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RESEARCH ARTICLE

COMPARISON STUDIES OF THE INTERACTIONS BETWEEN TESTOSTERONE AND PROGESTERONE WITH HUMAN SERUM ALBUMIN "HSA" : UV/FLUORESCENCE ABSORPTION

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ABSTRACT

The molecular interactions between HSA, Testosterone and Progesterone have been successfully investigated. In this study, the interaction between Testosterone, Progesterone and HSA has been investigated using UV-VIS absorption spectrophotometry. From UV absorption spectrophotometry it was shown that the absorption intensity decreasing with increasing the molecular ratios of Progesterone-HSA, while the absorption intensity increases with increasing the molecular ratios of Testosterone-HSA For VIS absorption spectrophotometer it was shown a decreasing in the absorption intensity with increasing the molecular ratios for both Progesterone and Testosterone. From both the UV-VIS spectrophotometer studies, is found that the value of the binding constant of testosterone to HSA, K equals 34.9×10^2 M^{-1}, While the binding constant (k) for the Progesterone is estimated as 6.56×10^2 M^{-1} at 293 K. From the results, it was observed that strong binding of the testosterone than that of progesterone.

INTRODUCTION

Hormones are the most familiar to the general public, due probably to the widespread pharmacological use and abuse of steroid hormones for diverse purposes, such as contraception and body building [1](Hardie, 1991). Steroids hormones mainly can act as a chemical messenger in a wide range of species and target tissues to produce both slow genomic responses and rapid non-genomic responses [2] (Norman *et al*. 2004). They have many physiological effects on human body; their incorrect concentration may cause abnormalities in human body [3] (Abu Teir *et al*. 2010). Steroid hormones help control metabolism, inflammation, immune functions, salt and water balance, development of sexual characteristics and the ability to withstand illness and injury[4] (Frye, CA 2009). In human all steroid hormones are derived from cholesterol and differ only in the ring structure and side chains attached to it[5] (Brandt 1999), so as cholesterol is a non-polar and hydrophobic molecule steroid hormones are insoluble in water but lipid soluble thus they have to be carried in the blood bound to specific carrier proteins such as sex hormone-binding globulin or corticosteroid-binding globulin. Sex steroid binding globulin carries testosterone and estradiol[4] (Frye, CA 2009).

Testosterone is a steroid hormone from the androgen group which is found in males and in smaller amount in female. It is 7-8 times concentrated in human males' plasma than in human females. The metabolic consumption of testosterone in males is greater. Testosterone is classified as a strong androgen and secreted primarily from the testicles of males and the ovaries of females, while small amounts of testosterone and weak androgens such as anabolic steroids are secreted by the adrenal gland [6](Reed WL *et al* 2006).

The chemical structure of testosterone is as shown in figure (1) ($C_{19}H_{28}O_2$) [5](Brandt., 1999). It is classified as Δ4 steroid as the double bond (un-saturation site) is located at 4-5 position. Testosterone chemical structure lacks the 2-carbon side-chain attached to the 17 position existed in the cholesterol structure, making it a 19-carbon steroid, also the side-chain has been replaced by a 17β-hydroxyl [5](Brandt 1999). Progesterone, is one of the steroid hormones, and belongs to a class of hormones called progestogens[3]. Progesterone is a C-21 steroid hormone; its chemical structure is shown in Figure 1 [4].

Human serum albumin HSA is an abundant plasma protein that binds a wide variety of hydrophobic ligands including fatty acids, bilirubin, thyroxine and hemin and also drugs[7,3] (Carter *et al*. 1989; Abu Teir *et al*. 2010). The most important physiological role for the protein is to bring such solutes into blood stream and then deliver them to the target organs, as well

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as to maintain the PH and osmotic pressure of plasma [2](Norman.A.W *et al*. 2004). HSA concentration in human plasma is 40 mg/ml [8] (Tushar *et al*. 2008). The molecular interactions between HSA and some compounds have been investigated successfully [9,10,11,3,12,13](Gudrum *et al*. 2002; Ouameur *et al*. 2004; Ji-Sook *et al*. 2006; Abu Teir *et al*. 2010; Abu Teir *et al*. 2014; Darwish *et al*. 2010). It has recently been proved that serum albumin plays a decisive role in the transport and disposition of variety of endogenous and exogenous compound such as fatty acids, hormones, bilirubin, drugs[14] (Tang *et al*. 2006).

