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SOME NEW DEVELOPMENTS IN THE KNOWLEDGE OF HUMAN PLACENTAL ESTRADIOL-17β DEHYDROGENASE

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SUMMARY

Since the discovery of a high estradiol-17β dehydrogenase (EDH) activity in human placenta [1] extensive work was carried out on this soluble enzyme by Engel's, Talalay's, Warren's groups and by ourselves, in order to gain information on its biological function. Moreover, it rapidly became apparent that it was a convenient model for the study of steroid–protein interactions, since this relatively abundant enzyme is now available in pure form. This enzyme is a dimer (34,000 × 2), with 6 Cys by monomer, 3 of which being buried in the dimeric structure. Each Cys is carried by a different chymotryptic or tryptic peptide (one of these tryptic peotides, carrying an essential Cys, belonging to the coenzyme binding site, has been sequenced): thus specific labelling, of these Cys residues can be easily studied. An attempt to determine the geometry of the substrate recognition site was made, according to the results of kinetic studies. Affinity labelling of this site, with more than 20 alkylating estradiol derivatives allowed the localization of an His in the A ring vicinity and probably above the β face of the ring. A Cys was also localized in the catalytic site; it is also labelled by 3 chloroacetyl pyridine adenine dinucleotide, an alkylating analog of NAD. The results suggest that the substrate is, in the evolutive ternary complex, oriented towards the coenzyme according to Prelog's hypothesis on the orientation of asymetric polycyclanic alcohols towards nicotinamide in dehydrogenases. Regarding the molecular mechanism of the catalysis, some new results suggest half-site reactivity.

In 1953, Ryan and Engel[1] reported that in human term placenta a soluble enzyme transformed 17β estradiol into estrone and *vice versa*. A first purification was described by Langer and Engel[2], which named the enzyme estradiol dehydrogenase owing to its high specificity [3] for this hormone (K_M = in the range of 10^{-6} M, with NAD as coenzyme).

Since 1958 this enzyme has been extensively studied in a number of laboratories, namely Engel's, Talalay's, Warren's and ourselves. At the present time, it is the most well known tissular enzyme of steroid hormone metabolism, it is a good tool for studying steroid–protein interactions and most of its physiological interest lies in the fact that it catalyses the inter-conversion of estradiol and estrone, a biologically active estrogen and a relatively inactive one. A brief survey of the status of our knowledge on this enzyme will be presented with special reference to our own contribution.

As demonstrated in the early studies using crude preparation, and as confirmed later with highly purified enzyme, the soluble estradiol dehydrogenase of human placenta has all the main characters of any hydroxysteroid oxydoreductase:

—It is a NAD^+ dependent enzyme which uses either NAD^+ or $NADP^+$ but with a lower K_M for $NADP^+$: $K_{M_{NAD^+}} = 13 \pm 2$ μM, $K_{M_{NADP^+}} = 1 \pm 1$ μM. Our results are in agreement with Jarabak's [4], Warren's[5] and with binding experiments [4] which reported dissociation constants of 1.9 μM and 0.051 μM respectively for NAD^+ and $NADP^+$;

—It behaves *in vitro* like an oxydoreductase able to catalyse, depending on the pH, the interconversion of a ketone and a secondary alcohol, as shown in Fig. 1;

—It behaves *in vitro* like an oxydoreductase able to a strict double stereospecificity: it is a "B" enzyme (transferring the hydrogen "pro S" of the nicotinamide) and leads to S alcohols [6, 7];

—It is highly selective, acting rather exclusively on the C_{17} carbon atom of steroids of the estrane series [3, 8, 9].

The main peculiarity of the estradiol dehydrogenase of human placenta is to act as a *trans-hydrogenase*: in the presence of catalytic amount of estrogens and $NADP^+$ the enzyme transfers hydrogen from a NADPH generating system to NAD^+ through the cyclic estrone and estradiol interconversion shown in Fig. 2, as suggested by Talalay *et al.*[10] and demonstrated by Engel *et al.*[11]. In this system the steroid probably remains bound to the enzyme as

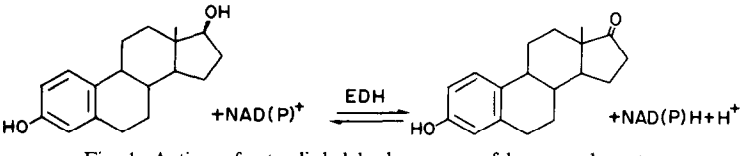

Fig. 1. Action of estradiol dehydrogenase of human placenta.

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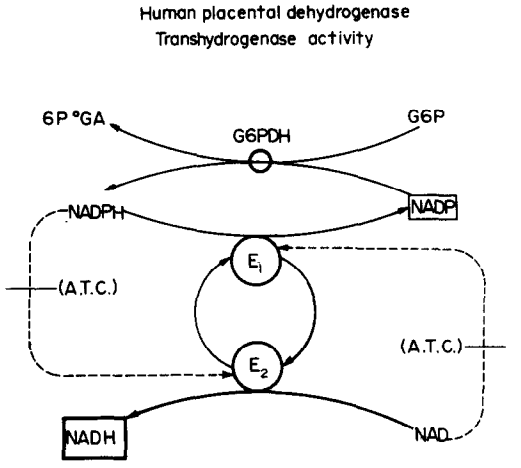


Fig. 2. The transhydrogenase function of the estradiol dehydrogenase. The NADPH formed by a generating system is continuously oxidized by catalytic amounts of estrone which are converted into estradiol by estradiol dehydrogenase. Estrone is regenerated by the same enzyme in the presence of NAD^+. The interrupted lines and the mention (A.T.C.) indicate the inability of the enzyme to form abortive ternary complexes. GP°GA = 6 phosphogluconic acid. G6P = glucose 6 phosphate. G6PDH = glucose 6 phosphate dehydrogenase. E_1 = estrone. E_2 = estradiol.

shown by its very high affinity for estradiol (K_M transhydrogenase = 0.2 μM). Of course, this trans-hydrogenase function implicates that neither NADPH in the presence of estradiol, nor NAD^+ in the presence of estrone bind to the enzyme. Such associations would be abortive ternary complexes and in fact they have been shown not to be possible with estradiol dehydrogenase [12]. Thus the trans-hydrogenase function appears to be a consequence of the coincidence of two properties of this dehydrogenase: ability to work with NAD^+ or $NADP^+$, and inability to form abortive ternary complexes.

The knowledge of the physicochemical properties of estradiol dehydrogenase needed pure enzyme. In three laboratories, Engel's, Jarabak's and ourselves', extensive purification resulted in the obtainment of homogenous estradiol dehydrogenase in sufficient amounts to perform amino acid analysis of the protein. The discovery of the stabilizing effect of estradiol or high concentrations of glycerol [2] against cold inactivation [13] allowed substantial ameliorations to the purification, but until 1972, the purification processes were "conventional" and rather tedious, yielding only small amounts of enzyme. Decisive progress needed other methods: in 1972, we synthetized an affinity column by coupling Sepharose-4B to carbone 3 of estrone with 2-aminocaproate as a "spacer group". Our studies on the geometry of the substrate binding site had previously demonstrated that such a spacer group could be easily accepted in the active site of the enzyme [9]. Elution was performed either with estrone-hemisuccinate or with a negative gradient of ammonium sulphate [14]. In combination with conventional procedures we now obtain pure estradiol dehydrogenase (S.A. 4 I.U./mg corresponding to about a 500-fold purification of the crude homogenate. About 300 kg of placenta were treated in our laboratory giving 3 g of pure enzyme used mainly for chemical investigation and affinity labelling. We don't use the affinity chromatography more recently proposed by Warren[15] since, in this method, the absorbent must be resynthetized after each operation.

