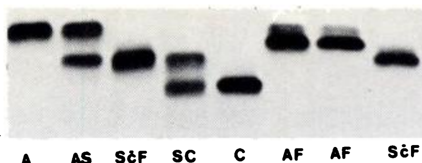

Fig. 1. Agarose-gel electrophoresis at pH 8.6, barbital buffer The horizontal line at left is the point of application

backgrounds. Most laboratories use solubility testing combined with hemoglobin electrophoresis on cellulose acetate membranes for this purpose (1). I would like to report our experience with hemoglobin electrophoresis on a "high-resolution" agarose-gel membrane at alkaline pH (Panagel; Worthington Diagnostics, Freehold, NJ 07728).

Clinical samples from ULCA Hospitals and Clinics were collected in tubes containing ethylenediaminetetraacetate as anticoagulant, washed in isotonic saline, and lysed with a commercial lysing diluent (Hemolysate Reagent, Gelman). The optimum hemoglobin dilution was 3–4 g/dL. Samples were then applied to the pre-prepared agarose-gel membrane (Worthington) and electrophoresed in a barbital buffer (pH 8.6 ± 0.5) at 200 V for 50 min. A cooling block was used to keep temperatures at about 25 °C. After electrophoresis, the slides were fixed for 10 min in a solution of 1.5 g of picric acid dissolved in 120 mL of dilute (170 mL/L) aqueous acetic acid, rinsed in 95% ethanol, dried, and stained with Amido Black stain. After destaining in tap water, the slides were again dried and scanned with a densitometer or visually inspected.

Commonly encountered abnormal hemoglobins were well resolved by the high-resolution electrophoresis system. These commonly encountered hemoglobins included: hemoglobin A with increased amounts of A_2, most often seen with B-thalassemia; hemoglobin S, either homozygous (SS) or heterozygous (AS); hemoglobin C, homozygous (CC) or heterozygous (AC); and hemoglobin F, in neonates and in various amounts in other hemoglobinopathies (Figure 1). These hemoglobins migrate in the same relative positions as on cellulose acetate: hemoglobin A followed by F, S, A_2, and C. Hemoglobins A_2 and C were not separated when samples supplemented with these were examined. Samples from infants containing significant amounts of hemoglobins F and A were reasonably well separated when the sample was fresh; in samples run after seven to 10 days storage, these bands were less distinct.

We tried to quantitate hemoglobin A_2, using densitometric scanning, but we found this technique unreliable because of an unacceptably high CV. Similar findings have been reported for electrophoresis on cellulose acetate (2).

According to national surveys, most clinical laboratories utilize cellulose acetate systems for hemoglobin electrophoresis (1). With the introduction of high-resolution electrophoretic systems in the study of serum, urine (3), and cerebrospinal fluid proteins (4), additional applications of agarose-gel electrophoresis have been suggested, including lipoprotein, lactate dehydrogenase isoenzymes, and hemoglobin electrophoresis. With this high-resolution system we found commonly encountered abnormal hemoglobins to be well separated, with electrophoretic mobilities essentially the same as in the more commonly used cellulose acetate systems, and we conclude that the system is useful in the laboratory evaluation of commonly encountered abnormal hemoglobins.

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Stephen E. Vernon[1]

Dept. of Pathol.
UCLA Hospitals and Clinics
Los Angeles, CA 90024

[1] Present address: Dept. of Pathol., St. Francis Hosp., 250 West 63rd St., Miami Beach, FL 33141.

Radioimmunoassay of Free Cortisol with Antiserum-coated Tubes and ^{125}I-Labeled Cortisol

To the Editor:

We have developed a new, highly sensitive method for determination of the apparent free-cortisol concentration (AFCC) in plasma, by means of a radioimmunoassay (RIA) method in which an antiserum is directly coated to the test tubes and ^{125}I-labeled cortisol is used as the tracer. Diffusible cortisol in the plasma dialysate after an equilibrium dialysis is measured directly by this method.

