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RATES OF DISSOCIATION OF STEROID AND THYROID HORMONES FROM HUMAN SERUM ALBUMIN

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Summary—A rapid filtration assay employing dextran-coated charcoal as acceptor particles for free hormone was used to measure rates of dissociation of steroid and thyroid hormones from human serum albumin. Modification of a previously described assay allowed measurements at 1-s intervals. Nevertheless, this still permitted only minimum estimates of the dissociation rate constants. The hormones studied were thyroxine, 3,5,3′-triiodothyronine, cortisol, corticosterone, testosterone, dihydrotestosterone, estradiol, progesterone, and aldosterone. The apparent dissociation rate constant of the thyroxine–albumin complex at 37°C was $1.3 \pm 0.2\,s^{-1}$ ($t_{1/2}$, 0.5 s). The apparent dissociation rate constants of the other hormone–albumin complexes at 37°C generally exceeded $2\,s^{-1}$ ($t_{1/2} < 0.35$ s). Apparent dissociation rate constants at 4°C were only slightly lower. These findings indicate that steroid and thyroid hormones dissociate from albumin rapidly compared with the 1-s capillary transit times that characterize many tissues.

INTRODUCTION

General models of hormone transport *in vivo* can be used to make predictions about which fraction(s) of hormone in plasma (i.e. free or protein-bound) affects biological response [1]. Such models, for their implementation, require knowledge of (among other factors) the rate constants governing the hormone–protein interactions in the plasma. Although it has generally been assumed that the rates of dissociation of steroid and thyroid hormones from albumin are very rapid, there is in fact little published information on this subject. In particular, it is unknown whether these rates are rapid compared with the 1-s capillary transit times that characterize many tissues. Hillier [2] reported that 3,5,3′-triiodothyronine (T3) dissociated from albumin with a half-time ($t_{1/2}$) at 37°C of less than 1 s. Mendel *et al.* [3] reported that thyroxine (T4) dissociated from albumin with a $t_{1/2}$ at 37°C of less than 1.5 s. Pardridge [4], in a review, cited studies reportedly showing that the $t_{1/2}$ of the testosterone–albumin complex at 37°C was less than 1 s. However, we have been unable to locate these data in the studies cited. Vigersky *et al.* [5] reported very slow ($t_{1/2} > 5$ min) rates of dissociation for testosterone, dihydrotestosterone, and estradiol from albumin at 37°C. Their methods were not extensively validated, however, and likely yielded erroneously low values for the rates of dissociation, as discussed elsewhere [6]. Thus, the present investigation was undertaken to measure the rates of dissociation of a wide variety of steroid and thyroid hormone complexes with albumin.

MATERIALS AND METHODS

Materials

[^{125}I]T4 (1100–1300 μCi/μg), [^{125}I]T3 (1100–1300 μCi/μg), [1,2,6,7,16,17-^{3}H(*N*)]testosterone (135–180 Ci/mmol), [2,4,6,7,-^{3}H(N)]estradiol (85–115 Ci/mmol), [1,2,6,7-^{3}H(*N*)]progesterone (90–115 Ci/mmol), and [1,2,6,7-^{3}H(*N*)]-corticosterone (80–105 Ci/mmol) were purchased from New England Nuclear (Boston, Mass). 5α-Dihydro-[1,2,4,5,6,7-^{3}H]testosterone (100–150 Ci/mmol), [1,2,6,7-^{3}H]cortisol (80–105 Ci/mmol) and [1,2,6,7-^{3}H]aldosterone (75–105 Ci/mmol) were purchased from Amersham (Arlington Heights, Ill.). Immediately before each use, the thyroid hormones were purified by chromatography with disposable Sep-Pak C-18 cartridges (Waters Associates, Milford, Mass) [7, 8] and the steroid hormones were

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Table 1. Purification method (celite chromatography) for steroid hormones