Our latest results, in agreement with Jarabak's[16] and Engel's[17] indicate that estradiol dehydrogenase is not a glycoprotein. Its molecular weight is 68,000 and it is a dimer with two subunits of the same molecular weight. The identity of the two subunits is based on the following arguments:

—There is a single N terminal pentapeptide [18];
—There are 12 cystein residues and we have shown as described below that chymotryptic hydrolysis gives only six different cysteinyl peptides;
—Each subunit has an essential cystein residue in its active site and this cystein belongs to a same tryptic peptide, as shown by Nicolas in Harris's laboratory [19].

However, against the identity of the two subunits it should be mentioned that using isoelectric focusing, a method which is known to have a high resolving power, Engel[20] found 5 bands, bearing enzymatic activity, which resolved into 3 bands in dissociating conditions. As estradiol dehydrogenase is not a glycoprotein, these results support the idea that this enzyme could be of an isozymic type.

There are some serious discrepancies in amino acid composition of estradiol dehydrogenase when determined on U.S. samples or French samples (Table 1): these discrepancies do not result from experimental errors, since samples have been exchanged with Engel's laboratory, and the first results were confirmed. As experiments were performed on pooled placentas, the hypothesis that heterogeneity and differences in amino acid composition come from genetic differences between populations, should be seriously considered as suggested in [20]. But, anyway, even if estradiol dehydrogenase is isozymic, it is highly probable, as it is of common observation with most of the known isozymic systems, that estradiol dehydrogenase subunits differ only by a minor modification of the primary structure, not affecting the active site. For this reason, it is very unlikely that such differences could alter the validity of the results obtained in the study of this active site.

At the beginning of our studies, only small quantities of relatively impure enzyme were available, and we could only do kinetic measurements such as those of Ringold with 3α hydroxysteroid dehydrogenase of *Pseudomonas testosteroni* [21, 22]. However, using this "kinetic method", and applying the theoretical concepts given by Prelog in the study of nicotinamide dehydrogenases acting on cyclic ketones [23], we were able to "design" the shape of the substrate binding site as follows: if we superimpose the C—O bond

Table 1. Amino acid composition of estradiol dehydrogenase prepared in U.S.A. and in France

Amino acid	U.S. Preparation analysed by			French preparation analysed by			
	Groman*	Burns[18]	Jarabak[17]	French workers	Groman*	French workers I[14]	II
Lys	10	10	11	10	12	12–14	15
Arg	23	22	21	22	19	18–20	17–18
Ile	5	4	4	5	8	8–9	10
Trp	1	1	1	—	—	2	2
His	7	7	6	7	8	7	7
Asp	22	21	23	(26)	26	25–28	27–28
Thr	15	16	16	16	16	16	16–17
Ser	19	19	19	18	22	18	20
Glu	29	28	29	32	32	34–35	34–35
Pro	20	19	18	19	22	19	20
Gly	29	31	29	28	30	25–28	24
Ala	35	36	31	34	35	30–34	27
1/2 Cys	6	6	6	6	6	6	6
Val	30	32	27	29	29	27–30	26
Met	3	4	3	5	3	6	6
Leu	42	41	39	39	(48)	34–39	33
Tyr	8	6	5	7	6–7	7–8	8
Phe	13	13	11	12	12	12	12
N =	317	316	299		334	314	312

The main differences affected the four amino acids (Lys–Arg–Ileu–Trp). * Personal communication.

to be transformed and the two neighbouring C–C bonds of a large series of "good" substrates of rigid conformation (which is the case here) we can obtain a kind of "mold" of the substrate binding site, since the lower the K_M, the better is the fitting in the site. Applying this method to estradiol dehydrogenase, and taking in consideration the fact that estradiol dehydrogenase is a B enzyme giving alcohol S (as shown in Fig. 3), we obtained the following results: The steroid binding site looks like a cleft with a large part receiving the C-D rings and a narrow one corresponding to the A-B ring region. This cleft is "closed" on one side by the coenzyme, and widely open on the opposite side. This model (Fig. 4), when proposed, was able to explain some biological properties of estradiol dehydrogenase, for instance its good activity on steroids conjugated in position 3. It gave also valuable indications for the coupling of steroids in order to build adsorbents for affinity chromatography and it appeared later to be consistent with the results of affinity labelling. Finally, it should be noted that this "strategy", in the design of a substrate binding site, was successfully applied to other steroid dehydrogenases [24, 25].

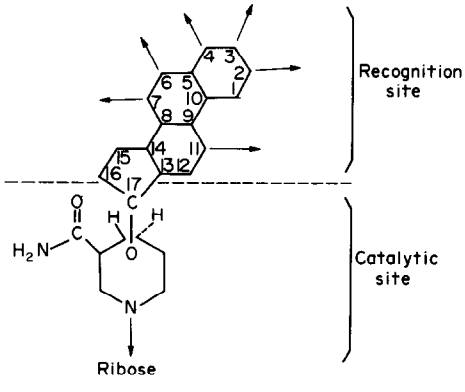


Fig. 3. Hypothetical transition state during oxidoreduction catalysed by estradiol dehydrogenase: the respective orientations of estradiol and NAD were inferred from the stereospecificity of the enzyme according to Prelog's hypothesis [23].

Like many other dehydrogenases, the estradiol dehydrogenase was known to be sensitive to thiol reagents. Thus, we developed a study of the cysteine residues of this enzyme with a special care to their reactivity and implication in enzyme activity. Amino acid analysis indicates that one mole of estradiol dehydrogenase (dimer) contains 12 cystein residues. These residues are not implicated in the formation of disulphide bridges since, in dissociating conditions (Urea 8 M), 12 thiol groups are titrated by Ellman's reagent. Neither the rates of reaction of the thiol groups, nor the amount of thiols titrated are identical when the enzyme is not completely unfolded by 8 M urea or 6 M guanidine–HCl. The overall reactivity of the titrated thiols is: denatured enzyme > native enzyme in aqueous buffer > enzyme in 20% glycerol. This glycerol concentration is necessary to protect the enzyme against cold inactivation and, in this condition, only 6 of the 12 cystein residues of the dimer can be titrated by Ellman's reagent. Furthermore, the rate of reaction of these 6 accessible residues is affected by the presence of estradiol and the coenzyme: estradiol faintly decreases the whole reactivity whereas the addition of 0.5 mM NADP protects 4 residues among the 6, and the enzyme activity is then fully retained (Fig. 5). With lower amounts of NADP (5 μM), the protective effect is reduced and the enzyme is slowly inhibited. Figure 6 shows the residual enzyme activity as a function of the number of

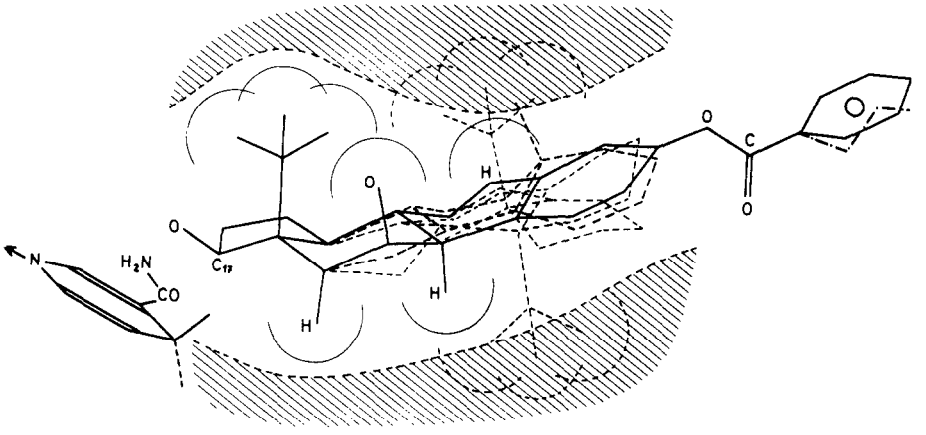


Fig. 4. The substrate binding site of estradiol dehydrogenase, as deduced from kinetic studies [9]. Hatched areas delineate regions of the active site forbidden to the substrate by aminoacids residues of the protein, bound water molecules, or part of the coenzyme... Full lines: "good substrates", dotted lines: molecules not accepted by the steroid binding site.

modified cystein residues: in these conditions, the modification of 4 residues per mol of estradiol dehydrogenase does not affect the enzyme activity, but the further chemical modification of the two remaining cystein residues results in simultaneous inactivation of the enzyme.

