Plasma Protein Binding of Triamcinolone-H₃
and Hydrocortisone-4-C¹⁴* 

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It has become increasingly apparent in recent years that certain structural alterations of corticosteroids cause an increased biological potency of the resultant steroid (2). A number of chemical and microbiological alteration products of hydrocortisone exhibit enhanced anti-inflammatory potency coupled with unchanged or depressed mineralocorticoid activity (2, 3). As a result, steroids bearing various combinations of the Δ¹, 6α-CH₃, 9α-F, 16α-OH, and 16α-CH₃ substituents on hydrocortisone are now produced in large quantities for the treatment of various types of inflammations.

We have been engaged in a series of studies on triamcinolone (8α-fluoro-11β,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3, 20-dione), a synthetic corticosteroid which has achieved widespread medical application. In the first paper of this series (4), we reported the effects of various substituents on the rate of disappearance of hydrocortisone derivatives from rat liver supernatant systems. The present study is concerned with the physicochemical state in which corticosteroids are transported in the plasma.

The binding of steroids by plasma proteins has been reviewed by several authors (5-7). Corticosteroids in plasma are bound by a low affinity, high capacity protein fraction (albumin) and a high affinity, low capacity protein fraction (designated corticosteroid-binding globulin (8, 9) or transcortin (10)). Binding of corticosteroids to each fraction depends on the number and kind of substituents present on the steroid skeleton (9, 11, 12). These reports prompted us to extend our investigation of the biochemical effects of multiple structural alterations of hydrocortisone to an examination of plasma protein binding of the synthetic steroids. Both the whole plasma and the albumin binding of triamcinolone were determined and compared with similar experiments on hydrocortisone. In addition, the effect of various substituents on transcortin-steroid binding was investigated in a series of experiments on the effects of hydrocortisone derivatives on the plasma binding of hydrocortisone.

Unlike hydrocortisone (8, 9), triamcinolone was not bound extensively to transcortin in human or dog plasma. In addition, triamcinolone was bound to a lesser extent than hydrocortisone by solutions of plasma albumin. Thus, at any given concentration, a greater percentage of triamcinolone than of hydrocortisone would be present in the unbound state. The relationship between plasma protein binding and the relative biological potencies of the substituted corticosteroids is discussed herein.

EXPERIMENTAL PROCEDURE

Radioactive Steroids—The preparation of tritium-labeled triamcinolone (4.7 μc per mg) by the Wilzbach technique (13) has been described in detail (14). Hydrocortisone-4-C¹⁴ was purchased from the New England Nuclear Corporation. Paper chromatography in three solvent systems showed no radioactive impurities in conditions under which a 0.2% impurity would have been detectable.

Equilibrium Dialysis—The apparatus and technique developed by Roepke for the measurement of sulfonamide binding in plasma (15) and used by Wozniak in determining tetracycline binding (16) was employed in these experiments. Aliquots of 1.5 ml of undiluted plasma or 4% human plasma albumin solution in buffer (0.15 M NaCl, 0.02 M phosphate, pH 7.4) were pipetted into 7.6-cm sacs of Visking Nojax 18/32 cellophane casing containing an 8-mm glass rod over which the ends of the casing were folded and secured with rubber bands. The bag was placed inside a 50-ml wide mouth centrifuge tube with a ground glass stopper, containing 10 ml of buffered 0.9% NaCl solution to which the steroid, dissolved in 0.05 to 0.10 ml of formamide, was added. The tube was secured in a hole in a vertical turntable at a 78° angle. Thus, the rotation of the turntable at 8 revolutions per minute imparted a continuous rocking and rolling motion to the tube. This, coupled with the high ratio of membrane surface to plasma volume, allowed attainment of equilibrium in 2 hours, as demonstrated by experiments in which binding versus time was observed after addition of steroid to either the inner (plasma) or outer (buffer) solution (see Fig. 1.). On the basis of this observation, all equilibrium dialysis determinations were run for 3 hours to assure that equilibrium had been reached. The entire apparatus was enclosed in a constant temperature oven which was maintained at 37 ± 1° in all experiments. Duplicate tubes were used for all determinations, and duplicate samples were taken for counting except at the lowest triamcinolone concentrations.

The effect of buffer composition was examined by determining the binding of hydrocortisone at high and at low concentrations (5.90 μg per ml and 0.07 μg per ml) in the phosphate-saline buffer described above, in Krebs-Ringer bicarbonate, and in Krebs-Ringer phosphate buffers (17). A variation of less than 2% bound was observed, so it was concluded that neither transcortin nor albumin binding was affected by buffer composition.

Radioactivity Determinations—All counting was carried out by
Binding is defined as the phenomenon or phenomena through which the toluene-phosphor mixture for counting.

The volumes and radioactivity concentrations of both plasma and buffer solutions were known at the end of each experiment; it was thus possible to calculate the recovery of radioactivity from each tube. Only those tubes for which a 95 to 105% recovery was obtained are reported.

