AN IMMUNOASSAY FOR PLASMA CORTISOL BASED ON CHEMILUMINESCENCE

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ABSTRACT

An immunoassay procedure for the determination of cortisol in human plasma is described, which utilizes chemiluminescence as the end point. A cortisol-isoluminol conjugate serves as the chemiluminescent marker. The light emission by this conjugate upon oxidation is delayed by prior incubation with anti-cortisol IgG, but not by unrelated γ-globulin. This delayed light emission was inhibited by cortisol in a dose-dependent manner, with a linear range of 20-1000 pg steroid/assay tube. A competitive protein binding assay based on this procedure was applied to methylene chloride extracts of cortisol from normal and pathological human plasma (2-40 μg/100 ml). Cortisol values obtained by this procedure agreed well with those obtained by radioimmunoassay, using the same antiserum with tritiated cortisol as the label (r = 0.98). The chemiluminescence immunoassay is comparable to radioimmunoassay with regard to sensitivity, specificity, precision and accuracy. The advantage of the new assay procedure is that it obviates the need for counting radioactivity and for separation of bound and free ligand.

INTRODUCTION

The clinical value of plasma cortisol determinations is now well established. The methods currently used to estimate cortisol in plasma include fluorometry [1], double isotope dilution [2], and a variety of competitive protein binding techniques such as radioimmunoassay (RIA) [3-10]. Although RIA is sensitive, reasonably specific and convenient, it possesses certain disadvantages inherent in the use of radioactive labels, such as the problem of radioactive waste disposal, and the relatively short shelf-life of the iodinated tracers [7-10]. Several approaches such as enzyme immunoassay [11-13] have been suggested as a possible alternative to RIA for the measurement of cortisol in biological fluids. Although enzyme-labeled cortisol conjugates are potentially

more stable than radiolabeled cortisol derivatives, the preparation and subsequent purification of the enzyme labeled-cortisol conjugate is difficult. Furthermore, a phase-separation step is usually required for both RIA and enzyme immunoassay procedures, with the exception of ELISA [14] and EMIT [14].

Recent studies from our laboratory [15,16] and elsewhere [17] have shown that chemiluminescence immunoassay can be a feasible alternative to RIA of steroids. The ligand-chemiluminescent marker conjugates are relatively easy to prepare, are stable and can be measured at pM levels [18]. Moreover, assays of this type can be designed that do not necessitate a phase separation step [15,16].

In the method described here, the chemiluminescent marker aminopentyl ethyl isoluminol (APEI) [18,19] is covalently attached through its free amino group to a carboxy derivative of cortisol (Fig. 1). The resulting cortisol-chemiluminescent marker conjugate emits light upon oxidation with microperoxidase and H_2O_2. When the cortisol-APEI conjugate is bound to anti-cortisol IgG, the peak light intensity of the conjugate is decreased, but the total light yield is slightly increased, due to an increase in the light production during the decay part (DP) of the reaction. The binding of cortisol-APEI and consequent shift of the light emission curve to the right is prevented by the additon of free cortisol in a competitive manner. These properties of the system were utilized for the development of an immunoassay for cortisol utilizing chemiluminescence as the end-point.

MATERIALS AND METHODS

Reagents. Cortisol-21-hemisuccinate [20] was obtained from Steraloids, Wilton, N.H.; microperoxidase (MP-11), carbodiimide, N-hydroxysuccinimide, bovine serum albumin (fraction V)(BSA) and thyroglobulin

(bovine) from Sigma, St. Louis, Mo.; DEAE-cellulose from Whatman, Springfield Mill, Kent; 30% hydrogen peroxide solution and silica gel G-60 from Merck, FRG; cortisol from Makor Chemicals, Jerusalem, Israel; spectral grade methylene chloride from Fluka, Buchs, Switzerland and tritiated cortisol from New England Nuclear, Boston, Mass.

Antiserum to cortisol was raised in rabbits, using a cortisol-21-hemisuccinate thyroglobulin conjugate as the immunogen.

Anti-cortisol IgG fraction was prepared by ammonium sulfate precipitation, followed by DEAE-52 cellulose column chromatography and dialysis against phosphate buffered saline [21]. The titer and specificity of anti-cortisol IgG fraction were determined by radioimmunoassay procedures [22] using as the assay buffer 0.05 M Tris-HCl pH 8 containing 0.1 M NaCl and 0.1% NaN_3. The amount of specific antibody in 1% IgG solution, determined according to [23], was 0.6 mg/ml.

