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Increased Unbound Cortisol in the Plasma of Estrogen-treated Subjects *

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The plasma cortisol [1] level is the sum of the cortisol bound to the plasma proteins and that which is unbound. During pregnancy or estrogen treatment the plasma cortisol and 17-hydroxycorticoid level rises several-fold (1–8). This rise is secondary to an increase in the plasma content of transcortin (6, 9), which is a normal α-globulin component of plasma (10) with high affinity for binding cortisol. Since the subject maintained on long term estrogen therapy does not develop the clinical stigmata of hyperadrenocorticism, it has generally been considered that the bound plasma cortisol is physiologically inactive (7, 11) and that the unbound plasma cortisol remains at normal concentration (12) in these subjects.

In the present investigation the plasma levels of bound and unbound cortisol were determined in a group of estrogen-treated subjects at different adrenal cortisol production rates and compared to those of a control group. At the beginning of this work, we postulated that if the unbound plasma cortisol level is the only factor which determines the extent of the plasma cortisol effect on cellular functions, then the unbound cortisol level should be the same in both groups of subjects when studied at rest. In addition, if only the unbound cortisol level controls pituitary ACTH inhibition via the negative feedback mechanism, then this level should be the same in both groups at equivalent adrenal cortisol production rates. Our results indicate that the unbound cortisol level cannot be the only factor which determines peripheral physiological activity. Estrogen-treated subjects were found to have relatively elevated levels of plasma unbound cortisol.

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[1] Cortisol: 11β,17α,21-trihydroxy-4-pregnene-3,20-dione.

Methods

Radioactive cortisol. Cortisol-1,2-H^3 (2.08 μc per μg) [2] was purified on two different paper chromatographic systems. This labeled cortisol did not show significant change in specific activity when added to carrier cortisol and recrystallized from three different solvent systems.

Ultrafiltration procedure. Details of this procedure have been published (13). Ten ml of heparinized plasma was adjusted to pH 7.4, and 0.04 μg of tritiated cortisol was then added. The plasma was incubated at 37.5° C for 0.5 hour while being slowly shaken and was then transferred to a cellophane casing and placed in a Toribara ultrafiltration apparatus (14). The sample was centrifuged initially for 15 minutes at 37.5° C. The 0.2-ml ultrafiltrate formed during this period was removed and discarded and the sample further centrifuged for 45 minutes. During this period 0.55 to 0.65 ml of ultrafiltrate formed. One-half ml of the ultrafiltrate was transferred to a liquid scintillator (15) and counted in a liquid scintillation spectrometer. The number of counts in the 0.5 ml of ultrafiltrate was considered representative of the proportion of unbound cortisol in the sample. The unbound cortisol in micrograms per 100 ml plasma was calculated from the total plasma cortisol value and the percentage of this cortisol which was found to be ultrafilterable by the above technique.

In preliminary experiments the validity of this technique, which was described by other investigators (12, 16), was confirmed. The cellophane membrane used in these experiments was not found to be a selective barrier to the free passage of either water or cortisol. The proportion of cortisol found to be ultrafilterable was not affected by the volume of plasma studied so long as the plasma was not allowed to become too concentrated during the ultrafiltration procedure. In addition, the half-hour preliminary incubation afforded equilibrium between the added tracer cortisol and the plasma cortisol; no significant alteration of the proportion ultrafilterable could be detected in plasma from normal or estrogen-treated subjects by prolonging the incubation time to 5 hours.

Clinical subjects. Thirty-four male subjects, hospitalized on a urological service, were studied. Their mean age was 52 years with a range of 16 to 81 years. As

[2] Purchased from New England Nuclear Corp., Boston, Mass.

a group, they were individuals in good general health who were well into their convalescence from an acute urological problem. Six subjects, ages 67 to 78, had prostatic carcinoma treated by orchiectomy and had been on stilbestrol (diethylstilbestrol) for at least 1 year at the time they were studied. None of these subjects had evidence of metastatic disease. The remaining estrogen-treated subjects were given 5 mg of stilbestrol daily for 1 to 3 weeks for the purposes of this study.

