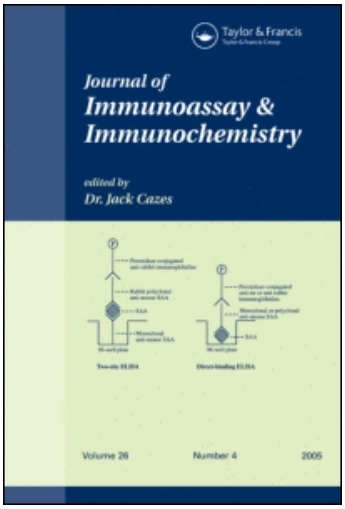

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A HOMOGENEOUS BIOELECTROCHEMICAL IMMUNOASSAY FOR THYROXINE

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ABSTRACT

Thyroxine has been modified with a ferrocene derivative to produce an immunologically reactive conjugate which remains capable of functioning as an electron transfer mediator, shuttling electrons between an oxido-reductase enzyme and an electrode. On binding with an anti-thyroxine antibody the conjugate's ability to function as a mediator is impaired. The conjugate has been used as a tracer in an homogeneous competitive assay to quantify the total thyroxine concentration in serum, the assay being measured electrochemically after the addition of a suitable oxidoreductase enzyme (glucose oxidase) and its substrate (glucose) on completion of antibody/antigen binding. This results in a simple and sensitive assay for thyroxine.

(**KEY WORDS**: Thyroxine, bioelectrochemical, homogeneous, ferrocene)

INTRODUCTION

Ever since Yalow and Berson's pioneering work on immunoassay (1,2), the technique of radioimmunoassay has become well established in clinical analysis, offering both high sensitivity and selectivity for a wide range of analytes. However, due to problems associated with the safe handling and disposal of radioisotopes, there has been a move from radioactive to non-radioactive labels in immunoassays. Amongst the commonly used non-isotopic labels are enzymes (3), chemiluminescent molecules (4) and fluorescent

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molecules (5). Immunoassay techiques which utilise electrochemical end point detection are beginning to be developed. In a discussion regarding the use of metal labels in immunoassay, Cais (6) suggested the possibility of detecting the metal label, and therefore quantifying the assay, electrochemically although he presented no data to substantiate the idea. Weber and Purdy (7) described an homogeneous voltammetric assay in which free, electroactive morphine was measured at a glassy carbon electrode, the electrochemical signal of the antibody-bound morphine being attenuated. Subsequent displacement of morphine from the antibody by codeine, which is electrochemically inactive, enabled the codeine to be quantified. Wehmeyer et. al. (8) demonstrated an assay for the electrochemically inert hormone estriol. After nitration, to impart some electrochemical behaviour to the molecule, the modified estriol was bound to an antibody, perturbing the estriol's electrochemistry. By displacing the nitrated estriol from the antibody with sample estriol, the concentration of estriol in the sample could be measured via the electrochemical current arising from the displaced nitrated hormone.

Electrochemical detection methods have been used to quantify enzyme immunoassays, replacing the more traditional optical systems. Doyle (9) described an assay of human orosomucoid in which the activity of the enzyme label, alkaline phosphatase, was monitored at a carbon paste electrode whilst glucose oxidase (10) and catalase (11) have been used in conjunction with oxygen electrodes for the quantification of immunoassays. A more comprehensive review of the electrochemical detection of enzyme immunoassays may be found elsewhere (12).

Ikariyama (13) produced a bioaffinity sensor for thyroxine (T4) using an enzyme (either glucose oxidase or catalase) conjugated to an anti-T4 antibody. Sample T4 competes with membrane bound T4 for the anti-T4 antibody, the assay being quantified using a galvanic oxygen detector covered by the T4 membrane. Hepler et. al. (14) described an assay for thyroid hormones in which the hormones are separated by reverse-phase high-performance

liquid chromatography and then quantified directly at a carbon working electrode.

Recent advances in the study of electron transport proteins, notably by Hill and coworkers (15), has led to the discovery of a range of compounds which will act as electron transfer mediators between proteins and electrodes. Many of these mediators (e.g. ferrocene and its derivatives) will also function as electron acceptors for oxido-reductase enzymes. The role of such mediators in the quantification of heterogeneous enzyme immunoassays has recently been described (16,17,18).

