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HYDROLYSIS OF STEROID HORMONE ESTERS BY AN UNSPECIFIC CARBOXYLESTERASE* FROM PIG LIVER MICROSOMES

CHRISTOPH SCHÖTTLER† and KLAUS KRISCH

Institut für Physiologische Chemie und Physikochemie der Christian-Albrechts-Universität, 23 Kiel, GFR

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Abstract—Twenty-two steroid esters, most of them therapeutically used as long-acting hormone preparations, were studied as *in vitro* substrates of an unspecific carboxylesterase from pig liver. All but four were hydrolysed. The activities were measured titrimetrically using the pH stat technique, and the kinetic parameters K_m and k_{cat} were determined. All Michaelis constants were found to be in the range of 10^{-5}–10^{-6} M. The enzymatic hydrolysis of most substrates followed Michaelis–Menten kinetics. Some deviations are discussed. Some experiments on the influence of acetonitrile, used as a solvent for all substrates, are reported.

IN ORDER to prolong their duration of action, many steroid hormones and analogous synthetic derivatives are therapeutically applied as esters. There is little information available, however, on the enzymatic hydrolysis of steroid esters *in vivo* and *in vitro*.

Bischoff *et al.*[1,2] described the enzymatic hydrolysis of several steroid esters by serum from rabbit, ox, and man. In 1953 Dirscherl and Krüskemper[3] demonstrated the occurrence of esterase activity toward desoxycorticosterone acetate, testosterone propionate, and cortisone acetate in mouse liver and kidney homogenates. These studies were extended by Dirscherl and Dardenne[4] who reported further results on the hydrolysis of steroid esters by homogenates of various tissues from rat, ox, horse, rabbit, and man. In 1972 Weisenberger and König[5] found that dexamethasone 21-isonicotinate is hydrolyzed very rapidly by serum from rats and rabbits, but rather slowly by human serum.

None of these studies, however, was carried out with purified and well-defined enzymes but with serum or crude tissue homogenates. Consequently, they could not answer the question whether specific steroid esterases exist or, alternatively, whether the hydrolysis of steroid esters is effected by the widely distributed unspecific carboxylesterases (EC 3.1.1.1). For the same reasons no kinetic studies on the cleavage of steroid esters have been done.

In this paper we report the results of investigations on the kinetics of the enzymatic hydrolysis of various steroid hormone esters by a highly purified unspecific carboxylesterase from pig liver microsomes (for a review see ref. 6).

* Carboxylic ester hydrolase, EC 3.1.1.1.

† The results reported in this paper are part of the doctoral thesis of Ch. Schöttler.
This paper is dedicated to Prof. Dr. Hans Netter on the occasion of his 75th birthday.

MATERIALS AND METHODS

Steroid hormone esters. The following compounds were used as substrates: (1) testosterone cyclopentylpropionate (Depovirin®); (2) testosterone propionate (Perandren®); (3) testosterone isobutyrate (Perandren® M); (4) testosterone oenanthate (Testoviron-Depot®); (5) testosterone benzoate; (6) testosterone acetate; (7) nortestosterone phenylproprionate (Durabolin®); (8) estradiol-17β-cyclopentylpropionate (Depofemin®); (9) estradiol-17β-valerate (Progynon-Depot®); (10) estradiol-3-benzoate (Progynon B oleosum®); (11) estradiol-3,17β-dipropionate (Ovocyclin®); (12) estradiol-17β-propionate; (13) estrone acetate; (14) 17α-hydroxyprogesterone-17-capronate (Proluton-Depot®); (15) cortisol-21-acetate; (16) cortisone acetate; (17) prednisolone acetate; (18) 16-methylprednisolone-21-diethylaminoacetate; (19) 11-deoxycorticosterone acetate; (20) cortisol-21-hemisuccinate; (21) prednisolone-21-hemisuccinate (Solu-Decortin H®); (22) dexamethasone isonicotinate (Auxiloson®).

The purity of all the compounds was checked by thin-layer chromatography. In all cases only one spot was obtained under the conditions described below. The steroid esters, indicated by the numbers used above, were kindly provided by the following companies: 2,3,11, Ciba-Geigy, Wehr/Baden; 5,6,13,16, Fluka, Neu-Ulm; 1,8, Farbwerke Hoechst, Frankfurt a.M.; 7, Organon, München; 4,9,10,12,14,17, Schering AG, Berlin; 17,18,19,21, Merck, Darmstadt; 15,20 Th. Schuchardt, München; 22, Karl Thomae, Biberach a.d.Riß.

