A SPECIFIC, NON-CROMATOGRAPHIC RADIOIMMUNOASSAY
FOR HUMAN PLASMA CORTISOL

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ABSTRACT

A radioimmunoassay technique has been developed for the measure-
ment of cortisol in a single methylene chloride extract of human plasma
without chromatography. The antiserum, obtained by immunizing rabbits
with cortisol-3-carboxymethyl-oxime conjugated to bovine serum albumin,
had a high affinity ($K_A = 1.8 \times 10^9/\text{mole}$) and capacity ($2.3 \times 10^{-6}$
moles/L undiluted serum) for cortisol. The minimum detectable amount
determined at the lower 95% confidence limit of the buffer control
tubes was $8.3 \pm 4.7 \text{ pg/tube}$ and a log dose - logit response standard
curve was linear between 20 pg and 20 ng/tube. The antiserum was high-
ly specific for cortisol with only corticosterone, cortisone, 11-deoxy-
cortisol and 21-deoxycortisol showing significant cross-reaction (12.4,
6.6, 3.8 and 3.7%, respectively). The cross-reaction for the other
tested naturally occurring and synthetic steroids did not exceed 1%.
Regression analysis of cortisol concentration estimates obtained on 20
samples before and after Sephadex LH-20 column chromatography gave a
coefficient of correlation ($r$) of 0.995 and a regression coefficient
($b$) of 1.04. Recovery of cortisol added to plasma samples was quanti-
tative. The intra-assay error was 8.5% and the inter-assay error aver-
aged 5.7%. The method is simple requiring a single solvent extraction
of plasma, therefore permitting large numbers of samples to be handled
efficiently by a single technician.

INTRODUCTION

Cortisol in plasma and urine is usually measured with either the
competitive protein binding (CPB) technique, originally described by
Murphy (6,7), the Porter-Silber color reaction (8) or fluorometry (9).
Cortisol binding globulins (CBG) from various species have high affin-
ity (10) but relatively low specificity for cortisol. To make the CPB technique more specific, prior chromatographic systems may be employed; but, in the absence of considerable care, these approaches can give rise to high plasma and urinary blank values. The Porter-Silber method and fluorometry also lack in specificity, unless prior chromatography of the samples is undertaken. Further, many drugs interfere with the Porter-Silber color reaction which makes this method unsuitable for random use (11). Ruder et al. (1972) have reported a radioimmunoassay procedure for plasma and urinary free cortisol, using antibodies against cortisol-21-hemisuccinate-bovine serum albumin (BSA) conjugate. The antiserum had a high affinity ($K_A = 2 \times 10^9$ l/mole) and binding capacity ($2 \times 10^{-6}$ moles/l of undiluted serum) but a low specificity for cortisol (12).

This communication describes the development of a sensitive, accurate and specific, non-chromatographic method for radioimmunoassay of cortisol in a single methylene chloride extract of plasma. The procedure uses an antiserum raised against a BSA conjugate of cortisol-3-carboxymethyl-oxime and tritiated cortisol ($^{3}\text{H}$-cortisol) as the tracer.

**METHODS AND MATERIALS**

All solvents used in the assay were glass distilled (Burdick-Jackson Laboratories) and were used without further purification. Cortisol-1,2,6,7,$^{3}\text{H}(n)$, purified by thin layer chromatography, with a specific activity of 107 Ci/m mole, was purchased from the Radiochemical Centre, Amersham. The cortisol standard was prepared by dissolving crystallized cortisol (Hydrocortisone, Sigma Chemical) in ethanol (1 mg /ml). The ethanol solution was diluted with 0.1% gel-PBS (0.1% gelatin in 0.14 M NaCl, 0.01 M NaPO$_4$ buffer and pH 7 with 1:1000 merthiolate) to a final concentration of 40 ng/ml, dispensed in 5 ml aliquots, quickly frozen by immersion in a dry ice-ethanol mixture, and stored at -20°C. Other steroid preparations used in validation of the procedure were identically prepared, dispensed and stored at final concentrations of 40 and 800 ng/ml. Prior to use, each solution was thawed by gently agitating the vial in lukewarm water. A small fraction of $^{3}\text{H}$-cortisol in benzene-ethanol (1:1 v/v) was pipetted into a 500 ml brown flask, the
solvent evaporated to dryness under nitrogen and the $^3$H-cortisol reconstituted in 0.1% gel-PBS to a final concentration of approximately 20,000 cpm/100 μl.