A further development of mediator based enzyme immunoassays is the modification of an antigen with the mediator using the antigen/mediator complex as a label in a competitive assay (19). If the unbound antigen/mediator conjugate is able to transfer electrons between a suitable enzyme and an electrode, with the ability to mediate being perturbed on antibody/antigen binding, a homogeneous, competitive antigen assay is feasible. This reaction sequence, in which thyroxine is the antigen, is shown diagrammatically in Fig. 1.

Such an assay for thyroxine (3,5,3',5'-tetraiodothyronine; T4), a thyroid hormone, is reported here; T4 was covalently coupled to ferrocene monocarboxylic acid, the resulting conjugate being used in a competitive T4 assay. The oxido-reductase enzyme glucose oxidase was used as an electron source and, in conjunction with the T4 blocking agent "fenclofenac", an assay for total T4 in serum over the clinically relevant range was obtained.

MATERIALS AND METHODS

Materials

Sephadex G-50 superfine was supplied by Pharmacia (GB) Ltd., Hounslow, U.K.; L-thyroxine, sodium salt, was supplied by Sigma (London) Ltd., Poole, Dorset, U.K., whilst the Aldrich Chemical Co. (Gillingham, Dorset, U.K.) supplied ferrocene monocarboxylic acid. Glucose oxidase (E.C. 1.1.3.4.) from Aspergillus niger came from the Boehringer Corporation (London) Ltd., Lewes, U.K.

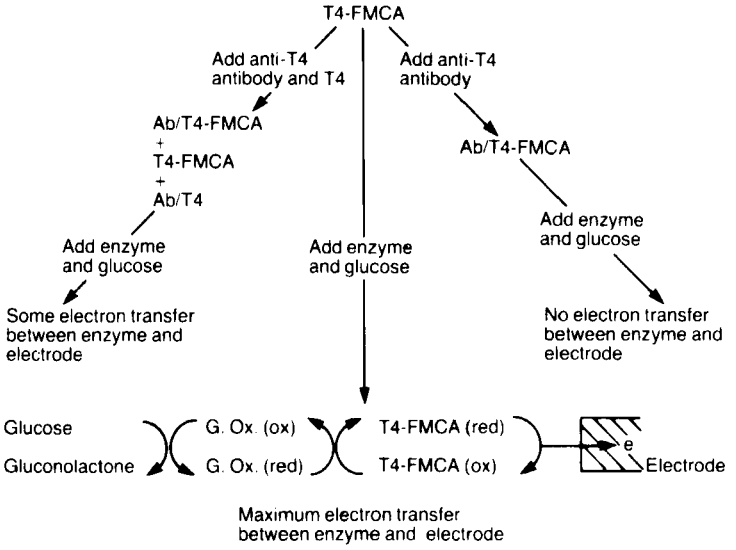

FIGURE 1. Diagrammatic representation of the homogeneous bioelectrochemical assay for thyroxine (Ab=antibody; e^-=electron; G.Ox.=glucose oxidase; (ox)=oxidised; (red)=reduced; T4=thyroxine; T4-FMCA=thyroxine-ferrocene monocarboxylic acid conjugate).

(grade II). All other chemicals were supplied by British Drug Houses, Dagenham, U.K. and were of "Analar" grade unless otherwise stated.

Silica gel plates (Merk) were supplied by British Drug Houses. Fenclofenac tablets were obtained from the Weil Organization (Pty) Ltd., Doornfontein, South Africa. The ultraviolet lamp was model UVSL-58 (Ultraviolet Products Inc., San Gabriel, California, USA).

Synthesis of thyroxine/ferrocene monocarboxylic acid (T4-FMCA) conjugate

57.5mg of ferrocene monocarboxylic acid were dissolved in 1ml of anhydrous dimethylformamide and the solution cooled to -10°C and stirred. Tri-n-butylamine and then isobutylchloroformate

were added (final concentrations of these reagents were 1.0M and 0.25M respectively) and allowed to react for 15 minutes at -10°C. The reaction was maintained at -10°C in an ice-salt bath. After raising the reaction temperature to ambient ($\sim$ 22°C), the reaction was allowed to proceed for a further 30 minutes to form the carboxycarbonic anhydride derivative of the ferrocene.

