THE CONJUGATION OF TESTOSTERONE WITH HORSERADISH PEROXIDASE AND A SENSITIVE ENZYME ASSAY FOR THE CONJUGATE

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ABSTRACT

The formation of a horseradish peroxidase-testosterone conjugate for the enzyme-linked immunoassay of testosterone was investigated, using tritiated testosterone to follow the reaction. The formation of testosterone-3-(carboxymethyl) oxime-peroxidase by the mixed anhydride method was found to give a conjugate of high enzymatic activity and with three molecules of testosterone per molecule of peroxidase.

The optimum conditions for the assay of peroxidase activity were studied and an assay capable of measuring 1 to 5 ng of the conjugate developed; the standard curve being virtually linear. The stability of the conjugate in solution and the effect of lyophilisation on enzymatic activity are also described. The peroxidase-testosterone conjugate was suitable for enzyme-linked immunoassay and the quantities measurable with the peroxidase assay covered the range necessary for a plasma testosterone assay. The stability of the conjugate was such that no particular precautions were necessary for its storage.

Enzyme-linked immunoassays developed over the past five years involve either antibody-enzyme (1-6) or antigen-hapten (7-16) conjugates. This work concerns a hapten-enzyme conjugate where the hapten is testosterone and the enzyme horseradish peroxydase.

Reports on the conjugation of steroids with enzymes (10, 12,14,15) have concerned coupling agents conventionally used for hapten-antigen conjugation in the preparation of antibodies, e.g. hemisuccinate 'bridges' formed using the

mixed anhydride method of Erlanger et al.(17). Thus the 'bridge' formation studied here is that of testosterone-3-(carboxymethyl)oxime-peroxidase, the antibody to be used was raised against testosterone-3-CMO-bovine serum albumin.

As, for maximum sensitivity, at least nanogram quantities of steroid-enzyme should be assayed by as simple a technique as possible, it is necessary to investigate the kinetics of the conjugated enzyme over long reaction times with substrate. Peroxidase is non-specific with respect to hydrogen donor (18,19) and a donor giving oxidation products with a comparatively high light absorption in the visible range had to be chosen. Finally, although peroxidase is a stable enzyme the stability of the peroxidase-testosterone conjugate remains unknown and so its stability in solution and when lyophilised has been tested.

MATERIAL AND METHODS

Testosterone was obtained from UCLAF Roussel (France) and 1α,2α-^{3}H-testosterone, 49 Ci/mmole, from the Radiochemical Centre,Amersham (UK). Organic solvents of analytical grade (Prolabo,Paris) were used without further purification except for dioxan which was chromatographed on a basic alumina (Woelm) column immediatly before use. Carboxymethoxylamine hydrochloride and 5-amino salicylic acid,95% (Aldrich Chem.Co.), tri-n-butylamine, isobutyl chloroformate and o-dianisidine (Merk), guaiacol and hydrogen peroxide (Prolabo) were used without further purification, the hydrogen peroxide (110 volumes) being titrated regularly with potassium permanganate. Norit powdered charcoal was supplied by Prolabo and pre-coated silicagel thin-layer plates (0.25 mm thickness) by Merck.
For the proteins: high specific activity (type VI, RZ app. 3.0) horseradish peroxidase (E.C.1.11.1.7) was supplied by Sigma. The rabbit antitestosterone-3-(carboxymethyl)oxime-BSA antibodies were prepared in this laboratory.
Antibodies were immobilised on cyanogen bromide (Aldrich) activated microcrystalline cellulose (Merk) by the method of Axen et al. (20) and Wide (21).

Kinetic studies and spectra were performed by means of a Perkin-Elmer Model 402 recording spectrophotometer.

The measurement of peroxidase activity: Optimum hydrogen donor
Enzymatic activities of peroxidase (0.1ml, about 40ng/ml in 0.1M phosphate buffer pH 7.0 containing 1g/l gelatine) were routinely measured in 2.9ml "Assay Buffer A" (0.02M phosphate buffer pH 6, 0.45mM hydrogen peroxyde, 2.5mM 5-amino salicylic acid), initial velocities being read at 470nm. This optimum condition was chosen after having assayed different hydrogen donors (guaiacol 30mM, o-dianisidine 0.34mM) which gave less sensitive colorations.

