CHEMICAL DETERMINATION OF 17-HYDROXYCORTICOSTEROIDS IN
THE BLOOD OF CATTLE AND SOME INDICATIONS OF
ITS PHYSIOLOGICAL SIGNIFICANCE

W. G. ROBERTSON AND J. P. MIXNER
New Jersey Agricultural Experiment Station, Sussex

Querido et al. (19) have stated that the level of blood 17-hydroxycorticoster-
oids is the best criterion of adrenal cortical function. Information in regard to
the blood levels of these substances in cattle is lacking, although a review of the
literature by Dorfman and Ungar (6) lists 27 steroids that have been isolated
and identified in bovine adrenal tissue. Hechter et al. (10) obtained both corti-
costerone and 17-hydroxycorticosterone from cattle blood, but the method in-
volved extensive column and paper chromatographic fractionations and as such
was not well suited to routine quantitative analysis.

A number of chemical assays have been proposed for determining 17-hydroxy-
corticosteroids in biological fluids. One group of assays (2, 3, 4, 5, 9, 13, 22, 24)
is dependent on the reducing property of the side chain at carbon 17 or in the
same manner on the reducing property of any nonsteroidal contaminant present
in the final extract. The results obtained with the reducing assays are reliable
only to the extent that the extraction and fractionation procedures employed are
specific for the 17-hydroxycorticosteroids.

Another type of chemical assay that has been used rather widely is the peri-
odic acid oxidation of the side chain at carbon 17 to produce formaldehyde. The
formaldehyde is then measured by various colorimetric procedures (7, 11, 12).
Here again, as in the reducing assays, there is a lack of specificity since many
nonsteroidal substances, which could very possibly contaminate a final extract,
will produce formaldehyde under these conditions.

This paper presents a quantitative method for the determination of total free
17-hydroxycorticosteroids in the blood plasma of dairy cattle, suitable for routine
analysis. A preliminary report on the method (20) has already been presented.
This method utilizes a micro-modification of the Porter-Silber color reaction, de-
pendent on the formation of phenylhydrazones in an acid solution, and is spe-
cific for 17-21-dihydroxy-20-ketones (18). Nelson and Samuels (16) have deter-
mined 17-hydroxycorticosteroid levels in human blood and plasma by using a
modification of this same reaction. Other workers also have utilized this reaction
to study relationships between plasma 17-hydroxycorticosteroid levels and vari-
ous clinical and experimental conditions in the human (1, 8, 15, 21).

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2 Recipient of the Ayerst, McKenna, and Harrison Fellowship of The Endocrine Society for
1955.
METHOD

Reagents:
3. Sodium chloride, C. P.
5. Dilute sulfuric acid, 310 ml. sulfuric acid C. P., and 190 ml. distilled water.
6. Phenylhydrazine HCl, C. P., recrystallized from ethanol four times.
7. Phenylhydrazine—dilute sulfuric acid reagent. 57 mg. of reagent 6 plus 50 ml. reagent 5. Make up fresh each day.

Extractions. Collect jugular vein blood under aseptic conditions and within 2 hours after collection, centrifuge and separate the plasma. Place the plasma in glass-stoppered flasks and chill to 4°C. Add 6 g. of sodium chloride to 80 ml. of chilled plasma and transfer to a prechilled, 250-ml. separatory funnel. Extract for precisely 30 seconds with 80 ml. of cold ethyl acetate (4°C.). Draw off the plasma, rechill, and extract with another 80 ml. of cold ethyl acetate in a prechilled separatory funnel. Combine the ethyl acetate extracts and wash with 3 × 10 ml. portions of saturated sodium chloride solution, 5 × 10 ml. portions of cold 0.1 N sodium hydroxide solution, and finally with 10-ml. portions of distilled water until the wash is neutral. Allow the ethyl acetate extract to stand over an excess of anhydrous sodium sulfate for 10 minutes and then filter and measure the ethyl acetate recovered. The plasma equivalent of the recovered ethyl acetate is determined by dividing the volume by two, since two volumes of ethyl acetate were used in the original extraction. Reduce the extract to dryness with the aid of a gentle stream of air in a water bath maintained at 50°C. Transfer the methanol soluble portion of the residue to a centrifuge separatory funnel with the aid of 3 × 5 ml. portions of 80% methanol. Partition the methanol three times with 15-ml. portions of hexane, using gentle centrifugation each time to aid in separation of the layers. Discard the hexane and transfer an aliquot of the methanol phase (adjusted to contain 20 to 30 ml. of plasma equivalent) to each of two glass-stoppered test tubes. There is a negligible loss of steroid incurred during the methanol-hexane partition. Reduce the extract to dryness with the aid of a gentle stream of air in a water bath maintained at 50°C.

