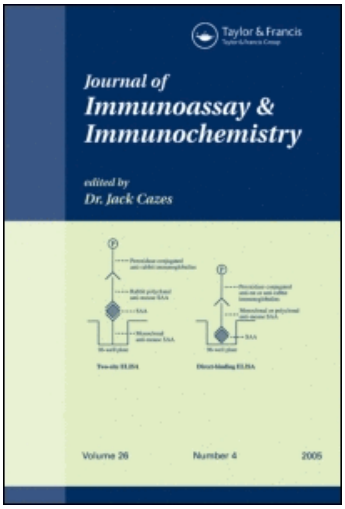

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DEVELOPMENT OF HETEROLOGOUS ENZYME LINKED IMMUNOSORBENT ASSAY FOR DEHYDROEPIANDROSTERONE IN SERUM

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□ *Homologous and heterologous combinations of enzyme conjugate with immunogen in steroid enzyme immunoassay (EIA) influence labeled steroid recognition by an antibody that affects the sensitivity of the assay. To develop dehydroepiandrosterone (DHEA) enzyme linked immunosorbent assay (ELISA), antibodies were generated against dehydroepiandrosterone-17-carboxymethyloxime-bovine serum albumin (DHEA-17-CMO-BSA) and dehydroepiandrosterone-7-carboxymethyloxime-bovine serum albumin (DHEA-7-CMO-BSA). Two dehydroepiandrosterone (DHEA) horse radish peroxidise (HRP) enzyme conjugates were prepared using two dehydroepiandrosterone derivatives (DHEA-17-CMO and DHEA-7-CMO). Four combinations of homologous and heterologous assays were evaluated. The use of heterologous combination improved the sensitivity of the assay.*

Keywords dehydroepiandrosterone, heterologous, homologous, immunoassay, site heterology

INTRODUCTION

Dehydroepiandrosterone (DHEA) is the most abundant C19 steroid secreted by the cortex region of the adrenal glands.[1] Often, DHEA is referred to as a weak androgen; however, there is no evidence that it binds to androgen receptors. Thus DHEA has little or no intrinsic androgen activity. However, it converts into androstenedione and then into potent

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androgens and estrogens in the liver and other target organs.[2] In both sexes, DHEA levels vary profoundly throughout the life cycle. Levels are high at birth but quickly decline within a few months. Levels start increasing in children 8 to 10 years of age, peaking by the middle or end of the second decade of life. Levels then decline by 10% per decade, plateauing after a person is older than 80 years. DHEA has been considered to be a biomarker of aging.[3–6] Adrenal DHEA secretion is episodic and follows a diurnal rhythm, similar to that of cortisol, under the stimulus of corticotropin releasing hormone (CRH) and adrenocorticotrophic hormone (ACTH).[4,7] In contrast to age-associated decline in adrenal DHEA, basal and ACTH-stimulated concentrations of cortisol change minimally with aging, suggesting an ACTH-independent, age-related diminution in adrenal DHEA secretion.[8] Circulating DHEA is bound to albumin in plasma, but no binding globulin has been identified.[9]

Measurement of serum DHEA is a useful marker of adrenal androgen synthesis. Lowered levels of DHEA have been associated with critical illness, emotional stress, and a variety of medical conditions including rheumatic disease,[10] cardiovascular disease,[11] immune system disorders,[12] and osteoporosis.[13] Elevated levels have been observed in connection with obesity and type II diabetes,[14] female hirsuitism,[15] and prolonged physical stress.[16] Since very little DHEA is produced by the gonads, measurement of DHEA levels may aid in the localization of the androgen source in virilizing conditions.

For measurement of DHEA in serum, commercial ELISA kits are available, but no single published article is available in the literature for development of ELISA for DHEA. The lack of an enzyme immunoassay (EIA) system for DHEA analysis has prompted us to carry out the present work. This article describes the development of heterologous ELISA for DHEA.

EXPERIMENTAL

This study was conducted according to the Institutional Ethical Committee's norms.

