J. Steroid Biochem. Molec. Biol. Vol. 37, No. 2, pp. 251–255, 1990
Printed in Great Britain. 

0960-0760/90 $3.00 + 0.00


# RATES OF DISSOCIATION OF SEX STEROID HORMONES FROM HUMAN SEX HORMONE-BINDING GLOBULIN: A REASSESSMENT

CARL M. MENDEL

Cardiovascular Research Institute and Department of Medicine, University of California, San Francisco, CA 94143-0130, U.S.A.

*(Received 23 April 1990)*

**Summary**—A rapid filtration assay employing dextran-coated charcoal as acceptor particles for free hormone was used to measure the rates of dissociation of dihydrotestosterone (DHT), testosterone (T), and estradiol ($E_2$) from their binding proteins in human serum at 37°C. Because measurements were begun after each hormone had fully (>99%) dissociated from albumin, the observed rates of dissociation correspond to the rates of dissociation of the sex hormone-binding globulin (SHBG)–hormone complexes. The dissociation rate constants of the hormone–SHBG complexes were determined to be $0.016 \pm 0.001$, $0.056 \pm 0.002$, and $0.083 \pm 0.003\ s^{-1}$ for DHT, T, and $E_2$, respectively, corresponding to half-times of dissociation ($t_{1/2}$) of 43, 12 and 8.4 s, respectively. The physiological significance of these findings can best be appreciated by comparing these $t_{1/2}$s with the capillary and sinusoidal transit times of various tissues (<1 s to ~10 s).

## 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 require knowledge of not only the equilibrium constants but also the kinetics constants governing the hormone–protein interactions in the plasma. Previous determinations of the dissociation rate constant at 37°C for the testosterone (T)–sex hormone-binding globulin (SHBG) complex have been in general agreement [2, 3]. Unfortunately, the small discrepancy between these results ($0.032\ s^{-1}$ [2] vs $0.056\ s^{-1}$ [3]*) falls within a range that is critical for the interpretation of certain biological data (see Discussion). In addition, the literature apparently contains no well-validated measurements of the dissociation rate constant of the estradiol ($E_2$)–SHBG complex at 37°C. The present investigation was designed to determine (or redetermine) these rate constants.

## MATERIALS AND METHODS

### *Materials*

[1,2,6,7,16,17-$^3$H(*N*)]Testosterone (135–180 Ci/mmol) and [2,4,6,7-$^3$H(*N*)]estradiol (85–115 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) was purchased from Amersham (Arlington Heights, Ill.). Each was purified before use by celite chromatography (companion paper). 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 (St Louis, Mo.). The pool of pregnant serum used had been obtained from healthy women in their third trimester of pregnancy and was a generous gift of Drs James T. Murai and Pentti K. Siiteri. It had been stored at −70°C for approx. 2 yr.

### *Methods*

The rates of dissociation of DHT, T, and $E_2$ from serum binding proteins were determined from the rates of transfer of each to acceptor particles (dextran-coated charcoal) [4, 5]. The theoretical basis and details of this method have been described in detail elsewhere [6]. Briefly, 5 μl of undiluted human serum (adjusted to pH 7.4 with 0.1 N HCl) that had been pre-incubated with 0.1–0.2 pmol of radiolabeled hormone for 30 min at 37°C was rapidly injected into 20 ml of a vigorously stirred slurry of dextran-coated charcoal (0.2%, w/v) in Krebs–tricine buffer (pH 7.4, 37°C) containing 1 mg/ml

*The reported value of Lata *et al.* [3] was for the dissociation rate constant at 37.5°C.

ovalbumin. The rate of transfer of hormone to the charcoal was then determined by periodic (5-s intervals) rapid filtration (GF/C glass fiber filters, Whatman Inc., Clifton, N.J.) of 1-ml aliquots of this slurry. 500-$\mu$l portions of each filtrate were assayed for radioactivity by liquid scintillation counting, and compared with the radioactivity in an unfiltered sample of the 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. Data for replicate experiments were analyzed separately, and the resulting parameter values were averaged to determine the mean and SD values presented in the text.