The antiserum was obtained by immunizing each of 6 New Zealand White female rabbits, approximately 4 months of age, in the foot pads with 2.5 mg of cortisol-3-carboxymethyl-oxime BSA conjugate (18-20 molecules of cortisol/molecule of albumin) emulsified in complete Freund's adjuvant (13). The adjuvant was prepared by mixing 15.5 mg cortisol-BSA conjugate in 3.1 ml of phosphate-buffered-saline (pH 7.4), 0.5 ml of Arlacel A (Atlas Chemical Industries) and 2.8 ml of Bayol F (Esso Petroleum Co.), together with 15 mg of desiccated M. tuberculosis, in the barrel of a 20 ml glass syringe using a high speed Vertis Macromixer, Model 45. The initial immunization was followed by 2 booster immunizations (M. tuberculosis normally not included in booster adjuvant) given at approximately 3 week intervals and a final booster given 2 1/2 months later. The rabbits were bled biweekly, from the marginal ear vein, following the second booster immunization. The bleeding selected for use in the assay system was obtained 3 weeks after the final immunization (22 weeks following the initial immunization). This bleeding showed the highest titer and since it proved to be highly specific, other bleedings from the same rabbit or other rabbits were not evaluated further. The antiserum (#891, 2-8-U), used in the radioimmunoassay at a final dilution of 1:20,000 in 0.1% gel-PBS, bound 38.5% of 88 pg of $^3$H-cortisol ($B/F = 0.63$).

The dextran-coated charcoal suspension, prepared by adding 250 mg of doubly washed charcoal (Mallinckrodt Chemical Works) and 25 mg of dextran-80 (Pharmacia Corp.) to 100 ml of de-ionized water, was used for separation of antibody-bound and free $^3$H-cortisol.

All glassware used in the radioimmunoassay was heated in the self-cleaning cycle of a Frigidaire self-cleaning oven prior to use. The entire assay procedure was carried out in 12 x 75 mm disposable glass tubes. Scintillation counting was done by using a solution containing toluene (analytical grade, Mallinckrodt Chemical Works), Triton X-100 and Permaflour (Packard Instruments) (1.0:0.95:0.16) in a refrigerated Packard Tricarb scintillation counter with a counting efficiency for $^3$H of 35.4%. Nitrogen used to evaporate the solvents was passed over Drierite, soda lime and a thick pad of glass wool.

**Assay Procedure.** The general outline of the assay is as follows:

Freshly thawed plasma (100μl), de-ionized water (500μl) and $^3$H-cortisol (5000-6000 cpm) in 100 μl of 0.1% gel-PBS were pipetted into a 16 x 150 mm culture tube and extracted with 3 ml of methylene chloride by thoroughly mixing the contents on a vortex mixer for about 2 min. The methylene chloride phase was transferred to a 12 x 75 mm tube and the solvent evaporated under nitrogen in a water bath kept at 45°C. The residue was then reconstituted in 3 ml of 0.1% gel-PBS. Aliquots (100 μl) of this were taken for assay and recovery estimates of $^3$H-cortisol. All assay results were corrected for procedural losses using calculated recovery rates of $^3$H-cortisol. The low levels of $^3$H-cortisol used for recovery were neglected in later calculations.

The samples were assayed in duplicate at two dose levels. The volume was brought to 500 μl by adding the requisite amount of 0.1% gel-PBS. Upon addition of 200 μl of a 1:2000 dilution of antiserum and 100 μl of $^3$H-cortisol (approx. 20,000 cpm) in 0.1% gel-PBS, the tubes
were mixed and incubated overnight (15-18 hrs) at 4°C (5). The bound and free \(^3\text{H}\)-cortisol were separated by addition of 1 ml dextran-coat charcoal suspension to each tube in batches of 30-40 and the incubation was continued for another 10 min. The tubes were then centrifuged a 600 x g in a refrigerated centrifuge (Model PR-J, International Equipment Co.) for 10 min. The supernatant was decanted into scintillation vials containing 10 ml of scintillation fluid and counted for one minute. An eleven point standard curve (0.02 - 20 ng/tube) was constructed for each assay and run in triplicate (at the beginning, middle and end of samples). Standard curves and unknown samples were processed simultaneously with a computer program which uses weighting procedure and logit-log dose transformation to obtain a linear regression curve. The program tests all sample curves for linearity and parallelism with the standard and then computes the weighted mean concentration ± 1 SE per tube (14). The values obtained for unknown samples were corrected for procedural losses based on the recovery of \(^3\text{H}\)-cortisol.