Experimental procedure. Intravenous infusions of 5% glucose in water were started at 9:00 a.m. If adrenocortical stimulation was desired, from 0.3 to 30 U of ACTH [3] was infused during the 7-hour experimental period. The amount of ACTH was varied from patient to patient in an attempt to provide a range of adrenal cortisol production rates. At 1:00 p.m. a 45-ml blood sample was drawn into a heparinized syringe. Ten ml of a standard solution of tritiated cortisol in 5% ethanol-saline was then administered intravenously within a 1-minute period. A total of 5.5 μg of cortisol was given which contained, at 16% counting efficiency, 4 million cpm. One-half hour after the cortisol was given a 20-ml heparinized blood sample was taken. Five additional blood samples were taken at one-half hour intervals in the estrogen-treated subjects and at 20-minute intervals in the other subjects. The volumes of these samples were also 20 ml except for the final one, which was 40 ml. Blood samples were immediately centrifuged, and the plasma was separated and frozen until analyzed.

Ten ml of plasma from the first sample was analyzed by the ultrafiltration procedure. The last plasma was divided into two equal aliquots, and the eight plasma samples were carried through the extraction procedure and paper chromatographic separation of cortisol previously described (17). The cortisol area from the paper chromatogram of the first and of the last samples was eluted and analyzed with the Porter-Silber reagent (18). The cortisol areas from the six other paper chromatograms were eluted, transferred to vials, and counted with an error of less than 5% in a Packard Tri-Carb liquid scintillation spectrometer.[4]

Calculation of the adrenal cortisol production rate was carried out by using established isotopic techniques (19–22). In those subjects in whom the cortisol level at the beginning and the end of the experiment fell within 5% of their mean level, the plasma cortisol was considered to be constant during the experimental period, and in these cases the adrenal cortisol production rate and the peripheral removal rate were equal. Cortisol radioactivity in counts per minute per milliliter of plasma was plotted on semilogarithmic co-ordinates against time and, with the occasional exception of the initial point, represented a single exponential curve. From the ordinate intercept, slope of this curve, knowledge of the dose of administered radioactivity, and the plasma cortisol level, the miscible pool, apparent distribution volume, and adrenal production rates were calculated.

In many of the patients, the plasma cortisol level did not remain constant during the experimental period. This was particularly true in those subjects who did not receive ACTH and whose level fell with the diurnal rhythm. Although these subjects were not, strictly speaking, in a steady state, their cortisol production and disappearance rates may be calculated independently if both of these rates are constant, although not equal, throughout the experimental period. To estimate the plasma cortisol level at intermediate experimental times, the initial and final plasma cortisol levels were noted on a semilogarithmic plot, and the cortisol level at intermediate times was obtained by interpolation. On three occasions, additional plasma samples were taken at intermediate times and analyzed for cortisol, and in these instances their analyzed levels fell within experimental error of the anticipated interpolated values. From the plasma cortisol levels and the plasma radioactivity levels the specific activity (counts per minute per microgram cortisol) could be calculated and plotted on semilogarithmic co-ordinates against time. Analysis of the specific activity curve provided the miscible pool size, distribution volume, and production rate values; the disappearance rate was calculated from the fractional turnover rate obtained from the plasma radioactivity (counts per minute per milliliter) curve. In all the data to be presented, it appeared justified to assume that the rate of cortisol production and the rate of disappearance were constant throughout the experimental period, since all the data used showed a single exponential fall.

Results

The data for the 21 control subjects and 13 stilbestrol-treated subjects are tabulated in Tables I and II, respectively. The mean experimental values have been calculated for those control and stilbestrol-treated subjects who were not given ACTH.

In Figure 1 the adrenal cortisol production rates are plotted against the total plasma cortisol concentration for the control subjects. The plasma cortisol level in this group of subjects provides a fair index of their adrenal cortisol production rate. The values for the stilbestrol-treated subjects are not plotted here, but they showed plasma cortisol levels that were markedly elevated relative to their cortisol production rates when compared to the control group.