This work will be limited to spectroscopy techniques that are usually used in studying the interaction of drugs and proteins, fluorescence and UV spectroscopy are commonly used because of their high sensitivity, rapidity and ease of implementation. [15,16,17](Wybranowski, T *et al*. 2008; Li, J *et al*. 2008; Li,Y *et al*. 2006).

The binding of testosterone, progesterone to HSA was investigated by means of UV-absorption spectroscopy, and fluorescence spectroscopy. Spectroscopic evidence regarding the drug binding mode, drug binding constant effects of testosterone and progesterone on the protein are provided in this work.

MATERIALS AND METHODS

Materials

HSA (fatty acid free), testosterone, progesterone in powder form were purchased from Sigma Aldrich chemical company and used without further purifications. The data were collected using samples in the form of liquid form for UV-VIS and fluorescence measurements.

Preparation of stock solutions

HSA was dissolved in phosphate buffer saline, at physiological pH 7.4, to a concentration of (80 mg/ml), to get a final concentration of (40 mg/ml) in the final hormone-HSA solution.

Testosterone with molecular weight (288.42 g/mol) was dissolved in phosphate buffer saline (0.7622 mg/ml), the phosphate buffer saline was at room temperature, the solution was placed on a shaker for one hour in order to dissolve the testosterone powder with buffer, then it was placed in ultrasonic water path (SIBATA AU-3T) for 8 hours to ensure that the entire amount of testosterone was completely dissolved. The solution was placed in a water path with a temperature range 37-40c° for one hour to let the solution completely dissolved and became homogenous. The final concentrations of HSA-Testosterone solutions were prepared by mixing equal volume of HSA and hormone. HSA concentration in all samples kept at 40 mg/ml. However, the concentration of hormone in the final protein hormone solutions was reduced such that the molecular ratios (HSA: Testosterone) are 10:18, 10:14, 10:10, 10:6 and 10:2. For Progesterone which has molecular weight of (314.47g/mol),the same preparation process was done for HSA-Progesterone solutions and the final protein Progesterone solutions was reduced such that the molecules ratio (HSA:Progesterone) are 10:18, 10:14, 10:10, 10:6, and 10:2.

UV-absorption spectroscopy

The absorption spectra were obtained by the use of a NanoDrop ND-1000 spectrophotometer. It is used to measure the absorption spectrum of the samples in the range between 220-750 nm, with high accuracy and reproducibility.

The absorption spectra of different ratios of testosterone/progesterone with fixed amount of HSA are displayed in figure (2). The figure shows that the UV intensity of HSA increases with the increasing of testosterone percentage, while the UV intensity decreases with the increasing of progesterone percentage, and that the absorption peaks of these solutions showed moderate shifts indicating that with the addition of testosterone and progesterone, the peptide strands of HSA molecules extended more and the hydrophobicity of the two hormones was decreased [18](Peng *et al*. 2008). The results indicated that an interaction occurred between testosterone, progesterone and HSA. Obviously, it was seen from the spectrum that pure hormone have little or no UV absorption. This result support that the peak shifts between free HSA solution and the two hormones-HSA complexes are due to the interaction between the two hormones and HSA. Repeated measurements were done for all the samples showing consistent results and no significant differences were observed.

Fluorescence spectrophotometer

The fluorescence measurements were performed by a NanoDrop ND-3300 Fluorospectrophotometer at 25°C. The excitation source comes from one of three solid-state light emitting diodes (LED's). The excitation source options include: UV LED with maximum excitation 365 nm, Blue LED with excitation 470 nm, and white LED from 500 to 650nm excitation. A 2048-element CCD array detector covering 400-750 nm, is connected by an optical fiber to the optical measurement surface. The excitation is done at the wavelength of 360 nm and the maximum emission wavelength is at 439 nm. In this work for HSA-Testosterone/Progesterone complexes excitation wavelength at 360nm was used, and the observed wavelength emission was at 439 nm. The fluorescence sensor is based on intramolecular charge transfer (ICT), which is highly sensitive to the polarity of microenvironment. Therefore, it is expected to act as fluorescent probe for some biochemical systems like proteins [19](Tian *et al*. 2003).

