A STEROID IMMUNOASSAY BASED ON ANTIBODY-ENHANCED HYDROLYSIS OF A STEROID–UMBELLIFERONE CONJUGATE

F. KOHEN, Z. HOLLANDER, J. F. BURD+ and R. C. BOGUSLASKI+

Department of Hormone Research The Weizmann Institute of Science, Rehovot, Israel and
+Ames Co., Division of Miles Laboratories, Inc., Elkhart, IN, USA

Received 9 January 1979

1. Introduction

We wish to report a new approach to monitoring steroid immunoassay based upon antibody-enhanced hydrolysis of a steroid–fluorescent dye conjugate [1]. In this method a carboxy derivative of a steroid is conjugate to a fluorescent dye (e.g., 7-hydroxy-coumarin (umbelliferone)) through an ester bond, and the steroid–umbelliferone (fluorescent dye) conjugate (fig.1) yields fluorescent products upon hydrolysis. The rate of hydrolysis of this conjugate is enhanced upon addition of antisteroidal IgG fraction to the solution. The antibody-enhanced hydrolysis of this steroidal embelliferone ester is specifically inhibited by the presence of free homologous steroid. Hence, a decrease in fluorescent intensity is proportional to the steroid concentration in the sample. Since only the liberated label is determined by fluorometry, no separation steps of bound and free hormone are necessary, and the immunoassay is thus homogeneous.

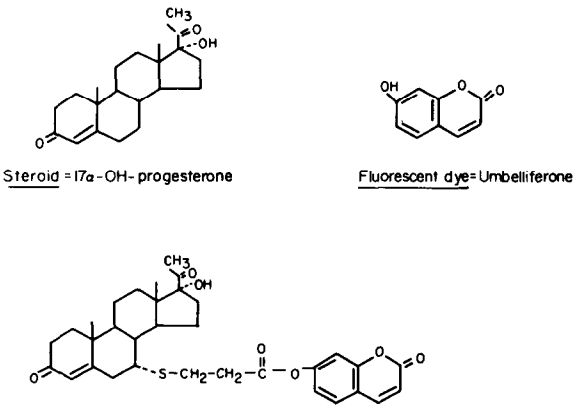

Fig.1. Structure of 17α-OH-progesterone-7α-carboxyethyl thioether–umbelliferone conjugate, the steroid–fluorescent dye conjugate.

A preliminary account of this work has appeared in the abstracts of the 8th Congress of the International Study Group for Steroid Hormones, Rome 5–7 December 1977

As an illustration of this method we have studied the reaction kinetics of antibody-enhanced hydrolysis of 17α-OH-progesterone-7α-carboxyethyl thioether umbelliferone (17-OH-P–U) conjugate (fig.1), the specificity of the reaction and the effect of competitive inhibitors on the hydrolysis reaction. The results that were obtained are given in the following sections.

2. Materials and methods

17-OH-Progesterone-7α-carboxyethyl thioether [2] (17-OH-P-CET) and cortisol-21-hemisuccinate were conjugated via a carbodiimide reaction in dimethyl formamide solution to umbelliferone (7-OH-coumarin) to yield 17α-OH-progesterone–umbelliferone (17-OH-P–U) and cortisol–umbelliferone (C–U) conjugates. The purity of the synthesized conjugates was ascertained by thin-layer chromatography, elementary analysis and spectroscopic methods.

Anti-steroidal sera were generated as in [2]. The IgG fractions of these antisera were prepared by ammonium sulfate precipitation, followed by DEAE-52 cellulose column chromatography, dialysis and lypholization. Upon reconstitution with distilled

water, the resulting antisera contained 10 mg protein/ml. Their properties (titer, specificity, affinity constants) were determined by radioimmunoassay procedures [2]. The amount of specific antibody in these IgG fractions was determined as in [3]. Fluorescence measurements of umbelliferone were made with an Aminco-Bowman spectrofluorometer using 364 nm and 448 nm for the excitation and emission wavelengths.