**Sephadex LH-20 column chromatography.** Sephadex LH-20 (lot 2764 Pharmacia Corp.) was equilibrated in a methylene chloride: methanol (98:2) mixture for 24 hrs. and then packed into 5 x 100 mm columns. Floating of the gel in the solvent system was eliminated by placing fine glass wool between 2 filter paper plates on the top of the gel columns. The solvent eluted from the column at a rate of 0.35 - 0.4 ml/min. Prior to use, each column was washed with 20 ml of the elute. The samples were prepared by extracting 300 μl of freshly thawed plasma and approximately 15,000 cpm \(^3\text{H}\)-cortisol in 100 μl of 0.1% gel-PBS with 3 ml of methylene chloride. Eight hundred μl of the methylene chloride extract was evaporated under nitrogen in a water bath at 45°C and the residue dissolved in 500 μl of the elution solvent for column chromatography. The samples were eluted in 500 μl fractions. The content of the 3-4 tubes containing cortisol were pooled, dried and reconstituted in 2 ml of 0.1% gel-PBS. Another 800 μl of the methylene chloride extract was dried and the contents reconstituted in 2 ml of 0.1% gel-PBS for assay. This allowed a comparison to be made of estimates of cortisol in a methylene chloride extract of a sample and the same extract after it had been subjected to chromatography.

Following use, the columns were washed with 100 ml of a methylene chloride: methanol (98:2) mixture to remove any residual radioactivity and stored in air-tight glass jars containing 150-200 ml of the chromatography solvent system.

**RESULTS**

**Immunoreactivity of the antiserum against cortisol-3-BSA.** The specificity of the antiserum used in the cortisol radioimmunoassay was assessed by examining the dose response curves for 19 different steroids. Commonly used glucocorticoids, dexamethasone and triamcinolone showed extremely low cross-reactivity (<.01%) in the assay system. Similar, nearly parallel dose response curves were obtained with pre
nisolone, cortisone, corticosterone, 11-deoxycortisol, 21-deoxycortisol and prednisone (Fig. 1). However, the relative activity of these com-

Fig. 1 Inhibition curves of various steroids, naturally occurring and synthetic, in cortisol-3-BSA radioimmunoassay.

pounds as determined at the 50% displacement of $^3$H-cortisol were negligible for all except prednisolone (28%), corticosterone (12.5%), cortisone (6.6%), 11-deoxycortisol (3.8%) and 21-deoxycortisol (3.7%) (Table 1).

The cortisol standard curve. Figure 2 shows the standard dose response curve for cortisol based on data from 8 assays (24 curves). The average index of precision ($\lambda$) based on a weighted least squares linear regression for the logit-log transformation of standard curves was 0.018 (range 0.012 - 0.026). The assay median variance ratio ($\mu$) ranged from 0.012 to 0.055 with an average of 0.025. The non-specific background (NSB) as a percent of the total counts added ($T$) ranged from 0.5-0.9% (0.6 ± 0.01%) and the radioactivity bound in the absence of unlabeled steroid (buffer control, $B_o$) expressed as a percent of the total activity added ($B_o$-NSB)/($T$-NSB) ranged from 32.7% - 40.4% (38.5 ± 2.6%).
Regression analysis of the concentration of cortisol bound (from the