Enzyme. Carboxylesterase was isolated from pig liver microsomes according to a previously described procedure.[7] The final preparation was subjected to ultrafiltration in order to remove salt and buffer ions which may interfere with the pH-stat titrations described below.

The protein concentration was determined by a microbiuret procedure.[8,9]

Thin-layer chromatography. For the qualitative detection of the enzymatic hydrolysis of steroid esters 100 nmoles of steroid ester were dissolved in 20 μl of acetonitrile and then added to an incubation mixture containing 200 μmoles of Tris–HCl pH 8·6, and 10 μl of pig liver esterase (0·935 μg protein) in a total volume of 2 ml. Incubation was carried out in glass-stoppered tubes for 60 min at 30°. The reaction was stopped by addition of 4 ml methylene chloride and shaking for 2–3 min. The organic phase was concentrated in a rotary evaporator and 5–10 μl samples applied to thin-layer plates. We used 20 × 20 × 0·4 cm glass plates covered with a layer (thickness 0·3 mm) of silica gel $HF_{254+366}$ with fluorescence indicator (E. Merck/Darmstadt). The plates were developed with the following solvent systems: (a) for testosterone and derivatives: methylene chloride–tetrahydrofurane (93:7, v/v); (b) for estrogens and derivatives: benzene–ethanol (9:1, v/v); (c) for corticosteroids and derivatives: chloroform–acetone (3:1, v/v).

The plates were examined under u.v. light and the steroids detected as dark spots on a green–yellow fluorescent underground. They were identified by comparison with the migration of reference substances.

Titration of acid equivalents by the pH-stat-technique. All titrations were carried out with an Autotitrator (TTT 1c) with automatic burette (ABU 13) and continuous registration (Titrigraph SER 2c; all from Radiometer, Copenhagen). Because of the poor water solubility of the steroid substrates it became necessary to increase the sensitivity of the assay. This was effected by a relatively large test volume, i.e. 25 ml, and by use of a highly diluted titrant, namely 0·004 N NaOH. Thereby the formation

of down to 10 nVal H^+ per minute could be conveniently measured. Interfering CO_2 was excluded by continuous bubbling of alkali-washed nitrogen through the reaction mixture. The pH was 8·0 and the temperature 30°. The reaction was recorded for at least 3 min. If not mentioned otherwise, the final acetonitrile concentration was always 2% (v/v), regardless of the substrate concentration. Six to eight different substrate concentrations were employed to measure the K_m. Because of the poor solubility of the steroid esters it was not possible to prepare substrate solutions of higher concentrations than 2×10^{-4} M. On the other hand, a minimal substrate concentration is required in order to obtain a linear reaction rate up to 3 min. (This also depends on a sufficiently low K_m.) In general, 10^{-6} M was the lowest possible substrate concentration; therefore most determinations of K_m and V were carried out in a range of substrate concentration from 1 to 200×10^{-6} M.

A linear relationship between the activity, expressed in acid equivalents liberated per minute, and the enzyme concentration in the range of 1–20 μg protein/25 ml was obtained. The time course of the reaction was linear for all substrates but estradiol dipropionate for at least 3 min. Initial velocities were calculated from the slopes of the curves. A non-enzymatic hydrolysis was not observed with any of the 22 steroid esters studies under the conditions specified, i.e. pH 8·0, a temperature of 30°, and within a time period of 5 min.

Evaluation of the data. Initial velocities were calculated from the slopes of the registered curves. K_m and V were obtained by graphical extrapolation from double-reciprocal plots according to Lineweaver and Burk.[10] The catalytic constant is defined as $k_{cat} = V/e_0$, where V is the maximal velocity and e_0 the amount of enzyme.[12] The values were related to the time-unit of 1 min and based on a molecular weight of 166,000.[13]

RESULTS

Kinetic parameters of the hydrolysis of steroid esters by pig liver esterase. After preliminary qualitative studies using thin-layer chromatography (see Methods) had revealed that almost all steroid hormone esters were rapidly hydrolysed by pig liver esterase we started a systematic study in order to determine the corresponding maximal velocities and Michaelis constants. Using the pH-stat method the initial rates of hydrolysis of a total of 19 steroid esters were studied as a function of the substrate concentration and plotted according to Lineweaver and Burk.[10] If not stated otherwise, no deviations from normal Michaelis–Menten kinetics were found. A summary of the kinetic data is given in Table 1.