In Figure 2 the adrenal cortisol production rates are plotted against the unbound plasma cortisol concentration for the two groups of subjects. The unbound cortisol concentration also appears

[3] Supplied as corticotropin solution by the Wilson Laboratories, Chicago, Ill.

[4] Packard Instrument Co., La Grange, Ill.

TABLE I

*Isotopic cortisol studies in control subjects**

No.	Initial cortisol†	Unbound cortisol	Final cortisol	Initial miscible pool	Initial distribution volume	Disappearance $t_{\frac{1}{2}}$	Disappearance rate	Production rate
	μg/100 ml	*μg/100 ml*	*μg/100 ml*	*μg*	*L*	*min*	*μg/min*	*μg/min*
1	9.6	0.85	5.7	2,340	24.4	68	23.8	11
2	8.9	0.98	5.3	1,900	21.4	70	19	12
3	10.5	1.68	7.9	2,760	26.2	84	23	14
4	12.3	2.1	8.3	2,520	20.4	85	20.5	15
5	8.6	0.94		2,580	30.0	100		18
6	8.0	0.96		1,730	20.2	57		21
Mean	9.6	1.25		2,305	23.8			15
7	14	2.1	9.3	4,980	36	85	40.6	21
8	18	2.9		3,830	21.3	90		30
9	21	3.8	27	3,810	18.3	80	33	40
10	16	2.3		2,940	17.7	50		40
11	30	6.6		10,900	35	86		88
12	19	3.2		7,450	39	58		89
13	44	8.8		11,900	37	93		89
14	40	9.9		11,400	29	72		109
15	36	7.0		10,450	29	65		111
16	36	9.0		10,000	27	56		123
17	43	11.6		18,000	42	92		135
18	35	7.7		16,900	48	82		142
19	42	10.5		20,540	49	71		200
20	45	13.5		34,300	76	98		240
21	50	16.7	64	35,400	71	77	318	378

* Subjects 7 to 21 were studied during iv infusion of supplemental ACTH.

† In those cases in which the initial and final plasma cortisol values fell within 5% of their mean value, the plasma cortisol concentration is considered to have stayed constant throughout the experimental period, and this mean value is given in this column.

to provide a fair index of adrenal cortisol production but does not appear to be superior to the plasma cortisol level in this respect. In the stilbestrol-treated subjects the relationship between the unbound cortisol level and the adrenal cortisol production rate was not the same as that seen in the control subjects. Equivalent adrenal cortisol production rates produced much higher plasma levels of unbound cortisol in the stilbestrol-treated subjects.

In this same respect, in Tables I and II the mean values may be compared for those control and stilbestrol-treated subjects who were not given ACTH. Both groups were producing equivalent quantities of cortisol, i.e., 15 and 15.2 μg per minute during the study period. Despite this, the unbound cortisol level in the stilbestrol-treated subjects was several times higher than that seen in the control subjects. These stilbestrol-treated subjects, while apparently maintaining a eucorticoid status, had appreciable elevation of their plasma unbound cortisol levels.

The initial miscible pool in the two groups of non-ACTH-treated subjects was also different. The mean pool size in the control subjects was 2.3 mg, whereas that in the stilbestrol-treated subjects was 3.8 mg, a difference of 1.5 mg. The difference in pool size apparently cannot be accounted for solely by the larger amount of cortisol present in the plasma of the stilbestrol-treated subjects. If one estimates a mean plasma volume of 3 L, the plasma cortisol content of the control group will be 3 L × 96 μg per L or about 0.3 mg, and the extraplasma miscible pool will be 2.3 mg minus 0.3 mg or 2.0 mg. By similar calculation, the extraplasma miscible pool for the stilbestrol-treated subjects will be 2.9 mg. Although the difference between the two extraplasma miscible pools is smaller than the difference between the total pools, it is still probably significant.