The identification of the residues essential for enzyme activity was effected through separation of the cysteine carrying peptides: the chymotryptic hydrolysate of estradiol dehydrogenase fully carboxymethylated with [2-^{14}C]-ICH_2COOH gave six radioactive peptides (Fig. 7). These peptides were separated by chromatography on Sephadex G 50 followed by electrophoresis at pH 6.5 Each peptide contained only one radiolabelled cystein residue; this result, which is in agreement with the dimeric structure, allows the identification of each cystein residue: C_1 and C_2 which are carried by two neutral peptides, and C_3, C_4, C_5, C_6 which are carried by peptides of increasing electrophoretic mobility. Thus the cysteins labelled by other reagents and more particularly with *N*-ethyl maleimide could be identified: in 20% glycerol, the cystein C_5 was by far the most reactive towards [2-^{14}C]-*N*-ethyl maleimide and a preferential labelling occurred with a stoichiometric inactivation of the enzyme (Fig. 8) showing the essential character of this residue. The sequence of the tryptic peptide containing this essential residue (Fig. 9) was determined in collaboration with Harris's laboratory [19]. Prolonged incubations in the same conditions resulted in alkylation of 6 cysteines per dimer which were identified as C_1, C_3 and C_5. It could be concluded that cys C_2, C_4 and C_6 were protected by the conformation favoured by the presenoe of glycerol.

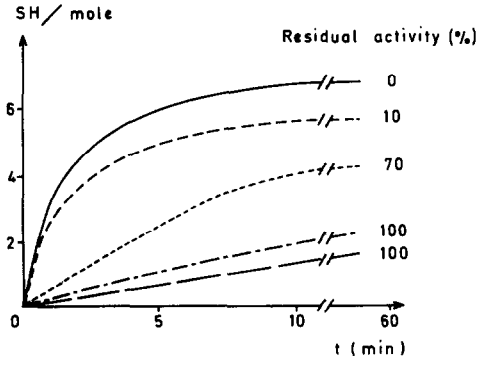


Fig. 5. Reactivity of the cystein residues of estradiol dehydrogenase, towards 5,5' dithiobis 2 nitrobenzoate (Ellman's reagent) in the presence of 20% glycerol and different concentrations of substrate and/or coenzyme. Estradiol dehydrogenase (0.2–0.3 mg/ml) was incubated in 0.1 M phosphate buffer pH 7.2, 20% glycerol, at 37°, with 5 mM Ellman's reagent. The reaction was followed spectrophotometrically at 412 nm by continuous monitoring of the absorbancy of the chromophore formed, against a blank from which the enzyme had been omitted. The reference and residual enzyme activities were measured at pH 7.2 because at an alcalin pH, Ellman's reagent undergoes decomposition leading to a chromophore interferring with enzyme activity measurements —— 1. Ellman reagent, –– 2. Ellman reagent + 50 μM estradiol, ----- 3. Ellman reagent + 50 μM NADP, —·— 4. Ellman reagent + 500 μM NADP, ——- 5. Ellman reagent + 500 μM NADP and 50 μM estradiol.

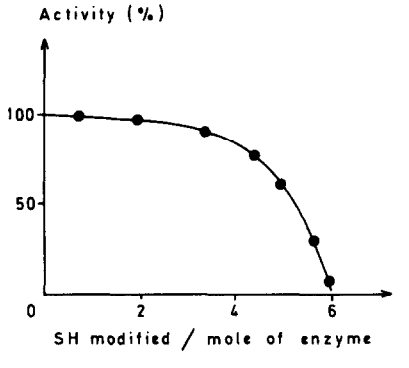


Fig. 6. Inhibition of estradiol dehydrogenase as a function of the number of cysteins modified by 5,5' dithio bis (2 nitrobenzoate) (5 mM) in the presence of NADP (5 μM). Same experimental conditions as in Fig. 5.

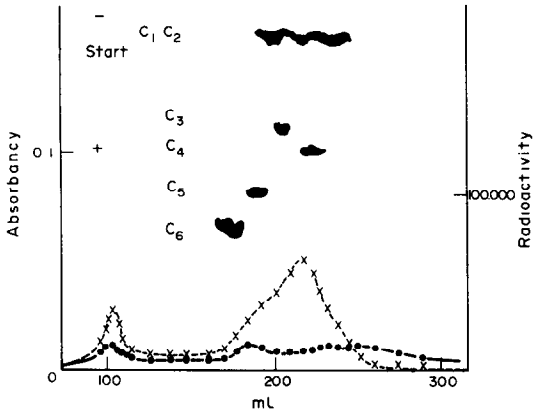


Fig. 7. The cystein containing peptides of estradiol dehydrogenase: electrophoretic separation after G50 Sephadex filtration. After dialysis against 100 vol. of 1 M Phosphate, 8 M urea buffer pH 8, the solution of estradiol dehydrogenase was treated for 3 h with 1 mM [2-^{14}C]-iodoacetic acid at 25° under nitrogen. 0.25 μM of the fully carboxymethylated enzyme were dialysed against 0.1 M ammonium bicarbonate buffer pH 8, then hydrolysed with 0.7 mg recrystallized α chymotrypsin (Worthington) for 4 H at 37°. After acidification (0.1 ml acetic acid) and lyophilisation the hydrolysate was filtered on a G50 Sephadex column. The elution diagram is shown in the lower part of the figure. Radioactivity (×——×), absorbance at 220 nm ●——●. The radioactive fractions were submitted to high voltage electrophoresis for 35 min (60 V/cm) on 3 MM Whatman paper at pH 6.5 in acetic acid–pyridine–water buffer (0.5:12.5:112, v/v). The [2-^{14}C]-carboxymethylated peptides were detected by autoradiography (24-72 H, Kodirex film). C_1 to C_6 refer to the six separated chymotryptic peptides each carrying a corresponding cystein.

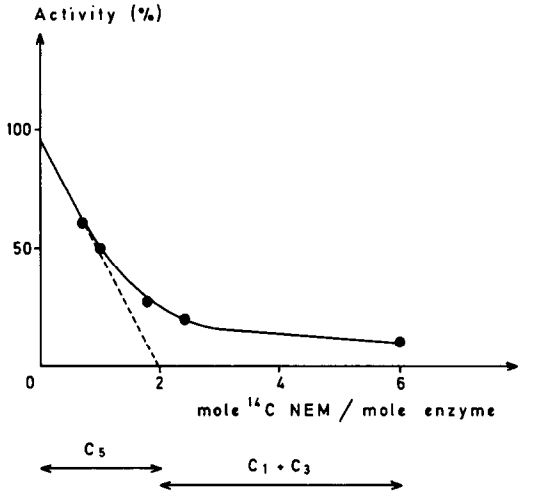


Fig. 8. Inhibition of estradiol dehydrogenase by [2-^{14}C]-*N*-ethyl-maleimide: percent residual activity as a function of the number of mole of *N*-ethyl-maleimide irreversibly bound by mole enzyme (dimer). The enzyme was incubated with 0.2 mM [2-^{14}C]-*N*-ethyl-maleimide (1 mC-/m-Mol) at pH 7.2 and 37° for several h. Aliquots were dialysed several times against 0.1 M Tris HCl buffer pH 8. The covalently bound radioactivity was determined by liquid scintillation counting after combustion of the dialysis bag in an "Oxymat" apparatus.