The assay buffer in the chemiluminescent reaction was 0.05 M sodium phosphate, pH 8.0,containing 6 g NaCl/l and bovine serum albumin (20 mg/l).

Stock solutions of steroids and chemiluminescent compounds were prepared in ethanol and diluted to the desired concentration in assay buffer when required.

Microperoxidase was dissolved at 1 mg/ml in 0.01 M Tris-HCl, pH 7.4; this stock solution was kept at 4°C. The working solution was obtained by diluting the stock solution in assay buffer to an enzyme concentration of 2.6 μM.

The oxidant solution was prepared by adding 30 μl of 30% H_2O_2 solution to 5 ml of borate buffer (pH 8.6, 0.06 M).

Synthesis of cortisol-aminopentyl ethyl isoluminol (APEI) conjugate. This conjugate was synthesized in two steps:

1. Cortisol-21-hemisuccinate (25 mg) was dissolved in dry dimethylformamide (0.5 ml). Carbodiimide (12 mg) and N-hydroxysuccinimide (6 mg) were added to the solution, and the reaction mixture was stirred overnight at room temperature. The resulting urea was filtered, and the eluate containing the activated N-succinimide ester of cortisol-21-hemisuccinate was used in the next step without any further purification.

2. 6[N-(6-aminopentyl)-N-ethyl]-amino-2,3-dihydrophthalazine-1,4-dione (aminopentyl ethyl isoluminol, APEI) was synthesized according to the procedure developed by Schroeder et al. [18] for the synthesis of aminobutyl ethyl isoluminol [18] and was added (10 mg) to the dimethylformamide solution (0.5 ml) containing the activated ester. $NaHCO_3$ solution (0.5 ml, 0.13 M) was then added. The reaction mixture was stirred for 4 h at room temperature; neutralized to pH 6, and extracted with ethyl acetate (2 x 20 ml). The organic phase was washed with 0.1 N HCl, water, and 0.1 M $NaHCO_3$ solution; dried with anhydrous Na_2SO_4, filtered and taken to dryness under reduced pressure. The residue was chromatographed on silica gel G-60 (35-70 mesh) using chloroform-methanol (60:40) as the developing solvent. The cortisol-aminopentyl ethyl isoluminol conjugate (Fig. 1) was eluted in the second 50 ml fraction (5 mg). It was crystallized from methanol-hexane: m.p.

182-185°C; ultraviolet absorption peaks (in ethanol) at 285 nm (ε = 16000), 310 nm (shoulder, ε = 12000) and 320 nm (shoulder, ε = 11000).

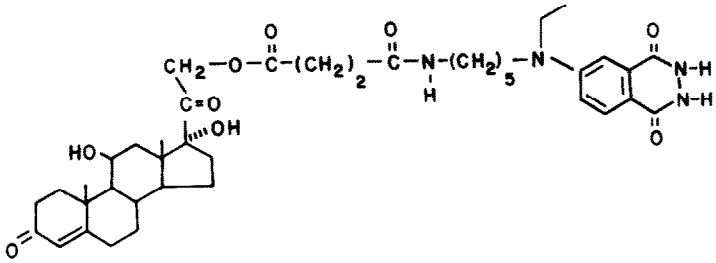

Fig. 1: Proposed structure for cortisol-aminopentyl ethyl isoluminol (APEI) conjugate.

Light measurements. Measurements of light emission were made with a Lumac Luminometer Model 2080 (Lumac Systems, Basel) using the automatic injection and integration modes of the instrument, and Lumacuvette P polystyrene test tubes (12 x 50 mm) as reaction vessel. The Luminometer was also connected to a storage oscilloscope (Type 5111, Tektronix, Beaverton, Oregon) in order to observe the curves generated in the light reaction. When the Luminometer was used with the automatic injection mode, readings on the Luminometer started two seconds after initiation of the light reaction. Since the peak light intensity of the chemiluminescent reaction was reached within one second, as indicated on the oscilloscope, the readings displayed on the Luminometer corresponded to the decay part of the reaction, hereafter referred to as "DP". The light emission was then measured for 10 seconds, and recorded as arbitrary light units.

Sample preparation. Plasma (0.1 ml) was extracted with 3 ml of spectral grade methylene chloride. The supernatant was aspirated, and the organic phase was washed with 0.1 N NaOH (0.5 ml) and water (0.5 ml). The organic extract was then dried under a stream of nitrogen, and assay buffer (5 ml) was added to the dried residue. Aliquots of this solution were subjected to the chemiluminescence immunoassay and to conventional radioimmunoassay [22], using the same batch of IgG.