Steroid hormone	Aqueous solvent	Organic solvent	Approximate volume for elution (ml)
Cortisol	Water	Isooctane:*t*-butanol (2:1)	7–9
Corticosterone	Water	Isooctane:*t*-butanol (20:7)	5–6
Testosterone	Methanol:water (2:1)	Isooctane:*t*-butanol (5:2)	16–18
Dihydrotestosterone	Methanol:water (2:1)	Isooctane:*t*-butanol (5:2)	8–10
Estradiol	Methanol:water (7:3)	Ethyl acetate:*n*-hexane (3:17)	14–18
Progesterone	Methanol:water (7:1)	Isooctane:*t*-butanol (11:1)	9–11
Aldosterone	Methanol:water (1:9)	Isooctane:*t*-butanol (2:1)	20–22

Two grams of celite is wetted with 1 ml of aqueous solvent and packed into a 5-ml disposable glass pipet. The tritiated steroid (dissolved in organic solvent) is applied to the column and eluted with organic solvent. Purity of the steroid is assessed by symmetry of the elution peak (evaluated by liquid scintillation counting of aliquots of 1-ml elution fractions). Most of the impurities generally elute prior to the steroid of interest. The purification scheme for each steroid was worked out empirically by one of us (PKS). In each case, it was shown that when unlabeled steroid hormone was added to the tritiated steroid and followed spectrophotometrically, the specific activity of the steroid remained constant over its elution peak.

purified by celite chromatography, using a modification of a previously described method ([9] and Table 1). Activated charcoal (Norit-A), dextran (average mol. wt 60,000–90,000), ovalbumin (grade V), and fatty acid-free human serum albumin (product No. A-3782) were purchased from Sigma. The human albumin used has previously been shown to be free of T4-binding globulin and T4-binding prealbumin by reverse-flow paper electrophoresis [10]; it was shown to be free of corticosteroid-binding globulin and sex hormone-binding globulin by the DEAE filter disc assay [11; J. T. Murai and P. K. Siiteri, unpublished observations].

Methods

The rates of dissociation of steroid and thyroid hormones from human serum albumin were determined from their rates of transfer to acceptor particles (dextran-coated charcoal). The theoretical basis of this method has been described in detail elsewhere [3, 12]. The present method is a modification of a previously described method [7]. Briefly, 10 μl of human serum albumin (50 g/l in Krebs–tricine buffer, pH 7.4) that had been preincubated with 0.1–0.2 pmol of radiolabeled hormone for 30 min at 37°C or 4°C was rapidly injected with a Hamilton syringe into 3 ml of a vigorously stirred slurry of dextran-coated charcoal (1%, w/v) in Krebs–tricine buffer [12], pH 7.4, containing 1 mg/ml ovalbumin. The slurry (prewarmed to 37°C or prechilled to 4°C) rested on a filter manifold inside of prewarmed or prechilled stainless steel weights and was stirred from above using a Dremel (Denver, Colo.) variable speed Moto-Tool with a serrated blade on setting No. 1 (Fig. 1). The rate of transfer of hormone to the charcoal was then determined by rapid filtration (GF/C glass fiber filters, Whatman Inc., Clifton, N.J.) of the slurry at various times following injection (1-s intervals). Filtration of the slurry took 1 s from the time the vacuum was applied. Therefore, the vacuum was applied 1/2 s before each specified time point. 500-μl aliquots of the filtrate (taken in triplicate) were assayed for radioactivity by liquid scintillation counting, and compared with the radioactivity in an unfiltered sample of the

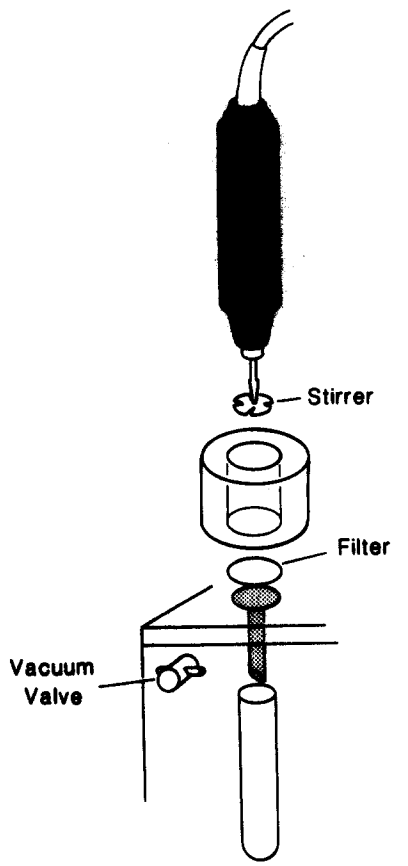

Fig. 1. Diagram of stirring/filtration apparatus used.