The fluorescence quenching spectra of HSA at various percentage of testosterone/progesterone is shown in figure (4). Obviously from the results, the fluorescence intensity of HSA gradually decreased while the peak position shows little change upon increasing the percentage of testosterone/progesterone, indicating that testosterone/progesterone binds to HSA. Under the same condition, no fluorescence of testosterone/progesterone was observed. Which indicates that

testosterone/progesterone could quench the auto fluorescence of HSA, and that the interaction between testosterone/progesterone and HSA exists, leading to a change in the microenvironment around the tryptophan residue and further exposure of tryptophan residue to the polar solvent [20,21](Petitpas *et al*. 2001; Wang. *et al*. 2007).

RESULTS AND DISCUSSION

Analysis of UV-absorption spectroscopy of HSA by Testosterone/Progesterone

Testosterone –HSA complexes binding constant were determined using UV-VIS spectrophotometer results according to published method[22,23,10] (Stephanos *et al*. 1996; Koltz *et al*. 1971; Ouameur *et al*. 2004) , by assuming that there is only one type of interaction between testosterone/progesterone and HSA in aqueous solution, which leads to establish equation (1) and (2) as follows:

HSA+Testosterone/Progesterone ⟷
Testosterone/Progesterone: HSA .....(1)

K=[Testosterone/Progesterone: HSA] /[Testosterone/Progesterone] [HSA] ……..(2)

The absorption data were treated using linear double reciprocal plots based on the following equation[24] (Lakowicz 2006) :

$$\frac{1}{A-Ao} = \frac{1}{A\infty-Ao} + \frac{1}{K[A\infty-Ao]} \times \frac{1}{L} \quad \text{……..(3)}$$

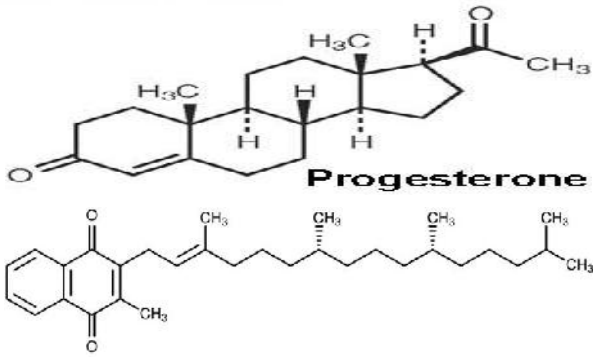


Figure 1 Chemical structures of Testosterone and Progesterone.

Where A_0 corresponds to the initial absorption of protein at 280 nm in the absence of ligand , A_∞ is the final absorption of the legated protein, and A is the recorded absorption different testosterone/progesterone concentrations (L). The double reciprocal plot of $1/(A-A_0)$ vs. 1/L is linear (figure 3) and the binding constant (K) can be estimated from the ratio of the intercept to the slope to be ($0.349 \times 10^4\ M^{-1}$) for testosterone-HSA complexes and ($6.56 \times 10^2\ M^{-1}$) for progesterone-HSA complexes. In spite that the strong interaction of Testosterone with HSA more than that of Progesterone with HSA, except that these values which are obtained in this work is an indication of a weak interaction of the two hormones with the protein with respect to the other drug-HSA complexes with binding constant in the range of 10^5 and $10^6\ M^{-1}$ [25](Kargh Hanse 1981).