Derivative formation
Tritiated testosterone was incorporated into the conjugation reactions with peroxidase, thus necessitating the preparation of a tritiated testosterone-3-(carboxymethyl)oxime derivative. Tritiated testosterone (approx. 2µg, 18.5×10^6 CPM) and testosterone (30 mg) were reacted with carboxymethoxylamine HCL by the method of Erlanger et al.(17). The purified product was dissolved in benzene:ethanol (5:1) and samples taken for counting and for verification of purity by TLC followed by radiochromatogram scanning and visualisation with iodine vapour. The product was 100% pure and had a melting point of 179-181°C. A recovery of 64.3% was obtained and the specific activity was 617 cpm/µg.

RESULTS

Testosterone-peroxidase conjugation

The following adaptation of the Erlanger method was used: testosterone-3-CMO (1.0mg) and ^{3}H-testosterone-3-CMO (0.8mg, 493×10^3 cpm) were taken up in 0.05 ml pure dioxan and tri-n-butylamine (1.2 µl) added with a Hamilton microsyringe. The mixture was cooled to approx. 10°C and isobutyl chloroformate (0.65 µl) added. The mixture was then held at 4°C with occasional shaking- with slight warming whenever the mixture froze - and then it was transferred in 10 µl aliquots, with agitation after each addition, to a solution of 10 mg peroxidase (type VI) in 0.18ml pure dioxan and 0.18ml 0.5% sodium bicarbonate at 4°C and left 2 hours at 4°C. Finally 1.77ml 0.1M phosphate buffer pH 7.0 containing

1 g/l gelatine was added to give a total volume of 2 ml. After reaction, the solution was dialysed twice again 2 liters 0.1M phosphate buffer pH 7.0 at 4°C. 50 µl aliquots were finally taken for counting, total volumes were measured by weight.

Enzyme activities were determined and total peroxidase estimated by its absorbance at 403 nm. Table I shows the recoveries of enzyme and enzyme activity and the amount of testosterone bound per mole of enzyme in the peroxidase-testosterone fraction

TABLE I - Characteristics of peroxidase after reaction with testosterone-3-(carboxymethyl)oxime.

Enzyme : percent recovery	38%
Specific activity : percent recovery	77,3%
Steroid/Enzyme : (mole/mole)	3.1

The amount of unreacted peroxidase remained unknown with these tests and had to be estimated from an antibody dilution curve. The dialysed peroxidase-testosterone fraction was diluted to 1/20000 and 0.1ml volumes incubated overnight at 4°C with 0.1ml volumes of a suspension of anti-testosterone immobilised on CNBr activated cellulose at various dilutions in 0.1M phosphate buffer pH 7.0. The tubes were then centrifuged at 3000 rpm for 10 minutes and 0.1ml volumes of supernatant taken for peroxidase activity assay- absorbances being read after 2 hours. The resulting dilution curve is shown in figure 1 and indicates that the enzyme is almost completely reacted.

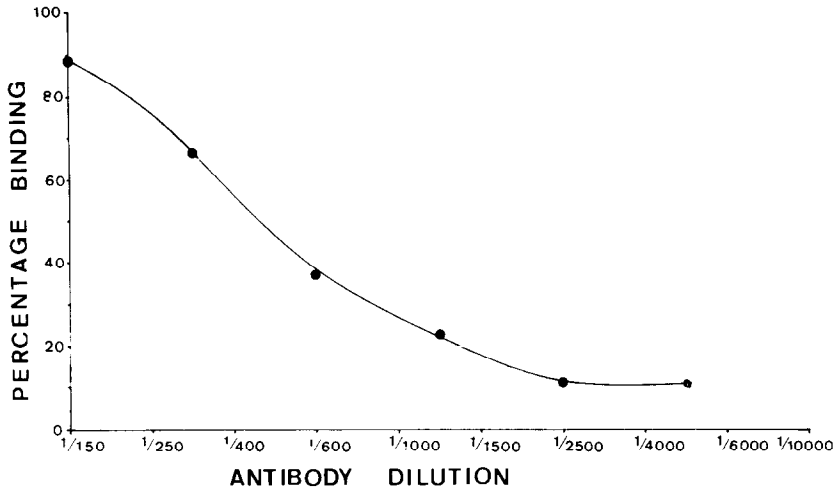

Fig.1 Antibody dilution curves with testosterone-3-CMO-peroxidase as ligand and rabbit anti-testosterone immobilised on cellulose