When NADP is added, the following results are obtained after 45 min incubation, with [2-^{14}C]-*N*-ethyl maleimide (Table 2):

—at a low NADP concentration (8 μM) 90% of enzyme activity remained while 0.8 mol of each cystein C_1, C_3, C_5 were labelled per mol of dimer, and the inactivation developed only with the further alkylation of the second mol of each of these residues. Since Cys C_5 is essential for enzymatic activity, and since 1 Cys C_5 by dimer was alkylated with retention of 90% activity, the "residual" enzymatic activity results from the non alkylated subunit. It can be concluded, from this observation, that at a low NADP concentration, the coenzyme has an asymetric protective effect towards the two subunits.

—At a higher NADP concentration ($\simeq 10^{-4}$ M), the protective effect of the coenzyme seems to be identical for the three cysteins C_1, C_3 and C_5, just as that observed with low concentrations (Table 2). But for a longer incubation time (24 H), the 2 mol of C_1 are alkylated while only 0.85 mol C_3 and 0.68 mol C_5 are modified, with a 25% loss of enzyme activity.

It could be concluded that C_1, which can be completely alkylated by *N*-ethyl maleimide in both subunits without loss of enzyme activity, is not an essential residue. On the other hand, at high concentrations of NADP (Table 3) or in absence of NADP, the alkylation of C_3 and C_5 is accompanied by an enzyme inactivation which is proportional to the alkylation of these residues in both subunits, and the asymmetric protective behaviour observed at low $NADP^+$ concentration is lost. It could not be concluded from these results whether C_3 was an essential residue or not.

Figure 10 summarizes the results of this study on cystein residues and emphasizes the difference in the

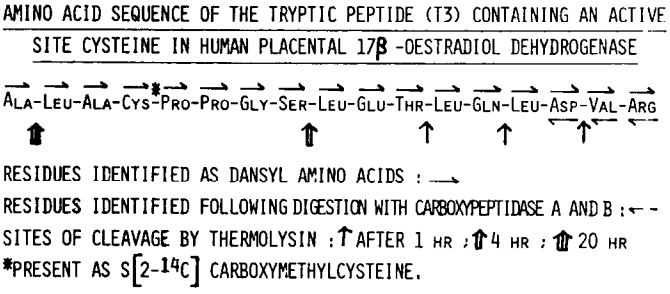


Fig. 9. Sequence of the tryptic peptide containing an essential cystein C_5.

Table 2. Relative labelling of cysteins C_1 C_3 C_5 by [2-^{14}C]-*N*-ethyl maleïmide in the presence of different concentrations of NADP

NADP concentration	$2\cdot10^{-6}$ M	$4\cdot10^{-6}$ M	$8\cdot10^{-6}$ M	$2\cdot10^{-5}$ M	$4\cdot10^{-5}$ M
Peptide					
C_1	1.6	1.24	0.84	0.76	0.75
C_3	1.6	1.2	0.76	0.64	0.60
C_5	1.7	1.4	0.80	0.65	0.50
Total	4.9	3.84	2.40	2.05	1.85
Residual activity	30%	60%	90%	100%	100%

1 ml of estradiol dehydrogenase (0.3 mg/ml) in presence of 20% glycerol and various amounts of NADP was incubated at pH 7.2 with $2\cdot10^{-4}$ M [2-^{14}C]-*N*-ethyl-maleïmide for 45 min. After precipitation by 10% trichloroacetic acid, the protein was hydrolysed by chymotrypsine for 4 h. The chymotryptic peptides were separated by paper electrophoresis at pH 6.5 and the radioactivity was determined by liquid scintillation counting.

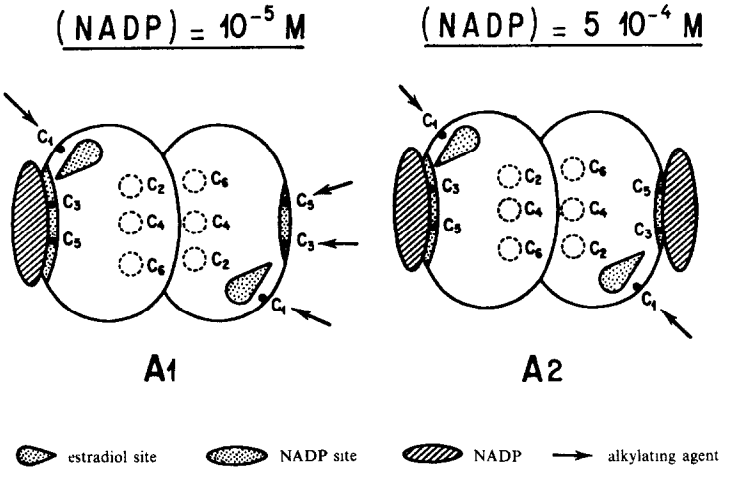


Fig. 10. Schematic interpretation of the differences between an alkylation by [2-^{14}C]-*N*-ethyl-maleimide when NADP is added in low $5\cdot10^{-5}$ M) or high ($5\cdot10^{-4}$ M) concentration. NADP, NADP site, estradiol site, → alkylation, ○ cystein residues: protected by 20% glycerol, ● cystein residues non protected by 20% glycerol. Differences in affinity for the coenzyme are expressed as the size of the binding areas.

protective effect of NADP at low and high concentrations. Further decisive progress in the knowledge of the active site and of the mechanism of action of estradiol dehydrogenase were done by using affinity labelling. Affinity labels, for enzymes, are analogs of substrate, or competitive inhibitors, able to react specifically and irreversibly with some amino acid residues in the active site. The mechanistic feature of the coenzyme and the relative rigidity of the substrate molecules make estradiol dehydrogenase a very suitable enzyme for affinity labelling: the substrate binding site can be explored using several analogs each carrying an alkylating substituent in different positions, as indicated in Fig. 11. Thus, the localisation of labelled amino acids in the binding site can be performed with some accuracy, provided the orientation of the substrate analog, during alkylation, is identical to that of the substrate during the transition state of the enzymic reaction.

Table 3. Relative labelling of cysteins C_1 C_3 C_5 by [2-^{14}C]-*N*-ethyl-maleïmide after various incubation times

Incubation time	9 min	30 min	100 min	24 h
C_1	0.21	0.41	0.66	1.9
C_3	0.05	0.13	0.33	0.85
C_5	0.07	0.14	0.21	0.68
Total	0.33	0.68	1.2	3.43
Residual activity	100%	100%	90%	75%

5 ml of estradiol dehydrogenase (0.3 mg/ml) were incubated in presence of 20% glycerol with 10^{-3} M NADP and $2\cdot10^{-4}$ M [2-^{14}C]-*N*-ethyl-maleïmide. Aliquots (1 ml) were taken at different times for chymotryptic hydrolysis. The peptides were separated by paper electrophoresis at pH 6.5 and the radioactivity was determined by liquid scintillation counting.

