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THE *IN VITRO* RED BLOOD CELL UPTAKE OF C^{14}–CORTISOL; STUDIES OF PLASMA PROTEIN BINDING OF CORTISOL IN NORMAL AND ABNORMAL STATES *

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The investigative work of Daughaday (1), Sandberg, Slaunwhite and Antoniades (2), and Slaunwhite and Sandberg (3) has established the presence of a plasma protein that plays an important role in the transport of blood cortisol.[1] This protein, called transcortin or corticosteroid-binding globulin, has been shown to have great affinity, but low capacity, for binding cortisol and certain other corticosteroids. In dialysis and ultrafiltration experiments on human plasma containing concentrations of cortisol less than 15 μg per 100 ml, only a small percentage of cortisol is found to be freely diffusible (1, 4, 5). This is attributed to the firm binding of the transcortin-cortisol complex. At higher concentrations of plasma cortisol, transcortin becomes saturated, and other plasma proteins, mainly albumin, assume a greater role in the binding equilibrium. Since albumin binds cortisol less firmly than transcortin, the diffusible fraction of plasma cortisol increases (1–4).

While much investigative work has centered around plasma factors in blood cortisol transport, relatively little attention has been given to the red cells. In this investigation the percentage of blood cortisol associated with the red blood cells *in vivo* and in an *in vitro* system has been studied. During the course of these studies it was found that the *in vitro* distribution of C^{14}-cortisol between plasma and red blood cells could be used to measure the cortisol-binding properties of plasma proteins. Although there are other methods of study, this procedure is advantageous in that the conditions used approach physiological conditions, and the techniques involved are relatively simple. In this communication the method is described, and the results with normal subjects and subjects with abnormal plasma protein binding of cortisol are reported. Particular emphasis is given to the abnormalities found in subjects with thyroid disorders.

METHODS

The term "RBC uptake" is used to denote the percentage of total C^{14}-cortisol found in association with the red blood cells. For the determination of the *in vitro* RBC uptake of C^{14}-cortisol, heparinized venous blood was drawn between 8 and 11 a.m. The microhematocrit was determined and the hematocrit was adjusted to 45 per cent by removing plasma or cells. Five-ml aliquots of blood were then pipetted into 25-ml Erlenmeyer flasks containing exactly 0.15 or 0.30 μg of 4-C^{14}-cortisol [2] (specific activities, 15.43 and 7.90 μc per μmole, respectively), with varying known amounts of unlabeled cortisol. One drop of aqueous penicillin was added to each flask. (The added penicillin did not affect the RBC uptake in control experiments.) The flasks were covered, placed in a "wrist-action" shaker, and partially immersed in a water bath at 37° C. After 1 hour of incubation each blood sample was centrifuged in a 12-ml conical centrifuge tube. Approximately 2.25 ml of plasma was cleanly separated from the packed cells, and an aliquot of the separated plasma (usually 2 ml, on rare occasions slightly less) was then pipetted into a 90-ml centrifuge tube. Ten ml of distilled water was added, and the pH was adjusted to 9.0 with a dilute lithium hydroxide solution [cortisol recovery appears to be more reproducible when lithium hydroxide is used for alkalinization (6)]. The dilute alkaline plasma was extracted three times with 1.5 vol of freshly redistilled dichloromethane. Mixing was accomplished with end-looped glass stirrers, and occasional emulsions were broken by centrifugation. A drop of 5 N acetic acid was added to the combined dichloromethane extract, and the solution was evaporated under reduced pressure at 40° C. The residue was quantitatively

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[1] The following trivial names are used. Cortisol: 11β,17α,21-trihydroxy-4-pregnene-3,20-dione. Cortisone: 17α,21-dihydroxy-4-pregnene-3,11,20-trione.

[2] The 4-C^{14}-cortisol was generously supplied by the Endocrine Study Section, National Institutes of Health. Purity was approximately 96 per cent.

transferred to a small tube with dichloromethane and the solvent removed with a stream of nitrogen. The residue was then redissolved in 0.7 ml of ethanol, and 0.3 ml of this solution was transferred to a stainless steel planchet. Planchets were dried and counted for 3 minutes in a gas-flow counter. The counts of duplicate planchets did not differ by more than 2 per cent from their means. Sample weights were sufficiently small so that correction for self-absorption was not necessary.