Assay procedure. 0.1 ml of standards or samples were transferred to Lumacuvettes and incubated with 0.1 ml of specific anti-cortisol IgG (0.23 pmol) for 20 min at room temperature and for 20 min at 4°C.

Cortisol-APEI conjugate (0.1 ml, 25 pg) was then added, and the incubation was continued for another 90 min at 4°C. The enzyme solution (0.1 ml) was added; the oxidant (0.1 ml) was injected after transfer of the tube into the light-proof cuvette housing and light emission (DP) was measured for 10 seconds, from the end of 2nd to the 12th second.

The following additional assay tubes are set up: the maximal emission (ME) tube contains all the reagents, but no sample or standard; a basal control (BC) tube contains enzyme and marker conjugate only but no antibody; standard tubes contain all the reagents plus known amounts of free cortisol. The decrease in DP light yield induced by standards or sample is expressed as a percentage of the difference ME-BC. From a plot of these percentages against log-concentration of the standard (20-1000 pg/tube), the cortisol content of unknown sample is read and the plasma cortisol concentration is calculated, taken into consideration the volume of the sample assayed and the dilution used. No correction for recovery was made since experiments with radiolabeled cortisol indicated that recovery of radioactivity was consistently >90%.

RESULTS

Light yield of cortisol-APEI conjugate. When varying amounts of cortisol-APEI conjugate were treated with microperoxidase and peroxide as described above, the DP light emission increased linearly with cortisol-APEI concentration up to 160 pg (cf. Fig. 4). The lower limit of detection was 4 pg/tube.

Effect of anti-cortisol IgG on the light yield produced by cortisol-APEI conjugate upon oxidation. Fig. 2 shows oscilloscope tracings of the light emission obtained upon oxidation of cortisol-APEI conjugate (25 pg) in the presence of specific antibody (0.23 pmol IgG) with or without addition of cortisol (625 pg). The antibody reduced the peak height of the light response, but enhanced the light emission during the decay portion (DP) of the reaction, with only a small increase in total light yield (curve 3 vs. curve 2). Addition of free cortisol (curve 4) reduced this effect.

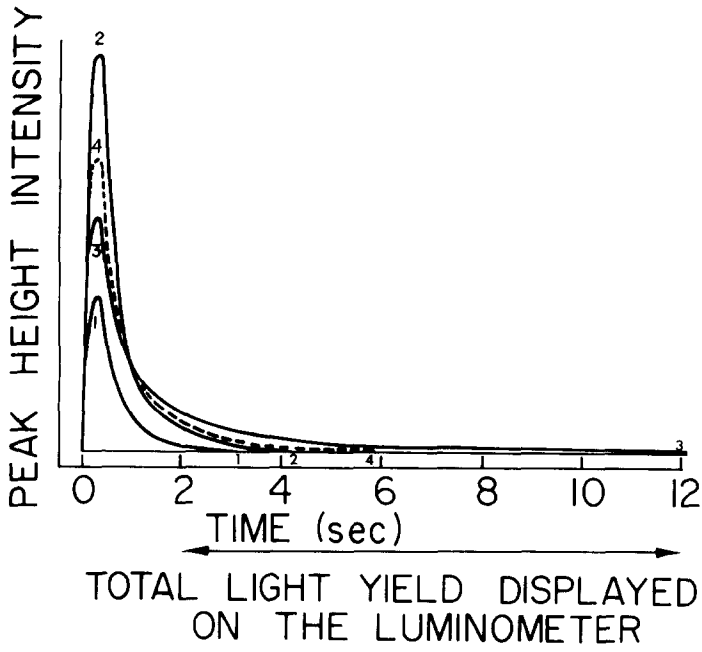

Fig. 2: Oscilloscope tracings of the light signal obtained upon oxidation of cortisol-APEI conjugate in the presence of specific antibody with or without addition of cortisol. Legend: (1) signal obtained when the enzyme solution only was mixed with the oxidant; (2) signal due to the oxidation of 25 pg of cortisol-APEI conjugate; (3) signal obtained when the conjugate (25 pg) was oxidized in the presence of anti-cortisol IgG (0.23 pmol/tube); (4) signal obtained when the conjugate (25 pg) was oxidized in the presence of anti-cortisol IgG (0.23 pmol/tube) and cortisol (625 pg). Scale used on the oscilloscope: speed: 1 sec/1.22 cm; sensitivity: 3 x 100 mV/1.22 cm.