The affinity labels we have synthetized are mainly haloacetate or haloacetamide derivatives of estrone or estradiol. The alkylating group has been introduced in 9 different positions, as indicated by the arrows on Figure 10. Four types of affinity labels were used, they differed by the nature of the bond between

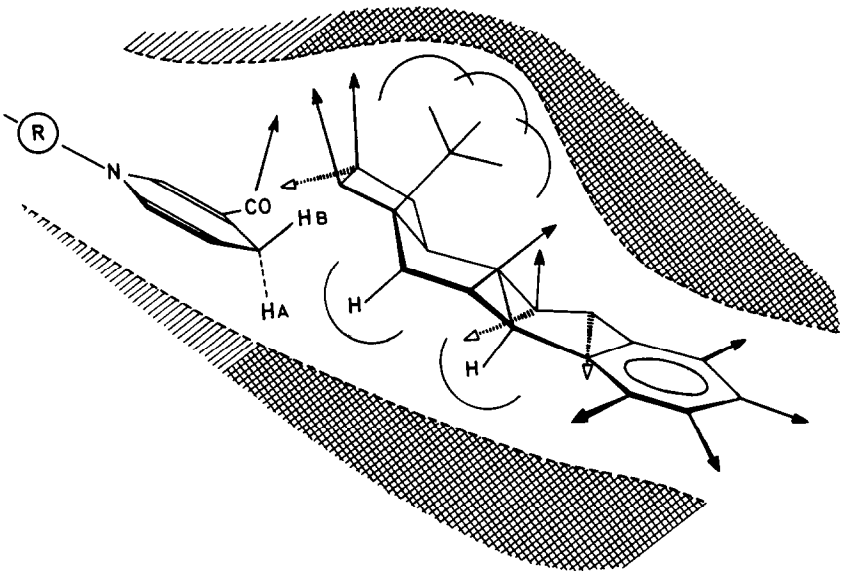


Fig. 11. Situation of the alkylating side chains of the affinity labels (arrows) used in this study. The scheme shows an hypothetic transition state inferred from our kinetic results [9] according to the stereospecificity of estradiol dehydrogenase and to Prelog's hypothesis [23].

the alkylating group and the carrying steroid: α haloketones, aromatic haloacetamides, alcoholic haloacetates, and phenolic haloacetates (Fig. 12). All can react, in aqueous solution, with nucleophilic aminoacid residues.

With affinity labels of types 1, 2 and 3 the labelled amino acid residue can be identified as its known carboxymethylated derivative(s), by aminoacid analysis. Moreover with affinity labels of types 1 and 2, the steroid nucleus can be readily split off in mild conditions, without hydrolysis of the peptide bond of the protein and even, with type 1, without denaturation of the enzyme. Enzymatic hydrolysis is then possible (it was impaired by the steroid), and the labelled (as a carboxymethylated derivative) amino acid can be localized on the tryptic or chymotryptic peptide.

With an affinity label of type 4, the chemical cleavage between the steroid and the labelled aminoacid cannot be obtained, and neither enzymatic hydrolysis nor identification of the labelled aminoacid is possible (the reference compounds, "steroid-aminoacids", not being available).

Most of these alkylating steroids have been radiolabelled either on the steroid itself, or on the alkylating side chain, so that we were able to follow the kinetic's of inactivation, the stoichiometry of alkylation and the hydrolysis and the release of the steroid

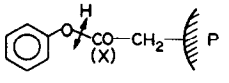
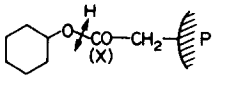
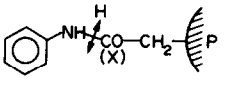
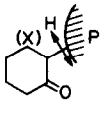
Affinity label (type)	Steroid release (H)	Enzymic hydrolysis of the protein
1 	A few hours at pH 7.2	Possible, after steroid release
2 	24 H in NaOH 0.5 N at 20°C	Possible, after steroid release
3 	24 H in HCL 6N at 110°C	Impossible, without steroid release, complete acid hydrolysis is only possible.
4 	Impossible	Impossible, and even after acid hydrolysis, the steroid remains covalently bound to the labelled amino-acid residue.

Fig. 12. Four types of affinity labels and their respective properties.

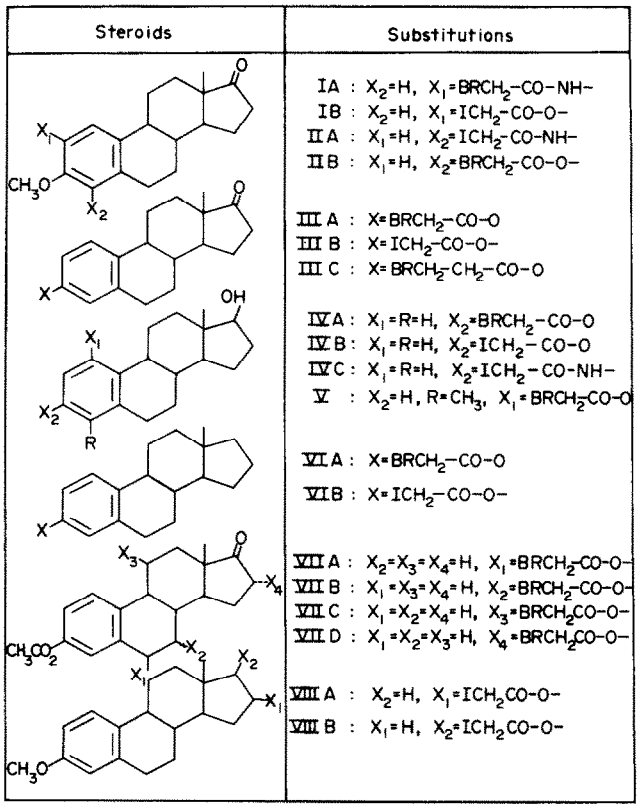


Fig. 13. Affinity labels of estradiol dehydrogenase: The radioactive alkylating side chain was introduced on the steroid nucleus by a reaction of esterification or amidification as described by Buzas[26, 27]. It allows the synthesis of small amounts of products (a few milligrams) with convenient yields. A selective enzymic oxidation of the 17β-hydroxyl group of triols was used for the synthesis of products (VIIA, VIIB, VIIC). This reaction is described (Fig. 14) for the selective oxydation of the 1,3,5(10)-estratriene-3, 11β, 17β triol.

when this phenomenon occurred. We have thus studied the action of 20 affinity labels, of which some are represented in Fig. 13; most of them were new compounds specially synthetized in the laboratory for this work [28]. The selective bromoacetylation or iodoacetylation in position 6, 7 or 11 required specific oxydation in position 17 of the triol which was the starting material. This specific oxydation was realised using the original enzymic conversion represented in Fig. 14.

The best affinity labels were revealed to be 3 iodoacetoxy estrone derivatives but interesting results

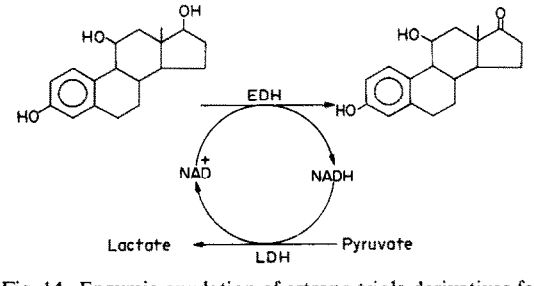


Fig. 14. Enzymic oxydation of estrane triols derivatives for the synthesis of affinity labels: 0.1 to 1 mM of steroid reacted estradiol dehydrogenase (20 I.U./1) and 0.15 mM NAD^+ in 0.1 M pyrophosphate buffer pH 9.5, 10% ethanol (v/v).

were also obtained with 16α and 16β estrone iodoacetates or bromoacetates. Complete inactivation was obtained with 3 iodoacetoxy estrone at pH 7.2, in less than 15 min, and in the absence of coenzyme (full line in Fig. 15). NADP (coenzyme in the same oxydation state as the inhibitor) has a strong protective effect (upper dotted line in Fig. 15). This protective effect of NADP versus inactivation by the oxydized inhibitor is not surprising because abortive ternary complexes cannot be formed by estradiol dehydrogenase, as previously shown by the kinetic results of Betz[12].