Kinetic anomalies. A number of steroid substrates deviated from the expected kinetic behaviour. Thus, estradiol cyclopentylpropionate, in the presence of 2% acetonitrile, caused a substrate inhibition of pig liver esterase at concentrations higher than 1×10^{-5} M (Fig. 1). In this case, K_m and V were obtained by extrapolating the linear part of the curve to infinite substrate concentration.

With estradiol dipropionate as a substrate the time course of the reaction was sigmoidal (Fig. 2) creating difficulties in determining exactly the initial velocity.

Both testosterone benzoate and testosterone acetate had a considerably lower substrate affinity than the other steroid esters. Therefore, the reaction rates were too slow for an evaluation at substrate concentrations below 2×10^{-5} M. Non-linear

TABLE 1. KINETIC PARAMETERS OF THE HYDROLYSIS OF STEROID HORMONE ESTERS BY PIG LIVER ESTERASE

Substrate	K_m (M)	k_{cat} (min^{-1})	k_{cat}/K_m (l. $mole^{-1}$ min^{-1})
Androgens			
Testosterone cyclopentyl propionate	$2{\cdot}0 \times 10^{-6}$	1250	$6{\cdot}25 \times 10^{8}$
Testosterone propionate	$1{\cdot}4 \times 10^{-5}$	1385	$9{\cdot}89 \times 10^{7}$
Testosterone isobutyrate	$4{\cdot}6 \times 10^{-6}$	935	$2{\cdot}03 \times 10^{8}$
Testosterone oenanthate	$4{\cdot}3 \times 10^{-6}$	3740	$8{\cdot}70 \times 10^{8}$
Anabolics			
Nortestosterone phenylpropionate	$4{\cdot}6 \times 10^{-6}$	3675	$8{\cdot}17 \times 10^{8}$
Estrogens			
Estradiol-17β-cyclopentylpropionate	$3{\cdot}5 \times 10^{-6}$	735	$2{\cdot}10 \times 10^{8}$
Estradiol-17β-valerate	$5{\cdot}4 \times 10^{-6}$	1765	$3{\cdot}27 \times 10^{8}$
Estradiol-3-benzoate	$3{\cdot}0 \times 10^{-6}$	5020	$1{\cdot}67 \times 10^{9}$
Estradiol-17β-propionate	$1{\cdot}0 \times 10^{-5}$	5020	$5{\cdot}02 \times 10^{8}$
Estrone acetate	$4{\cdot}2 \times 10^{-5}$	8350	$1{\cdot}99 \times 10^{8}$
Corticosteroids and derivatives			
16-Methyl-prednisolone-21-diethylaminoacetate	$8{\cdot}3 \times 10^{-5}$	885	$1{\cdot}07 \times 10^{7}$
Corticosterone acetate	$1{\cdot}3 \times 10^{-5}$	1250	$9{\cdot}62 \times 10^{7}$
11-Deoxycorticosterone acetate	$1{\cdot}3 \times 10^{-5}$	5020	$3{\cdot}85 \times 10^{8}$

Lineweaver–Burk plots were obtained with the acetic acid esters of cortisol, cortisone and prednisolone (see example given in Fig. 3). The downward deflection of the curve indicates a substrate activation, a phenomenon described repeatedly for some other substrates of pig liver esterase.[13–17] Because of the difficulties in the quantitative evaluation of the data, none of the substrates mentioned in this paragraph are included in Table 1.

Dexamethasone isonicotinate and 17α-hydroxyprogesterone-17-capronate were not measurably hydrolysed by pig liver esterase. In addition, no hydrolysis was found with cortisol-21-hemisuccinate and prednisolone-21-hemisuccinate.