When varying amounts of anti-cortisol IgG were incubated with 25 pg of cortisol-APEI conjugate, the DP light emission produced upon oxidation of the conjugate increased as a function of IgG concentration (Fig. 3). The

antibody-induced delay in light production was specific and was inhibited by the addition of the homologous ligand cortisol. Heterologous IgG, e.g. anti-progesterone IgG, had no effect on either peak light intensity or the decay portion (DP) of the light signal produced by cortisol-APEI conjugate upon oxidation. Conversely, anti-cortisol IgG had no effect on the light yield produced by progesterone-aminobutyl ethyl isoluminol [24] conjugate upon oxidation. When a fixed amount of the homologous ligand cortisol (625 pg) was added to the reaction tubes, the anti-cortisol IgG induced increase in DP light emission produced by cortisol-APEI conjugate during oxidation was reduced to less than half over the range of 0.18 to 0.27 pmol anti-cortisol IgG/tube. At higher IgG concentrations the inhibition caused by the addition of cortisol was minimal (Fig. 3).

In additional experiments, varying levels of cortisol-APEI conjugate were incubated with a fixed amount of anti-cortisol IgG (0.23 pmol) with or without cortisol (1 ng). In the presence of anti-cortisol IgG, the light yield increased steeply with concentration of cortisol-APEI conjugate up to 160 pg/tube. The antibody-induced enhancement in DP light yield over the range of 10-40 pg cortisol-APEI was about 4-fold, and in the presence of the homologous competitor (1 ng free cortisol), DP light emission was reduced by 70% (Fig. 4).

In other experiments, 0.23 pmol of anti-cortisol IgG was incubated with or without a fixed amount of cortisol (625 pg) for 15 min at room temperature and for 15 min at 4°C. Cortisol-APEI conjugate (25 pg)was added to each tube and incubation at 4°C was continued for various time intervals after which the enzyme solution was added and the oxidant was injected in the dark. In the absence of added cortisol

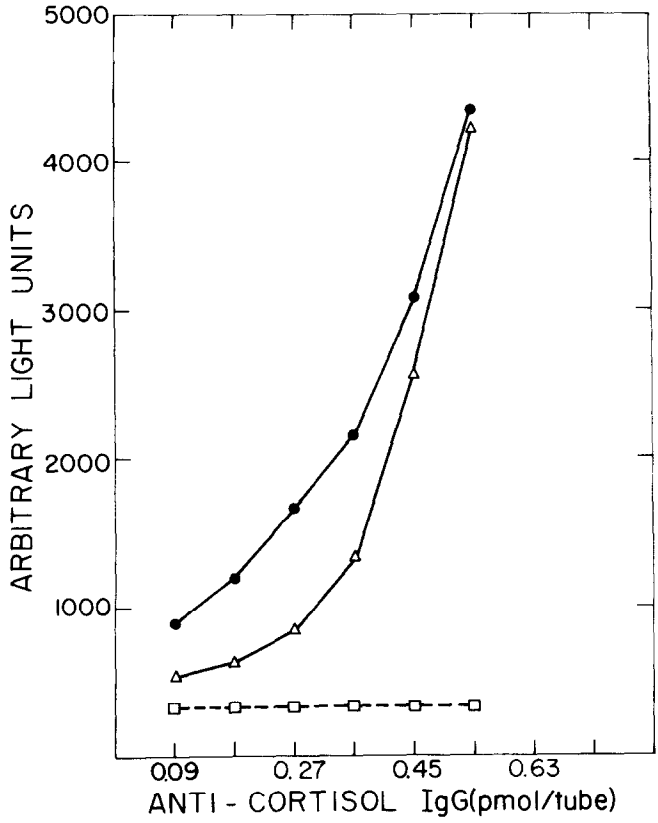

Fig. 3: Effect of anti-cortisol IgG concentration on the DP light yield produced by cortisol-aminopentyl ethyl isoluminol (APEI) conjugate upon oxidation. Varying levels of anti-cortisol IgG were incubated with (Δ——Δ) or without (●——●) a fixed amount of cortisol (625 pg) for 15 min at room temperature and for 15 min at 4°C in a total volume of 200 µl of assay buffer. Cortisol-APEI conjugate (25 pg in 100 µl of assay buffer) was added to each tube, and the incubation was continued for another h at 4°C. □ --- □ , Cortisol-APEI only. Microperoxidase (100 µl of a 2.6 µM solution) was added; the reaction tube was then introduced into the Luminometer and 100 µl of the oxidant (0.19% of H_2O_2 solution in borate buffer, pH 8.6, 0.06 M) was injected. Light emission was integrated over the period 2-12 sec after adding the last reagent and is expressed in arbitrary units.