RESULTS

The results of equilibration time determinations for hydrocortisone-4-C\textsuperscript{14} in dog plasma are shown in Fig. 1. Two sets of tubes were prepared. In one set, labeled steroid was included with the plasma inside the dialysis sac; in the other, the labeled steroid was added to the outer buffer solution. It is apparent from Fig. 1 that equilibrium was achieved in 2 hours and that the binding at equilibrium was not affected by the starting point of the steroid. Similar results were obtained when triamcinolone-H\textsuperscript{3} was studied. Paper chromatography of extracts of these solutions revealed no additional radioactive components in incubations of either triamcinolone-H\textsuperscript{3} or hydrocortisone-4-C\textsuperscript{14} up to 4 hours. At 6 hours, approximately 0.5% of a more polar component was detected in the hydrocortisone incubation mixture. Metabolism of hydrocortisone was expected; Rongone et al. (21) have reported that the 3-keto-\Delta\textsuperscript{4}-groups of various corticosteroids are reduced upon incubation with plasma proteins.

The binding of hydrocortisone-4-C\textsuperscript{14} and triamcinolone-H\textsuperscript{3} was investigated first in dog plasma; the results are presented in Fig. 2. Over the range of concentrations employed, there was a striking difference in the binding of the two compounds. Triamcinolone binding did not change appreciably with concentration, whereas hydrocortisone binding decreased as the hydrocortisone concentration increased. This result suggested that triamcinolone was not bound by transcortin. Inasmuch as it has been reported that human blood contains a much higher level of transcortin than dog blood (8, 10), the investigation was extended to human plasma.

The results of the human plasma studies are shown in Fig. 3. The binding of hydrocortisone and triamcinolone to human plasma albumin solutions of approximately the same concentration as the albumin in the plasma pool being studied (4%) was also determined.

Unlike hydrocortisone, triamcinolone showed no indication of transcortin binding in human plasma at the concentration range studied. The binding of triamcinolone to whole plasma corresponded very closely to that observed in albumin solutions. As would be expected from previous reports (11, 12), the inclusion of polar \(\alpha\)-substituents in triamcinolone caused it to be less highly bound to albumin than was cortisol. Thus, at all concentrations triamcinolone was bound to a lesser extent than cortisol by plasma proteins. At the low plasma steroid concentrations corresponding to normal hydrocortisone levels, at least 5 times as much triamcinolone would be present in the unbound state as would hydrocortisone at the same plasma concentration. If one may assume that only the unbound steroid is biologically active, this observation may contribute to an understanding of the enhanced activity of triamcinolone as compared to hydrocortisone. Following this line of reasoning, it was of interest to compare the plasma binding properties of a series of substituted hydrocortisone derivatives, some of which have exhibited interesting biological activities. Since these compounds bearing radioisotopic labels were not available, it was not possible to determine their plasma binding directly. However, the competition experiments by which Daughaday (8) investigated the

\[ \text{% bound} = \frac{[\text{Total steroid in plasma}] - [\text{unbound steroid in plasma}]}{[\text{Total steroid in plasma}]} \]

In which

\[ [\text{Unbound steroid in plasma}] = [\text{Steroid in buffer}] \times \frac{[\text{plasma water}]}{[\text{buffer water}]} \]

Final plasma concentrations of steroid were calculated from the observed radioactivity concentrations and the initial specific activities of the steroids. No correction for endogenous cortisol was made.

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specificity of transcortin suggested an alternative way of observing whether these highly substituted corticosteroids were extensively bound by transcortin.

The binding of hydrocortisone-4-C\textsuperscript{14} was determined in human plasma and in albumin solutions in the presence of unlabeled hydrocortisone derivatives. The results of a series of such experiments are presented in Table I. It is apparent that these compounds differed markedly in their effects on hydrocortisone binding by plasma, and that these effects were on the binding by transcortin, rather than by albumin. In general, increasing the number of substituents decreased the competition with transcortin binding of hydrocortisone and, presumably, the binding of the competitor to transcortin. Incorporation of a 1,2-double bond reversed this trend. For instance, $\Delta^1$, 9\alpha-fluorohydrocortisone was a more potent competitor than 9\alpha-fluorohydrocortisone. This relationship was observed throughout the series of compounds reported here.

The decrease in hydrocortisone-4-C\textsuperscript{14} binding with increasing unlabeled steroid concentration indicates that the suppression of hydrocortisone binding was a competitive phenomenon which depended upon the relative concentrations of the two steroids present. The existence of this competition for transcortin but not albumin binding is indicative of the relatively small number of steroid-binding sites provided by transcortin as compared to albumin in plasma.