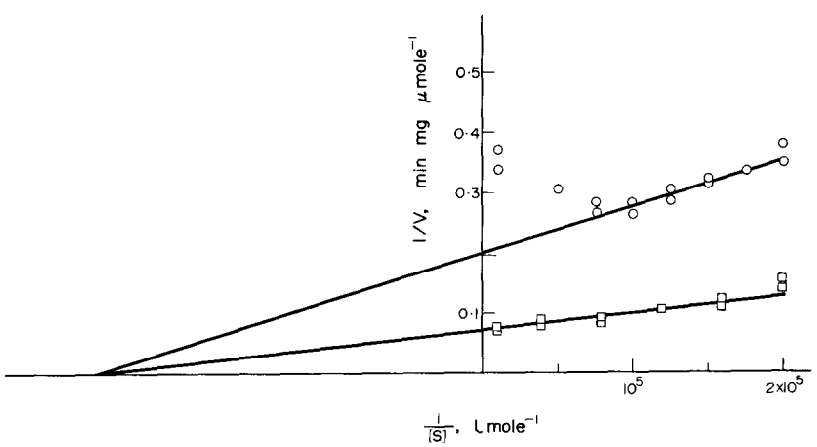

FIG. 1. Kinetics of the enzymatic hydrolysis of estradiol cyclopentylpropionate in the presence of 2% (○) and 10% (□) acetonitrile (v/v). Lineweaver–Burk plot.

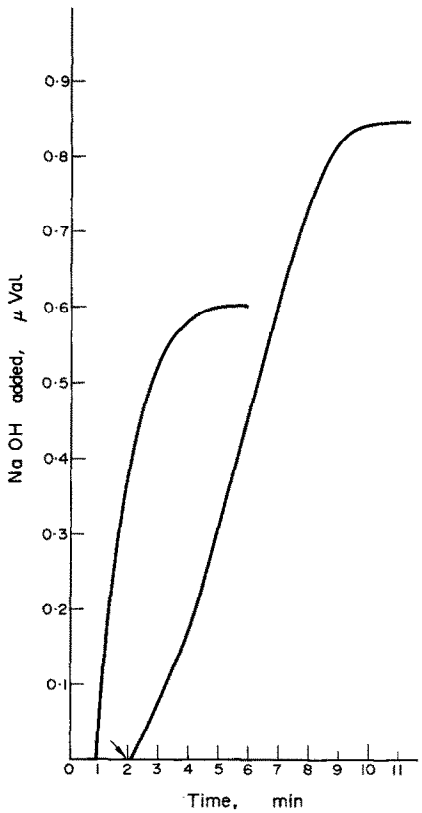

FIG. 2. Time course of the enzymatic hydrolysis of estradiol dipropionate by pig liver esterase (right curve). Original recording. The left curve represents the unspecific NaOH consumption for adjusting the reaction pH to 8·0. The start of the reaction by addition of enzyme is indicated by an arrow.

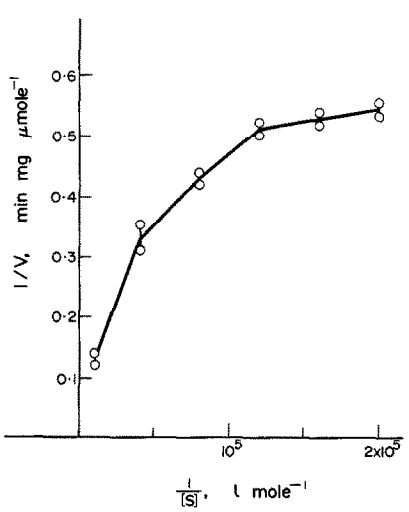

FIG. 3. Substrate activation of pig liver esterase by cortisol acetate. Lineweaver–Burk plot.

TABLE 2. HYDROLYSIS OF STEROID HORMONE ESTERS BY PIG LIVER ESTERASE. INFLUENCE OF ACETONITRILE ON THE RELATIVE REACTION RATE

	$V_{obs}/V_{1\%}^{*}$	Optimal concn of acetonitrile (%)
Substrates whose hydrolysis is activated by acetonitrile ($V_{obs} > V_{1\%}$)		
Testosterone cyclopentylpropionate	8·9	9·0
Testosterone oenanthate	5·5	9·0
Nortestosterone phenylpropionate	10·0	9·0
Estradiol-17β-cyclopentylpropionate	6·6	11·0
Estradiol-17β-valerate	6·0	6·0
Estradiol-3-benzoate	8·4	11·0
Estrone acetate	9·7	7·0
Substrates whose hydrolysis is inhibited by acetonitrile ($V_{obs} < V_{1\%}$)		
Testosterone propionate		
Testosterone isobutyrate		
Testosterone acetate		

The substrate concentration was 5×10^{-5} M throughout.
The acetonitrile concentration was varied from 1 to 19% (v/v) with intervals of 1%.
* Activity observed in the presence of the optimal concentration of acetonitrile divided by the activity with 1% acetonitrile.