Tris-buffer (pH 8, 0.1 M) was used throughout the experiments. The kinetic parameters were computed from initial rates over a suitable range of substrate concentration, and the Lineweaver-Burk plots were evaluated by the least square method.

3. Results

3.1. *Rates of enzymatic and antibody-enhanced hydrolysis of 17-OH-progesterone–umbelliferone conjugate*

The effects of varying levels of esterase, anti-17-OH-progesterone IgG and of normal, non-immune rabbit IgG on the rate of hydrolysis of 17-OH-progesterone–umbelliferone conjugate (17-OH-P–U) are shown in fig.2,3. The rates measured using 2.5 nM

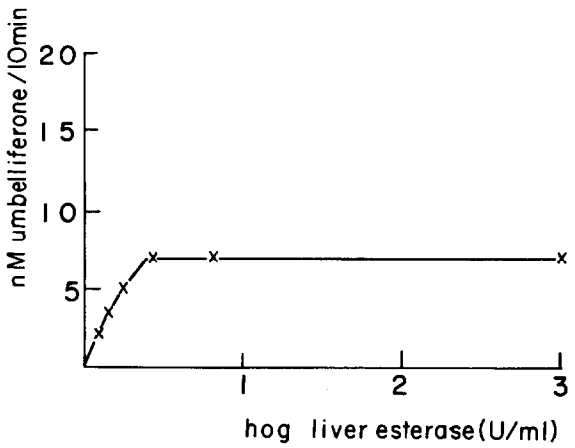

Fig.2. Rates of enzymatic hydrolysis of 17-OH-progesterone–umbelliferone conjugate. Varying levels of esterase were incubated with 17-OH-progesterone–umbelliferone conjugate (final conc. 24 nM) in 0.1 ml total vol. buffer for 10 min at 37°C. Buffer (0.4 ml) was then added, and the liberated umbelliferone was measured fluorometrically. The background hydrolysis rate was subtracted in each case.

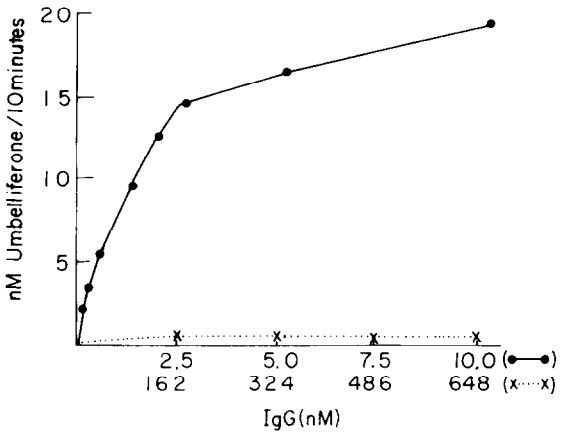

Fig.3. Rates of antibody-enhanced 17-OH-progesterone–umbelliferone conjugate. Varying levels of anti-17-OH-progesterone IgG (•——•) or rabbit IgG (X. . .X) were incubated with 17-OH-progesterone–umbelliferone conjugate (final conc. 24 nM) for 10 min at 37°C in 0.1 ml total vol. buffer. Buffer (0.4 ml) was then added, and the liberated umbelliferone was measured fluorometrically. The background hydrolysis rate was subtracted in each case.

of anti-17-OH-progesterone IgG fraction per 0.5 ml reaction mixture were twice as high as those of enzymatic hydrolysis using hog liver esterase at 0.25 U/0.5 ml. In all three cases the final concentration of 17-OH-P–U was 24 nM.

3.2. *Rates of antibody-enhanced hydrolysis of 17-OH-progesterone–umbelliferone conjugate as a function of time*

The rate of antibody-enhanced hydrolysis of 17-OH-P–U conjugate was examined as a function of time, and the results are shown in fig.4. A plateau was reached after 20 min reaction.