The RBC uptake was calculated by the following equation:

$$\% \text{ RBC uptake} = \frac{\text{total cpm} - \text{cpm in plasma}}{\text{total cpm}} \times 100,$$

where total cpm was accurately known, and cpm in plasma was determined experimentally.

Initial plasma cortisol levels were determined by the method of Plager, Cushman and Chase (6) in which cortisol is separated by paper chromatography. Since a relatively large amount of cortisol was added to each blood sample, the final plasma cortisol concentration was calculated by the following equation:[3]

Final plasma cortisol concentration = (initial plasma cortisol concentration + added cortisol concentration) × (100 − % RBC uptake)

With each subject the *in vitro* RBC uptake was measured in duplicate at two or more plasma cortisol concentrations. Less than 10 per cent of all duplicate determinations differed from their mean value by more than 3 percentage points, and the mean difference was 2 percentage points.

The *in vivo* RBC uptake of cortisol was determined in 5 subjects. One μc (specific activity, 15.43 μc per μmole; or approximately 23 μg) of C^{14}-cortisol was administered intravenously to Subjects 1 and 2. After 10 minutes, during which time relatively little metabolism of the labeled cortisol would be expected to occur, blood was drawn and 10-ml aliquots were centrifuged. The plasma was separated from the cells and the plasma volume was measured. The plasma was then alkalinized, extracted, and counted for radioactivity, by the methods already described. The cells (plus "trapped" plasma) were diluted with 3 vol distilled water, and this mixture was alkalinized and extracted three times with 1.5 vol dichloromethane. The extracts were subsequently treated as were the plasma extracts and measured for radioactivity. Counting in these experiments was also done at infinite thinness. The respective counts per minute in plasma and red cells were calculated, allowing for the calculated volume of plasma "trapped" with the red cells. The RBC uptake was then determined by the equation:

$$\% \text{ RBC uptake} = \frac{\text{cpm in red blood cells}}{\text{total cpm}} \times 100$$

In Subject 2, 3.5 mg of unlabeled cortisol was intravenously injected immediately after the withdrawal of blood for the first *in vivo* RBC uptake determination. Five minutes later another blood sample was obtained for determination of another *in vivo* RBC uptake at a higher plasma cortisol concentration. This subject had received 12.5 mg of cortisone 12 hours prior to the experiment, and an unknown amount of this substance was present in his plasma.

With Subjects 3, 4, and 5 the *in vivo* RBC uptake was measured by separating cells and plasma soon after venipuncture and determining the cortisol levels in each by paper chromatographic methods. Subject 3 had been on estrogen therapy and, consequently, had an elevated plasma cortisol concentration. In all instances the *in vivo* RBC uptake was determined by extracting unwashed red cells, and calculations were carried out in accordance with the principles outlined by Migeon, Lawrence, Bertrand and Holman (7).

RESULTS

In vitro experiments

Recovery experiments. The recovery of added C^{14}-cortisol from plasma was 82 ± 2 per cent (mean ± SD; range, 78 to 89 per cent). Recovery from red blood cells was 79 ± 4 per cent (range, 69 to 84), and the recovery from saline was 96 ± 2 per cent (range, 93 to 100). The following results are corrected for these mean recoveries.

To validate the assumption that the RBC uptake can be determined by extracting the plasma alone, five incubation experiments were conducted in which the red blood cells were extracted in addition to the plasma. All of the labeled cortisol added to each blood sample was completely accounted for by adding the counts per minute in the red blood cells and in the plasma, allowing for known recoveries. Calculation of the RBC uptake, using the counts per minute in either red blood cells or plasma, yielded the same result. Adsorption of labeled cortisol to glassware was not apparent.