Chemicals and Reagents

All solvents, chemicals, and salts used in the present study were of analytical grade and were used without prior purification. All steroids used for the synthesis and cross-reactivity were obtained from Sterloids, Inc., Newport, CT, USA. Bovine serum albumin, N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), complete Freund's adjuvant, gelatin, and thimerosal were purchased from

Sigma Chemical Company, St. Louis, MO, USA. Horseradish peroxidase and tetramethyl-benzidine/hydrogen peroxide (TMB/H_2O_2) solution were purchased from Bangalore Genei, Bangalore, India, and Arista Biochemical, Allentown, PA, USA, respectively. Microtiter plates were procured from Greiner, Frickenhausen, Germany.

BUFFERS

The most frequently used buffer was 10 mM phosphate (10 mM PB), pH 7.0, (Na_2HPO_4 $2H_2O$: 0.895 g/L and NaH_2PO_4 $2H_2O$: 0.39 g/L) containing 0.9% NaCl (10 mM PBS) and 0.1% NaN_3.

The HRP conjugate dilution buffer was 10 mM acetate buffer (10 mM AB), pH 5.6, (CH_3COONa: 0.84 g/L and 1 N CH_3COOH 1.5 mL/L), containing 0.1% thimerosal and dextran T-70, 0.3% BSA.

The microtiter well blocking and stabilizing buffer was 10 mM PB containing 0.9% NaCl, 0.2% BSA, 0.1% gelatin, thimerosal, dextran T-70, ethylene diamine tetra acetic acid:di-potassium salt (EDTA:K salt), and 0.01% gentamicin sulfate.

METHODS

Preparation of DHEA-17-CMO-BSA and DHEA-7-CMO-BSA

DHEA-17-CMO and DHEA-7-CMO were coupled to BSA by an N-hydroxy succinimide ester method with modification, described elsewhere.[17] To 10 mg DHEA-17-CMO or DHEA-7-CMO 400 µl of each, dioxan and dimethyl formamide were added. To this solution, 100 µl of water containing 20 mg of NHS and 40 mg of EDAC was added. The reaction mixture was vortex-mixed and kept for overnight at 4°C for activation. The activated steroid was added slowly to the aqueous solution of BSA (1 mg/0.3 mL) with swirling. The reaction mixture was further vortex-mixed and kept for overnight at 4°C. The DHEA-17-CMO-BSA or DHEA-7-CMO-BSA conjugate was then dialyzed against three changes of distilled water. The dialysate was centrifuged to remove any precipitate formed. It was thereafter lyophilized and kept at 4°C in aliquots of 1 mg for immunization.

Immunization of Rabbit and Collection of Antiserum

The antibody against DHEA-17-CMO-BSA or DHEA-7-CMO-BSA was generated in New Zealand white rabbits according to the method by Shrivastav et al.[17] In brief, an emulsion of 0.5 mL of Freund's complete adjuvant in 0.5 mL of saline containing 1 mg of DHEA-17-CMO-BSA or

DHEA-7-CMO-BSA immunogen was freshly prepared. 250 μL of emulsion was injected intramuscularly in each limb of a New Zealand white rabbit. Intramuscular injections of DHEA-BSA emulsions were repeated on days 7, 14, 21, and 28. Following the five primary injections, booster injections were given every 30 days thereafter. Blood was regularly collected between 9–14 days after booster injection, starting from the first booster injection. Antiserum was collected after centrifugation at 750× g for 10 min and stored at −30°C in 1 mL aliquots.

Collection of Normal Rabbit Serum (NRS)

NRS was collected from non-immunized New Zealand white rabbits and stored at −30°C.

Generation of Anti-Rabbit Gamma Globulin (ARGG)

A one-year-old Sirohi goat was immunized with non-immunized rabbit IgG following the method by Shrivastav et al.[17] After immunization, goat serum was collected and stored at −30°C.

Preparation of DHEA-7-CMO-HRP and DHEA-17-CMO-HRP

DHEA-7-CMO or DHEA-17-CMO was coupled to HRP by an active ester method with some modification following the method of Shrivastav et al.[17] To 5 mg of DHEA-7-CMO or DHEA-17-CMO, 200 μL of each of dioxan and dimethyl formamide were added. To this solution, 100 μL of water containing 10 mg NHS and 20 mg EDAC was added. The reaction mixture was activated for 24 h at 4°C. Activated DHEA-17-CMO or DHEA-7-CMO solution was added to the aqueous solution of HRP (1 mg/mL) and kept for 24 h at 4°C. Then the reaction mixture was passed through a G-25 column, previously equilibrated with 10 mM PBS containing 0.1% thimerosal. The brown colored fractions containing enzyme activity were pooled, and to them 1% of sucrose, ammonium sulfate, BSA, and an equal volume of ethylene glycol were added. The solution was kept at −30°C in aliquots for future use.