Phenylhydrazine assay. Dissolve the dried steroid in each of the two tubes with 0.2 ml. of methanol. Use one tube as an acid plasma blank and the other as a reaction tube. To the acid plasma blank add 0.8 ml. of dilute sulfuric acid. To the reaction tube add 0.8 ml. of the phenylhydrazine-dilute sulfuric acid reagent. Shake to obtain thorough mixing and place the tubes in a water bath maintained at 55°C for 20 minutes. Remove, cool in tap water for 2 minutes, and transfer to cuvettes (10 mm. light path) and determine the optical density in a Beckman Model B spectrophotometer at 410 μμ.

Anticoagulant is composed of 24 g. of ammonium oxalate, 16 g. of potassium oxalate in 1 l. of water. Citrate anticoagulant is not satisfactory as it interferes with the assay. Five ml. of anticoagulant dried down in a flask is sufficient for 100 ml. of blood.
To accommodate the volumes used in the reaction, a special plate was designed to attach to the side of the absorption cell holder. The plate cuts the height of the incident light to 5 mm., and the cuvettes containing 1.0 ml. of solution are then centered in this area, retaining the 10 mm. light path, with no limitation on the width of the incident light except those inherent in the instrument.

**EXPERIMENTAL PROCEDURE**

*Ringbom analyses.* In order to determine the lowest amount of 17-hydroxycorticosterone (Compound F) which could be properly accommodated by the 1.0 ml. phenylhydrazine reaction, a Ringbom plot was prepared (25). An absolute 1% photometric error is assumed in this presentation and would result from the limits of reproducibility of the particular instrument being used. The reaction was studied over the range of 0.5 $\gamma$ through 10.0 $\gamma$ of standard material, as shown in Figure 1. As indicated by the point of tangency, the range over which a relative analysis error of 5% or less may be expected is from 2.0 to 10.0 $\gamma$.

*Standard curve.* To test both the linearity of the dose-response curve and the repeatability inherent in the phenylhydrazine reaction, a standard curve was made that would fall within the range limitations established by the Ringbom plot. Tubes were prepared containing 2.0, 4.0, 6.0, 8.0, and 10.0 $\gamma$ of 17-hydroxycorticosterone. Six series were run with each dosage in duplicate in each series;

![Fig. 1. A Ringbom plot of the phenylhydrazine reaction showing that 2-10 $\gamma$ of 17-hydroxycorticosterone may be assayed with a relative analysis error of 5% or less.](image-url)
thus each point in Figure 2 is the mean of 12 determinations. It will be noted that the data are strictly linear over the dosage range covered.

An analysis of variance of the data (Table 1) showed that the series effect was not significant, indicating good repeatability of the reaction. When series, series × dosage, and residual effects were all included in the error term, a coefficient of variation of 4.47% and a standard error of a single observation of 0.258 µ in terms of 17-hydroxycorticosterone were obtained. A regression equation was also derived by using the least squares method, and this equation was used in calculating plasma corticoid values.

Absorption spectra. A comparison of the absorption spectra obtained with 17-hydroxycorticosterone and with a plasma extract (28.0 ml. of plasma equivalent) is shown in Figure 3. Peak absorption in both instances occurs at 410 mµ and is specific for the 17,21-dihydroxy-20-ketone grouping.