Effect of pH and incubation time. The blood pH at the end of incubation was approximately 7.8. In three instances a mixture of 95 per cent O_2 and 5 per cent CO_2 was delivered constantly into the flasks during incubation; the pH at the end of these incubations was 7.5. The mean RBC

[3] A slight discrepancy is inherent in this calculation, since the concentration of cortisol in the red cells prior to the addition of exogenous cortisol was not measured or allowed for in the equation; however, under the experimental conditions, this factor is exceedingly small and does not alter the final plasma cortisol concentration by more than 1 μg per 100 ml.

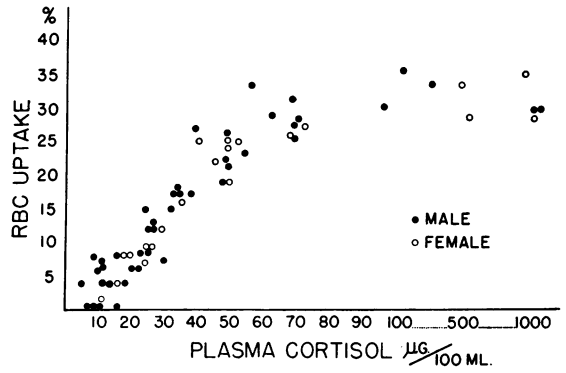

FIG. 1. THE *in vitro* RBC UPTAKE OF C^{14}-CORTISOL IN NORMAL MALES AND FEMALES. Each value of RBC uptake is the mean value of duplicate determinations and is plotted against the corresponding total plasma cortisol concentration, protein-bound and unbound.

uptake in these three samples differed by only 2 per cent from the mean of their duplicates which were incubated in air. This observation correlates with that of Mills (8) who reported that the binding of cortisol by plasma proteins at 37° C does not change appreciably through the pH range of 7.4 to 8.2.

The distribution of labeled cortisol between plasma and red blood cells reached equilibrium during the hour of incubation. Prolonging the incubation for 2 or 3 hours caused no further change in the cortisol distribution.

Stability of cortisol during incubation. Paper chromatographic analyses of the plasma extracts after incubation of labeled cortisol with whole blood revealed that the radioactivity was virtually limited to the cortisol area, indicating that the cortisol was not altered during the experiment. Peterson and co-workers (9) have made similar observations.

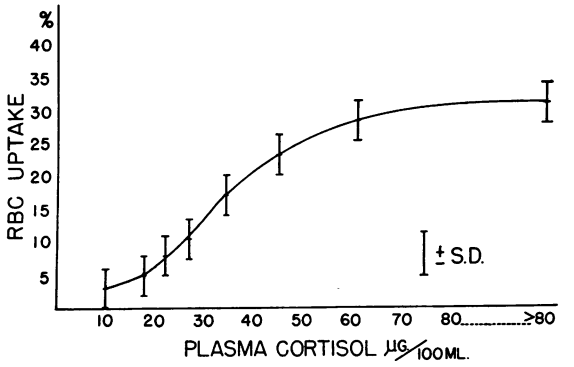

FIG. 2. THE NORMAL MEAN RBC UPTAKE CURVE ± 1 SD. The plasma cortisol represents the total concentration of cortisol present in the plasma, both protein-bound and unbound.

Normal subjects. The *in vitro* RBC uptake of C^{14}-cortisol was measured in 21 normal subjects. There were 9 females and 12 males in this group. Nine (4 females and 5 males) were between 45 and 80 years old and 12 (5 females and 7 males) were less than 45. The RBC uptake was determined between 8 and 11 a.m. in all but 5 subjects. In these, blood was drawn at 8 p.m. in order to determine the RBC uptake at lower plasma cortisol concentrations.