slurry*. Dissociation rate constants were estimated by fitting the sum of one or more exponential functions to the data by computerized nonlinear least-squares analysis. All data were best fitted by two exponential functions. The steeper function was assumed to represent the rate constant of interest, because the shallower function usually described less than 10% of the total curve and has previously been shown to reflect the equilibrium distribution of hormone between the charcoal and the buffer [3]. Data for replicate experiments were analyzed separately, and the resulting parameter values were averaged to determine the mean and SE values presented in the text.

When the amount of albumin added to the slurry was doubled, the apparent dissociation rate constant for each hormone was unchanged, indicating that the albumin did not compete significantly with the charcoal for free hormone. [^{125}I]albumin (human) did not bind (<5%) to the charcoal under the experimental conditions employed.

All glassware was treated with dimethyldichlorosilane (Pierce, Rockford, Ill.) as previously described [13]. No (<5%) binding of the hormones to glassware or pipet tips could be detected under the experimental conditions employed. Dextran-coated charcoal (dextran:charcoal, 1:10, w/w) was prepared as previously described [13]. Ultrafiltration-dialysis and equilibrium dialysis were performed as described previously [10, 14]. Albumin was iodinated by the chloramine-T method [15] to a level of not more than one iodine per molecule of albumin.

Calculations

In previous studies of this type from our laboratory, the observed rates of binding of hormone by acceptor particles were shown or assumed to be very rapid compared with the rates of dissociation of the hormone–protein complexes studied [3, 6, 7]. Therefore, observed rates of transfer of hormone to the acceptor particles were considered to represent rates of dissociation of the hormone–protein complexes. In the present investigation, however, the observed rates of dissociation of the hormone–protein complexes were so rapid that the rates of transfer of hormone to the acceptor particles were partially limited by the rates of binding of hormone by the acceptor particles (see Results). The following calculations, taken essentially from Hillier [2], were designed to address this potential methodological limitation.

For each hormone, the apparent rate constant for the disappearance of free hormone from bulk solution (due to binding to the charcoal) (k) was determined (see Results). The fraction of initially free hormone in solution expected to be bound by the charcoal at time t (F) was then calculated from the exponential equation

$$F = 1 - e^{-kt}. \quad (1)$$

The fraction of hormone unbound to charcoal measured at 1 s (U_1) was corrected to the protein-bound fraction at 1 s (V_1) by

$$V_1 = 1 - [(1 - U_1)/F_1] \quad (2)$$

where $F_1 = F$ at $t = 1$ s.† The fraction of hormone unbound to charcoal measured at 2 s (U_2) was corrected to the protein-bound fraction at 2 s (V_2) by

$$V_2 = V_1 - [U_1 - U_2 - (1 - V_1)(F_2 - F_1)]/F_1 \quad (3)$$

where $F_2 = F$ at $t = 2$ s (the term in brackets represents total hormone bound to charcoal during the second interval minus hormone bound to charcoal during the second interval that is accounted for by hormone released from protein during the first interval [2]). Further corrections (for V_3, V_4 and V_5) were found to be quantitatively unimportant. Curve fitting as described above (Methods) was then carried out with V_1 and V_2 substituted for U_1 and U_2. The fraction of hormone that was protein-bound at time 0 was set at the equilibrium value, determined by ultrafiltration-dialysis or equilibrium dialysis.