Checkerboard: Coating of Antibody to Microtiter Plates

The 96-well microtiter plate was coated using the immunobridge technique for primary antibody immobilization according to the method of Shrivastav et al.[17] In brief, 250 μL of NRS diluted (1:250) in water was dispensed into each well and incubated at 37°C overnight. Following

incubation, the plate was washed under running tap water 15–20 times. To the NRS-coated wells, 250 µL of 1:1000 diluted goat anti-rabbit gamma globulin (ARGG) was added and incubated for 2 h at 37°C. Thereafter, plate content was decanted and washed under running tap water.

The DHEA antiserum against DHEA-17-CMO-BSA or DHEA-7-CMO-BSA was serially diluted in phosphate buffer containing 0.1% NaN3 and 0.3% gelatin (1:500, 1:1000, 1:2000, 1:4000, and 1:8000), and 200 µL was added into the wells (one dilution per eight-well strip); for non-specific binding (NSB), 200 µL of phosphate buffer was added in a separate eight-well strip and incubated for 1 h at 37°C. Then the plate content was decanted and washed under running tap water.

For blocking of unoccupied sites of the microwell surface, microwell blocking and stabilizing buffer, 250 µL, was added to all the wells and incubated for 1 h at 37°C.

Determination of Optimal Loading of Primary Antibody Using DHEA-7-CMO-HRP and DHEA-17-CMO-HRP Conjugates

The amount of primary antibody (DHEA-17-CMO-BSA or DHEA-7-CMO-BSA antiserum) immobilized on microtiter wells was determined by adding 100 µL of enzyme conjugate (DHEA-17-CMO-HRP and DHEA-17-CMO-HRP) diluted serially in conjugate dilution buffer, 1:500, 1:1000, 1:2000, and 1:4000, in wells (one dilution per two wells in vertical fashion). This was incubated for 1 h at 37°C.

After incubation, the contents of the wells were decanted and washed in running tap water five to six times by filling, decanting, and tapping. Finally, for measuring bound enzyme activity (which is a direct function of primary antibody), 100 µL of TMB/H_2O_2 substrate solution was added to all the wells and incubated for 15 min at 37°C. The reaction was stopped by adding 100 µL of 0.5M H_2SO_4, and the color intensity was measured at 450 nm in a Tecan-Spectra ELISA plate reader.

Preparation of DHEA Standard

Seven DHEA-working standards (0 ng/mL, 1.0 ng/mL, 3 ng/mL, 5 ng/mL, 10 ng/mL, 25 ng/mL, and 50 ng/mL) were prepared in stripped pooled serum.

Standard Displacement Assay

To the antibody-coated wells, 100 µL of different concentrations of standard were added in duplicate. 100 µL of the working dilution of the

enzyme conjugate (DHEA-17-CMO-HRP/DHEA-17-CMO-HRP) were added to all of the wells, which were then incubated for 1 h at 37°C. After incubation, the contents of the wells were decanted, and the wells were washed in running tap water. Finally, to measure the bound enzyme activity, 100 µL of TMB/H_2O_2 substrate was added to all the wells and kept for 20 min at rt. Thereafter 100 µL of 0.5 M H_2SO_4 was added to all the wells. The developed yellow color was read at 450 nm in a Tecan Spectra micro-plate reader.