Influence of acetonitrile on the reaction rate and K_m. Because of the poor solubility of most steroid substrates in water, the addition of organic solvents to enzymatic reaction mixtures cannot be avoided. If not mentioned otherwise, we have used acetonitrile in a final concentration of 2 per cent (0·38 M) throughout all experiments.

The influence of increasing concentrations of acetonitrile in the range of 1–19 per cent on the activity of pig liver esterase with various substrates is shown in Table 2. It is obvious, that an increase in the acetonitrile concentration leads to a 5- to 10-fold activation using most steroid esters as substrates. The optimal acetonitrile con-

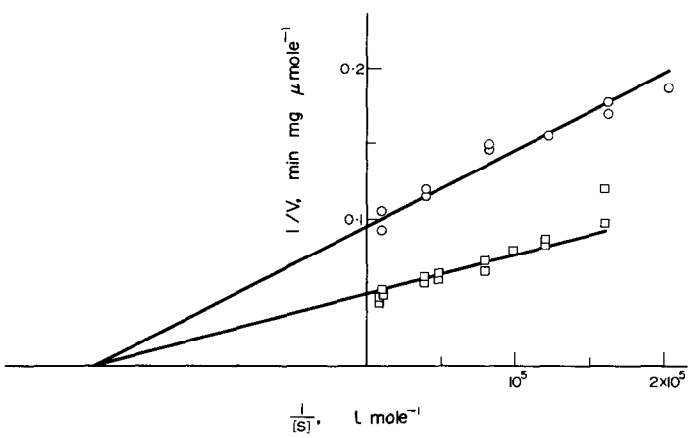

FIG. 4. Determination of the K_m of pig liver esterase with estradiol valerate in the presence of two different concentrations of acetonitrile. (○) 2% Acetonitrile (v/v); (□) 4% acetonitrile (v/v).

centration varies between 6 and 11 per cent depending on the substrate, while the activity falls off steeply at higher concentrations of the solvent. In contrast, when 3 esters of testosterone were studied as substrates, an immediate decrease of activity occurred with an increase in the acetonitrile concentration to above 1 per cent.

Since some organic solvents are known to influence the kinetics of pig liver esterase,[17,18] the effect of elevated acetonitrile concentrations on the Michaelis constant was studied. In these experiments the K_m of pig liver esterase for estradiol cyclopentylpropionate was determined in the presence of 2 and 10% acetonitrile, the latter being the optimal concentration for activation. The result is shown in Fig. 1. It is evident that the K_m is not altered under these conditions. It is interesting that the substrate inhibition is abolished at the higher acetonitrile concentration. The same result, i.e. no change in K_m, was obtained with estradiol valerate in the presence of 2 and 4% acetonitrile (Fig. 4).

DISCUSSION

The results of this study clearly show that many steroid hormone esters are effectively hydrolysed by pig liver carboxylesterase (EC 3.1.1.1). This is another example of the remarkably low substrate specificity of this enzyme.[6] Its substrates may contain alcohol moieties of such different structure and size as methanol, *p*-nitrophenol, retinol,[19] or various steroids. The steroid esters found to be substrates for pig liver esterase comprise androgens, estrogens and corticosteroids or their derivatives. Many of them are used therapeutically as long-acting "depot" preparations. It is generally believed that steroid esters have to be hydrolyzed before they exert their physiological or pharmacodynamical actions. There are, however, some exceptions. Thus cyproterone acetate is more effective than unesterified cyproterone and it is neither hydrolyzed *in vivo* by man nor *in vitro* by rat liver and intestinal mucosa.[20,21]