The dissociation assay was validated for each hormone by the following studies: (1) When the amount of serum added to the slurry was doubled, the apparent dissociation rate constant was unchanged. This indicates that the serum binding proteins added to the slurry did not significantly compete with the charcoal for free hormone. (2) When the hormones were added to the charcoal slurry in the absence of binding proteins, the rate constants describing the binding of each hormone to the charcoal were all greater than 0.5 $s^{-1}$. Because this rate of binding is rapid compared with the sampling intervals, and is much more rapid than the dissociation rates observed for the complexes (see Results), it can be concluded that the observed rate of transfer of hormone to the charcoal was not significantly limited by the rate of binding of free hormone to the charcoal. Under both of these sets of conditions, the observed rate of transfer of hormone to the charcoal should represent the rate of dissociation of hormone from binding proteins. In addition, it was shown that various serum hormone-binding proteins ([$^{125}$I]albumin (human) and [$^{125}$I]corticosteroid-binding globulin (both rat and human), prepared as previously described [5]) did not bind (<5%) to the charcoal under the experimental conditions employed, and that none of the hormones (DHT, T, $E_2$) were bound by 0.1% ovalbumin (added to the buffer in the dissociation assay to prevent nonspecific binding), as assessed by ultrafiltration-dialysis [7].

All glassware was treated with dimethyldichlorosilane (Pierce, Rockford, Ill.). as previously described [5]. 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 described previously [5].

## RESULTS

The rates of dissociation of DHT, T, and $E_2$ from binding proteins in human serum were examined at 37°C. Serum collected during the third trimester of pregnancy was used in these studies to maximize the binding of each of these hormones to SHBG [8]. The first time-point for sampling was at 5 s. Because the dissociation rate constants of DHT, T, and $E_2$ from human serum albumin at 37°C are all greater than 1 $s^{-1}$ [9], more than 99% of each of these hormones initially bound to albumin is expected to have dissociated by this time. Thus, the rates of dissociation of hormone from serum proteins observed from 5 s on should correspond to the rates of dissociation of the hormone–SHBG complexes.

The dissociation rate constants from SHBG for DHT, T, and $E_2$ at 37°C were $0.016 \pm 0.001$ ($\pm$SD, $n = 4$), $0.056 \pm 0.002$ and $0.083 \pm 0.003$ $s^{-1}$, respectively, corresponding to half times of dissociation of 43, 12 and 8.4 s, respectively (Fig. 1). The exponential components of the curves corresponding to these rate constants disappeared when the serum was heated to 60°C for 1 h (data not shown), as would be expected if they represented binding to SHBG [10]. The second exponential component observed in each curve (accounting for 10% or less of the total radioactivity) has been observed previously in these types of experiments [4, 6, 11] and likely reflects the equilibrium distribution of hormone between the buffer and the charcoal [11]. The composite curves (Fig. 1) can be extrapolated back to time 0 to yield the fraction of hormone in third trimester pregnant serum carried by SHBG. This value was 88, 84 and 70% for DHT, T, and $E_2$, respectively. These values are slightly lower than those predicted by Dunn *et al.* [8].

*Because of the strong quenching effect of the charcoal, radioactivity was determined (in pentuplicate) in 25-$\mu$l aliquots of the unfiltered slurry (with 475 $\mu$l of aqueous solution added to each sample to maintain the same quenching as in the filtrates). This amount of charcoal caused <2% quenching of the samples. The volume change caused by the addition of the charcoal (0.2%) to the buffer was <1%.