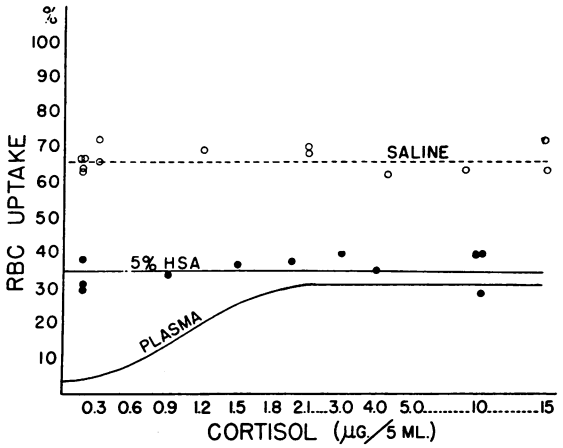

FIG. 3. THE RBC UPTAKE OF C^{14}-CORTISOL FROM VARIOUS INCUBATION MEDIA. Incubations were carried out at a hematocrit of 45 per cent with saline, 5 per cent human serum albumin (5% HSA), and plasma. The cortisol concentrations are expressed as µg per 5 ml of incubation mixture. Individual values of RBC uptake from saline and 5 per cent human serum albumin are shown by open circles and closed circles, respectively.

The RBC uptake of normal subjects is plotted against the corresponding plasma cortisol concentrations in Figure 1. In Figure 2 the mean RBC uptake curve ± 1 SD is depicted. The RBC uptake is 3 ± 3 per cent when the plasma cortisol concentration is 10 µg per 100 ml. As the plasma cortisol concentration increases, the RBC uptake increases curvilinearly to 31 ± 3 per cent at plasma cortisol concentrations above 80 µg per 100 ml. Males and females are compared in Figure 1 and show no apparent differences. The results with subjects above and below the age of 45 are also comparable.

The RBC uptake of cortisol from various incubation media. In Figure 3 the RBC uptakes of C^{14}-cortisol from various incubation media are compared. When red blood cells are incubated with saline, the mean RBC uptake is 66 per cent (range, 62 to 72) and stays constant as the cortisol concentration increases. In the system of red blood cells and 5 per cent human serum albumin, the mean RBC uptake is 35 per cent (range, 27 to 40) and, similarly, does not change with increasing cortisol concentration. For comparison, the mean curve of whole blood (plasma curve) is also shown in Figure 3. In contrast to the other systems, the RBC uptake in whole blood ranges from 3 to 31 per cent and is dependent upon the cortisol concentration. The RBC uptake in whole blood is slightly less than the RBC uptake in the 5 per cent human serum albumin system at higher cortisol concentrations.

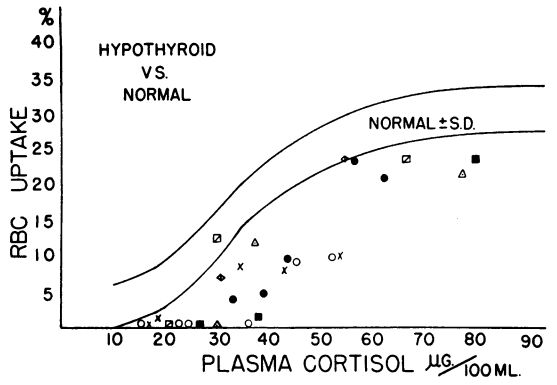

FIG. 4. THE *in vitro* RBC UPTAKE OF C^{14}-CORTISOL IN HYPOTHYROIDISM. The values of each subject are represented by a specific symbol. The plasma cortisol represents the total concentration of cortisol present in the plasma, both protein-bound and unbound.

Hypothyroidism. Seven hypothyroid subjects were studied. All displayed signs and symptoms of moderately severe hypothyroidism, and the diagnosis was confirmed by measurement of the serum protein-bound iodine and the thyroid uptake of I^{131}. Five of the group had primary hypothyroidism. The other two had hypothyroidism secondary to hypopituitarism as judged by clinical features, the thyroid response to thyroid-stimulating hormone administration, and the urinary gonadotropin assay. Both hypopituitary subjects had normal plasma cortisol concentrations (9 and 10 µg per 100 ml) at the time of testing. All seven subjects had an abnormally low RBC uptake of labeled cortisol at multiple plasma cortisol concentrations; 25 of 27 values are displaced to the right of the normal curve, as illustrated in Figure 4.

TABLE I

Incubation of abnormal plasma with normal red blood cells

Plasma type	Plasma cortisol conc.	RBC uptake* of C^{14}-cortisol using	
		Subjects' RBC	Normal RBC
	µg/100 ml	%	%
Hypothyroid	33†	4	5
Hypothyroid	27†	0	1
Metastatic carcinoma	14	16	19

* Each value of RBC uptake is the mean of duplicate determinations.