In the stilbestrol-treated subjects studied without supplemental ACTH infusion, it was unclear whether the elevation of plasma unbound cortisol might represent some degree of excitement secondary to the experimental procedure. For this reason, a single blood sample was taken from ten subjects who had been on long-term stilbestrol therapy and from five control subjects. These

TABLE II

*Isotopic cortisol studies in stilbesterol-treated subjects**

No.	Initial cortisol†	Unbound cortisol	Final cortisol	Initial miscible pool	Initial distribution volume	Disappearance $t_{\frac{1}{2}}$	Disappearance rate	Production rate
	μg/100 ml	*μg/100 ml*	*μg/100 ml*	*μg*	*L*	*min*	*μg/min*	*μg/min*
1	22	4.2		2,600	11.6	155		11.6
2	32	5.7		4,700	14.6	235		12
3	35	2.5		1,690	4.8	90		13
4	37	5.2		3,300	8.9	160		14
5	29	3.8	21	3,350	11.5	88	26	15
6	27	3.6	20	4,350	15.8	110	27	20
7	37	5.6	24	6,900	18.4	125	38	21
Mean	31	4.4		3,841	12.2			15.2
8	47	8.5		10,600	22.5	170		43
9	83	14.2		13,900	16.8	135		71
10	71	16.3		8,950	12.6	76		81
11	75	17		16,200	21.6	120		94
12	74	18.3		20,400	27.5	110		128
13	127	34.4		55,500	44.0	135		285

* Subjects 8 to 13 were studied during iv infusion of supplemental ACTH.

† In those cases in which the initial and final plasma cortisol values fell within 5% of their mean value, the plasma cortisol concentration is considered to have stayed constant throughout the experimental period, and this mean value is given in this column.

samples were taken at a local county home between 2 and 3 p.m. The subjects were ambulatory, and the procedure appeared to be accepted with equanimity. The data from these fifteen subjects are presented in Table III and are consistent with values presented in Tables I and II. The mean unbound cortisol value for the control subjects was 1.1 μg per 100 ml and for the stilbestrol-treated subjects 4.0 μg per 100 ml.

The previous data show that neither the level of bound nor of unbound cortisol similarly reflects the adrenal cortisol production rate in both the estrogen-treated and control subjects. Inspection of the percentages of unbound cortisol in Table III suggested that these values were similar in the two groups of subjects. The data from Tables I and II were therefore plotted as the percentage of cortisol unbound (ultrafilterable) against the adrenal cortisol production rate (Figure 3). This plot, and the additional data in

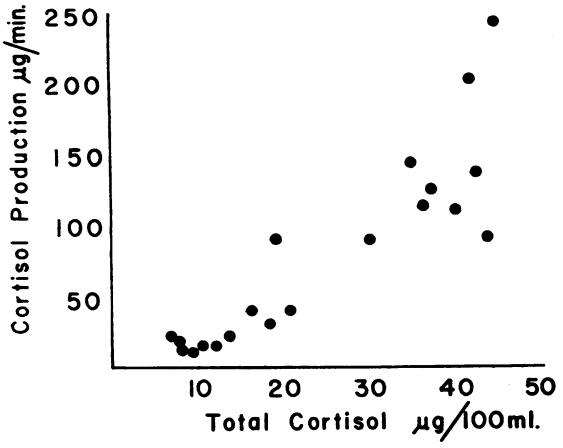

FIG. 1. TOTAL CORTISOL IN THE PERIPHERAL PLASMA OF CONTROL SUBJECTS AS A FUNCTION OF THE ADRENAL CORTISOL PRODUCTION RATE.