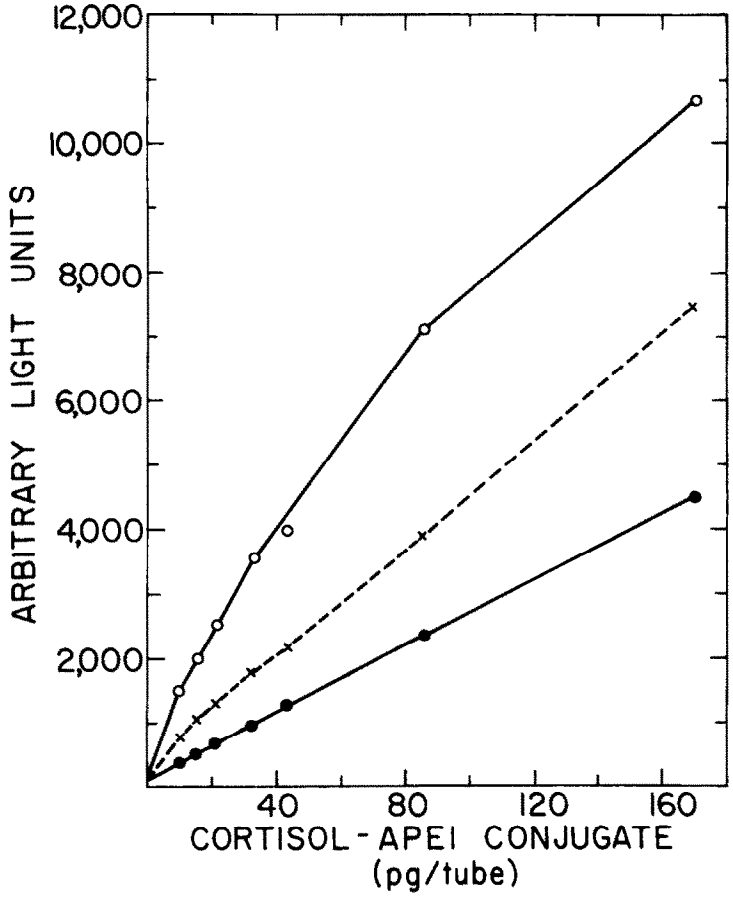

Fig. 4: Effect of cortisol-APEI conjugate concentration on IgG-enhanced light emission. Reaction mixtures containing varying levels of cortisol-APEI conjugate were incubated with a fixed amount of anti-cortisol IgG (0.23 pmol/tube) in the presence (x---x) or absence (o——o) of cortisol (1 ng/tube). Further treatment as described in legend to Fig. 3. ●——●, cortisol-APEI only.

antibody-induced light enhancement of the DP light yield increased with the time of incubation of the conjugate with antibody and reached a plateau after 60 minutes (Fig. 5). After incubation with cortisol (625 pg), the antibody-induced light emission was reduced to a constant degree, irrespective of the duration of incubation at 4°C beyond the

initial 15 min. Light production was routinely determined after 90 min of incubation of specific antibody with the cortisol-chemiluminescent marker conjugate.