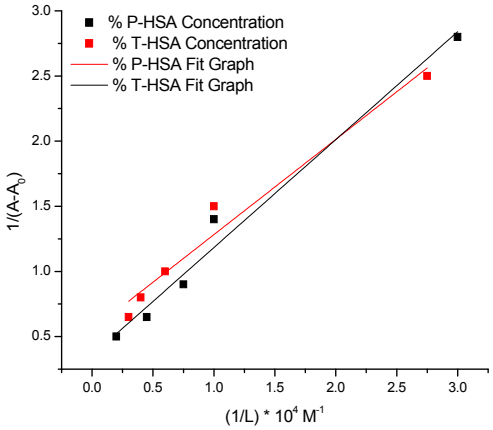
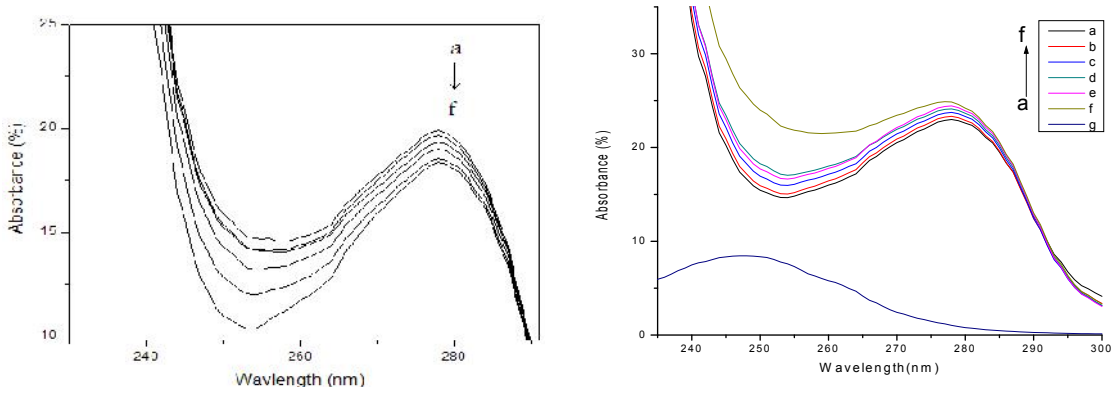


Figure 3 The plot of $1/(A-A_0)$ vs 1/L for HSA with different concentrations of progesterone and testosterone.

Analysis of Fluorescence spectroscopy of HSA by Testosterone/Progesterone

Fluorescence quenching can be defined as a bimolecular process that reduces the fluorescence intensity without changing the fluorescence emission spectrum; it can result from transient excited-state interactions (collisional quenching) or from formation of non-fluorescent ground-state species.



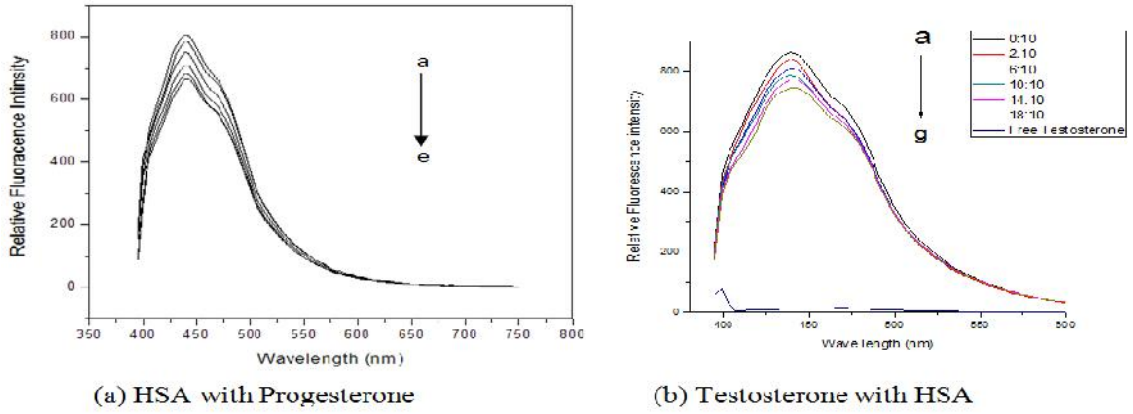
(a) HSA with Progesterone (b) Testosterone with HSA

Figure2 (a) UV-Absorbance spectra of HSA with different contents of progesterone (a=0:10, b=2:10, c=6:10, d=10:10, e=14:10, f=18:10). (b) UV-absorbance spectra of HSA with different molar ratios of testosterone (a=0:10, b=2:10, c=6:10, d=10:10, e=14:10, f=18:10, g= free testosterone)

As discussed in chapter two assuming dynamic quenching is dominating, then the decreased in intensity is described by the well-known Stern-Volmer equation:

$$F_0/F = 1+ K_q \tau_0 (L) = 1+ K_{sv}(L) \quad \text{……..(4)}$$

In this expression F and F_0 are the fluorescence intensities with and without quencher, K_{sv} is the Stern-Volmer quenching constant, K_q is the bimolecular quenching constant, τ_0 is average lifetime of the biomolecule without quencher, and (L) is the quencher concentration. The Stern-Volmer quenching constant K_{sv} indicates the sensitivity of the fluorophore to a quencher.