Selection of the Best Combination of Antiserum and Enzyme Conjugate

Two DHEA antibodies were generated (DHEA-17-CMO-BSA and DHEA-7-CMO-BSA), and two enzyme conjugates (DHEA-17-CMO-HRP and DHEA-7-CMO-HRP) were prepared. Using these antibodies and enzyme conjugates, four different combinations (heterologous and homologous) of DHEA assay were formulated, as follows:

Combination 1: Antibody DHEA-17-CMO-BSA and enzyme conjugate DHEA-17-CMO-HRP (homologous assay)
Combination 2: Antibody DHEA-17-CMO-BSA and enzyme conjugate DHEA-7-CMO-HRP (site heterologous assay)
Combination 3: Antibody DHEA-7-CMO-BSA and enzyme conjugate DHEA-7-CMO-HRP (homologous assay)
Combination 4: Antibody DHEA-7-CMO-BSA and enzyme conjugate DHEA-17-CMO-HRP (site heterologous assay)

The above-mentioned four assay combinations were tested in the standard displacement assay as described above. The combination of antiserum and DHEA–enzyme conjugate, which displayed the best affinity, slope of curve, sensitivity, and specificity, was chosen.

DATA ANALYSIS

Preparation of Standard Curve, Determination of Affinity Constant and Sensitivity

The standard curve was prepared using MS Excel. The concentration was plotted in the *X*-axis (log scale) and the A/A0 × 100 in the *Y*-axis. Values of the unknown samples were calculated by an in-house–developed PC program written in QBASIC language using the logit–log linear regression method according to the method of Rodbard.[18] The affinity constant of the DHEA antibody for the antigen was estimated by the Scatchard plot

according to method of Feldman and Rodbard.[19] The sensitivity of the developed method was determined according to the method of Rodbard.[20]

Method Comparison

Method comparison was tested by analyzing the extracted and non-extracted same samples by the developed ELISA.

Statistical Analysis

The statistical analyses, such as mean, standard deviations, logit log transformation, and correlation coefficient, of the data were done using the MS Excel. The results follow.

Standard Curve

Both the homologous combinations (DHEA-7-CMO antiserum with DHEA-7-CMO enzyme conjugate and DHEA-17-CMO antiserum with DHEA-17-CMO enzyme conjugate) and one heterologous combination (DHEA-7-CMO antiserum with DHEA-17-CMO enzyme conjugate) did not show displacement. The composite dose–response curve of the heterologous combination (DHEA-17-CMO antiserum with DHEA-7-CMO enzyme conjugate) of DHEA ELISA is shown in Figure 1. The CVs for the A/A0 ratio of each standard ranged from 2.85% to 5.65% for the enzyme immunosorbent assay. Thus the standard curves obtained over several assays remained stable and precise. When logit-log transformation of the standard curve was conducted, the equation for the relationship for ELISA was $y = -1.776x + 1.689$ ng/mL (Figure 2). The association constant (Ka) of the DHEA antibody towards DHEA was estimated by using a Scatchard plot and was found to be 1.25×10^{-8} L/mol.

Sensitivity

The assay sensitivity is usually expressed in terms of its lower detection dose (LDD) and the effective displacement at 50% (ED_{50}). The LDD is the lowest concentration of analyte (A) that gives a response statistically different from that observed in the absence of analyte (A0). It is calculated as A0−2SD, after 32-fold determination of A0. The ED_{50} is the effective concentration at which 50% of inhibition in the binding of enzyme conjugates occurs in assays in the presence of analyte. It is calculated as $ED_{50} \pm SD$, after 8 times determination of ED_{50}. The LDD and the ED_{50} of the present assay are 0.5 ng/mL and 8.5 ng/mL, respectively.

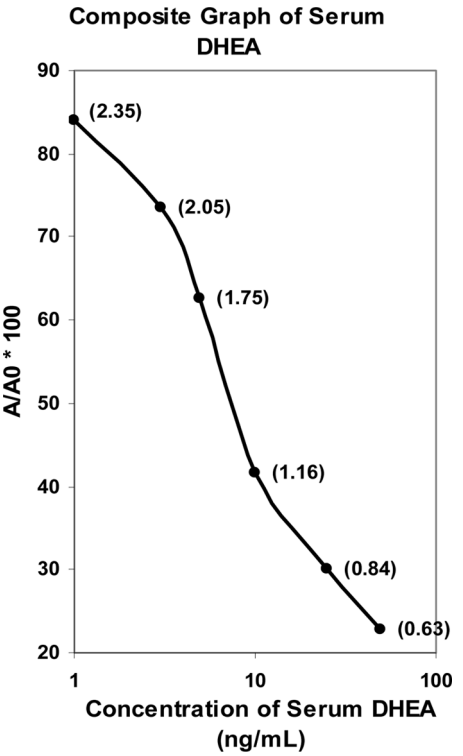

FIGURE 1 Composite dose–response curve of DHEA site heterologous ELISA using DHEA-17-CMO antiserum and DHEA-7-CMO enzyme conjugate. Each value is a mean ± SD of eight assays (in duplicate). The coefficient of variation at each concentration is shown in parentheses.