These observations on competition with hydrocortisone binding led to the suggestion that some of the compounds, other than steroids, used in the relief of arthritis and its symptoms might act by releasing bound hydrocortisone, thus increasing the effective corticosteroid concentration. This was examined by carrying out competitive experiments with aspirin (O-acetyl salicylic acid) at 5.26 to 5260 \mu g per tube and phenylbutazone (3,5-pyrazolidinedione, 4-butyl-1,2-diphenyl-butan-1-ol) at 1.31 to 1310 \mu g per tube; the hydrocortisone-4-C\textsuperscript{14} concentration was held constant at 0.13 \mu g per tube. Under these conditions, hydrocortisone binding varied less than ±1.7% from the 88.5% binding observed in control tubes. Thus, at very great excesses of aspirin or phenylbutazone, no decrease in binding of hydrocortisone to plasma protein was detected. On the basis of these data in vitro, it seems unlikely that the release of bound hydrocortisone is a contributing mechanism by which these drugs exert their antiarthritic effects.

**DISCUSSION**

The results of these studies on the binding of hydrocortisone by human plasma and albumin are in substantial agreement with previous reports (5-12), although we found somewhat less binding of cortisone at low concentrations. This difference may be attributable to the decrease in transcortin binding with increase in temperature reported by Sandberg and Slaunwhite (10) (our dialyses were run at 37° and the earlier studies were conducted at 4°) or perhaps to a difference in the endogenous hydrocortisone concentration of the plasma samples. The striking similarity of our results on the albumin and whole plasma binding of hydrocortisone (Fig. 3) and those of Mills et al. (22, Fig. 7), in which binding was determined by ultrafiltration, should be noted. The discrepancy between our observations that dog plasma contains approximately as much transcortin as human plasma and previous reports (9, 10) that dog plasma contains substantially less transcortin cannot be explained by the data available at this time.

The observation that triamcinolone is not bound extensively by transcortin in dog or human plasma and the demonstration that the affinity of transcortin for corticosteroids depends on the degree of substitution of these compounds suggests one means by which the biological activity of synthetic corticoster-
oids is enhanced (2, 3). If corticosteroids bound to plasma proteins are biologically inactive, then the substituted corticosteroids may exert enhanced activity as a result of their lesser binding to transcortin and albumin. Direct measurement of the biological activities of transcortin-bound steroids is not possible in the absence of a satisfactory system for measuring corticosteroid biological activities in vitro. However, recent reports on hydrocortisone and transcortin levels in plasma during pregnancy and after estrogen administration (22-25) present indirect evidence that transcortin-bound hydrocortisone is inactive in suppressing adrenocorticotrophic hormone release from the pituitary, in relieving rheumatoid arthritis, in inducing the symptoms of hypercortisolism, and in causing eosinophil depression. If binding to transcortin reduces the availability of hydrocortisone to these systems, the lesser binding of triamcinolone to transcortin may result in a greater concentration of this steroid at the sites of activity. Thus, less triamcinolone than hydrocortisone would be required to achieve a given concentration at the active sites; this may be simply an alternative definition of enhanced potency. Assuming that the competition experiments provide an indication of the affinity of transcortin for a steroid, we can apply the same line of reasoning to other synthetic corticosteroids.

The enhancement of activity which occurs upon proper alteration of the corticosteroid molecule cannot be attributable solely to lesser albumin and transcortin binding of the substituted steroid. Binding to albumin and transcortin is only one of several factors which may govern the physiological availability of a steroid. More rapid absorption from the gastrointestinal tract, slower metabolism and excretion by the liver (4, 26-28), and the resultant longer plasma half-life (1, 28-34) would also contribute to a higher concentration of steroid at the sites of activity. A comparison of the effects of various substituents on some of these properties of corticosteroids will be the subject of a later report.

Steroid structural alterations do not always result in enhancement of a desirable biological activity. There are many instances in which one type of activity is increased while another is unchanged or even depressed (3, 3). Thus, it appears that some structural modifications decrease the affinity of the steroid for one or more of the target systems as well as for transcortin, albumin, and the metabolizing enzymes. Obviously, if a substituted steroid has no inherent biological activity, its concentration at the sites of corticosteroid activity is of no biological significance.

SUMMARY

The binding of triamcinolone-21H and hydrocortisone-4-C14 to dog and human plasma and to 4% human albumin solutions has been determined by the equilibrium dialysis technique over a wide range of steroid concentrations. Hydrocortisone was bound by albumin and by a second plasma protein, presumably the corticosteroid-binding globulin, transcortin, which has been described by Daughaday and by Sandberg and Slawhite. Triamcinolone, on the other hand, was bound almost exclusively to albumin, and was bound to a lesser extent than hydrocortisone to albumin. Thus, triamcinolone was present to a greater extent than hydrocortisone in the unbound state at all concentrations; when either steroid was present at 0.01 μg per ml in plasma, 5 to 6 times as much triamcinolone as hydrocortisone was unbound. Determination of hydrocortisone-4-C14 binding in the presence of a series of hydrocortisone derivatives revealed that the 6α-CH3, 9α-F, 16α-OH, and 16α-CH3 substituents all decreased the competition of steroids with hydrocortisone binding. Dehydrogenation at the 1,2 positions partially reversed this effect. These observations led to the suggestion that the lesser plasma binding of the synthetic substituted corticosteroids accounts in part for their enhanced biological activities.

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