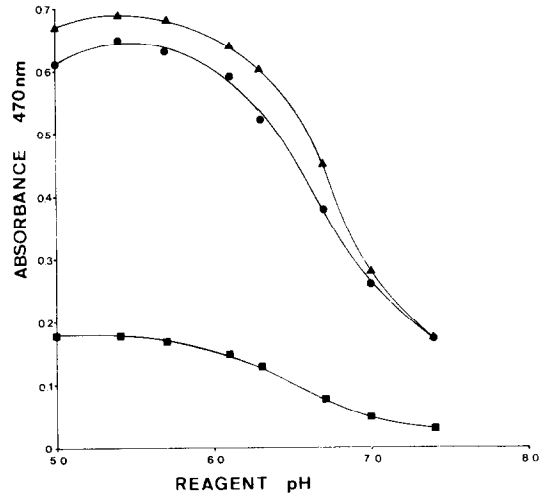

Fig.3 The effect of reagent pH on peroxidase (4 ng -▲-) and peroxidase-testosterone (1,6 ng -●- and 4,7ng -■-) activity (concentration in 3 ml final volume)

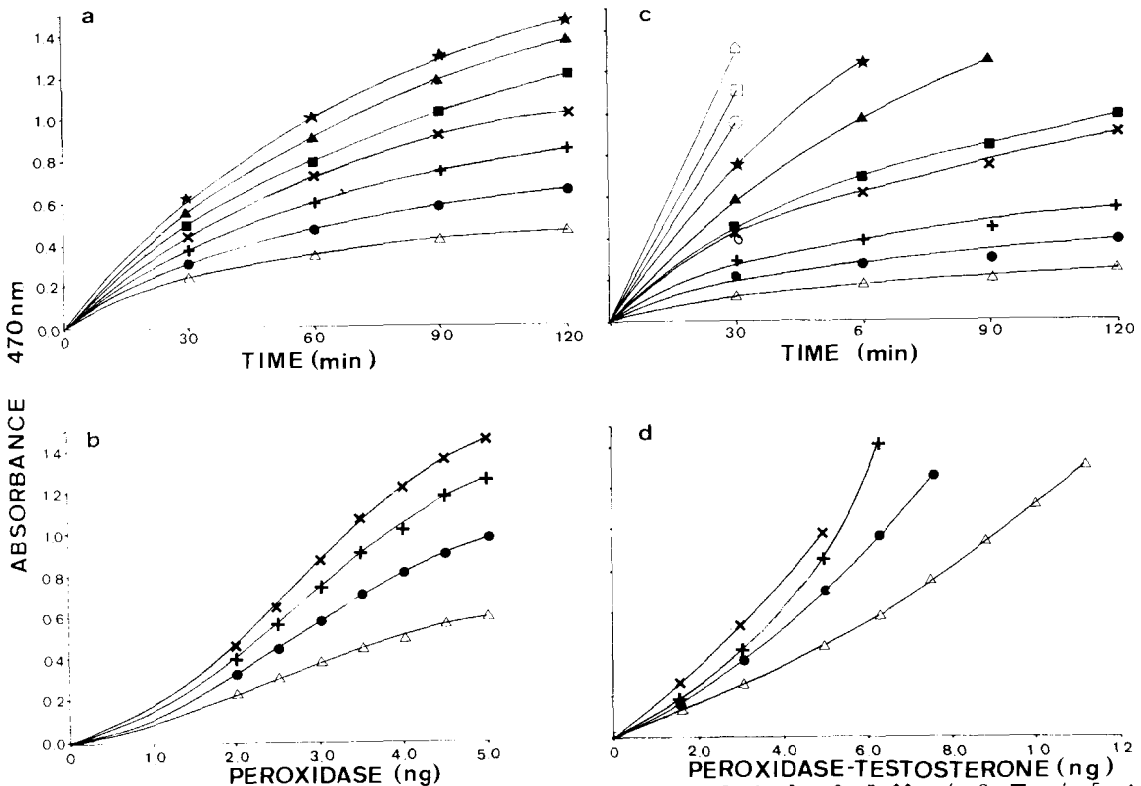

Fig.2 a.Time courses for peroxidase (2.0-△-,2.5-●-,3.0-✚-,3.5-✖-,4.0-■-,4.5-▲- and 5.0 -★- ng in 3 ml final volume

b. Standard curves for peroxidase after reaction time of 30(△),60(●)90(✚)and 120 min✖

c. Time courses for peroxidase-testosterone: 1.6(△),2.3(●),3.1(✚),4.6(✖),5.0(■), 6.3(▲) 7.5(★),8.8(○),10.0(□) and 11.2(⌂) ng in 3 ml final volume

d. Standard curves for peroxidase-testosterone after reaction times of 30(△),60(●), 90(✚) and 120(✖) minutes

Assay time course and standard curves

As the quantities of peroxidase to be assayed were smaller than those for which the initial velocity of the reaction was measurable the time course of the reaction with different enzyme concentrations had to be known. Figure 2 shows the time course of reactions with 0.1ml volumes of high specific activity peroxidase (20-50 ng/ml) and peroxidase-testosterone (16-120 ng/ml) using 5-amino salicylic acid reagent (Assay Buffer "A", see material and methods).

Absorbances were read after 30,60,90 and 120 minutes and the results are shown both as a function of time and of enzyme/enzyme-steroid concentration.

Optimum pH