<table>
<thead>
<tr>
<th>Steroid</th>
<th>-21 Hemisuccinate (Ruder et al, 1972)</th>
<th>-3 Carboxy-methyl-oxime (Present Study)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>($K_A=2 \times 10^9$ l/mole)</td>
<td>($K_A=1.8 \times 10^9$ l/mole)</td>
</tr>
<tr>
<td>Cortisol</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>-</td>
<td>28.5%</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>-</td>
<td>12.5%</td>
</tr>
<tr>
<td>Cortisone</td>
<td>40%</td>
<td>6.6%</td>
</tr>
<tr>
<td>11-deoxycortisol</td>
<td>-</td>
<td>3.8%</td>
</tr>
<tr>
<td>21-deoxycortisol</td>
<td>-</td>
<td>3.7%</td>
</tr>
<tr>
<td>Prednisone</td>
<td>16%</td>
<td>0.7%</td>
</tr>
<tr>
<td>11-dehydrocorticosterone</td>
<td>46%</td>
<td>0.1%</td>
</tr>
<tr>
<td>11-deoxycorticosterone</td>
<td>46%</td>
<td>0.06%</td>
</tr>
<tr>
<td>6β-cortisol</td>
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<td>0.05%</td>
</tr>
<tr>
<td>Tetrahydrocortisol</td>
<td>-</td>
<td>0.01%</td>
</tr>
<tr>
<td>Progesterone</td>
<td>28%</td>
<td>0.001%</td>
</tr>
<tr>
<td>17α-OH-progesterone</td>
<td>56%</td>
<td>0.06%</td>
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<tr>
<td>Dexamethasone</td>
<td>-</td>
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<tr>
<td>Triamcinolone</td>
<td>-</td>
<td>0.01%</td>
</tr>
<tr>
<td>Testosterone</td>
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<tr>
<td>Estradiol-17β</td>
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<td>0.001%</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>0.01%</td>
<td>0.001%</td>
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</table>

*Determined at 50% displacement = \( \frac{\text{ng of cortisol}}{\text{ng of the steroid}} \times 100 \)

Fig. 2 Standard dose response curve, mean ± 1 standard deviation from 24 individual standard curves.

above results) versus the bound to free ratio, according to the method
of Scatchard (15), revealed a linear relationship and indicated that the antiserum possessed an equilibrium association constant of $1.8 \times 10^9$ l/mole and a capacity of $2.3 \times 10^{-6}$ moles/l of undiluted serum.

**Sensitivity.** The smallest quantity of cortisol which could be distinguished from "no cortisol" determined at the lower 95% confidence limit of the buffer control tubes, was $8.3 \pm 4.7$ pg/tube. The log-log transformation was linear between 20 pg and 20 ng with a useful range of 80 pg to 10 ng/tube.

**Precision.** The intra-assay error, determined as the coefficient of variation of 58 assay replicates of 40 plasma samples, (each run in duplicate at two levels), was 8.5%. The inter-assay error measured as coefficient of variation of reference plasma included in each assay averaged 5.7% (n=6).

**Recovery of $^3$H-cortisol.** Recovery of $^3$H-cortisol added to the unknown samples prior to extraction with 30 volumes of methylene chloride averaged 93.5 ± 4.3%.

**Accuracy.**

1. **Recovery of unlabelled cortisol.** To 100 µl aliquots of plasma from a fasting male and a female in the second trimester of pregnancy, 0, 5, 10, 20 and 40 ng of unlabelled cortisol were added, and incubated for one hour at room temperature. The samples were then extracted with methylene chloride for radioimmunoassay. Figure 3 shows the correlation between the "observed" and the "expected" cortisol concentration/tube. The agreement between the two was assessed by linear regression analysis. The correlation coefficient ($r$) and slope ($b$) were 0.999 and 0.94 for male plasma and 0.998 and 1.10 for pregnancy plasma respectively. This indicates no influence of increased levels of cortisol bind-
ing globulin (CBG) on recovery of unlabeled cortisol.

Fig. 3 Recovery of known amounts of cortisol added to human plasma. Observed values were calculated by difference between enriched samples and original sample.