† The hypothyroid subjects had relatively large amounts of cortisol added to their incubation flasks.

Several experiments were performed to exclude the possibility that the low RBC uptake might be due to a red blood cell abnormality rather than to increased plasma protein binding. In two instances normal red blood cells were incubated with the plasma of hypothyroid patients. The RBC uptake under these circumstances was similar to the results observed when we used the patients' own cells (Table I). In three instances the red blood cells of a hypothyroid subject were incubated

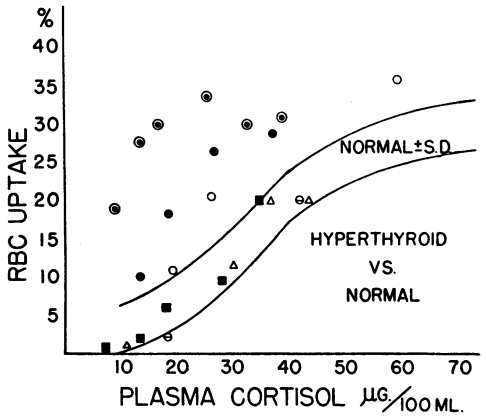

FIG. 5. THE *in vitro* RBC UPTAKE OF C^{14}-CORTISOL IN HYPERTHYROIDISM. The values of each subject are represented by a specific symbol. The plasma cortisol represents the total concentration of cortisol present in the plasma, both protein-bound and unbound.

with saline and the RBC uptake of C^{14}-cortisol was measured. These results were within normal range (65, 65, and 67 per cent).

Hyperthyroidism. Six subjects with clinical and laboratory evidence of hyperthyroidism were studied. Four had diffuse toxic goiter and two had toxic thyroid nodules. The RBC uptake of labeled cortisol was abnormally high in three subjects, while the remaining subjects had normal values (Figure 5). Of the subjects who displayed an elevated uptake, two had diffuse toxic goiter and one had a toxic nodule. The differences in RBC uptake could not be correlated with clinical findings.

Pregnancy and estrogen therapy. Four females in the last trimester of pregnancy and two elderly

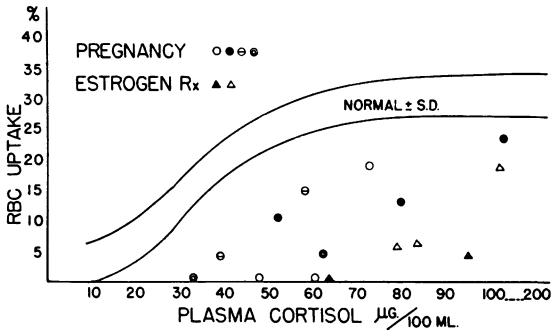

FIG. 6. THE *in vitro* RBC UPTAKE OF C^{14}-CORTISOL IN PREGNANCY AND ESTROGEN TREATMENT. The values of each subject are represented by a specific symbol. The plasma cortisol represents the total concentration of cortisol present in the plasma, both protein-bound and unbound.

males who were receiving diethylstilbestrol for metastatic carcinoma of the prostate were studied. All had elevated plasma cortisol concentrations which ranged from 23 to 73 µg per 100 ml. The RBC uptake of C^{14}-cortisol was extremely low in all instances (Figure 6). This agrees with the observation of others that plasma protein binding of cortisol is increased in these states (3, 4, 10, 11).

Rheumatoid arthritis. In their early studies Sandberg and Slaunwhite (10) reported that plasmas from subjects with rheumatoid arthritis showed a slight increase of cortisol binding. Since their results were equivocal, six subjects with this disorder were studied. One of these had a low RBC uptake of C^{14}-cortisol at multiple plasma cortisol concentrations, but the other five had normal values. These data indicate that plasma cortisol binding is usually normal in this disorder.