On the contrary, there is an increase of the inactivation rate when the evolutive ternary complex is formed (lower dotted line in Fig. 15) [29, 30]. This could be considered as an "indirect" argument for a "correct" orientation of the affinity label in the binding site. But indeed the best argument was given by Engel[31] independently of our work: working in similar conditions, but with 3 bromoacetoxy estrone instead of 3 iodoacetoxy estrone, he could demonstrate that even when covalently bound in the active site, estrone can be reduced by NADH. Furthermore, the same stereospecificity, both for the substrate and for the coenzyme is maintained. This possibility, which Engel named "catalytic competence", is, till now, the best proof that the affinity label is not only

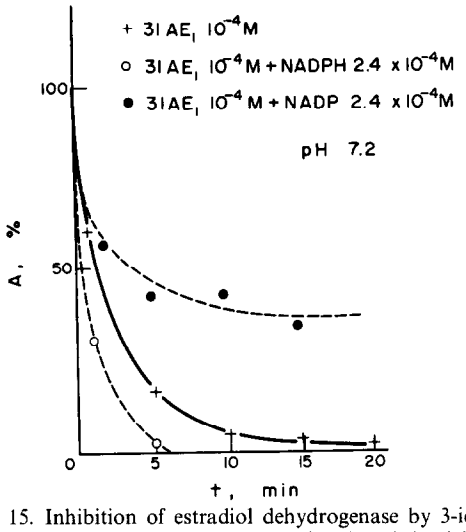


Fig. 15. Inhibition of estradiol dehydrogenase by 3-iodoacetoxy estrone. The enzyme was incubated in 0.03 M phosphate buffer pH 7.2 20% glycerol at 37° with: +——+ 0.1 mM inhibitor, o---o 0.1 mM inhibitor + 0.24 mM NADH, •---• 0.1 mM inhibitor + 0.24 mM $NADP^+$.

in the binding site, but is also in good "functional" orientation towards the coenzyme. But in our opinion this is a relatively rare situation since we have arguments which show that tiny shifts of the substrate with respect to the coenzyme are sufficient to dramatically decrease the oxydoreduction rate.

When complete inactivation is performed by the 3-iodo [2'-^{14}C]-acetoxy estrone *in the presence of NADPH*, a single peptide is radiolabelled and the label is recovered as 3 carboxymethyl histidine. In mild conditions, the steroid nucleus could be released by hydrolysis of the affinity label and we obtained a carboxymethylated enzyme which is specifically labelled on this single histidine residue. As the hydrolysis of the affinity label proceeds, the steroid is released and the active site is progressively rid of the steroid. Simultaneously the enzyme activity is recovered, and moreover, at the end of the recovery this specifically "histidine carboxymethylated" estradiol dehydrogenase looks like a "super enzyme" since its activity towards estradiol is 3 times the initial value of the native enzyme. The overall process of inactivation and reactivation is shown in Fig. 16.

When estradiol dehydrogenase was 100% inhibited by 3-iodoacetoxy estrone or 3-iodoacetoxy estradiol *in the absence of coenzyme*, three aminoacid residues were labelled: two cysteins and one histidine. The histidine residue is carried by the same peptide mentioned above. The two cysteins are cystein C_3 and C_5. Since cystein C_5 and/or cystein C_3 were demonstrated to be essential for catalytic activity, it is not surprising that the enzyme could not be reactivated, even if the steroid was released by mild hydrolysis. The labelling occurred in the active site as indicated by the stoichiometry of the inactivation and the protective effect of the substrate. But the distribution of the label between three different aminoacid residues strongly suggests that in the absence of coenzyme the affinity label could have access to the coenzyme binding site. This is not surprising since cysteins C_3 and C_5 are protected by NADP and since this coenzyme binding site is well known to be hydrophobic [32].

Many results obtained with other affinity labels

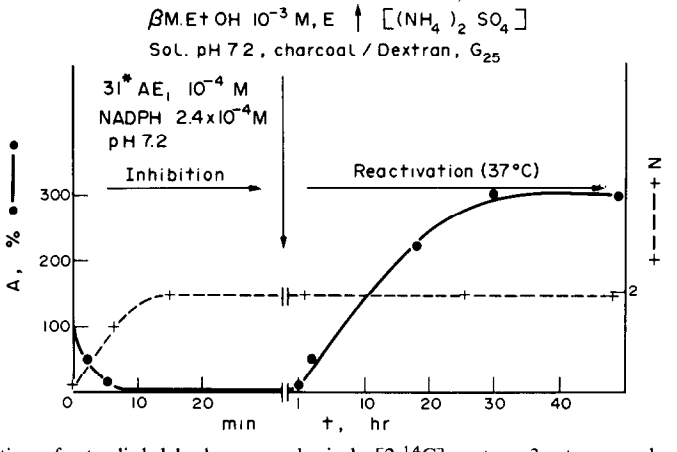
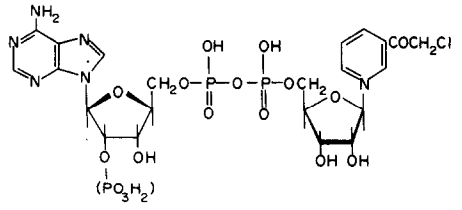
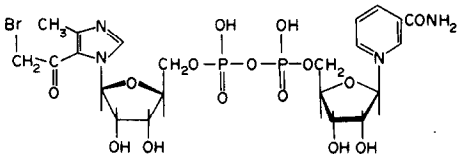


Fig. 16. Inhibition of estradiol dehydrogenase by iodo [2-^{14}C]-acetoxy-3-estrone and reactivation after release of the steroid. The enzyme was inhibited by a 20 min incubation at 37° with 0.1 mM inhibitor and 0.24 mM NADPH in 0.03 M phosphate buffer pH 7.2, 20% glycerol (buffer I). The excess of inhibitor was destroyed by addition of 1 mM mercaptoethanol and the enzyme was precipitated by one vol. of saturated ammonium sulfate solution. The precipitate was dissolved in 1 ml of buffer I. The remaining free radiolabelled material was removed first by incubation with 1 ml of a Dextran charcoal suspension and secondly by Sephadex filtration. After centrifugation of the charcoal suspension, the supernatant was filtered on a G25 Sephadex column in buffer I. The radioactive alkylated enzyme was eluted in the exclusion vol. Its radioactivity was determined on aliquots by liquid scintillation counting. The remaining solution was incubated again at 37° for 48 h in buffer I. The enzyme activity and the radioactivity were measured on aliquots all along the reactivation process. •——• Enzyme activity expressed as % of activity of native enzyme, +---+ bound ^{14}C radioactivity expressed as mol of carboxymethyl/enzyme dimer.



3 Chloro acetyl-pyridine-adenine dinucleotide (phosphate)



Nicotinamide-(5-bromoacetyl-4-methyl) imidazole-dinucleotide

Fig. 17. Alkylating coenzyme analogues used as affinity labels of estradiol dehydrogenase: 17a: nicotinamide [5 bromo acetyl 4-methyl imidazole]-dinucleotide [33]. 17b: 3-chloroacetyl pyridine adenine dinucleotide and 3 chloroacetyl pyridine adenine dinucleotide (phosphate).

lead to the same conclusion: in the absence of coenzyme, there is a lack of selectivity in the labelling, because of the free motion of the label in the whole active site. It is suggested that in the absence of coenzyme, affinity labelling could become frequently "hydrophobic labelling".

The results obtained with all the affinity labels we have used were discussed with respect to the presence or absence of coenzymes and to the position and the conformation of the alkylating side chain of the steroid nucleus.

Four aminoacid residues have been identified: three in the active site, (one histidin, cystein C_3 and cystein C_5), one (cystein C_1) close to the active site.