2. Comparison of the estimates of cortisol by the present method with and without chromatography. Plasma samples with high, normal and low cortisol levels obtained from 3 normal subjects at 8 a.m. (fasting).
and 4 p.m., 2 patients with controlled Cushing’s Syndrome before and after ACTH infusion, 5 subjects with normal pregnancy and 5 newborns of different gestational age were processed by (a) the present method (simple methylene chloride extraction) and (b) a methylene chloride extraction followed by Sephadex LH-20 column chromatography. Under the chromatographic conditions approximately 70% of cortisol could be eluted in fractions 10-12 (5-6.5 ml). Figure 4 shows the correlation between the estimates of cortisol obtained with and without chromatography, all estimates were corrected for recovery of $^3$H cortisol; the correlation coefficient ($r$) was 0.995.

3. Comparison of the present method with the Porter-Silber method.

![Graph](image)

Forty-one plasma samples with a wide range of cortisol concentrations were measured by radioimmunoassay and the Porter-Silber method (16). The values obtained by radioimmunoassay ranged from 0.4 - 62.3 μg/100 ml and the corresponding values, without recovery corrections by the Porter-Silber method (approximately 80% recovery) 0.9 - 63.7 μg/100 ml. In
general, the values obtained by the Porter-Silber method (Y) were slightly higher than those of radioimmunoassay (X), \( Y = 1.05 \) (X), the correlation coefficient (r) being 0.962 (Fig. 5).

4. Plasma cortisol levels in normal subjects and patients with treated Cushing's Syndrome. Figure 6 shows the diurnal rhythm of corti-

![Plasma cortisol levels in normal subjects and in patients with treated Cushing's Syndrome.](image)

Fig. 6 Plasma cortisol levels in normal subjects and in patients with treated Cushing's Syndrome.

Sol secretion in 8 normal subjects and 3 patients with Cushing's Syndrome (ACTH dependent) before and after treatment with Lysodrine (O'P' DDD; 2, 2-bis (2-chlorophenyl-4-chlorophenyl)-1, 1-dichlorethane 2 mg orally). In normal fasting subjects the 8 a.m. plasma cortisol ranged from 10.6 - 25.0 \( \mu \text{g/dl} \) (17.7 ± 5.0) and 4 p.m. estimates were 3.0 - 16.1 \( \mu \text{g/dl} \) (7.7 ± 4.2). These values are similar to those reported in the literature (12,17). In patients adequately treated for Cushing's Syndrome, the resting plasma cortisol levels were normal, but the expected diurnal rhythm was absent. These patients, however, did respond normally to ACTH infusion (25 U ACTH over 8 hrs) and dexamethasone suppression.
Radioimmunoassays have the potential of being used for quantitation of steroids in raw or extracted plasma or serum samples without additional chromatography and have the advantage over competitive-protein-binding (CPB) assays, provided a truly specific antiserum is available. Earlier data have shown that specificity of antisera against steroids are largely dependent on the site at which the carrier protein is conjugated (17). Conjugation of the protein to a site remote from the sites of the unique functional groups on the steroid molecule results in an increase in antibody specificity. This statement is amply documented by the observations that specificity of the antibodies against testosterone-3, progesterone-11, and 17β-estradiol-11 BSA conjugates are much greater than those of antibodies against testosterone-17, progesterone-3, or 17β-estradiol-3 or 17 BSA conjugates (18-20). Cortisol differs structurally from other steroids by the presence of hydroxy groups at C-11, C-17 and C-21. Therefore, an antibody prepared against a cortisol-21-BSA conjugate should be relatively less specific than one against a cortisol-3-BSA conjugate, since in the latter the unique functional groups are available for antibody induction and recognition. This is further supported by comparing the specific activity of 11 different steroids in two different radioimmunoassay procedures (Table 1). While the majority of steroids cross-reacted with the cortisol-21-BSA antiserum, few did so against cortisol-3-BSA antiserum (the cross-reaction of prednisolone was expected as it is structurally analogous to cortisol with only the insertion of a double-bond at C 1). Extraction of serum samples is required, however, to remove endogenous binding protein and cross-reacting water soluble compounds including
cortisol-3-conjugates. It should be apparent that the assay has not been validated for sera from animals, nor for sera from patients with certain rare diseases such as congenital adrenal hyperplasia with 17-hydroxylase deficiency.