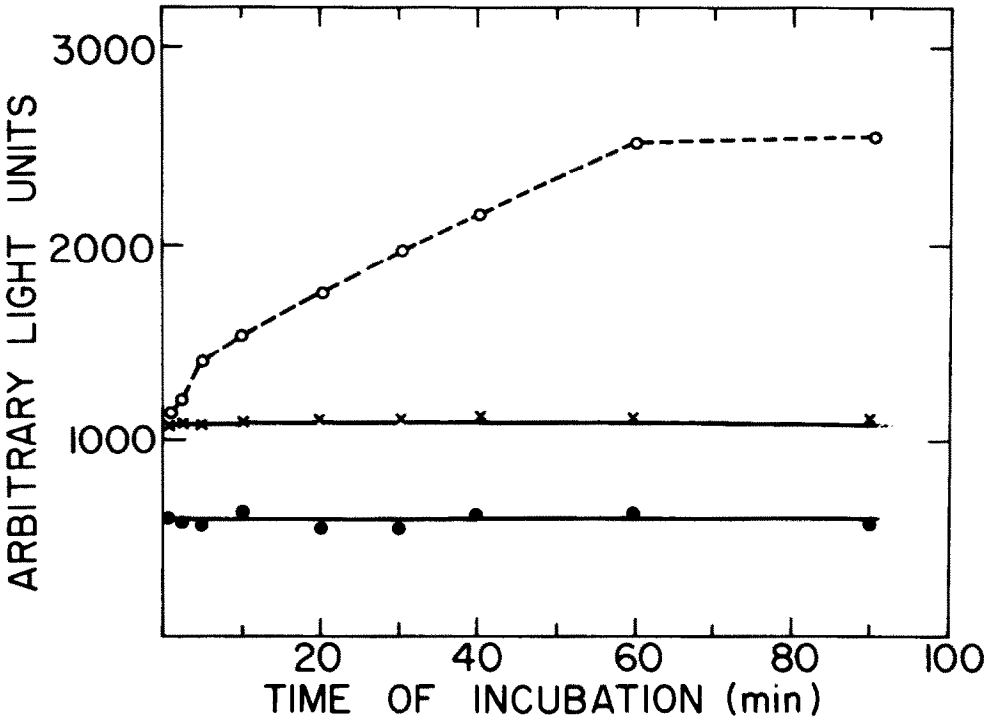

Fig. 5: Effect of incubation time of antibody with cortisol-APEI conjugate on DP light yield upon oxidation in the presence of anti-cortisol IgG. Anti-cortisol IgG (0.23 pmol) was incubated with (x——x) or without (o---o) a fixed amount of cortisol (625 pg) for 15 min at room temperature and for 15 min at 4°C in 200 µl of assay buffer. Cortisol-APEI conjugate (25 pg in 100 µl of assay buffer) was added to each tube and incubation continued at 4°C for the time indicated on the abcissa. Enzyme solution was then added and the oxidant was injected in the dark. The ordinate shows the light readings recorded on the Luminometer. ●——●, Light signal generated by oxidation of the conjugate without IgG.

Sensitivity of chemiluminescence immunoassay for cortisol.

Competitive protein binding reactions were carried out by incubating varying amounts of free cortisol with a fixed amount of anti-cortisol IgG, as described under Assay Procedure (see Methods sections). The DP light yield measured over 10 sec decreased with the log mass of added cortisol over the range of 10-1250 pg per assay tube (Fig. 6). The least amount of cortisol that could be distinguished from zero ($p<0.05$) was 20 pg/tube.

Reliability of the chemiluminescence immunoassay for cortisol.

The recovery of tritiated cortisol added to plasma by the extraction procedure described in the Methods section was 96.3% + 3.1 S.D. (n = 10). The specificity of the immunoassay using anti-cortisol IgG and chemiluminescence was similar to that observed when using the same antiserum in RIA: light emission was unaffected by addition of progesterone, estradiol, testosterone, dexamethasone, spironolactone or bethamethasone; minor cross reaction was observed in the chemiluminescence immunoassay with 11-deoxycortisol (1%), corticosterone (3%), 11-deoxycorticosterone (0.1%), 17-hydroxyprogesterone (14%) and prednisolone (1%) [19]. These compounds gave a similar degree of cross-reaction in the RIA.

A pool of human male plasma was serially diluted with the assay buffer and extracted with methylene chloride. The extracts were then assayed for cortisol by chemiluminescence immunoassay. The regression of dilution factor on measured cortisol content was linear ($y = 1408x - 3.6$) and the correlation coefficient was $r = 0.99$.