3.3. *Reaction kinetics*

The antibody enhanced hydrolysis of 17-OH-P–U conjugate followed first-order kinetics during the initial 5 min of the reaction. The K_m for 17-OH-P–U and V_{max} of the reaction, determined according to the Lineweaver-Burk plot, were 3.8×10^{-7} M and 3×10^{-9} M.min^{-1}, respectively. The rate constant (k) was 0.0052 min^{-1}. The turnover number of the antibodies was <1 under saturating substrate concentrations ($S > 100\ K_m$), and at equilibrium 1 nmol

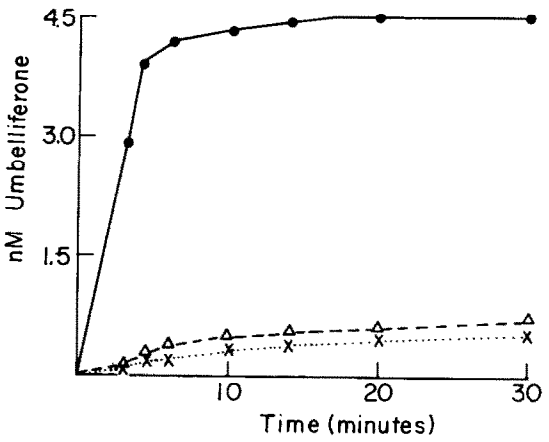

Fig.4. The rate of antibody-enhanced hydrolysis of 17-OH-progesterone–umbelliferone conjugate as a function of time. Anti-17-OH-progesterone IgG fraction (0.6 nM) was incubated with 17-OH-progesterone–umbelliferone conjugate (24 nM) at 37°C, and the fluorescent intensity of the liberated umbelliferone (•——•) was measured at the time indicated on the abcissa. Control reactions (X . . .X) containing buffer instead of anti-17-OH-progesterone IgG and rabbit IgG-induced hydrolysis (△- - -△) reaction of 17-OH-progesterone–umbelliferone conjugate are also shown.

antibody affected the hydrolysis of 22 nmol 17-OH-P–U conjugate.

3.4. *Specificity of the antibody-enhanced hydrolysis reaction*

The effect of various anti-steroidal IgG fractions and rabbit IgG on the rate of hydrolysis of 17-OH-P–U and C–U conjugates was examined. A striking selectivity in the hydrolysis reaction was observed. Antisteroidal IgG fractions generated with antigens conjugated to carrier at position 7 (e.g., anti-testosterone-7α-carboxyethyl thioether BSA and anti-17-OH-progesterone-7α carboxyethyl thioether BSA) were equally effective in the rate hydrolysis of 17-OH-P–U conjugate, but had no appreciable effect on the rate hydrolysis of C–U conjugate, and ethyl butyrate, a model substrate for hog liver esterase. Similarly anti-steroidal IgG fractions generated with antigens conjugated to carrier at position 21 (e.g., anti-cortisol-21–BSA IgG) or 3 (anti-deoxycortisol-3-BSA IgG) and rabbit IgG from a non-immunized rabbit did not effect the rate of hydrolysis of 17-OH-P–U conjugate. These results indicated that the