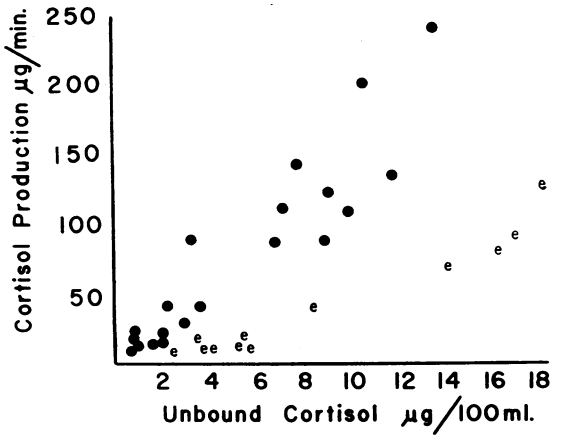

FIG. 2. UNBOUND CORTISOL LEVEL IN PERIPHERAL PLASMA AS A FUNCTION OF THE ADRENAL CORTISOL PRODUCTION RATE. The control subjects are designated by closed circles and the stilbesterol-treated subjects by the letter e.

TABLE III

The total cortisol and unbound cortisol in the plasma of a group of estrogen-treated and control subjects

No.	Plasma cortisol	Unbound cortisol	
Control	*μg/100 ml*	%	*μg/100 ml*
1	4.4	9.8	0.43
2	12.0	10.3	1.24
3	8	13.9	1.11
4	7.9	14.4	1.14
5	17.0	9.3	1.6
Estrogen-treated			
1	30	13.2	3.8
2	34	9.8	3.4
3	44	7.6	3.4
4	39	14.5	5.7
5	30	16.7	5.0
6	54	11	5.9
7	59	7.6	4.5
8	30	8.6	2.6
9	27	8.8	2.4
10	31	9.7	3.0

Table III, suggest that this proportion is similarly related to adrenal cortisol production in both the estrogen-treated and control subjects. Other workers have reported that both the percentage of ultrafilterable cortisol in the plasma and the 24-hour urinary corticoid excretion tend to decrease during estrogen administration (12). This relationship would be consistent with the present observation that the percentage of cortisol that is ultrafilterable appears to decrease with a decreasing adrenal cortisol production rate.

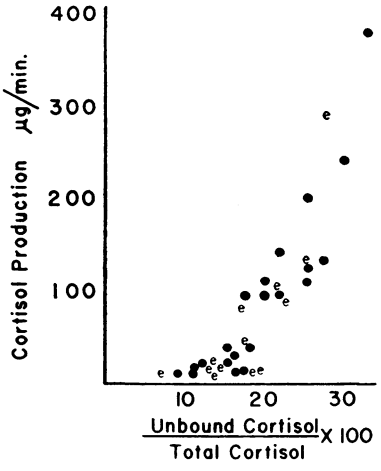

FIG. 3. PERCENTAGE OF UNBOUND PLASMA CORTISOL AS A FUNCTION OF THE ADRENAL CORTISOL PRODUCTION RATE. The control subjects are designated by closed circles and the stilbestrol-treated subjects by the letter e.

Discussion

Cortisol circulates in human plasma in at least three forms: *1*) bound to albumin, *2*) bound to transcortin, and *3*) in unbound form. During pregnancy or estrogen administration the total plasma cortisol rises (5, 7, 8) owing primarily to a marked increase in transcortin-bound cortisol (8). Since the pregnant woman or estrogen-treated man does not develop the clinical stigmata associated with increased adrenal production of glucocorticoids (7), it has been suggested that the transcortin-bound cortisol is physiologically inactive (7, 11) and that the plasma unbound cortisol is the active plasma cortisol form and is in normal concentration in the estrogen-treated subject (12). If the plasma unbound cortisol is the only moiety controlling cortisol activity in the tissues, for homeostasis to be maintained this moiety should also control pituitary ACTH inhibition and the hepatic reduction and removal of cortisol. One would therefore anticipate that while the estrogen-treated subject is secreting the equivalent quantity of cortisol as the normal person, the unbound cortisol levels in their plasmas will be similar.