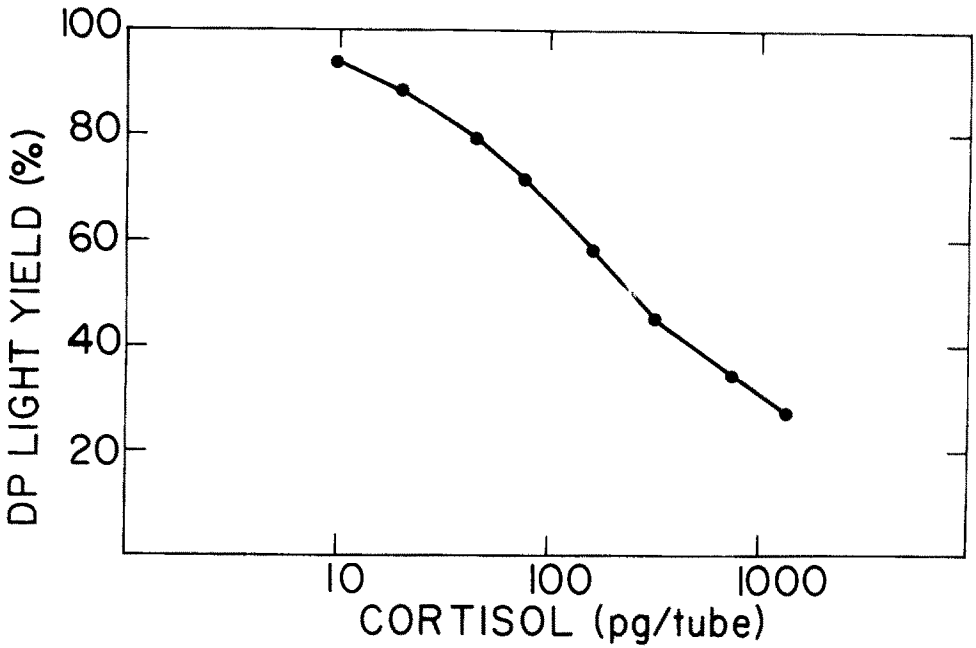

Fig. 6: Representative dose-response curve for cortisol measured by chemiluminescence immunoassay. Varying amounts of cortisol were incubated with 0.23 pmol of anti-cortisol IgG in a total volume of 200 µl of assay buffer for 20 min at room temperature and 20 min at 4°C. Cortisol-APEI conjugate (25 pg in 100 µl of assay buffer) was then added, and the incubation was continued for another 90 min at 4 C before adding the enzyme solution (100 µl) and oxidant. The difference in DP light yield ME-BC (see Methods section) is taken as 100% and is plotted against log-dose of cortisol.

The reproducibility of the assay was evaluated by performing replicate determinations using 0.1 ml samples from one pool of normal male plasma on the same day or repeated determinations on separate occasions. The within-assay coefficient of variation was 8.3% with a mean cortisol content (µg/100 ml plasma) of 7.5 $\pm$ 0.62 S.D. (n = 6). The between assay coefficient of variation was 18% with a mean value of 8.6 ± 1.55 S.D. (n = 11). Addition of methylene chloride extracts from 0.1 ml plasma to the enzyme solution and oxidant in the absence of cortisol-APEI conjugate generated no light signal. Likewise,

methylene chloride extracts of distilled water samples gave no significant light emission in the assay (<4pg/tube).

Comparison of plasma cortisol levels as determined by chemiluminescence immunoassay or RIA. Plasma samples (n = 20) from normal individuals, from patients with Cushing's Syndrome or with Addison's Disease and from patients under ACTH treatment or dexamethasone suppression were extracted with methylene chloride, and the extracts were assayed for cortisol by radioimmunoassay and by chemiluminescence immunoassay, using the same anti-serum. The results of the two methods agreed well: r = 0.98; y = 0.96 x - 0.28, where y represents values determined by chemiluminescence immunoassay (Fig. 7).

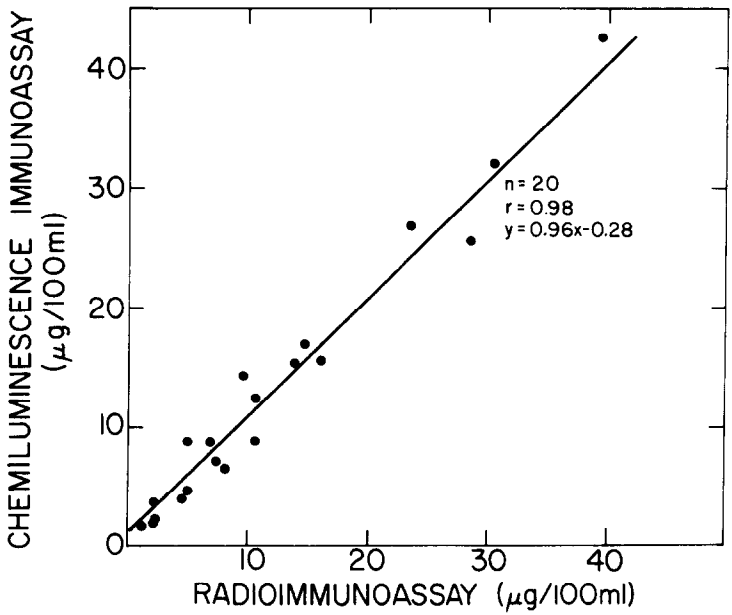

Fig. 7: Comparison of plasma cortisol levels as determined by RIA or chemiluminescence immunoassay.