Two of these residues were localized with some accuracy: the histidin residue is very close to the A ring of the substrate, probably above it (on the "β face" of the steroid), since its labelling is reduced when the alkylating side chain is shifted towards the B or C ring and since it is much more specifically labelled when the alkylating side chain is in a β position. Thus, this histidine residue could be implicated in the narrowing of the binding site in the neighbourhood of cycles A and B; the narrowing is shown in Fig. 4:

—The cystein C_3 was localized at the junction of the steroid and the coenzyme binding sites. This conclusion was drawn from the following observations:

—The only alkylating steroids which labelled cystein C_3 were bearing the alkylating side chain in position 16α or 16β;

—As shown in collaboration with Biellman's laboratory (Strasbourg) [33] the same cystein residue could also be alkylated by 3-chloroacetyl pyridine adenine dinucleotide, a coenzyme analogue which directs an alkylating group towards the catalytic site of dehydrogenases [34].

Another radioactive analogue of the coenzyme, bearing the same alkylating group, in the same position on the nicotinamide (Fig. 17a): 3-chloroacetyl pyridine adenine dinucleotide phosphate, was very recently synthesized by Biellman, Goulas *et al.* (Strasbourg; unpublished results). This affinity label had a better affinity for estradiol dehydrogenase ($K_M = 2 \cdot 10^{-6}$ M) than the NAD analogue and *labelled only* cystein C_5. Thus, this residue should be located nearer the catalytic site than cystein C_3.

Cystein C_1 could not be localized with precision, since it was not specifically labelled by any of the analogues used in this study, even by another NAD analogue bearing an alkylating group substituted to the adenine nucleus: nicotinamide [5-Bromoacetyl-4-methyl imidazole] dinucleotide (Fig. 17b) [35].

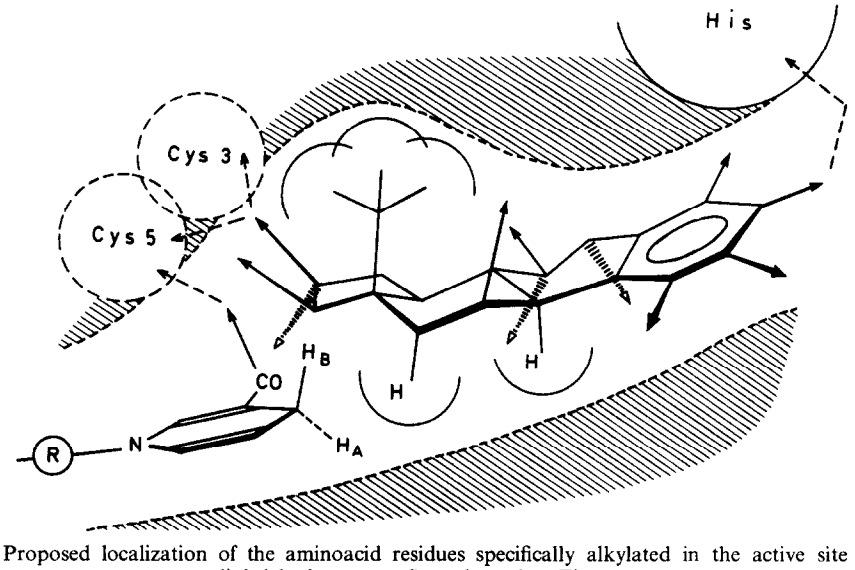


Fig. 18. Proposed localization of the aminoacid residues specifically alkylated in the active site of estradiol dehydrogenase. Same legend as Fig. 11.

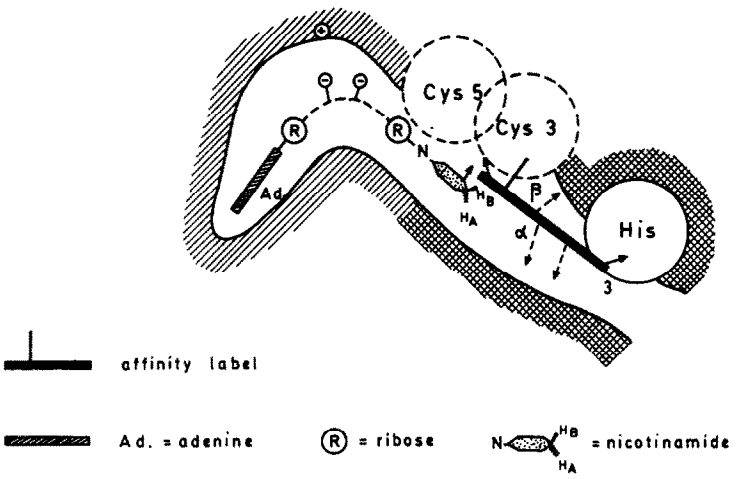


Fig. 19. Affinity labelling of the estradiol dehydrogenase by a substrate analogue in the presence of coenzyme allowing an evolutive ternary complex to be formed. The alkylating groups oriented towards a nucleophilic aminoacid residue (arrow in full lines) give specific and rapid alkylation. In the evolutive ternary complex, some other alkylating groups can be held rather far from the nucleophilic residues (arrows in dotted lines) and the enzyme is protected by the coenzyme against inactivation and (or) alkylation by these affinity labels according to [30]. Substrate analogue, coenzyme binding site, substrate binding site.

Thus, on the evolutive ternary complex, the substrate should be oriented towards the coenzyme as shown in Fig. 18. These results give the first direct demonstration of the validity of Prelog's hypothesis on the orientation of asymetric polyclanic alcohols towards nicotinamide, in corresponding nicotinamide dehydrogenase.

The Figs 19 and 20 summarize our concept of the action of an affinity label in the presence or in the absence of coenzyme. They are lateral views of the active site deduced from the models of the coenzyme binding site described by Rossman for the lactico dehydrogenase and of the substrate binding site of the estradiol dehydrogenase we have previously described:

—*In the presence of coenzyme*, only the steroid binding site is easily accessible to the substrate analogues, the orientation of which is specifically fixed by the formation of *an evolutive ternary complex*. Thus, if the alkylating group is specifically oriented near a nucleophilic aminoacid, the alkylation is *specific* and *much more rapid* than in the absence of coenzyme. If the alkylating group is removed from the nucleophilic residue, the enzyme is paradoxically protected against inactivation, as shown by a lower inhibition rate;

—*In the absence of coenzyme*, the alkylating steroid can be bound in several positions in the whole active site: a corresponding lack of specificity results, since multiple orientations are now available for the alkylating side chain: such a situation could be named "hydrophobic labelling".

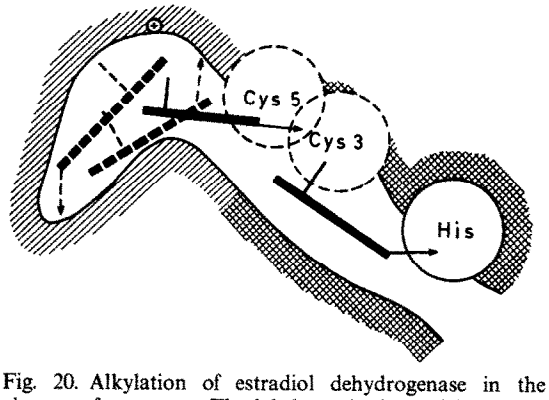


Fig. 20. Alkylation of estradiol dehydrogenase in the absence of coenzyme. The label can be located in "active positions" (in full lines) or in inactive positions (dotted lines). In the "active positions" the alkylating group shown by an arrow in full line is drawn near a nucleophilic residue which is alkylated. In the "inactive positions" the alkylating group (arrow in dotted line) is remote from the nucleophilic residues and alkylation does not occur.

It should be observed that with about twenty affinity labels, only two or three aminoacid residues could be localized with accuracy. But Warren did not have better results in affinity labelling of cortisol reductase [36, 37, 38]. However, these scanty results should not be surprising, since most of the aminoacid residues of the substrate binding site are probably hydrophobic (the whole site is itself hydrophobic) and the hydrophobic aminoacids do not react with this kind of affinity labels.