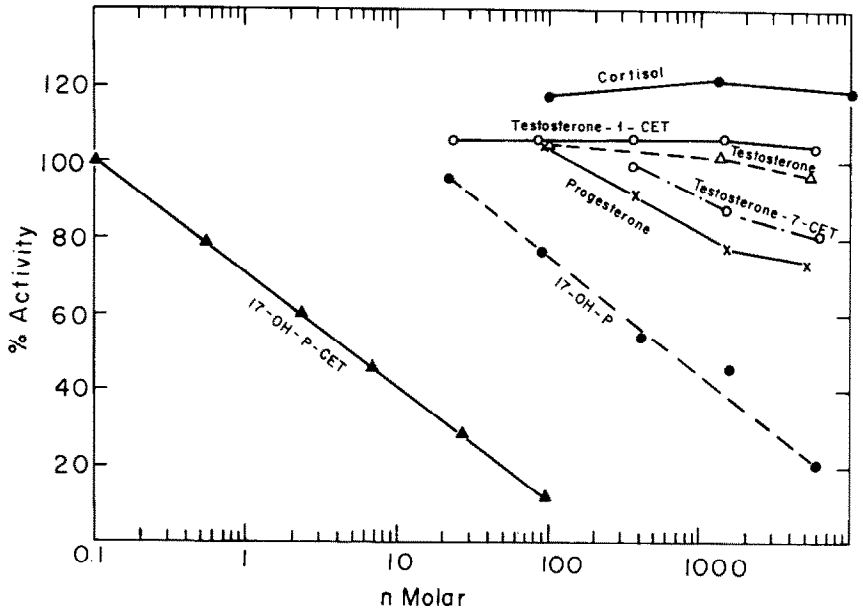

Fig.5. Effect of competitors on the antibody-enhanced hydrolysis of 17-OH-progesterone–umbelliferone conjugate. Anti-17-OH-progesterone IgG fraction (2 nM) was incubated with varying levels of competitors in 50 μl buffer for 20 min at 37°C. 17-OH-progesterone–umbelliferone conjugate (50 μl, final conc. 24 nM) was then added, and the reactions were incubated for another 30 min at 37°C. Buffer (0.4 ml) was then added and the liberated umbelliferone was measured.

hydrolysis of 17-OH-P–U conjugate by anti-steroidal IgG fractions was selective, and that the antibodies recognized the nature of the link between the steroid and the dye, and the position on the steroid molecule where the linking group was introduced.

3.5. *Binding assay for 17α-OH-progesterone*

A binding assay for 17α-OH-progesterone was developed by using anti-17-OH-progesterone IgG fraction, 17-OH-P–U conjugate as a label, 17-OH-progesterone as a competitor and by monitoring the assay fluorometrically. A linear relationship was observed between inhibition of the hydrolysis of 17-OH-progesterone–umbelliferone conjugate and 17-OH-progesterone within 0.02–6 μM (fig.5). Closely related steroids such as testosterone, progesterone, cortisol and estradiol did not significantly reverse the antibody-enhanced hydrolysis of 17-OH-progesterone–umbelliferone conjugate. On the other hand, 17-OH-progesterone-7α-carboxyethyl thioether, the hapten used for the preparation of the immunogen, was a more potent inhibitor than 17-OH-progesterone (~100-times).

4. Discussion

This assay permitted the determination of free 17α-OH-progesterone at a maximum sensitivity of 4 ng/tube, using short reaction times (50 min), without the need for physical separation of the bound and free forms of the ligand or for expensive instrumentation. Furthermore, this assay eliminated the need for enzymic hydrolysis of the steroid–fluorescent marker conjugate, thus reducing the number of reagents in the system. This method is one of the simplest techniques described so far for homogeneous immunoassay, utilizing an enzyme-like property of antibody and a steroid–fluorescent dye conjugate.

We are presently continuing our studies in the 'esterase' like properties of anti-steroidal IgG fractions with a view to gaining an insight into the mechanism of the antibody enhanced hydrolysis of steroid–fluorescent dye conjugates, and the results will be reported shortly.

Acknowledgements

We are grateful to Mrs J. Ausher for technical assistance, to Professor H. R. Lindner for valuable discussions and to Mrs M. Kopelowitz for secretarial assistance.

References

[1] Kohen, F., Hollander, Z., Burd, J. F. and Boguslaski, R. C. (1977) J. Steroid Biochem. 8, abst. 10, p. ix.
[2] Kohen, F., Bauminger, S. and Lindner, H. R. (1975) in: Steroid Immunoassay, (Cameron, E. H. D. et al. eds) pp. 11–32, Alpha Omega, Cardiff.
[3] Abraham, G. E. (1975) in: Steroid Immunoassay (Cameron, E. H. D. et al. eds) pp. 68–78, Alpha Omega, Cardiff.