2.9ml phosphate buffers (0.1M) of various pH from 5.0 to 7.4 containing 0.45mM hydrogen peroxide and 2.6mM 5-amino-salicylic acid were reacted with 0.1ml volumes of peroxidase (40 ng/ml) and peroxidase-testosterone (16 and 47 ng/ml) for 1 hour at room temperature in the dark. The effect of reagent pH on the enzyme activity is shown in figure 3. The optimum pH for the reagent was 5.4, however the buffering capacity of phosphate buffer at this pH is very low and so a reagent pH of 6,0 was used routinely.

Optimum salicylic acid concentration

Peroxidase (40ng/ml) and peroxidase-testosterone (16 and 47 ng/ml) were assayed in 0.1ml volumes with 2.9ml assay buffer "A" containing 0.1 to 0.6 mg/ml 5-amino salicylic

acid. Absorbances (470 nm) were read after 1 hour and the results are shown in figure 4a as a function of hydrogen donor concentration. Although the optimum hydrogen donor concentration was over 0.6mg/ml it was not possible to dissolve the compound to a concentration over 0.5mg/ml without heating, thus the latter concentration was used routinely.

Optimum hydrogen peroxide concentration

The same enzyme and enzyme-steroid solutions were assayed with reagent containing 0.5mg/ml 5-amino salicylic and 0.17 to 1.33 mM hydrogen peroxide (2-15 µl of solution at 110 vol /100ml) in 0.02 M phosphate buffer pH 6.0. Absorbances were read after 1 hour and are shown as a function of hydrogen peroxide concentration in figure 4b. Although the peroxidase is inhibited by a hydrogen peroxide concentration of 1 mM the peroxidase-testosterone is inhibited by a concentration of 0.50 mM. A hydrogen peroxide concentration of 0.45 mM (5 µl/100 ml) was used routinely.