*Because of the considerable quenching caused by charcoal, samples containing charcoal were not assessed directly for radioactivity. Instead, the radioactivity in an unfiltered (3-ml) sample of the slurry was assumed to be that which was added to the slurry with the Hamilton syringe, measured in pentuplicate (CV < 1%; 490 μl of aqueous solution was added to each of these samples before liquid scintillation counting to maintain the same quenching as in the filtrates). The volume change caused by the addition of the charcoal to the aqueous solution was approximately 1% and was taken into account when comparing the radioactivity in filtered and unfiltered samples.

†Dividing by F_1 in this (and the subsequent) equation to take into account the rate of binding of hormone to the charcoal assumes that the hormone released from protein during each (1-s) interval is released instantaneously from the protein at the beginning of the interval. Because this assumption cannot be correct, these calculations will result in underestimation of the rate of dissociation.

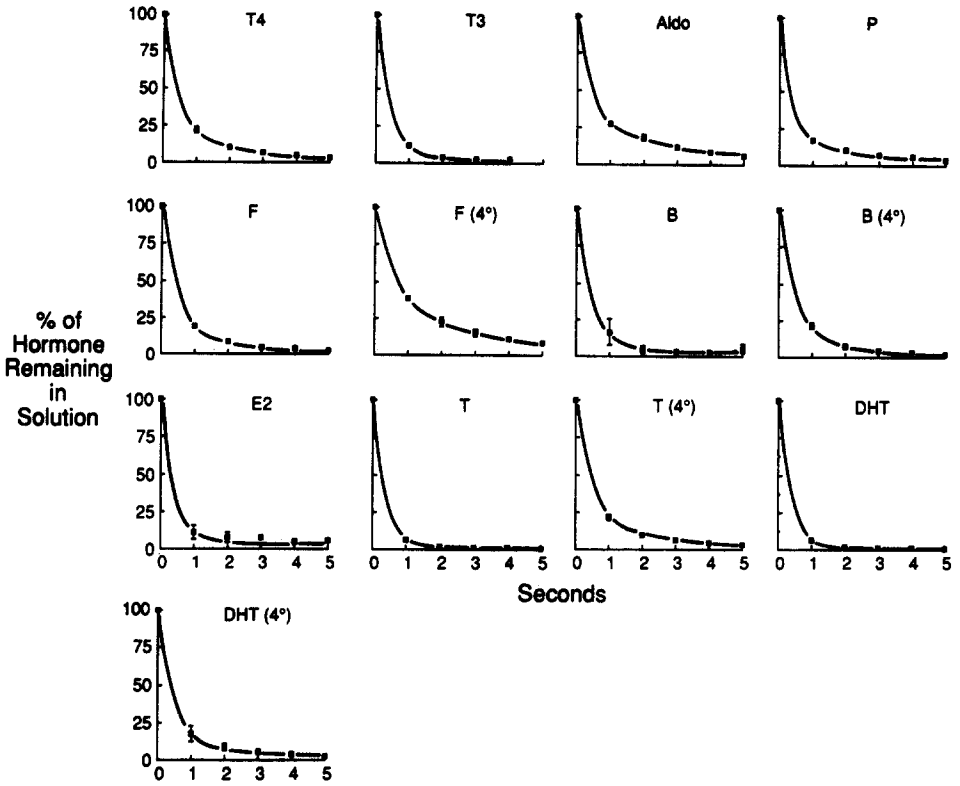

Fig. 2. Disappearance of hormone from bulk solution (due to binding by charcoal) in the absence of albumin. Each point shown is the mean ± SE of data obtained in three separate experiments. For some points, the SE bars were too small to show. Data were collected at 37°C unless otherwise indicated. T4 = thyroxine, T3 = 3,5,3′-triiodothyronine, Aldo = aldosterone, P = progesterone, F = cortisol, B = corticosterone, E2 = estradiol, T = testosterone, DHT = dihydrotestosterone.