L-thyroxine (T4) free acid (194.2mg) was dissolved in 4ml of 0.1M/L sodium hydroxide/ethanol (1:1) and cooled to 4°C whilst stirring. The carboxycarbonic anhydride derivative of the ferrocene was added dropwise to the T4 solution and allowed to react for 2 hours at 4°C then overnight at room temperature, continuously stirring. After concentrating the solution by rotary evaporation the resulting precipitate was washed three times with carbon tetrachloride, discarding the washings and finally redissolving the insoluble conjugate in 5ml of methanol.

The conjugate was purified by applying 1ml of conjugate solution to a preparative silica gel plate (2mm thick) and eluting with the solvent system chloroform:methanol:acetic acid (9:1:0.1). The band with an approximate R_f value of 0.4 was removed and extracted three times with 5ml of methanol by centrifugation. After pooling the supernatants and concentrating them by rotary evaporation about 20mg of the conjugate was recovered($\sim$ 40% yield).

The T4-FMCA conjugate was characterised electrochemically at a pyrolytic graphite electrode and its immunoreactivity measured by using radioimmunoassay.

Extraction and purification of ([2-(2, 4-dichlorophenoxy) phenyl] acetic acid-"fenclofenac")

46 tablets of fenclofenac (300mg of fenclofenac/tablet) were ground up and the powder added to 200ml of saturated ammonium carbonate solution and stirred at room temperature for 1 hour. After sequential extractions from both basic and acidic solution, the product was extracted with ethyl acetate (3 x 100ml). The organic fractions were pooled and washed with distilled water (3 x 50ml), the final washing having a pH of between 6 and 7,

and dried with anhydrous sodium sulphate. After filtration, the filter cake was washed with ethyl acetate (3 x 10ml) and the solution dried by rotary evaporation. Following dissolution of the product in a minimal amount of propan-2-ol at 80°C, the mixture was left overnight at room temperature, stored at 4°C for 8 to 10 hours and then kept at -20°C overnight. The large crystals were collected by filtering on a buchner funnel and then dried, the product being checked for purity by thin layer chromatography in the following systems:

chloroform : methanol : acetic acid	(90:10:1)
chloroform : methanol : ammonia	(60:40:1)
ethylacetate : methanol	(90:10)

Only one spot was visible under a short wavelength U.V. lamp.

Preparation of anti-T4 antibody

Anti-T4 antibody was a conventional polyclonal antiserum obtained by immunising sheep with T4 conjugated to a high molecular weight protein. Sodium sulphate (1.8g) was added to 10ml of antiserum and the mixture rolled for 30 minutes at room temperature. After centrifugation (1600g for 30 minutes at room temperature) the supernatant was discarded and the precipitate redissolved in 10ml of doubly distilled water and the above procedure repeated. The antibody was then purified on a gel filtration column (Sephadex G-25) pre-equilibrated with 10mM/L Tris/HCl buffer, pH 7.4.

Immunoreactivity of T4-FMCA conjugate

Serial dilutions of the T4-FMCA conjugate and unmodified T4, were made in buffer (50mM/L Tris/HCl pH 7.5) and then assayed in duplicate using a commercially available total T4 radioimmunoassay kit (Total T4 MAIA kit, code 12825, Serono Diagnostics Ltd.) following the manufacturer's protocol. The binding of the sample was expressed as the ratio of bound counts in the sample (B) to the bound counts in the zero standard (Bo).

Electrochemical apparatus and techniques

The methods and equipment used have been described in detail elsewhere (17). During cyclic voltammetry the d.c. voltage scan

rate was 5mV/s over a potential range of 0 to +550mV versus a saturated calomel electrode (S.C.E.).

The working electrode comprised of a disc of pyrolytic graphite foil 4mm in diameter (supplied by Union Carbide) attached to a brass rod by silver loaded epoxy resin and encapsulated with heat shrinkable polyolefin tubing (RS Components Ltd., Corby, U.K.). The graphite surface was polished smooth with an alumina/water slurry after each use.