Metastatic carcinoma. Twelve patients with various types of metastatic carcinoma were studied, and nine had normal RBC uptake values. Three had abnormally high uptakes. While these three had metastatic liver involvement, many of the subjects who had a normal RBC uptake also had liver involvement. The plasma of one of the subjects with an abnormally high RBC uptake of C^{14}-cortisol was incubated with normal red blood cells. The uptake under these circumstances was comparably elevated, confirming that the elevated RBC uptake was due to decreased plasma protein binding of cortisol (Table I).

Sandberg, Slaunwhite and Carter (12) have observed normal or increased plasma protein binding of cortisol in subjects with metastatic breast carcinoma. Only one of our group had metastatic breast carcinoma, and this subject had a normal RBC uptake. The subjects with an elevated RBC uptake had metastatic carcinoma of the stomach, lung, and pancreas.

In vivo experiments

In Table II the *in vivo* and *in vitro* RBC uptakes of cortisol are compared. With each subject the experiments were performed simultaneously at comparable, but slightly different, plasma cortisol levels. From inspection of the values there appears to be reasonably good agreement between *in vivo* and *in vitro* results.

TABLE II

Comparison of in vivo and in vitro results

Subject	*In vivo* RBC uptake of cortisol	*In vivo* Plasma cortisol concentration	*In vitro* RBC uptake of cortisol	*In vitro* Plasma cortisol concentration
	%	*µg/100 ml*	%	*µg/100 ml*
1	0	2	0	7
2a	14	9*	17	18*
2b	31	37*	29	49*
3†	0	73	0	78
4	6	10	8	15
5	5	13	4	18

* An unknown amount of cortisone was present in this plasma.

† Estrogen-treated.

DISCUSSION

These studies demonstrate that human red blood cells have the capacity to "bind" relatively large amounts of cortisol. The nature of this binding is unknown, but it is evident that the binding affinity of the red blood cells for cortisol is less than that of albumin, and much less than that of transcortin. From these studies a reasonable model of the physical state of blood cortisol may be constructed. The circulating cortisol apparently exists in several physical forms—namely, bound to transcortin, bound to other plasma proteins, associated with the red blood cells, and free in solution. An equilibrium exists among these forms, and the amount of cortisol present in each is dependent upon the relative binding affinities, the binding capacities, and the total amount of cortisol present. When the plasma cortisol concentration is less than 20 μg per 100 ml, the binding to transcortin predominates, and the RBC uptake of cortisol is low. With increasing concentrations of plasma cortisol, transcortin becomes saturated, increasing amounts of cortisol enter into nontranscortin-bound forms, and the RBC uptake increases. When the plasma cortisol concentration is above 80 μg per 100 ml, transcortin appears to be completely saturated and is no longer a significant factor in the binding equilibrium. The distribution of cortisol among the other plasma proteins (chiefly albumin) and red blood cells then stays proportionally constant even with relatively large amounts of cortisol present.

The fraction of blood cortisol that is freely diffusible and physiologically active has not been accurately defined. It has generally been accepted that the diffusible fraction of plasma cortisol, as determined by dialysis or ultrafiltration experiments, is an accurate measure of the physiologically active fraction of blood cortisol. While it is undoubtedly representative of the physiologically active fraction of circulating cortisol, it must be recognized that the red blood cells also enter into the binding equilibrium and affect the freely diffusible and physiologically active fraction of blood cortisol. Consequently, considerable doubt is cast upon the calculation of the renal clearance of cortisol from results obtained with plasma alone (13, 14), since it is presumably the freely diffusible fraction of whole blood that is available for glomerular filtration.

This scheme depicting the physical state of blood cortisol may clarify some of the conflicting reports concerning the role of the red blood cells in cortisol transport. Samuels and co-workers (15) observed that as much as 30 per cent of the 17-hydroxycorticosteroids of adrenal venous blood was associated with red blood cells, while in peripheral blood none was found to be associated with red blood cells. This observation is probably explained by the high levels of 17-hydroxycorticosteroids in adrenal venous blood. Peterson and colleagues (9) reported that approximately 20 to 25 per cent of labeled cortisol was associated with the red blood cells in *in vivo* and *in vitro* experiments. However, in these experiments relatively large amounts of cortisol were used, and the red blood cell uptake was consequently high.