RESULTS

The apparent rate constants for the disappearance of free hormone from bulk solution (due to binding to the charcoal) were determined (Fig. 2 and Table 2). Bound and free fractions of hormone in a solution of human serum albumin (50 g/l in Krebs–tricine buffer, pH 7.4) were determined by ultrafiltration-dialysis (steroid hormones) or equilibrium dialysis (thyroid hormones) (Table 3). Rates of transfer of hormone from the hormone–albumin complexes to the charcoal were determined (Fig. 3). Using the data shown in Table 2, apparent dissociation rate constants were derived from the data shown in Fig. 3, as described in Calculations. These apparent dissociation rate constants are shown in Table 4.

Table 2. Rate constants for the disappearance of free hormone from solution (due to binding to the charcoal) at 37°C (except where other temperature specified)

Hormone	Rate constant (s^{-1})[1]
T4	2.7 ± 0.3
T3	2.5 ± 0.1
Aldosterone	1.5 ± 0.1
Progesterone	2.1 ± 0.2
Cortisol	1.8 ± 0.1
Cortisol (4°C)	1.1 ± 0.1
Corticosterone	2.2 ± 0.5
Corticosterone (4°C)	1.8 ± 0.1
Estradiol	3.1 ± 0.6
Testosterone	3.0 ± 0.2
Testosterone (4°C)	1.7 ± 0.1
Dihydrotestosterone	2.9 ± 0.2
Dihydrotestosterone (4°C)	2.0 ± 0.4

[1]Mean ± SE, $n = 3$.

Table 3. Fraction of hormone unbound in a solution of human serum albumin (50 g/l) at 37°C (except where other temperature specified)

Hormone	Fraction unbound (%)[1]
T4	<1
T3	<1
Aldosterone	42.2 ± 1.1
Progesterone	4.9 ± 0.6
Cortisol	42.0 ± 0.3
Cortisol (4°C)	43.7 ± 0.3
Corticosterone	29.7 ± 3.1
Corticosterone (4°C)	27.5 ± 1.5
Estradiol	2.4 ± 0.1
Testosterone	7.4 ± 0.8
Testosterone (4°C)	5.0 ± 0.3
Dihydrotestosterone	5.2 ± 0.9
Dihydrotestosterone (4°C)	2.8 ± 0.2

[1]Mean ± SE, $n = 3$.