The major role of the free (non-protein-bound) moiety in determining the biological activity of steroid hormones is now widely accepted. Therefore, assay of free steroids in plasma appears to supply more reliable information about the functional setting of such hormones, especially when the binding capacity of plasma proteins is altered, whether increased, as in pregnancy or during estrogen therapy, or decreased, as in some liver and kidney diseases.

Recently, we described a RIA for determination of AFCC in plasma or serum samples (1, 2). This technique involved use of a solid-phase system (antiserum bound to cellulose) to separate the free from the bound antigen, with [^{3}H]cortisol as the tracer. However, use of gamma-emitting tracer in the RIA of steroids results in higher specific activity, greater practicability in counting, and greater ease of automation than with tritiated tracers (3). Moreover, the introduction of recent RIAs with antiserum coated directly onto the test tube walls to facilitate the separation of the free from the bound phase has markedly improved the laboratory feasibility and shortened the time of the assay (4). Therefore, we have developed a new coated-tube RIA for determination of diffusible cortisol in the dialysate after an equilibrium dialysis of plasma samples.

To determine total cortisol concentration and AFCC, we used a commercial kit, "SPAC Cortisol," kindly supplied by Byk Gulden Italia S.p.A., Cormano (MI), Italy. This RIA method involves ^{125}I-labeled cortisol (about 1 μCi for 50 test tubes) and antiserum-coated test tubes. Binding of cortisol to plasma proteins was inhibited by working at low pH. Figure 1 shows the mean standard curve (±1 SD) of seven assays performed during six months.

The assay for total cortisol was performed according to the directions of the supplier. Essentially, 5 μL of standard cortisol solution or the plasma sample was added to test tubes containing 1 mL of ^{125}I-cortisol solution (in pH 3.5 citrate buffer), gently stirred with a vortex-type mixer, incubated at 20–25 °C for 3 h, and the liquid then aspirated. The insides of the tubes were then washed once with 1.5 mL of saline solution, and their

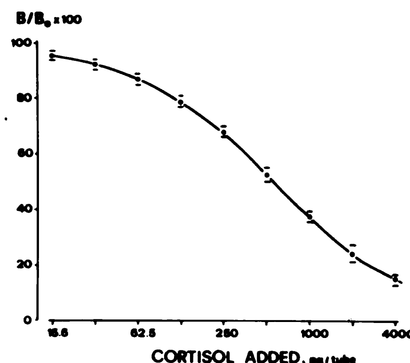

Figure 1. Mean standard curve (seven assays)

radioactivity was counted with a well-type scintillation counter for 1–2 min.

For the AFCC assay, standard cortisol (50 μg/vial; Sigma Chemical Co., Saint Louis, MO 63178) was dissolved in ethanol and then adequately diluted with the same buffer as used for equilibrium dialysis to set up the standard curve; 50 μL of the standard solution containing 15.63 pg to 2.0 ng of cortisol was used in the assay. AFCC was calculated by a simple formula, from the results of both the total-cortisol assay and those of the determination of hormone concentration in the dialysate, as previously described (*1*).

To assess sensitivity, we considered the value for total cortisol or AFCC corresponding in the standard curve to a decrease of $(B/T)_0$ ratio equal to 5% and 50%. For total cortisol, the respective values were 20.8 (SD 1.9) and 568 (SD 44) pg/tube; for AFCC, 13.1 (SD 3.1) and 383 (SD 120) pg/tube. From these data, the short incubation (3 h at 20–25 °C) seems always preferable, even for the AFCC assay, if maximum sensitivity is not required. In fact, the increase in sensitivity obtained with the incubation for 12 h is not sufficient to compensate for the longer incubation.

When a plasma sample was diluted with buffer or with plasma that had been depleted of steroid by treatment with charcoal (*5*), the correlation between concentration and response was linear (r = 0.999). Moreover, analytical recovery of exogenous cortisol added to cortisol-depleted plasma was quantitative from 25 to 800 μg/L ($y = 5.31 + 1.03x$, r = 0.998). On measuring AFCC in four different dialysates at two dilutions (using, respectively, 50 and 25 μL of dialysate, adjusted to a constant volume with 25 μL of buffer) we obtained an average agreement equal to 98.3% (SD 5.5%) between the two dilutions. A linear response to concentrations was also found when dialysate was diluted with phosphate buffer (r = 0.998).