Efficiency of extraction. The efficiency of the extraction procedure was assessed by adding 17-hydroxycorticosterone (free alcohol) to plasma and calculating recovery. Blood samples were obtained and centrifuged, and the plasma

<table>
<thead>
<tr>
<th>Variance source</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>59</td>
<td>0.338118*</td>
</tr>
<tr>
<td>Dosage</td>
<td>4</td>
<td>0.000429</td>
</tr>
<tr>
<td>Series</td>
<td>5</td>
<td>0.000232</td>
</tr>
<tr>
<td>Dosage × series</td>
<td>20</td>
<td>0.000133</td>
</tr>
<tr>
<td>Residual</td>
<td>30</td>
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</table>

* Highly significant effect (P < 0.01).
Fig. 3. A comparison of absorption spectra obtained with a plasma extract and 17-hydroxycorticosterone in the phenylhydrazine reaction.

was separated. Samples of plasma (80 ml.) were placed in each of two glass-stoppered 125-ml. flasks. One of the samples served as a control, and 17-hydroxycorticosterone was added to the other. The samples were then placed in a refrigerator and held at 4° C. overnight. Extraction and assay were accomplished the next day. A mean recovery of 81.0% of the added steroid was obtained with no significant variation in recovery over the range of steroid concentrations studied (Table 2). This performance agrees well with the results of Nelson (15), who reported a mean recovery of 80% of added 17-hydroxycorticosterone from human plasma.

**Effect of ACTH on plasma 17-hydroxycorticosteroids.** To assess the physiological significance of the plasma extract fraction, six dairy cows were injected

<table>
<thead>
<tr>
<th>Plasma sample No.</th>
<th>F added (γ %)</th>
<th>F recovered (γ %)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.5</td>
<td>13.88</td>
<td>79.3</td>
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<td>2</td>
<td>12.5</td>
<td>10.26</td>
<td>82.1</td>
</tr>
<tr>
<td>3</td>
<td>10.0</td>
<td>7.70</td>
<td>77.0</td>
</tr>
<tr>
<td>4</td>
<td>10.0</td>
<td>8.28</td>
<td>82.8</td>
</tr>
<tr>
<td>5</td>
<td>10.0</td>
<td>8.47</td>
<td>84.7</td>
</tr>
<tr>
<td>6</td>
<td>7.5</td>
<td>6.37</td>
<td>84.9</td>
</tr>
<tr>
<td>7</td>
<td>5.0</td>
<td>3.89</td>
<td>77.8</td>
</tr>
<tr>
<td>8</td>
<td>5.0</td>
<td>3.97</td>
<td>79.5</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td></td>
<td></td>
<td><strong>81.0</strong></td>
</tr>
</tbody>
</table>
intramuscularly with 600 Armour Veterinary Units of ACTH. Blood samples were collected from the animals immediately before and 2 hours after the ACTH administration. A mean increase of 120% in the level of circulating 17-hydroxycorticosteroids was obtained in these trials (Table 3) as a consequence of ACTH administration. These results are very similar to those in the human reported by Nelson et al. (17), who found a 125% increase in the level of circulating 17-hydroxycorticosteroids 2 hours after the intramuscular injection of 25 mg of ACTH.

**Plasma 17-hydroxycorticosteroid levels in normal dairy cows.** To further assess the physiological significance of the plasma extract fraction, blood samples were obtained from 23 dry, pregnant cows within the 6-week period prior to parturition and from 20 milking, nonpregnant cows within the 6-week period after parturition. The plasma 17-hydroxycorticosteroid values for the dry, pregnant group ranged from 6.90 to 17.62 with a mean value of 9.77 γ %, whereas the values for the milking, nonpregnant group ranged from 2.14 to 8.40 with a mean of 4.58 γ % (Table 4). The increase in plasma 17-hydroxycorticosteroid levels accompanying pregnancy is in agreement with that in the human reported by Gemzell (8), who found 100% increase in the plasma steroid levels during pregnancy.