From our kinetic data a connection between the structure and hormonal action of the steroid esters on the one hand, and the rate of their hydrolysis by pig liver esterase on the other hand is not immediately evident. With regard to k_{cat} and the specificity constant k_{cat}/K_m estrone acetate and estradiol benzoate can be considered to be the best substrates, but the data of most other esters are in the same order of magnitude. Compared with those of other substrates[6,7] the Michaelis constants of steroid esters are remarkably low, i.e. in the range of 10^{-6}–10^{-5} M. All catalytic constants of pig liver esterase with steroid hormone esters are in the range of 735 (estradiol cyclopentylpropionate) to 8350 (estrone acetate). Thus, most steroid hormone esters are relatively good substrates for the esterase when both, activity and affinity, are considered. There are, however, some exceptions: cortisol-21-hemisuccinate, prednisolone-21-hemisuccinate, 17α-hydroxyprogesterone-17-capronate, which is the only 17α-hydroxyester studied, and dexamethasone isonicotinate were not measurably hydrolysed. With the relatively water-soluble hemisuccinates this negative result is not surprising, since it is known that substrates with a negatively charged carboxylate group are not or are poorly hydrolysed by pig liver esterase.[6,16] For the two other substrates the reasons are less obvious although for an explanation steric reasons may be discussed. Gerhards *et al.* recently found that C_{17} esters of derivatives of 17α-hydroxyprogesterone are not hydrolyzed by liver, adipose tissue, and intestinal mucosa of the rat.[20,21]

In some cases K_m and k_{cat} could not be determined because of technical difficulties. With estradiol-3,17-dipropionate the time-course of the reaction was not linear but sigmoidal (Fig. 2). This may be explained by the assumption that the two acyl groups are split off at different rates. That both ester bonds of estradiol dipropionate are cleaved by pig liver esterase is also obvious from the following stoichiometric consideration: in the experiment shown in Fig. 2, after complete hydrolysis a total uptake of NaOH of 1·0 μVal is expected if both acyl residues would be split off. As can be seen the reaction rate flattens off at about 0·85 μVal NaOH. The 15 per cent deviation from the theoretical is probably due to technical difficulties in determining the exact starting point of the reaction in autotitrator experiments. But it is possible as well, that equilibrium is reached before the hydrolysis is complete.

An interesting deviation from Michaelis–Menten kinetics, i.e. a downward deflection of the Lineweaver–Burk curve, was observed with the acetates of cortisol, cortisone, and prednisolone (Fig. 3). This "substrate activation" has been found by several authors with some, but by no means all, substrates of pig liver esterase[13–17] and may be attributed to the existence of two interacting binding sites on the enzyme. One of them is assumed to be the substrate-binding active site and the other a lipophilic modifier site, which may bind either a second substrate molecule or another lipophilic compound. Some authors[17,18] obtained a linear Lineweaver–Burk plot when binding the second site with benzene or dioxane, whereas in our experiments the substrate activation remained when increasing the concentration of acetonitrile. In the case of estradiol-17β-cyclopentylpropionate, however, the substrate inhibition was abolished by increasing the concentration of acetonitrile up to 10 per cent (Fig. 1). By increasing the acetonitrile concentrations a pronounced enzyme activation is found with most substrates, whereas with three testosterone esters an inhibition occurred. The reasons for the differing behaviour of these testosterone esters are not yet understood. Some organic solvents such as methanol, are known to influence esterase-catalysed reactions by nucleophilic competition for the acyl enzyme.[18] Such a mechanism, however, is improbable as an explanation of the effect of acetonitrile, which is rather an inert compound and cannot serve as acyl acceptor. In contrast to the activities, the K_m values are obviously not dependent on the acetonitrile concentration (Figs. 1 and 4). It should be mentioned, however, that the interpretation of kinetic results obtained in the presence of organic solvents is always somewhat ambiguous because alterations in the physical state of sparingly water-soluble substrates, such as microprecipitation, formation of micelles etc. can hardly be ruled out with certainty.

Our results have shown that an unspecific carboxylesterase from pig liver hydrolyses numerous steroid hormone esters. Further work will be required to clarify whether esterase preparations from species other than pig[6] are also able to hydrolyse steroid hormone esters and whether unspecific carboxylesterases are responsible for the cleavage of steroid esters *in vivo* as well.

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