The accuracy of the present method was examined in part by the recovery of various amounts (5-40 ng/tube) of unlabeled cortisol from two different types of plasma, one with normal and the other with an excess of human CBG. Excess CBG in plasma did not interfere with recovery and the coefficient of correlation did not differ between the two plasma specimens. Finding slightly higher values by the Porter-Silber method also lends support to the accuracy of measuring cortisol by the present method. Studies of different physiological states such as variations due to diurnal rhythm, as well as results of ACTH stimulation and dexamethasone suppression, suggests that the plasma cortisol can be reliably estimated over a wide range. Further, the excellent correlation between chromatographed and non-chromatographed cortisol estimates in plasma samples justifies application of this procedure for clinical and research purposes. The procedure prior to radioimmunoassay requires less than one hour for completion, the technique is not complex, and the time used in completion of the assay is reasonable and can be varied to meet individual needs (in emergencies the assay can be completed in about 2 hours using 37°C incubation conditions). The RIA described is more sensitive than the CPB methods, has much greater specificity and is equivalent in ease and convenience of set-up. Most of the synthetic or naturally occurring steroids that might be found in patient specimens (which would invalidate a CPB assay not involving chromatography) do not interfere with the radioimmunoassay described herein. Those steroids
that do cross-react in the RIA include a small number of synthetic steroids (prednisolone, cortisone and to a minor extent prednisone) and only one of the tested naturally occurring steroids (corticosterone). Other naturally occurring steroids do not cross-react as shown by the lack of difference between chromatographed and non-chromatographed samples.

With the described procedure, it is possible for one technician to assay about 50 different samples in duplicate and at 2 dose levels or a greater number of samples at only 1 dilution. The assay can be set up in the afternoon, allowed to incubate overnight and results obtained the next day. This schedule would be acceptable in most clinical laboratories where specimens are collected and run at intervals in large batches.

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REFERENCES

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(2) Career Development Awardee of the National Institute of Child Health and Human Development.

(3) Present address: Department of Physiology, Colorado State University, Fort Collins, Colorado 80521.

(4) The following trivial names have been used:

cortisol (11β, 17α, 21-trihydroxypregn-4-ene-3,20-dione),
corticosterone (11β, 21-dihydroxypregn-4-ene-3,20-dione),
aldosterone (11β, 21-dihydroxy-3, 20-dioxopregn-4-ene-18-al),
cortisone (17α, 21-dihydroxypregn-4-ene-3,11,20-trione),
progesterone (pregn-4-ene-3,20-dione),
17α-hydroxyprogesterone (17α-hydroxypregn-4-ene-3,20-dione),
deoxycorticosterone (21-hydroxypregn-4-ene-3,20-dione),
17α-estradiol (1,3,5(10)-estratriene-3,17α-diol),
17 β-estradiol (1,3,5,(10)-estratriene-3,17 β-diol),
testosterone (17 β-hydroxyandrost-4-ene-3-one),
dehydroisoandrosterone (3 β-hydroxyandrost-5-ene-17-one),
tetrahydrocortisol (3 α, 11 β, 17 α, 21-tetrahydroxy-5 β-pregnane-20-one),
triamcinolone (11 β, 16 α, 17 α, 21-tetrahydroxy-9 α-fluoro-pregn-1,4-diene-3,20-dione),
dexamethasone (11 β, 17 α, 21-trihydroxy-9 α-fluoro-16 α-methylpregn-1,4-diene-3,20-dione),
prednisone (17 α, 21 dihydroxypregn-1,4-diene-3,11,20-trione),
prednisolone (11 β, 17 α, 21-trihydroxypregn-1,4-diene-3,20-dione)
6 α-hydroxycortisol (6 α, 11 β, 17 α, 21-tetrahydroxypreg-4-ene-3,20-dione),
6 β-hydroxycortisol (6 β, 11 β, 17 α, 21-tetrahydroxy-pregn-4-ene-3,20-dione),
11-deoxycortisol (17 α, 21-dihydroxypreg-4-ene-3,20-dione)
21-deoxycortisol (11 β, 17 α-dihydroxypreg-4-ene-3,20-dione).

The incubation is now being carried out at room temperature for 1 hours using a 1:4000 dilution of the antiserum. No change in specificity has been observed however the assay is more sensitive.


(18) Niswender, G.D., and A.R. Midgley, Jr., In Peron, F.G., and Cald-