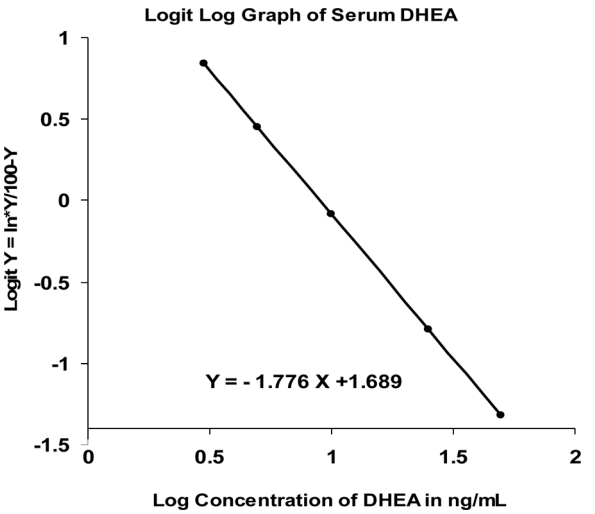

FIGURE 2 Logit-log transformation of composite dose–response curve of DHEA site heterologous ELISA using DHEA-17-CMO antiserum and DHEA-7-CMO enzyme conjugate.

Specificity

The specificity of the DHEA-17-CMO-BSA antibody was estimated as the percentage of cross-reaction with 47 commercially available steroids with analogous structure. The DHEA-17-CMO-BSA antibody showed 15.38% and 16.66% cross-reaction with androstenediol and testosterone, respectively, and less than 0.1% cross-reaction with other structurally related steroids. The percent cross-reaction was calculated from the following formula:

$$\%\text{cross-reaction} = \frac{\begin{array}{c}\text{Concentration of DHEA, required to}\\ \text{give 50\% inhibition}\end{array}}{\begin{array}{c}\text{Concentration of related steroid,}\\ \text{required to give 50\% inhibition}\end{array}} \times 100$$

Recovery

The ability of an assay to accurately quantify DHEA in serum was tested. Table 1 represents the percent recoveries of known amounts of DHEA added to four 10-mL aliquots of serum pools. It ranged from 98.21–102.31%.

Precision

Serum specimens containing approximately the same concentrations of DHEA were combined to form five pools with different concentrations. We analyzed each pool six times within the assay and also in six separate assays. Table 2 depicts the intra- and interassay coefficient variations. The intra- and interassay coefficient variations ($n = 6$, replicate of each pool) were $<11.5\%$.

Correlation Coefficient

The correlation coefficient for values of DHEA in extracted and non-extracted serum samples ($n = 50$) measured by developed ELISA are

TABLE 1 Recovery of DHEA from Exogenously Spiked Serum Pools

Human Serum Pools	DHEA Added (ng/mL)	DHEA Observed (ng/mL)	DHEA Expected (ng/mL)	% Recovery
Pool A (Basal)	–	1.24	–	–
Pool B	1.0	2.20	2.24	98.21
Pool C	3.0	4.34	4.24	102.32
Pool D	5.0	6.29	6.24	100.80
Pool E	10.0	11.21	11.24	99.73

TABLE 2 Intra- and Interassay Coefficient of Variation for the Measurement of DHEA in Human Serum Pools

Variation	Sample Value ng/mL (mean ± S.D.)	% Coefficient of Variation
Intra-assay $n=6$	1.24 ± 0.08	6.76
	2.20 ± 0.09	4.67
	4.34 ± 0.13	3.04
	6.29 ± 0.38	6.07
	11.21 ± 1.25	11.15
Inter-assay $N=6$	2.57 ± 0.25	9.96
	3.66 ± 0.21	5.93
	5.48 ± 0.33	6.07
	7.60 ± 0.21	2.80
	12.27 ± 0.48	3.97

n = Number of times same sample was analyzed for intraassay variation; N = number of times assays were carried out for interassay variation.