(a) HSA with Progesterone (b) Testosterone with HSA

Figure4 (a) Fluorescence emission spectra of HSA in the absence and presence of progesterone in these ratios (P:HSA a=0:10, b=2:10, c=6:10, d=10:10, e=14:10, f=18:10). (b) Fluorescence emission spectra of HSA in the absence and presence of testosterone in these ratios (T: HSA a=0:10, b=2:10, c=6:10, d=10:10, e=14:10, f=18:10, g=free testosterone).

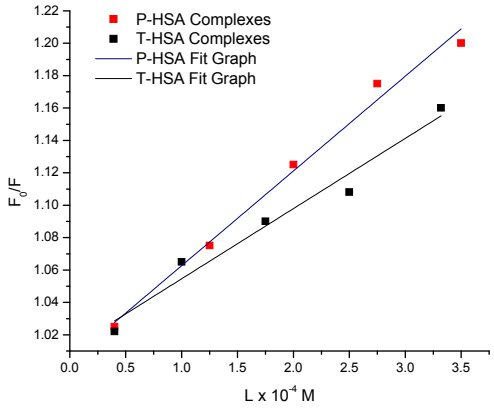


Figure 5 The Stern Volmer plot for Progesterone and Testosterone –HSA complexes.

Linear curves were plotted according to the Stern-Volmer equation as shown in figure (5) for testosterone/progesterone-HSA complexes. The Stern-Volmer quenching constant K_{sv} was obtained by the slope of the curves obtained in figure (5), and its value equals (0.0456×10^4 L mol^{-1}) for testosterone-HSA complexes. From equation 4 the value of $K_{sv} = K_q \tau_0$, from which we can calculate the value of K_q using the fluorescence life time of 10^{-8} s for HSA [26](Cheng *et al*), to obtain K_q values of (4.5×10^{10} L $mol^{-1}s^{-1}$) for testosterone-HSA complexes, while for the progesterone-HSA complexes, the Stern-Volmer quenching constant K_{sv} was obtained by the slope of the curves obtained in figure (5), and its value equals (6.26×10^2 L mol^{-1}). From equation 4 the value of $K_{sv} = K_q \tau_0$, from which we can calculate the value of K_q using the fluorescence life time of 10^{-8} s for HSA [26](Cheng *et al*), to obtain K_q values of (6.2×10^{10} L $mol^{-1}s^{-1}$) for progesterone-HSA complexes Which is larger than the maximum dynamic quenching constant for various quenchers with biopolymer (2×10^{10} L mol^{-1} s^{-1}) [24](Lakowicz 2006). This implies that the quenching is not initiated by dynamic collision but from formation of a complex, so static quenching is dominant [21] (Wang *et al*. 2008).

When static quenching is dominant the modified Stern-Volmer equation could be used [27](Yang, *et al* , 1994)

$$\frac{1}{Fo-F} = \frac{1}{FoKL} + \frac{1}{Fo} \quad \text{……..(5)}$$

Where K is the binding constant of testosterone with HSA, and can be calculated by plotting 1/ (F_0-F) vs 1/L, see figure (6). The value of K equals the ratio of the intercept to the slope. The obtained value of K for testosterone-HSA complexes equals (0.382×10^4 M^{-1}) while the obtained values of K equals 6.56×10^2 M^{-1} for progesterone- HSA complexes as shown from Figure(3) which agrees well with the value obtained earlier by

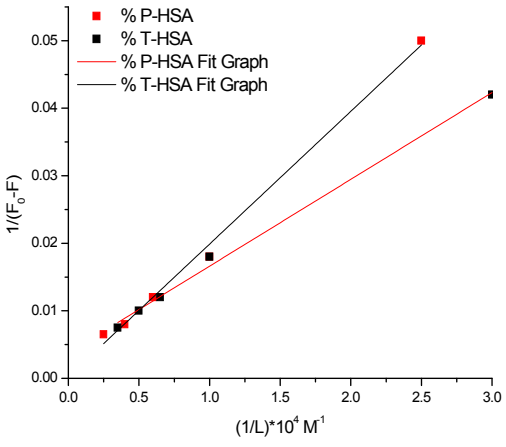


Figure 6 The plot of 1/(F_0-F) vs (1/L) $\times10^4$ for Progesterone and Testosterone –HSA complexes

UV spectroscopy and supports the effective role of static quenching from Figure(6). The highly effective quenching constant in this case has lead to a lower value of binding constant between the hormone and HSA, due to an effective hydrogen bonding between testosterone/progesterone and HSA [13](Darwish *et al*. 2010).