Table 1. Between-assay Variability of the Assay for Total Cortisol

No. assays	Mean (SD), μg/L	CV, %	Theoretical value (mean and SD),ᵃ μg/L
	Normal pool		
13	150 (16)	10.7	—
	Pregnancy pool		
20	319 (45)	14.1	—
	Control I		
7	57 (12)	21.0	58 (20)
	Control II		
7	189 (25)	13.2	175 (39)
	Control III		
7	405 (55)	13.6	388 (95)

ᵃ Calculated from the means of the different reported methods.

We measured the amount of cortisol in heparinized venous blood samples collected from 42 apparently normal subjects of both sexes at 0800–0900 hours. We also collected blood every 4 h (except not at 0400 hours) for 24 h in one patient affected by adrenal insufficiency. In addition, we prepared pooled plasma samples from women in various stages of gestation and from normal subjects. Results were compared with radioassay control serum in various concentrations, supplied by Environmental Chemical Specialties Inc., Anaheim, CA 92807. All plasma samples were frozen immediately after separation, and stored at −20 °C until assay. The results (Table 1) demonstrate the close agreement between the theoretical values and the results found by the described method.

To assess between-assay variability for the total-cortisol assay, we repeatedly measured the cortisol concentration in plasma samples and control sera containing various amounts of the steroid hormone (Table 1). The mean CV for repeated assays (n = 13) of AFCC in the pooled plasma from pregnant women was 10.9%.

Total cortisol, as measured in the plasma samples from the 42 apparently normal subjects, was 137 (SD 44) μg/L; mean AFCC in the same plasma samples was 8.0 (SD 3.5) μg/L. AFCC concentrations in a patient with adrenal insufficiency ranged from 0.6 to 1.8 μg/L, with an apparent circadian rhythm.

This high degree of sensitivity makes this method particularly suitable for the determination of diffusible cortisol present in the plasma dialysate after an equilibrium dialysis. In addition, the precision and the normal range obtained with this assay for AFCC compare well with those found by a previous RIA method, in which antiserum bound to cellulose was used and [³H]cortisol as the tracer (2). Equilibrium dialysis has the disadvantage of a long incubation with respect to other techniques available for the measurement of the free steroid fraction (e.g., gel-equilibration, ultrafiltration, and ultracentrifugation). However, its main advantage is its thermodynamic validity (*6*); moreover, in the equilibrium dialysis system used for this study, no radiolabeled tracers are introduced, thus eliminating the need for chromatographic purifications of the tracers themselves. Consequently, this procedure may provide more nearly accurate and more reliable results than previously described techniques. In addition, we have recently described the possibility of measuring different free-steroid concentrations (cortisol, progesterone, estradiol) in a plasma pool of pregnant women (*7*). This feature (multiple free-hormone determinations) may fully compensate for the relatively time-consuming step of equilibrium dialysis, and makes this method preferable in clinical research, especially when different free steroid concentrations must be measured in the same plasma sample.

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Maria Grazia Del Chicca
Aldo Clerico[1]
Marco Ferdeghini
Giuliano Mariani[2]
Antonio Boldrini[3]
Carlo Cipolloni[3]

C.N.R. Instit. of Clin. Physiol.
[2]2nd Med. Clinic, and [3]Perinatal Unit
University of Pisa
Via Savi 8
56100 Pisa, Italy

[1] Address correspondence to this author.

Improved Ion-Chromatographic Method for Determining Mg^{2+} and Ca^{2+} in Serum

To the Editor:

Anderson (*1*) described the application of a relatively new technique, ion chromatography (IC), to determination of inorganic and small organic ions in biological fluids. By this method the divalent cations Mg^{2+} and Ca^{2+} were