**DISCUSSION**

The micro-modification of the phenylhydrazine reaction used in this investigation has several very favorable aspects. The specificity of the reaction is par-

<table>
<thead>
<tr>
<th>Cow No.</th>
<th>Initial F (γ %)</th>
<th>2-hour F (γ %)</th>
<th>Increase (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>17.62</td>
<td>29.79</td>
<td>67</td>
</tr>
<tr>
<td>2</td>
<td>12.85</td>
<td>29.74</td>
<td>116</td>
</tr>
<tr>
<td>3</td>
<td>9.64</td>
<td>19.73</td>
<td>105</td>
</tr>
<tr>
<td>4</td>
<td>7.00</td>
<td>14.38</td>
<td>105</td>
</tr>
<tr>
<td>5</td>
<td>5.10</td>
<td>13.80</td>
<td>170</td>
</tr>
<tr>
<td>6</td>
<td>2.98</td>
<td>7.74</td>
<td>159</td>
</tr>
<tr>
<td>Mean</td>
<td>9.20</td>
<td>18.86</td>
<td>120</td>
</tr>
</tbody>
</table>
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particularly desirable when applied to extracts of biological fluids. Of 14 steroids tested by Porter and Silber (18), only those containing the 17,21-dihydroxy-20-ketone grouping reacted to produce solutions exhibiting an absorption maximum at 410 m\(\mu\). These steroids did not produce appreciable color in sulfuric acid alone. Silber and Porter (23), in a further examination of this reaction, determined that steroids which bear the 21-hydroxy-20-ketone grouping but not the 17-hydroxyl function, such as corticosterone, reacted with the reagents but the absorption peaks for these substances occurred at 340 to 360 m\(\mu\). Very little absorption was evident at 410 m\(\mu\). Nitrogen analysis of purified products from the reaction of phenylhydrazine with cortisolone and 17-hydroxy cortisolosterone indicates that the color is due to the formation of bisphenylhydrazones.

It would appear that certain specie differences are involved in regard to the amount and types of chromogenic material found in blood or plasma. The method of Nelson and Samuels (16) proved adequate for the determination of 17-hydroxy cortisolosteroids in human plasma. This method was not found satisfactory for the determination of these substances in dairy cattle plasma in this investigation. There appears to be considerably more nonspecific chromogenic material capable of reacting with dilute sulfuric acid in the plasma of cattle. This material was not removed by the method of Nelson and Samuels (16) and caused extremely high background absorption in the phenylhydrazine assay such that the absorption maximum at 410 m\(\mu\) due to the steroid was entirely obliterated.

The use of a cold ethyl acetate extraction for short periods of time is a departure from the usual means of removing 17-hydroxy cortisolosteroids from biological fluids. Meyer (14) pointed out the advantages of using ethyl acetate under these conditions. A number of organic solvents and solvent mixtures were tested by extracting 40 ml. of whole citrated blood. The solvents were rated by examining the ratio of the weight of the cortisolone recovered and the weight of the total residue. Ethyl acetate was found to be the most satisfactory of those investigated in that it removed cortisolone efficiently and at the same time removed less extraneous material.

The method proposed in this investigation should prove useful in assessing adrenal cortical physiology in dairy cattle as shown by plasma steroid response to ACTH and also by differences exhibited in plasma steroid levels of pregnant nonlactating and lactating nonpregnant animals.

The recovery of standard material compares very favorably with that obtained by Nelson (15). The specificity and sensitivity of the 1.0 ml. phenylhydrazine reaction are particularly advantageous when applied to relatively crude plasma extracts. The extracts resulting from this method contain no more nonspecific chromogenic material capable of interfering with the phenylhydrazine assay than those resulting from a more tedious silica gel chromatographic fractionation procedure.

**SUMMARY**

A method for the chemical determination of 17-hydroxy cortisolosteroids in the plasma of dairy cattle was developed and evaluated. The method employs the
extraction of cold plasma by cold ethyl acetate and assay of the steroids by using the Porter-Silber color reaction. The efficiency of the extraction procedure was judged to be 81% as determined from experiments involving the recovery of steroid added to plasma. A mean increase of 120% in plasma 17-hydroxycorticosteroids was obtained 2 hours after the intramuscular administration of 600 Armour Veterinary Units of ACTH to six cows. Plasma 17-hydroxycorticosterone values for 23 dry, pregnant cows ranged from 6.90 to 17.62 with a mean of 9.77 γ %. Similar values for 20 milking, nonpregnant cows ranged from 2.14 to 8.40 with a mean of 4.58 γ %. These experiments indicate that the method has considerable physiological significance in assessing adrenal cortical function in dairy cows.

ACKNOWLEDGMENTS

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REFERENCES

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