Activation of peroxidase by ammonium salts

Several enzyme-steroid solutions (peroxidase-testosterone 9-47ng/ml) were assayed with assay buffer A containing various concentrations (0.025-0.05 and 0.10 M)ammonium sulphate. Spectra from 350 to 510nm were performed after 2 hours incubation and showed that the ammonium sulphate causes a shift in the λ_{max} from 470 to 490 nm. Figure 5 shows the absorbance (read at the λ_{max}) as a function of peroxidase-testosterone concentration. It can be seen that the enzyme is stimulated by ammonium sulphate and that the

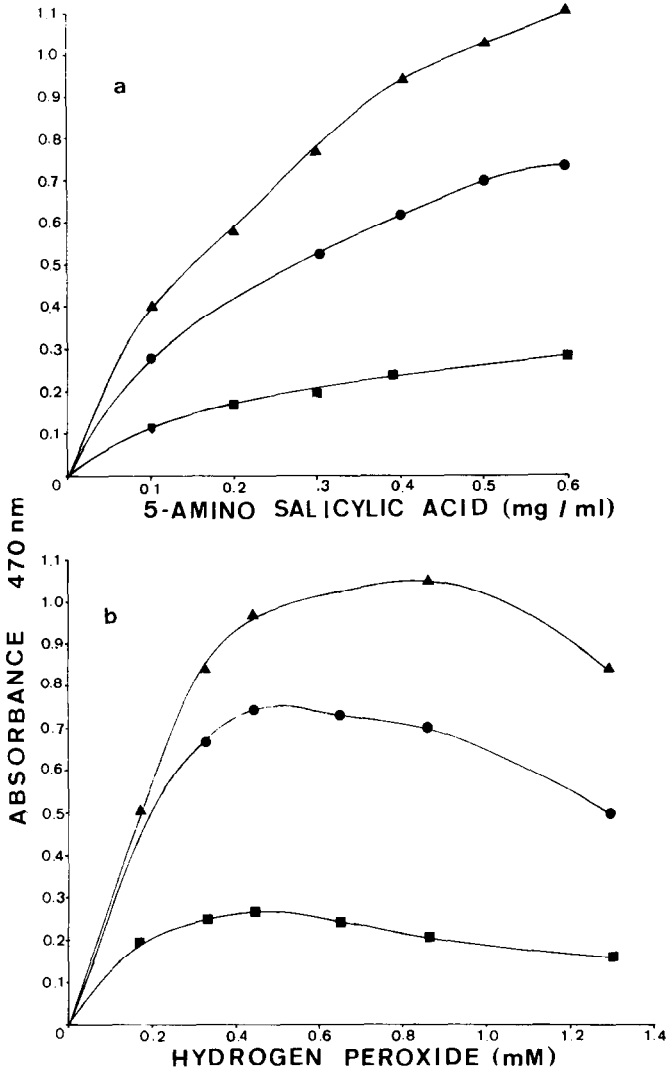

Fig.4 a. The effect of hydrogen donor concentration on peroxidase (4.0 ng -▲-) and peroxidase-testosterone (1.6 ng-■- , 4.7 ng-●-) in 3 ml assay buffer A.
b. The effect of hydrogen peroxide concentration on the same peroxidase and peroxidase-testosterone solutions.

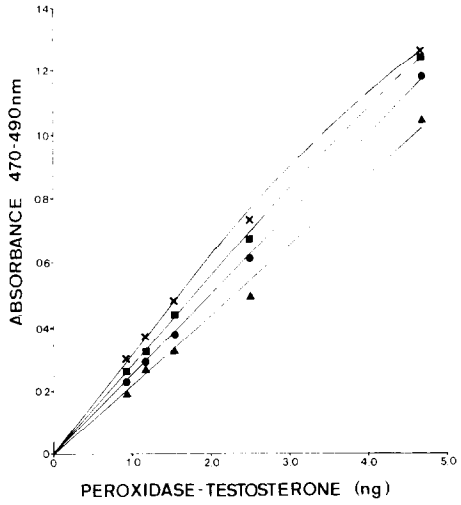

Fig.5 Standard curves for peroxidase-testosterone measurement in the presence of 0 (▲), 0.025 (●) 0.05 (■) and 0.1(✖)M ammonium sulphate. Absorbances read at the λmax. 470 or 490 nm were read after two hours