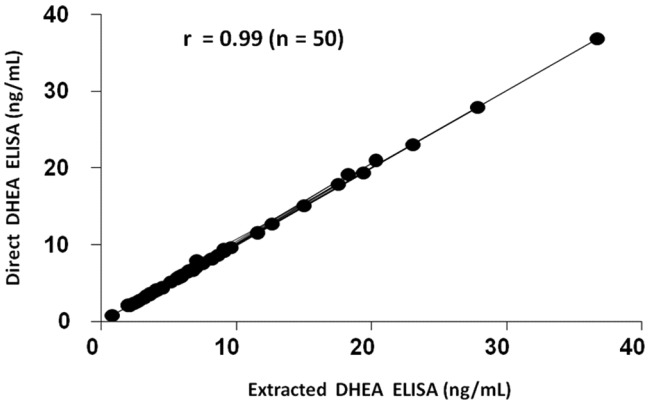

FIGURE 3 The correlation between direct and extracted DHEA estimated from serum by site heterologous ELISA using DHEA-17-CMO antiserum and DHEA-7-CMO enzyme conjugate. The linear regression of the correlated data was plotted by Graph Pad Prism Version 5 for Windows. The correlation coefficient, $r=0.99$ ($n=50$).

found to be 0.99, i.e., $r=0.99$. The linear regression curve of the correlated data (plotted by Graph Pad Prism Version 5 for Windows) is given in Figure 3.

DISCUSSION

In steroid enzyme immunoassays (EIAs), the association of hapten–enzyme conjugate with the antibody varies from that of the hapten itself. This has been recognized by Van Weemen and Schuurs,[21] who classified

EIAs as homologous or heterologous assays. In the former systems, the hapten derivative used for labeling the enzyme and for conjugation with the carrier protein for developing antibodies is the same, in which the antibody, being more specific, has a greater affinity for the hapten–enzyme conjugate, making it less sensitive. In heterologous systems, minor structural variations in the hapten used for enzyme labeling and that of hapten–protein conjugates used for antibody production proved to be more sensitive.[21–29] On the other hand some sensitive homologous systems have also been reported;[29–31] in such assays, apparently the affinity characteristics of the antibody influenced the sensitivity of the EIA. In addition, specificity of the antibody to measure a particular hapten in question may also change with the different hapten derivatives used for labeling with the enzyme.

The earlier observations of Van Weemen and Schuurs[21] and Exley and Abuknesha[22] on the importance of heterologous EIAs for developing sensitive assays for estrogens seemed to be supported by the present findings on the influence of homologous and heterologous systems on the sensitivity of ELISA for DHEA. While the results of the present study confirm that all homologous EIAs are less sensitive compared with heterologous systems, the latter assay system in certain combinations sometimes became non-functional in determining the analyte.[32] The results of present study confirm that certain combinations in heterologous systems are less sensitive. It has been observed that site heterologous combination, DHEA-7-CMO antiserum with DHEA-17-CMO enzyme conjugate, did not show displacement. This may be due to a change in the confirmation of DHEA-17-CMO after coupling with HRP that may cause a tight fit in the binding pocket of DHEA-7-CMO antibody, resulting in nondisplacement of labeled DHEA with unlabeled DHEA.

To the best of our knowledge, this is for the first time that the ELISA for DHEA has been demonstrated using HRP as a label. It requires 100 μL of serum, and the assay can be completed in 1.5 h. The association constant (Ka) of DHEA antibody towards DHEA in the present assay is found to be 1.25×10^{-8} L/mol. The DHEA-17-CMO-BSA antibody showed 15.38% and 16.66% cross-reaction with androstenediol and testosterone, respectively, and less than 0.1% cross-reaction with other structurally related steroids. The developed assay is sensitive; the LDD and the ED_{50} are 0.5 ng/mL and 8.5 ng/mL, respectively. The percent recoveries of known amounts of DHEA added to serum pools range from 98.21–102.31%. The intra- and interassay coefficient variations ($n = 6$, replicate of each pool) are $<11.5\%$.

In conclusion, a sensitive and viable direct assay for serum DHEA has been developed that requires 100 μL of serum sample and assay can be completed in 1.5 h.

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