>6</td>
<td>98.61</td>
</tr>
<tr>
<td>Area below origin</td>
<td>7</td>
<td>88.10</td>
<td>Below spot</td>
<td>23</td>
<td>0.82</td>
</tr>
<tr>
<td>Area below spot</td>
<td>12</td>
<td>0.78</td>
<td>Solvent in beaker</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The percentage radiochemical purity of the original progesterone-4-C\textsuperscript{14} was calculated as follows: Since rechromatography of the progesterone area of the 10-ml. sample indicated a small amount of trailing of the pure progesterone-4-C\textsuperscript{14} between the origin and the progesterone-4-C\textsuperscript{14} spot (Table 1), it was assumed that all of the radioactivity in the second area and some of the radioactivity in the first area (origin) of the first chromatogram (Table 1) was due to this trailing. The concentrations of radioactivity corresponding to trailing of pure progesterone-4-C\textsuperscript{14} were determined from the second area. This quantity was subtracted from this first area (origin). The amount of radioactivity in excess of that due to progesterone-4-C\textsuperscript{14} trailing was ascribed to an impurity. The degree of radiochemical purity as determined in this way by four experiments was 93\% in three cases and 94\% in one case.

A series of four planchets was covered with a single-thickness disc of lens paper; 500 \textmu l of progesterone-4-C\textsuperscript{14} was delivered into each, and counted. The counts from these planchets were averaged and corrected for purity. In this manner, it was possible to determine the counts per minute of progesterone-4-C\textsuperscript{14} added to each assay made.

Analysis of losses of progesterone-4-C\textsuperscript{14}. The average recovery of progesterone-4-C\textsuperscript{14} for a total of 224 bovine samples assayed was 40\%, with a standard error of 0.74\%. There was no significant difference between recoveries for the different cow tissues. Corpora lutea averaged 41\%, ovaries 40\%, and adrenals 38\%. The average recovery of progesterone-4-C\textsuperscript{14} for 114 samples of sow tissues was 51.4\%, which was significantly higher than for cow tissues. For sow corpora lutea, the average recovery was 56\% as compared with 51\% for the ovaries and 48\% for the adrenals (P < 0.01). The sow tissues characteristically showed fewer interfering substances after initial extraction than did extracts of cow tissues. For each species, the most difficulty was encountered in purifying adrenal extracts and the least for luteal tissue. In the Bush system, rechromatography of sow luteal tissue appears unnecessary.

Another species difference was the absence of detectable quantities of 20 \textbeta\textbeta-ol in the corpora lutea, ovaries, and adrenals of the sow. The substance from cow adrenals which chromatographed like 20 \textbeta\textbeta-ol has not been further identified.

An analysis of the method for losses incurred explains the low recoveries for progesterone-4-C\textsuperscript{14}. Approximately 3 to 4\% of the isotope was lost in the actual extraction procedures. The greatest loss occurred during elution through failure to remove all of the progesterone from the paper chromatogram (Table 2).

Identification of progestins in bovine ovaries and adrenals. Extracts were made of corpora lutea, stroma, ovaries, and adrenals collected from pregnant cows. These extracts were chromatographed in the Bush Skellysolve B–95\% methanol system and the compounds showing identical mobility to that of authentic progesterone and 20 \textbeta\textbeta-ol were eluted for further identification tests. Exhaustive identification tests were not performed on the compounds isolated from corpora lutea or residual ovaries since Gorski et al. (13) have identified progesterone and 20 \textbeta\textbeta-ol to be the progestins associated with these glands. These
TABLE 2
Determination (approximate) of loss encountered in method using 500 λ of progesterone-4-C\textsuperscript{3} in 4.6 g. of corpus luteum and ovary-stroma.

<table>
<thead>
<tr>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Progesterone-4-C\textsuperscript{3}</td>
</tr>
<tr>
<td>2. First chromatogram</td>
</tr>
<tr>
<td>(a) Rewash of eluted area</td>
</tr>
<tr>
<td>(b) Area below and directly above detected progesterone spot</td>
</tr>
<tr>
<td>(c) Area above and below Δ 4-pregnene-20 β-ol-3-one spot and rewash of eluted Δ 4-pregnene-20 β-ol-3-one spot</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>3. Rechromatogram</td>
</tr>
<tr>
<td>(a) Rewash of eluted area</td>
</tr>
<tr>
<td>(b) Area below and directly above progesterone spot</td>
</tr>
<tr>
<td>(c) Area above and below Δ 4-pregnene-20 β-ol-3-one spot and rewash of eluted Δ 4-pregnene-20 β-ol-3-one spot</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

compounds were chromatographed in the Skellysolve B-formamide system for 3.5 to 4 hr. and showed the same mobility as authentic progesterone and 20 β-ol. The material showing the same mobility rate as authentic 20 β-ol was oxidized and chromatographed in both the Skellysolve B-formamide and Bush Skellysolve B-95% methanol systems. In such cases, it showed the same mobility as authentic progesterone and oxidized 20 β-ol. An acetate of this material chromatographed in both systems showed identical mobility to that of authentic acetylated 20 β-ol. The ultraviolet spectrum in methanol of the compounds under consideration showed a single peak at 240-242 μ. similar to authentic progesterone and 20 β-ol.