T4 assay protocol

Replicate 40µl samples of T4 in serum were added to the electrochemical cell along with 40µl of T4-FMCA in buffer (1mM/L), 40µl fenclofenac (10.2mM/L in buffer) and 40µl of anti-T4 antibody. After mixing, the reagents were incubated for 15 minutes at 37 ± 0.5°C and then 40µl of glucose (1M/L containing 100mM/L magnesium chloride) and 160µl of buffer (50mM/L Tris/HCl, pH 7.4) were added. After mixing and allowing to come to thermal equilibrium the electrochemical current was measured. Following the addition of 40µl of glucose oxidase (1mg/ml), the electrochemical current was remeasured. The electrode was polished after each measurement.

After correcting the measured electrochemical current in the absence of glucose oxidase for the dilution effect of adding the enzyme, the electrochemical signal was calculated using the following equation:

$$\text{Signal (units)} = \frac{i_{340mV} + \text{glucose oxidase}}{i_{340mV} - \text{glucose oxidase}} \qquad - (1)$$

This compensates for any interference from electrochemical oxidation of T4 at the graphite electrode.

RESULTS

Cyclic voltammetry of the T4-FMCA conjugate produced two peaks on the forward wave at oxidising potentials (Fig. 2). The first peak (at + 295mV. vs S.C.E.) represents the oxidation of the ferrocene group in the conjugate and has a corresponding peak on the return wave (at + 235mV vs S.C.E.) indicating that

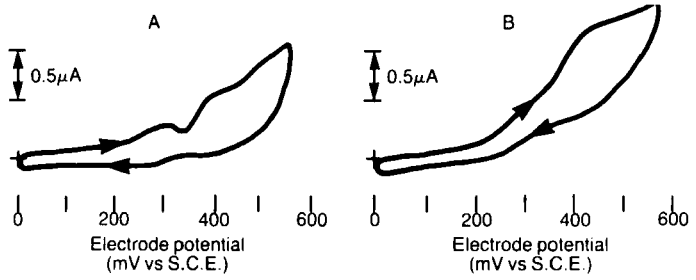

FIGURE 2. Cyclic voltammograms of A) 3mM/L thyroxine-ferrocene monocarboxylic acid in 50mM/L Tris/HCl buffer, pH 7.5, containing 100mM/L glucose and 10mM/L magnesium chloride. B) as A) but with the addition of 25.4 International Units of glucose oxidase/mL (temperature = 37 $\pm$ 0.5°C; voltage scan rate = 5mV/s; pyrolytic graphite electrode).

the ferrocene is still capable of undergoing redox electrochemical reactions. The second peak in the forward wave (at + 395mV vs S.C.E.) is the oxidation of T4. On addition of glucose and glucose oxidase to the conjugate there is a change in the voltammogram, the return wave disappearing and the forward wave at the lower electrode potential increasing in magnitude, indicative of the flow of a catalytic current (Fig. 2). For maximum sensitivity the catalytic current was measured at + 340mV vs S.C.E., thus reducing the error arising from the electrochemical oxidation of T4.

Binding of anti-T4 antibody to the conjugate perturbed its ability to function as an electron transfer mediator for glucose oxidase (see Table 1), whilst a mixture of T4, anti-T4 antibody and T4-FMCA gave intermediate currents (Table 1). These data suggest that, after adjusting the concentrations and volumes of the reagents, a competitive T4 assay, using T4-FMCA as a tracer, should be practicable.

The immunoreactivity of the T4-FMCA was compared with that of the T4 using radioimmunoassay. The results showed that the T4-FMCA is somewhat less immunologically active than T4 (see Fig. 3) but still suitable for use as a tracer in an immunoassay.

TABLE 1

Data Demonstrating the Ability of a Thyroxine/Ferrocene Monocarboxylic Acid Conjugate to Act as an Electon Transfer Mediator for Glucose Oxidase and the Effect of Binding the Conjugate to Anti-thyroxine Antibody.