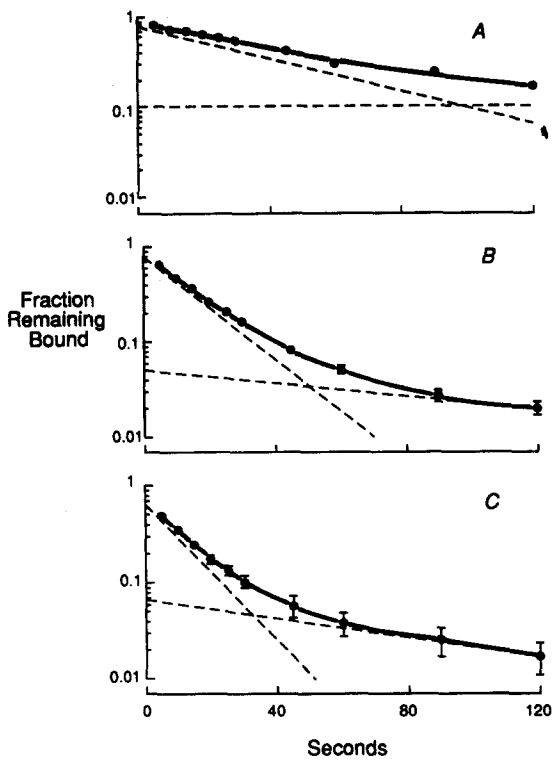


Fig. 1. Dissociation of DHT (A), T(B), and $E_2$ (C) from binding proteins in (pregnant) human serum at 37°C. Under the experimental conditions employed, the observed dissociation rates represent rates of dissociation from SHBG (see Methods). Each point shown is the mean ± SD of data obtained in four separate experiments. For some points the SD bars were too small to show. For each curve, two components (dashed lines) were defined by nonlinear regression analysis; the steeper component corresponds to the dissociation rate constant (see Results).

## DISCUSSION

The rate constant for the dissociation of T from SHBG at 37°C determined in the present study ($0.056 \pm 0.002\ s^{-1}$) is in close agreement with the corresponding value of $0.053 \pm 0.006\ s^{-1}$ previously reported by Lata *et al.** [3] using an entirely different method. It is greater than the value of $0.032\ s^{-1}$ reported by Heyns and De Moor [2], however. The rate constant for the dissociation of DHT from SHBG at 37°C determined in the present study ($0.016 \pm 0.001\ s^{-1}$) is similarly greater than the corresponding value of $0.007\ s^{-1}$ reported by Heyns and De Moor [2]. The reason for these discrepancies is not entirely clear. The method used by Heyns and De Moor, ammonium sulfate precipitation of SHBG-bound hormone in serum at timed intervals following the addition of excess unlabeled hormone, was carefully and appropriately validated in that study. Nevertheless, it seems possible that some free hormone was trapped in the (bulky) ammonium sulfate precipitate, leading to overestimation of the amount of hormone bound to SHBG. This overestimation would be quantitatively more important at later time points, when much less of the hormone is bound to SHBG and much more is free. Thus, such an effect would be expected to cause a flattening of the dissociation curve and, thereby, underestimation of the dissociation rate constant.

The rate constant for the dissociation of $E_2$ from SHBG at 37°C was determined in the present study to be $0.083 \pm 0.003\ s^{-1}$. Heyns and De Moor [2] previously determined this rate constant at 0°C to be $0.0035\ s^{-1}$. Assuming that the $E_2$–SHBG complex had the same activation energy as the T–SHBG complex, they extrapolated this value to 37°C and suggested that the dissociation rate constant of the $E_2$–SHBG complex at 37°C might be approx. $0.14\ s^{-1}$, a value remarkably similar to the presently reported value. To my knowledge, the only other previously reported value for this rate constant at this temperature is the study of Vigersky *et al.* [12], in which the methods used were not extensively validated,† and the rate constants obtained for the dissociation of DHT, T, and $E_2$ from serum binding proteins were orders of magnitude smaller than those reported here.

It is of interest to recall that Westphal [13] observed that for those hormones that bound progesterone-binding globulin with equilibrium association constants ($K_a$) greater than $10^8\ M^{-1}\ s^{-1}$, the association rate constants were fairly constant, yielding an inverse relationship between the dissociation rate constants and the $K_a$ values. Although it cannot be concluded that this relationship must hold for complexes of hormones with other binding proteins [14], it is nevertheless of interest to note that the ratios of the $K_a$ values for the binding of T and $E_2$ to SHBG reported by several (but not all) groups are very similar to the ratio of the dissociation rate constants of $E_2$ and T for SHBG (1.5) reported in the present study (Table 1).

Because of the wide species variation of not only the serum concentration but also various physicochemical parameters (including the $K_a$ value at 37°C) of SHBG [19], it is important not to extrapolate the present results obtained using

*The reported value of Lata *et al.* [3] was for the dissociation rate constant at 37.5°C.