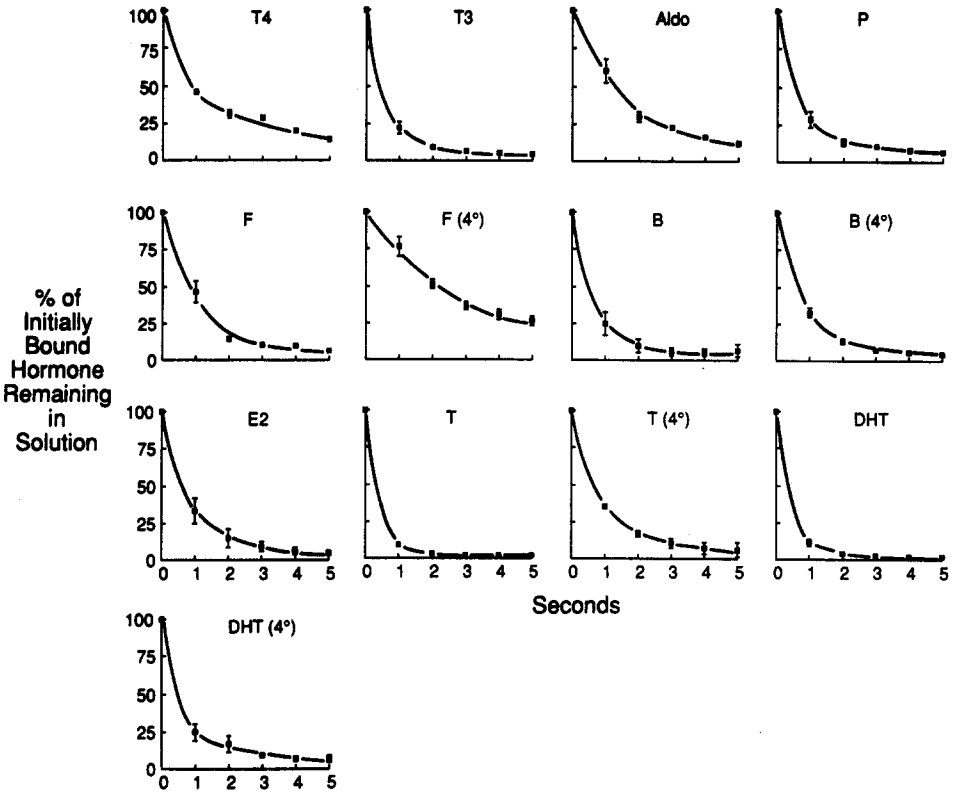

Fig. 3. Disappearance of hormone from bulk solution (due to binding by charcoal) in the presence of human serum albumin (50 g/l). Each point shown is the mean ± SE of data obtained in three separate experiments. For some points the SE bars were too small to show. Data were collected at 37°C unless otherwise indicated. Symbols are the same as in Fig. 2.

DISCUSSION

In the present investigation, we found that for all nine of the steroid and thyroid hormones studied, the rates of dissociation from albumin were very rapid. The apparent dissociation rate constant at 37°C of the T4–albumin complex was $1.3 \pm 0.2\ s^{-1}$ ($t_{1/2}$, 0.5 s). That of the other hormone–albumin complexes generally exceeded $2\ s^{-1}$ ($t_{1/2} < 0.35$ s) at 37°C (Table 4). For four of the hormones studied, the apparent dissociation rate constants of the hormone–albumin complexes were also determined at 4°C, and were found to be only slightly lower than those at 37°C. Limitations in our methods necessitate that all of these rate constants be considered only approximations of the true values. The most important of these limitations were that our 1-s sampling intervals were long compared with the $t_{1/2}$s of dissociation observed, and that, although we could take samples at 1-s intervals, our sampling procedure took one full second. In at least some ways, these limitations are expected to cause underestimation of the derived rate constants (see footnote). Thus, it is possible that the presently reported values for these rate constants are significant underestimates of the true values. Nevertheless, the present data provide the best resolution yet available on the rates of dissociation of thyroid and steroid hormones from albumin.

Table 4. Estimates[1] of the rate constants for dissociation of hormone from human serum albumin at 37°C (except where other temperature specified)

Hormone	Rate constant (s^{-1})[2]
T4	1.3 ± 0.2
T3	2.2 ± 0.3
Aldosterone	1.9 ± 0.6
Progesterone	2.2 ± 0.5
Cortisol	2.2 ± 0.5
Cortisol (4°C)	2.6 ± 0.1
Corticosterone	3.3 ± 0.5
Corticosterone (4°C)	3.1 ± 0.2
Estradiol	1.8 ± 0.4
Testosterone	3.5 ± 0.4
Testosterone (4°C)	2.6 ± 0.6
Dihydrotestosterone	3.4 ± 0.4
Dihydrotestosterone (4°C)	2.2 ± 0.4

[1]See text.
[2]Mean ± SE, $n = 3$.

General models of hormone transport can be used to make useful predictions about the *in vivo* state, but their implementation requires knowledge of (among other factors) the rate constants governing the protein–hormone interactions in the plasma [1]. The present investigation

indicates that the rates of dissociation of thyroid and steroid hormones from albumin are rapid compared with capillary transit times in most or all organs, which apparently range from 0.7 to 9 s [1]. Thus, under appropriate conditions (i.e. when the influx rate constant and the elimination rate constant are both high enough and the rate of binding of free hormone to other serum proteins is low enough [1]), all tissues should be considered at least potentially able to clear the majority of the albumin-bound hormone presented to them.

In other studies, it has been shown that free fatty acids can affect the rates of interaction of small ligands with albumin [16]. In the present study, this phenomenon was not addressed (fatty acid-free albumin was used). In general, however, free fatty acids tend to decrease the affinities of thyroid and steroid hormones for albumin [1]. Therefore, if lipids do affect the rates of dissociation of thyroid and steroid hormones from albumin, they are likely to increase these rates. Thus, our conclusion that these rates are rapid compared with capillary transit times in most or all organs should remain valid in the setting of this consideration as well.

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