The acting forces between a small molecule substance and macromolecule mainly include hydrogen bond, van der- waals force, electrostatic force and hydrophobic interaction force. It was more likely that hydrophobic and electrostatic interactions were involved in the binding process.

However, testosterone/progesterone might be largely unionized under the experimental conditions, as expected from its structures. Hence, electrostatic interaction could be precluded from the binding process. Thus, the binding of testosterone/progesterone to HSA includes the hydrophobic interaction. Hydrophobic interaction is mostly an entropic effect originating from the disruption of highly dynamic hydrogen bonds between molecules of liquid water by the nonpolar solutes. Minimizing the number of hydrophobic side chains exposed to water is the principal driving force behind the folding process [28](Pace C *et al* 1996). Formation of hydrogen bonds within the protein stabilizes protein structure [29](Rose G, *et al* 2006).

Previous experiment have been applied to investigate the interaction of cholesterol with HSA [3,30](Abu Teir *et al* 2010; Abu Teir *et al* 2012), by UV –VIS spectroscopy in addition to investigation of the fluorescence quenching for both hormones. In this experiment the same experimental procedure has been applied. The constant obtained were the binding constant k using UV-VIS spectrophotometer, the Stern-Volmer quenching constant K_{sv} and the binding constant using fluorescence spectrophotometer. Those constants for the two hormones are listed in table 1.

Table 1 Comparison between Testosterone and Progesterone Binding Constants: UV/Fluorescence absorptions.

Hormone	k_s-Stern Constant (L Mol^{-1})	k_q-Quenching Constant (L $Mol^{-1}s^{-1}$)	K-Binding Constant (UV) (M^{-1})	K-Binding Constant (Fluorescence) (M^{-1})
Testosterone	4.5×10^2	4.5×10^{10}	34.9×10^2	38.23×10^2
Progesterone	6.26×10^2	6.2×10^{10}	6.354×10^2	6.56×10^2

CONCLUSION

In this work, the interaction between testosterone (male hormone) and progesterone (female hormone) with HSA Albumin (the universal hydrophobic molecule carrier) have been investigated using spectroscopic techniques (UV-VIS spectrophotometers). Our experimental work showed relatively high binding affinity between testosterone and HSA relatively of progesterone and HSA. Referring to UV spectrum the calculated binding constant for testosterone-HSA is ($K= 34.9\times10^2\ M^{-1}$) while for progesterone-HSA is ($6.354\times10^2 M^{-1}$). The analysis of fluorescence spectrum yield binding constant for testosterone-HSA interaction, it has been measured to be ($K=38.23\times10^2\ M^{-1}$) while for progesterone-HSA it has been measured to be ($6.56\times10^2\ M^{-1}$). The binding constant obtained by different methods has very close values. Comparing the binding constant for testosterone with the previously measured binding constants of cholesterol and progesterone, regarding to that one can conclude, that testosterone-HSA interaction is the strongest as it is considered to be both hydrogen donor and acceptor. Cholesterol interaction with albumin was substantially weaker than testosterone-HSA interaction but stronger than progesterone-HSA interaction. The value of Stern-Volmer quenching constant and quenching rate constant for testosterone have been measured to be ($K_{sv}= 4.5\times10^2$ L Mol^{-1}, $K_q= 4.5\times10^{10}$ L $Mol^{-1}s^{-1}$), while the value of Stern-Volmer quenching constant and quenching rate constant for progesterone have been measured to be ($K_{sv}= 6.26\times10^2$ L Mol^{-1}, $K_q= 6.2\times10^{10}$ L $Mol^{-1}s^{-1}$). These experimental results static quenching is responsible for the fluorescence quenching (decrease of intensity). This is an indication for complex formation between the protein and hormone.

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