†In particular, it seems likely that there was a great deal of unrecognized rebinding of dissociated hormone to serum proteins in that system.

Table 1. Equilibrium association constants ($nM^{-1}$) for sex steroid hormone complexes with human SHBG at 37°C

| DHT | T | $E_2$ | $T/E_2$ | Ref. No. |
|---|---|---|---|---|
| | 1.9 | 0.6 | 3.2 | 15 |
| 0.99 | 0.35 | 0.22 | 1.6 | 16 |
| | 0.8 | 0.6 | 1.3 | 17 |
| 5.5 | 1.6 | 0.68 | 2.4 | 8 |
| | 0.76 | 0.45 | 1.7 | 10 |
| 0.45 | 0.27 | 0.22 | 1.2 | 18 |

Adapted from Westphal [19]. Only studies in which equilibrium association constants were determined at 37°C for both T and $E_2$ are included here.

human SHBG to the SHBGs of other species. In this regard, we have recently shown, for example, that the dissociation rate constant for thyroxine from human thyroid hormone-binding prealbumin (TBPA) is approx. 5-fold greater than the corresponding dissociation rate constant from rat TBPA (C. M. Mendel and R. A. Weisiger, manuscript in preparation).

Knowledge of the rates of dissociation of hormone–protein complexes is important in at least two contexts. The question of whether hormones are taken up by tissues exclusively via the free pool (the free hormone transport hypothesis [1]) or also via one or more protein-bound pools has been actively debated in recent years. Predictions about how much tissue uptake of hormone can be accounted for by the free pool cannot be made without knowledge of the relevant dissociation rate constants. For example, in a recent review [1] I pointed out that in no published study had the rate of tissue uptake of hormone exceeded the rate of spontaneous dissociation of hormone from its plasma binding proteins. I used this observation to argue in favor of the free hormone transport hypothesis. However, one possible exception was a study in which the fractional uptake by rat liver of DHT in human female serum was reported to be 34%, whereas the maximal predicted uptake via the free pool (based on rates of dissociation of the hormone-protein complexes in serum) was calculated as being only 26% (see Table 3 of Ref. [1]). Although I considered these two numbers to be within experimental error of each other, it is of interest to note that when these calculations [1] are repeated using the presently obtained value for the rate constant for the dissociation of DHT from SHBG, rather than the value of Heyns and De Moor [1, 2], the maximal predicted uptake via the free pool becomes 32%, which is much more clearly within experimental error of the measured value of 34% reported in that study.

Another context in which knowledge of hormone-protein dissociation rate constants is critical is in the use of general models of hormone transport *in vivo*. Such models can be used to make predictions about which fraction(s) of hormone in plasma affects biological response, but require knowledge of these (and other) rate constants to do so [1]. Thus, using the value of Heyns and De Moor for the rate constant for the dissociation of T from SHBG, I recently [1] concluded that the hepatic uptake of T from plasma in humans is likely to be limited by the rate of dissociation of T from SHBG. Under dissociation-limited conditions, the free hormone hypothesis, which states that intracellular hormone concentrations are affected by the free rather than the protein-bound hormone concentration in the plasma, will not hold [1]. However, if the presently determined value for the rate constant for the dissociation of T from SHBG is used in these calculations instead, it becomes less certain that the hepatic uptake of T in humans *in vivo* is dissociation-limited. Thus, the free hormone hypothesis may hold for T with respect to the liver, as it seems to with respect to other tissues [1, 20].

*Acknowledgements*—I thank Martin B. Miller for expert technical assistance, Jimmy Choy and Pentti K. Siiteri for purifying the steroid hormones, and Robert W. Kuhn for helpful discussions related to this work. This work was supported by National Institutes of Health Grants HL 01546 (Clinical Investigator Award), HL 14237 Arteriosclerosis Specialized Center of Research) and DK 40355.