DISCUSSION

This paper describes the development of an immunoassay procedure for plasma cortisol based on chemiluminescence. We have recently described a chemiluminescence assay procedure for the determination of progesterone [15] and estriol-16α-glucuronide [16]. In the latter two systems, the homologous antibody caused an increase in both the peak height of light emission and the total light yield of the steroid-isoluminol conjugate. The system described in the present paper behaves differently in that the peak height of the light response is not augmented, but on the contrary, reduced and total light yield is only slightly increased; however, the antibody caused a delay in light emission, resulting in a shift of the response curve to the right. This antibody effect forms the basis of the assay described here. Its theoretical basis remains to be elucidated. Thus, we do not know whether the discrepant behavior is a peculiar property of the particular steroid conjugate used as a labeled ligand or is due to the type of antibody used. In experiments with estradiol-isoluminol conjugate, for instance, a homologous rabbit IgG gave enhancement of both peak height and total light yield, whereas a monoclonal mouse antibody enhanced total light yield only, without a significant effect on peak height [unpublished data]. It would appear then that the detailed assay procedure will have to be adopted in each case to the type of antibody used, or else the antibody to be used will have to be rigidly standardized, e.g. by using the hybridoma technique [25].

Another variable in the behaviour of chemiluminescence immunoassays of the type described here is the length of the alkyl chain connecting the luminescent marker to the steroid molecule. This factor has been system-

atically explored with regard to the progesterone assay, and it was found that a 4-carbon chain conjugate gave a lower basal light yield butmaximal antibody induced enhancement [24]. The choice of a 5-methylene steroid-marker link in the present study was arbitrary, and it is possible that further improvement of the cortisol assay can be achieved.

Greater flexibility in selecting optimal assay conditions may be attained by using an instrument in which the period during which the light emission is integrated is not fixed, as is the case with the Luminometer used in these experiments, but can be varied to meet the needs of each assay system. In the cortisol system the ratio between the decay portion and the peak light yield would probably yield a more sensitive end-point than the one used in the present paper. Such refinements may help reduce the inter-assay variation which at this stage is still somewhat high (18%).

The cortisol assay described here can be accomplished within 2 h. The sensitivity achieved (20 pg/tube) approaches that obtained by radio-immunoassay, and is satisfactory for determination of cortisol in human plasma. The cortisol-chemiluminescent marker conjugate is stable, can be characterized by physical means, and can be measured in <10s. Chemi-luminescence immunoassay eliminate isotope disposal problems inherent in RIA. The apparatus needed, though not at present part of the routine equipment of most clinical laboratories, can probably be pro-duced more cheaply than a scintillation counter. The fact that no phase-separation step is required for the assay should facilitate the development of automated procedures.

ACKNOWLEDGEMENTS

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20. The following trivial names will be used: cortisol-21-hemisuccinate= 11β,17,21-trihydroxy-4-pregnene-3,20-dione 21-hemisuccinate; 11-deoxycortisol = 17,21-dihydroxy-4-pregnene-3,20-dione; 11-deoxy-corticosterone = 21-hydroxy-4-pregnene-3,20-dione; corticosterone= 11β,21-dihydroxy-4-pregnene-3,20-dione; dexamathasone = 9-fluoro-16α-methyl -11β,17,21-trihydroxy-1,4-pregnadiene-3,20-dione; prednisolone = 11β,17,21-trihydroxy-1,4-pregnadiene-3,20-dione; pentyl ethyl isoluminol (APEI) = 6[N-(4-aminopentyl)-N-ethyl]-amino-2,3-dihydrophthalazine-1,4-dione; aminobutyl ethyl isoluminol (ABEI) = 6[N-(4-aminobutyl)-N-ethyl]-amino-2,3-dihydrophthalazine-1: estriol-16α glucuronide= 3,17β-dihydroxy-1,3,5 (10)-estratrien-16α-yl β-D-glucopyranosiduronic acid.

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