Methods of identification similar to those above were performed on the compound extracted from adrenal glands. This compound showed the same mobility as authentic progesterone on chromatograms. The results of these tests were identical to those described for progesterone in the previous section.

The true identity of the compound extracted from adrenal glands of the cow which showed mobility identical to that of authentic 20 β-ol when chromatographed in the Bush Skellysolve B-95% methanol could not be established. The compound in question showed maximum absorption at 240 μ. When a large sample of adrenal tissue (200 g.) was extracted, difficulty was encountered in separating this compound from the large quantities of tissue residue. In chromatography, this tissue residue impeded the flow of solvent over the strip, in comparison with that of the control strip containing the authentic 20 β-ol, thus making it impossible to locate with certainty the compound on the chromatogram. Hence, sufficient quantities of this steroid could not be obtained for further identification tests.

GENERAL DISCUSSION

The use of radioactive progesterone as a measure of recovery for each assay at least partially corrects for variations in techniques and variations in tissues from different animals. This technique measures primarily the loss of progestins
initially extracted from the tissues and not the percentage of the total which may be actually present in the tissues. There is no reliable procedure by which the efficiency of the initial extraction can be determined, although the use of radioactive tracers may be an aid. There is considerable evidence that steroids in body tissues and fluids may be conjugated with various materials. Steroids in urine have been shown to be conjugated with glucuronic or sulfuric acids (26). This is not believed to be the case in most tissues, except perhaps in the liver (3).

One of the major difficulties in purifying progestins from tissue sources is separating them from the lipid material. Since paper chromatography alone will not satisfactorily separate progestins from large quantities of lipids, a procedure for removing these substances is essential.

Allen (1), in his early work on the isolation of progesterone, separated this hormone from fat by precipitating the fat from a 70% methanol solution at $-15^\circ$C. and removing it by filtration in the cold. Butt et al. (7) modified this method by centrifugation in the cold rather than filtering. Partition between Skellysolve B, 70% methanol, and benzene successfully eliminated a major portion of these extraneous materials in our method.

Loy et al. (15) used column adsorption chromatography on aluminum oxide, followed by separatory funnel counter-current distribution as a means of separating progesterone from corpora lutea of the cow and sow. A solution of 20% chloroform–Skellysolve B eluted all material from the column which had an absorption maximum near 240 m\(\mu\). Since 20 \(\beta\)-ol is probably also measured by this method (15), comparisons with our method should include both the progesterone and 20 \(\beta\)-ol. Chromatography in the Bush Skellysolve B–95% methanol system allows adequate separation between progesterone and 20 \(\beta\)-ol. The Bush system will not separate \(\Delta^4\)-pregnene-20 \(\alpha\)-ol-3-one, \(\Delta^4\) androstene 3, 17-dione, or 20 \(\beta\)-ol because of similar migration rates in this system. The Zaffaroni (27) formamide system is an appropriate one. Rakes et al. (19) used one paper chromatogram in the Bush petroleum ether–80% methanol system to make the final separation of progesterone from bovine tissues. Rechromatography was found necessary in our method in obtaining a final purified eluate of progestins, even with prior treatment of the extract with organic solvent to remove tissue residues.

Recoveries of progesterone-4-C\(^{14}\) ranged from 20 to 60%. Gorski et al. (14), using rechromatography, reported an average recovery of 59% for progesterone separated from bovine tissues. An average recovery of 78% was reported with one chromatogram when bovine corpora lutea were analyzed for progesterone (25). The values reported for recovery of progesterone from blood (11, 18, 24) are generally near the maximum value of our range. Variation in recovery rate is not critical when a radioactive tracer is used. This is in contrast with the methods which depend on a constant recovery rate to achieve quantitative assays.

The limit of accurate quantitative measurement of progestins was about 3 \(\mu\)g. in the total purified sample. This is above the 0.15 \(\mu\)g. limit of sensitivity reported by Edgar and Ronaldson (11) for the analysis of blood.
The total time required to perform all operations of this procedure approaches 24 hr. Although this seems relatively long in comparison to some methods, one technician can run six to eight samples concurrently.

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REFERENCES

DETERMINING PROGESTINS IN THE COW AND SOW