CONDITIONS	ELECTROCHEMICAL CURRENT (μA)
T4-FMCA + Glucose	0.58
T4-FMCA + glucose + glucose oxidase	1.58 ± 0.004
T4-FMCA + glucose + glucose oxidase + anti-T4 antibody	1.35 ± 0.01
T4-FMCA + glucose + glucose oxidase + T4 + anti-T4 antibody	1.53 ± 0.05

T4-FMCA = thyroxine/ferrocene monocarboxylic acid conjugate
Final concentrations of reagents:
T4-FMCA = 3×10^{-7} M/L
glucose = 100mM/L
glucose oxidase = 6.25×10^{-7} M/L
T4 = 5×10^{-5} M/L

T4 is normally present in the blood stream in large amounts, strongly and reversibly bound to serum proteins, mainly to thyroxine binding globulin (TBG), prealbumin and albumin. In order to measure total T4 in a serum sample using an electrochemical technique, all the bound T4 needs to be displaced from the serum proteins, the T4 being replaced by a blocking agent which exhibits no significant electrochemistry in the potential range of interest (0 to +550mV vs S.C.E.). The nonsteroidal anti-inflammatory drug fenclofenac ([2-(2,4-dichlorophenoxy) phenyl] acetic acid; fenclofenac) has been shown to displace T4 from serum proteins (20). Cyclic voltammetry on a solution of fenclofenac (2.5mM/L) showed that the drug has very little electrochemical activity in the potential range of interest (0 to +550mV vs S.C.E.) (Fig. 4) (i_{340mV} -fenclofenac = 0.135μA; i_{340mV} +fenclofenac = 0.160μA)

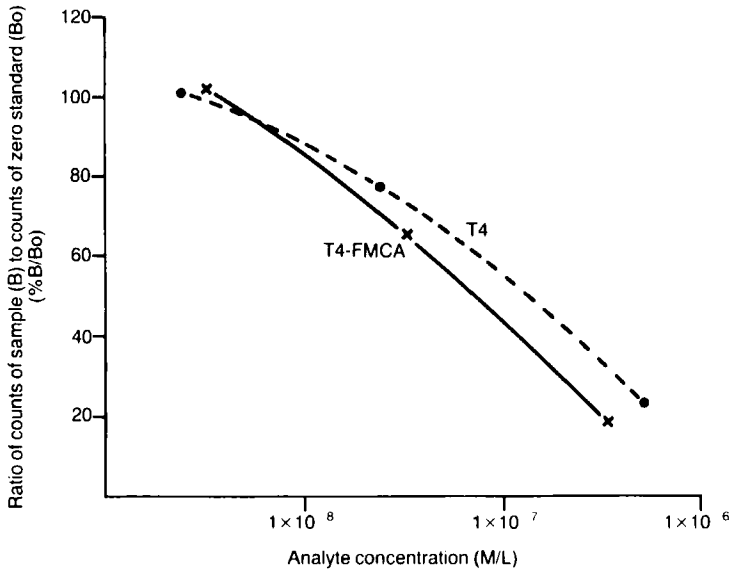

FIGURE 3. Data comparing the immunoreactivity of thyroxine (T4) and the thyroxine-ferrocene monocarboxylic acid conjugate (T4-FMCA).

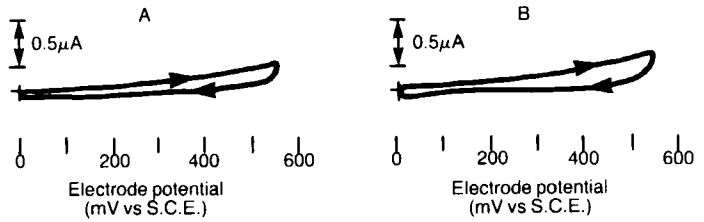

FIGURE 4. Cyclic voltammograms showing the electrochemistry of fenclofenac. A) is 50mM/L Tris/HCl, buffer, pH 7.5; B) as A) but with the addition of 10mM/L fenclofenac. (temperature = 37 $\pm$ 0.5°C; voltage scan rate = 5mV/s; pyrolytic graphite electrode).