It is clear from the presented data that at equivalent adrenal cortisol production rates the plasma unbound cortisol level in the estrogen-treated subject is appreciably higher than that of the control subject. Since the estrogen-treated subject does not develop stigmata of Cushing's syndrome, and since the urinary corticoid excretion of the estrogen-treated subject stays within normal range or decreases (4, 7), the plasma unbound cortisol level per se is probably not necessarily directly proportional to its evident peripheral physiological activity or to the rate of cortisol removal by the liver. In addition, since the stilbestrol-treated subjects had a plasma unbound cortisol level three to four times higher than the control group during a time in which they were secreting equivalent quantities of adrenal cortisol, the plasma unbound cortisol level per se was probably not controlling pituitary ACTH release in both groups. The only parameter which appeared to be similarly related to the adrenal cortisol production rate in both estrogen-treated and control subjects was the percentage of the total cortisol represented by the unbound fraction. This proportion of unbound

to total cortisol in the plasma appears to most closely approximate a physiologically significant index. Why this should be so is not completely clear at the present time. At low adrenal cortisol production rates the plasma transcortin in the control and estrogen-treated subjects is probably only partially saturated. Since the association constant for transcortin will be the same in both groups of subjects, their percentages of unbound cortisol will also be approximately the same. This would probably account for the flat part of the curve that is evident in Figure 3 at low cortisol production rates. At elevated adrenal cortisol production rates, transcortin is saturated, and there is a steep rise in the percentage of unbound cortisol as seen in Figure 3. In both the estrogen-treated and control subjects, this rise should represent a predominant binding equilibrium with plasma proteins of weaker binding affinity. The cortisol capacity and affinity of the weaker binding plasma proteins probably do not differ in the two groups of subjects.

The mean cortisol miscible pool was 2.3 mg and 3.8 mg in the control subjects and in the estrogen-treated subjects, respectively. As calculated above, the extraplasma miscible pool was approximately 2.0 mg and 2.9 mg in these two groups, respectively. Appreciable quantities of transcortin-like binding have been reported present in thoracic duct lymph, and its binding capacity has been stated to be slightly below that of the peripheral plasma (23). Although this observation does not necessarily mean that transcortin is present in appreciable concentration in the extracellular fluid, it does suggest the presence of transcortin outside the vascular compartment and that its concentration in estrogen-treated subjects may be higher than in normal subjects. In addition, recent experiments in which I^{125}-labeled transcortin was administered intravenously to normal and estrogen-treated subjects indicated that 50% of the transcortin miscible pool was outside of the plasma (24). The difference in total miscible pool between the estrogen-treated and control subjects was 1.5 mg, but this difference became 0.9 mg when the plasma cortisol content was subtracted from each group. This difference in cortisol pool may become negligible at the tissue level if there is another area of increased transcortin binding, besides the plasma, in the estrogen-treated subjects. In other words, despite elevated levels of bound and unbound cortisol in the plasma of the estrogen-treated subject, the total amount of cortisol in the tissues may be equivalent to that found in the control subject. The form in which cortisol exerts its physiological activity in the tissues is not known.

Failure to observe evidence of hyperadrenocorticism in estrogen-treated subjects may be related to a direct effect of estrogens on cellular enzyme activity, on cellular and vascular permeability for substances besides transcortin and cortisol, on the rate of cortisol catabolism, or on other metabolic parameters.

Summary

Data have been presented which test the postulate that the unbound level of plasma cortisol, irrespective of what the total level may be, is the deciding factor in determining whether the human subject will show clinical evidence of normal or abnormal tissue cortisol concentration. This postulate was studied in control and estrogen-treated human subjects and was not found to be consistent with the following observations: *1*) Both the bound and unbound plasma cortisol levels were found to be appreciably elevated in a group of clinically eucorticoid, estrogen-treated subjects, when compared to the control group. *2*) Both the plasma bound and unbound cortisol levels are a fair index of the adrenal cortisol production rate in the nonestrogen-treated subject, but these two parameters are relatively elevated in the estrogen-treated subject at equivalent rates of adrenal cortisol production. *3*) The data indicate that the proportion of unbound to total plasma cortisol bears a closer relationship to adrenal cortisol production in the combined group of estrogen-and nonestrogen-treated subjects than does either of these parameters independently.

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