Sandberg and co-workers (2) had also made preliminary observations on the binding of various steroids to red blood cells. They found that, *in vitro,* approximately 7 per cent of C^{14}-cortisol was associated with the red cells, but their results are not meaningful since unknown amounts of cortisol were removed from the red cells in the washing procedure, and the total amount of cortisol present was not recorded.

Migeon and co-workers (7) found that 17 to 36.5 per cent of the blood 17-hydroxycorticosteroids was associated with the red blood cells. In comparing their findings with ours, a simple explanation for the differences is not apparent. Their work largely dealt with the determination of the *in vivo* distribution of 17-hydroxycorticosteroids between plasma and red blood cells before and after the intravenous administration of large quantities of various 17-hydroxycorticosteroids. While the percentage of 17-hydroxycorticosteroids associated with the red blood cells after such administration would necessarily be high, it is not apparent why comparably high percentages were found in the control blood samples which presumably had normal 17-hydroxycorticosteroid levels.

The findings in estrogen-treated subjects and pregnant women clearly portray the effects of increased plasma protein binding of cortisol on the RBC uptake. All values of uptake are displaced to the right of the normal curve. While the plasma

cortisol concentrations are markedly elevated in these states, our data support the contention of Slaunwhite and Sandberg (3) that the freely diffusible fraction (and physiologically active fraction) of blood cortisol is either normal or only slightly increased.

In states characterized by increased plasma protein binding of cortisol (3, 9, 12), there is a concomitant increase in the total plasma cortisol concentration. This increase in total plasma cortisol, in effect, tends to saturate transcortin so that a new equilibrium is established between bound and unbound cortisol, and a normal concentration of unbound (or physiologically active) cortisol results. Consequently, the movement of cortisol from blood to tissues probably proceeds at a normal rate in these states despite the presence of elevated total plasma cortisol levels. In hypothyroidism, the total plasma cortisol concentration has been reported to be normal (16). If this is the case, then an increase in plasma protein binding would cause an increase in the protein-bound fraction of plasma cortisol at the expense of the unbound fraction. Conceivably, these circumstances might cause a decreased rate of movement of cortisol from blood stream to tissues and result in a state of tissue cortisol deficiency. This might explain some of the clinical similarities found in hypothyroidism and hypoadrenalism, and might also be operative in the decreased rate of turnover of cortisol in hypothyroidism (16, 17), in addition to the proposed decreased rate of hepatic degradation. Along similar lines, Sandberg and associates (2) have shown that, *in vitro,* plasma protein binding of steroid (estrogen) may compete successfully against liver protein binding. The significance of increased plasma protein binding of cortisol in hypothyroidism must await further investigation.

Transcortin and thyroxine-binding globulin are both thought to be glycoproteins with similar electrophoretic properties (18). After estrogen treatment, during pregnancy, and in metastatic breast carcinoma, the binding by both of these globulins is reportedly increased (3, 10, 12, 19–21). The present study indicates that the increase in thyroxine-binding globulin, which occurs in some hypothyroid subjects (20, 22), may be associated with a similar increase in transcortin.

Because decreased plasma binding of thyroxine has been observed in hyperthyroidism and in metastatic carcinoma (22, 23), these states were also studied. Our results indicate that similar abnormalities may occur in plasma cortisol binding.

SUMMARY

1. The proportion of blood cortisol associated with the red blood cells has been studied *in vivo* and *in vitro,* and a model describing the physical state of blood cortisol has been constructed. In this model the red blood cell uptake of cortisol in normal subjects ranges from 3 to 31 per cent and is dependent upon the binding of cortisol by plasma proteins and the plasma cortisol concentration.

2. A useful method for measuring the cortisol-binding properties of plasma proteins is described, and results in normal subjects and subjects with abnormal plasma binding of cortisol are reported.

3. In hypothyroidism, increased binding of cortisol by plasma proteins has been observed, and the significance of this finding is discussed.

4. Decreased binding of cortisol by plasma proteins occurs in some patients with hyperthyroidism and metastatic carcinoma.

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