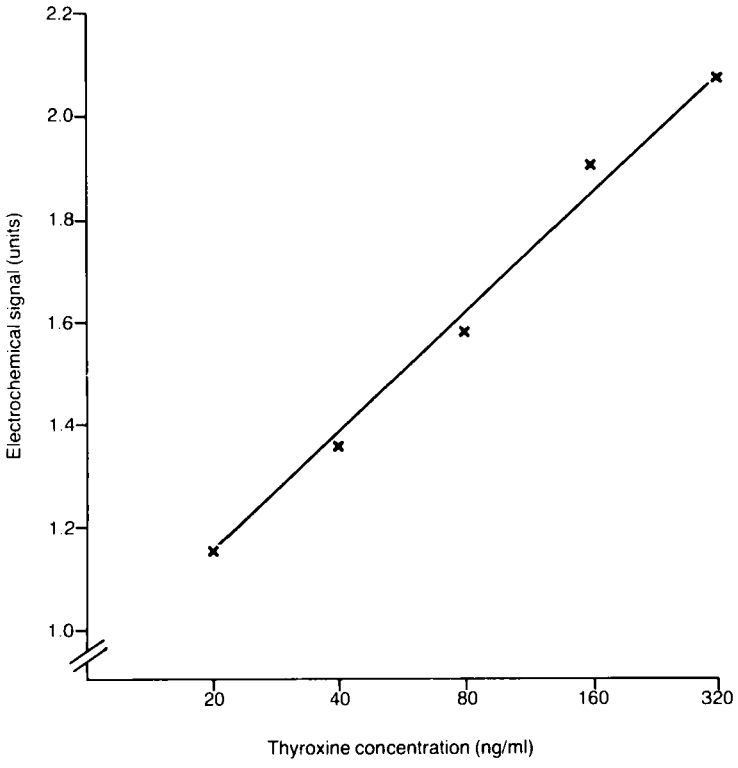

FIGURE 5. Plot of electrochemical signal versus serum thyroxine concentration.

and therefore should make a suitable T4 blocking agent for use in a bioelectrochemical T4 assay.

A dose response curve for total T4 in serum was produced, using a final concentration of 2.5mM/L fenclofenac as blocking agent. The assay gave a linear relationship over the clinically important concentration range (20ng T4/ml to 320ng T4/ml (2.5×10^{-8}M/L to 4.1×10^{-7}M/L)) (Fig. 5), demonstrating the applicability of an homogeneous electrochemical immunoassay for total T4 in serum.

DISCUSSION

These results demonstrate the general principle of homogeneous electrochemical immunoassays for small analyte molecules. Amplification of the normal redox chemistry of the ferrocene molecule is provided by the catalytic activity of an oxido-reductase enzyme (in this case glucose oxidase). Bioelectrochemical monitoring of an immunoassay is attractive because not only are the problems associated with the use of radioactive labels removed but also there is no need for the expensive items of optical equipment (e.g. fluorimeters, luminometers or spectrophotometers) or potentially carcinogenic chromophores which are normally associated with non-isotopic immunoassays. Advantageously, amperometric bioelectrochemistry generates a high current, low impedance signal directly, such a signal permitting easy data processing.

The bioelectrochemical T4 assay is much simpler to perform than the electrochemical assays described in the literature (7,8) as there is no displacement of an electroactive molecule from an antibody by the electrochemically inert molecule to be measured. The assay is also much less complicated than the affinity electrode for T4 as described by Ikariyama (13) because no complex membrane synthesisis required. As the electrode potential at which the catalytic current is measured in the bioelectrochemical assay is low (+340mV vs S.C.E.) there is little interference from electrochemically active components present in serum. Also the ferrocene monocarboxylic acid/glucose oxidase system is not significantly affected by the partial pressure of oxygen in the sample (15) further simplifying the assay as the oxygen tension in the samples does not have to be strictly controlled. The lack of a separation step reduces errors normally associated with the decanting and aspirating steps of heterogeneous assays.

The sensitivity of the assay may be improved by use of an oxido-reductase enzyme which has a higher turnover number than glucose oxidase (e.g. glucose dehydrogenase (E.C. 1.1.99.17)). From previous studies (15) other ferrocene derivatives have been shown to function as better mediators for glucose oxidase (e.g.

(dimethylamino) methyl ferrocene) and, therefore, producing antigen/mediator conjugates from such compounds may enhance the enzyme electrochemistry. The introduction of a spacer molecule between the antigen and mediator may facilitate electron transfer between the enzyme and mediator, again improving assay sensitivity.

Thus a homogeneous bioelectrochemical assay for a low molecular weight antigen has been demonstrated but the technique, at the moment, is of theoretical interest only. Although the antigen of interest was a hormone, the assay could find applicability to theraputic drug monitoring and may offer the possibility of automation, permitting use outside centralised clinical laboratories, especially if part of the assay can be confined to the electrode surface.

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