## REFERENCES

1. Mendel C. M.: The free hormone hypothesis: a physiologically based mathematical model. *Endocr. Rev.* **10** (1989) 232–274.
2. Heyns W. and De Moor P.: Kinetics of dissociation of 17β-hydroxysteroids from the steroid binding β-globulin of human plasma. *J. Clin. Endocr. Metab.* **32** (1971) 147–154.
3. Lata G. F., Hu H.-K., Bagshaw G. and Tucker R. F.: Equilibrium and kinetic characteristics of steroid interactions with human plasma sex steroid binding protein. *Arch. Biochem. Biophys.* **199** (1980) 220–227.
4. Mendel C. M., Weisiger R. A. and Cavalieri R. R.: Uptake of 3,5,3′-triiodothyronine by the perfused rat liver: return to the free hormone hypothesis. *Endocrinology* **123** (1988) 1817–1824.
5. Mendel C. M., Kuhn R. W., Weisiger R. A., Cavalieri R. R., Siiteri P. K., Cunha G. R. and Murai J. T.: Uptake of cortisol by the perfused rat liver: validity of the free hormone hypothesis applied to cortisol. *Endocrinology* **124** (1989) 468–476.
6. Weisiger R. A. and Ma W.-L.: Uptake of oleate from albumin solutions by rat liver: failure to detect catalysis of the dissociation of oleate from albumin by an albumin receptor. *J. Clin. Invest.* **79** (1987) 1070–1077.
7. Hammond G. L., Nisker J. A., Jones L. A. and Siiteri P. K.: Estimation of the percentage of free steroid

in undiluted serum by centrifugal ultrafiltration-dialysis. *J. Biol. Chem.* **255** (1980) 5023–5026.
8. Dunn J. F., Nisula B. C. and Rodbard D.: Transport of steroid hormones: binding of 21 endogeneous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. *J. Clin Endocr. Metab.* **53** (1981) 58–68.
9. Mendel C. M., Miller M. B., Siiteri P. K. and Murai J. T.: Rates of dissociation of steroid and thyroid hormones from human serum albumin. *J. Steroid Biochem. Molec. Biol.* **37** (1990) 245–250.
10. Siiteri P. K., Murai J. T., Hammond G. L., Nisker J. A., Raymoure W. J. and Kuhn R. W.: The serum transport of steroid hormones. *Recent Prog. Horm. Res.* **38** (1982) 457–503.
11. Mendel C. M., Cavalieri R. R. and Weisiger R. A.: Uptake of thyroxine by the perfused rat liver: implications for the free hormone hypothesis. *Am. J. Physiol.* **255** (1988) E110–E119.
12. Vigersky R. A., Kono S., Sauer M., Lipsett M. D. and Loriaux D. L.: Relative binding of testosterone and estradiol to testosterone-estradiol-binding globulin. *J. Clin. Endocr. Metab.* **49** (1979) 899–904.
13. Westphal U.: Steroid-binding serum globulins: Recent results. In Receptors and Hormone Action (Edited by B. W. O'Malley and L. Birnbaum). Academic Press, New York, Vol. 2 (1978) pp. 443–472.
14. Stroupe S. D., Harding G. B., Forsthoefel M. W. and Westphal U.: Kinetic and equilibrium studies on steroid interaction with human corticosteroid-binding globulin. *Biochemistry* **17** (1978) 177–182.
15. Burke C. W. and Anderson D. C.: Sex-hormone-binding globulin is an oestrogen amplifier. *Nature* **240** (1972) 39–41.
16. Rosner W. and Smith R. N.: Isolation and characterization of the testosterone-estradiol-binding globulin from human plasma. Use of a novel affinity column. *Biochemistry* **14** (1975) 4813–4820.
17. Vermeulen A.: Transport and distribution of androgens at different ages. In *Androgens and Antiandrogens* (Edited by L. Martini and M. Motta). Raven Press, New York (1977) pp. 53–65.
18. Strel'chenok O. A., Survilo L. I., Tsapelik G. Z. and Sviridov O. V.: Purification and physicochemical properties of the sex steroid-binding globulin of human blood plasma. *Biokhimiya* **48** (1983) 756–762.
19. Westphal U.: *Steroid-Protein Interactions II.* Springer, New York (1986) pp. 198–264.
20. Mendel C. M., Murai J. T., Siiteri P. K., Monroe S. E. and Inoue M.: Conservation of free but not total or non-sex-hormone-binding-globulin-bound testosterone in serum from Nagase analbuminemic rats. *Endocrinology* **124** (1989) 3128–3130.