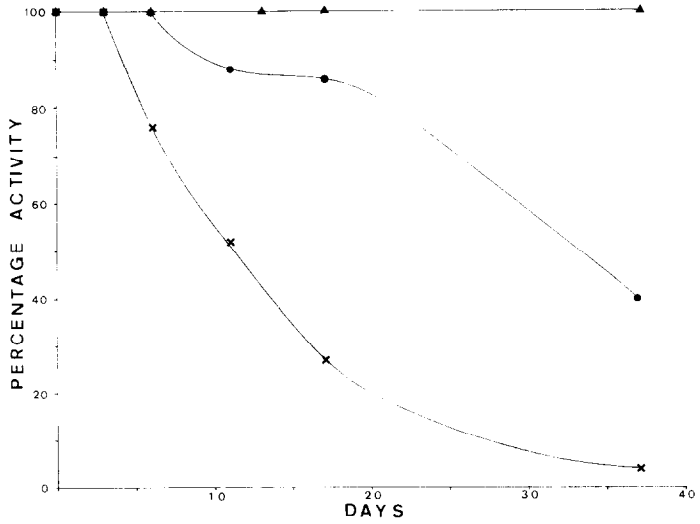

Fig.6 The effect of storage at 5°C on the activity of peroxidase-testosterone at 930 ng/ml (▲), 93 ng/ml (●)and 16 ng/ml (✖).

assay in the presence of 0.1M ammonium sulphate is enhanced in sensitivity.

Stability of peroxidase-testosterone

The conjugate solution was diluted to give 3 solutions at 930, 93 and 16 ng/ml with 0.1M phosphate buffer pH 7.0 containing 1g/l gelatine. Aliquots of each solution were assayed for enzyme activity and the solutions were stored at 5°C for 3 days and re-assayed. This was continued for 37 days with frequent assaying. The results (Fig.6) show that the enzyme-steroid solution at 930ng/ml is stable at 5°C for over a month and that the solution at 93ng/ml is stable at least 6 days at this temperature. Solutions of the enzyme-steroid at higher dilutions should be made up immediately before use.

The effect of lyophilisation was studied by lyophilising a 10µl sample of peroxidase-testosterone (930µl) in phosphate buffer without prior dialysis. The lyophilised peroxidase-testosterone was then stored one week at -15°C and then assayed for enzyme activity. It was found that the enzyme retained 98% of its activity, thus this method of storage could be used.

DISCUSSION

High specific activity horseradish peroxidase may be conjugated with testosterone-3-(carboxymethyl)oxime by the anhydride method, 38% of the enzyme is recovered having 77% of its activity. 3 molecules of testosterone are conjugated, on average, to each molecule of peroxidase under the conditions used here.

It should be noted here that the reactions, dilutions, assays etc... were carried out in glass tubes as it was found that the enzymereacted to form coloured (fluorescent red) products with the polystyrene tubes available. This was probably due to other compounds present in the plastic- peroxidase is known to react with 5-hydroxytryptamine etc...(24).

The hydrogen donor concentration used was higher than that used by Van Weeman et al.(9,10) and by Stimson and Sinclair (3)(0.4mg/ml), though Ruitenberg et al. used 0.8mg/ml, heating to 70°C to dissolve the compound. The hydrogen peroxide concentration optimum for the enzyme-steroid conjugate was 0.45mM, the same quantity as used by Stimson and Sinclair. The difference between the conjugated and unconjugated enzyme again resembled the differences found by Bartling et al. (25) with matrix-bound peroxidase.

Fridovitch (26) found that peroxidase activity was stimulated by ammonium salts when the hydrogen donor was o-dianosidine, and such stimulation was found here with 5-amino-salicylic acid as the hydrogen donor (though it was not so great).

Overall peroxidase-testosterone may be assayed in 1-5ng quantities with a reagent containing 0.5mg/ml 5-amino-salicylic acid, 0.45mM hydrogen peroxide (5µl 110vol.H_2O_2/100ml) and 0.1M ammonium sulphate in 0.02M phosphate buffer pH 6.0, absorbances being read at 490nm after 2 hours. The standard surve is close enough to linearity to be satisfactory.

The enzyme-steroid may ne lyophilised and stored in this form and solutions of 1µg/ml phosphate buffer made up monthly.

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