Further information on the nature of the active site could come from photo affinity labelling experiments which induces in the active site nitrenes or carbenes, reagents able to react with such hydrophobic aminoacids. Other informations could come from X-ray analysis if the first report of crystallization by Chin *et al.*[39] could enable X-ray crystallography to do such an analysis.

Now, the biological role of estradiol dehydrogenase in human placenta has not been definitively eluci-

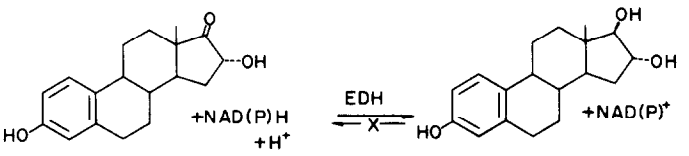


Fig. 21. The irreversible conversion of 16α-hydroxy estrone into estriol by estradiol dehydrogenase according to Engel [20].

dated. However the properties of the enzyme indicate that at least three possibilities should be considered:

—The enzyme could be involved in estradiol biosynthesis and in the equilibrium between estrone and estradiol concentrations since in human placental microsomes, aromatization proceeds "*via*" the androstene dione pathway ("17 oxo pathway") rather than by the testosterone pathway [40]. Thus, the main estrogen formed is estrone which can be easily reduced into estradiol by estradiol dehydrogenase, whereas the strict specificity of this enzyme for the estrane nucleus completely rules out the event of testosterone biosynthesis by simultaneous reduction of androstene dione ("17 hydroxy pathway"). Moreover the interconversion between estrone and estradiol has been demonstrated in perfused placenta [41] and there is little doubt, if any, that estradiol dehydrogenase is responsible for the equilibrium between these two steroids [41];

—The estrogen-mediated transhydrogenase function cannot be retained as a general mechanism of action of estrogens. However in the placenta, owing to the high estrogen content of this tissue [42], the possibility of an action of estrogens *via* the trans-hydrogenase function should not be ruled out. The hydrogen transfer from NADPH to NAD through this system should be operative at both oestrogen and NADP concentrations of the tissue owing to the low Km of estradiol when estradiol dehydrogenase acts as a trans-hydrogenase. In these conditions, the NADPH formed by the glucose-6-phosphate–glucose-6 phosphate dehydrogenase system could become available for the NAD respiratory chain with a specific modulation of this transfer by estrogens;

—Finally, the recent demonstration by Engel[20] that estradiol dehydrogenase reduces irreversibly 16α hydroxy estrone into estriol implicates that this enzyme catalyses the last step of estriol biosynthesis (Fig. 21) through the 16 hydroxy pathway (16α hydroxy dehydroepiandrosterone → 16α hydroxy androstene dione → 16α hydroxy estrone → estriol).

This last hypothesis suggests that the main substrate of estradiol dehydrogenase should not be estrone or estradiol but actually 16α hydroxy estrone. However the three possibilities suggested above are not mutually exclusive, the *in vitro* data being compatible with each of these events.

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REFERENCES

1. Ryan K. J. and Engel L. L.: The interconversion of estrone and estradiol by human tissue slices. *Endocrinology* **52** (1953) 287–291.
2. Langer L. J. and Engel L. L.: Human placental estradiol-17β dehydrogenase. I—concentration, characterization and assay. *J. biol. Chem.* **233** (1958) 583–588.
3. Langer L. J., Alexander J. A. and Engel L. L.: Human placental estradiol-17β dehydrogenase. II—Kinetics and substrate specificities. *J. biol. Chem.* **234** (1959) 2609–2614.
4. Jarabak J. and Sack G. H. Jr.: A soluble 17-β hydroxysteroid dehydrogenase from human placenta. The binding of pyridine nucleotides and steroids. *Biochemistry* **8** (1969) 2203–2212.
5. Warren J. C. and Crist R. D.: Site specificity and mechanism of human placental 17β-hydroxysteroid dehydrogenase. *Archs Biochem. Biophys.* **118** (1967) 577–584.
6. Jarabak J. and Talalay P.: Stereospecificity of hydrogen transfer by pyridine nucleotide linked hydroxysteroid dehydrogenases. *J. biol. Chem.* **235** (1960) 2147–2151.
7. Warren J. C., Gonzalez M. C. and Soria J.: Characteristics of the steroid binding site: A method to evaluate stereospecificity of hydrogen transfer by steroid dehydrogenases. *Endocrinology* **80** (1967) 884–888.
8. Adams J. A., Jarabak J. and Talalay P.: The steroid specificity of the 17β-hydroxysteroid dehydrogenase of human placenta. *J. biol. Chem.* **237** (1962) 3069–3073.
9. Descomps B. and Crastes de Paulet A.: Etude du site actif de la 17β-hydroxysteroide: NAD(P) oxydoreductase soluble du placenta humain. *Bull. Soc. Chim. biol.* **51** (1969) 1591–1611.
10. Talalay P., Hurlock B. and Williams-Ashman H. G.: On a coenzymatic function of estradiol 17β-dehydrogenase. *Proc. natn. Acad. Sci. U.S.A.* **44** (1958) 862–884.
11. Karavolas H. J., Baedecker M. L. and Engel L. L.: Human placental 17β estradiol dehydrogenase. V: Purification and partial characterization of the diphosphopyridine nucleotide (triphosphopyridine nucleotide) linked enzyme. *J. biol. Chem.* **245** (1970) 4948–4952.
12. Betz G.: Reaction mechanism of 17β-estradiol dehydrogenase determined by equilibrium rate exchange. *J. biol. Chem.* **246** (1971) 2063–2068.
13. Jarabak J., Seeds A. E. Jr. and Talalay P.: Reversible cold inactivation of a 17β hydroxysteroid dehydrogenase of human placenta. Protective effect of glycerol. *Biochemistry* **5** (1966) 1269–1278.
14. Nicolas J. C., Pons M., Descomps B. and Crastes de Paulet A.: Affinity chromatography: Large scale purification of the soluble estradiol-17β dehydrogenase of human placenta. *FEBS Lett.* **23** (1972) 175–179.
15. Chin C. C. and Warren J. C.: Purification of estradiol-17β dehydrogenase from human placenta by affinity chromatography. *Steroids* **22** (1973) 373–378.

16. Burns D. J. W., Engel L. L. and Bethune J. L.: The subunit structure of human placental 17β-estradiol dehydrogenase. *Biochem. biophys. Res. Commun.* **44** (1971) 786–792.
17. Jarabak J. and Street M. A.: Studies on the soluble 17β-hydroxysteroid dehydrogenase from human placenta. Evidence for a subunit structure. *Biochemistry* **10** (1971) 3831–3834.
18. Burns D. J. W., Engel L. L. and Bethune J. L.: Amino acid composition and subunit structure: Human placental 17β-estradiol dehydrogenase. *Biochemistry* **11** (1972) 2699–2703.
19. Nicolas J. C. and Harris J. I.: Human placental 17β-estradiol dehydrogenase: Sequence of a tryptic peptide containing an essential cysteine. *FEBS Lett.* **29** (1973) 173–176.
20. Engel L. L. and Groman E. V.: Human placental 17β-estradiol dehydrogenase: Characterization and structural studies. *Recent Prog. Horm. Res.* **30** (1974) 139–169.
21. Ringold H. J., Graves J. M. H., Clark A. and Bellas T.: Experimental strategy for delineation of the enzyme region involved in substrate recognition. *Proc. natn. Acad. Sci. U.S.A.* **56** (1966) 255–261.
22. Ringold H. J., Graves J. M. H., Clark A. and Bellas T.: Studies with the 3α-hydroxy steroid dehydrogenase from *Pseudomonas testosteroni*. Enzyme substrate complementary as the basis of selectivity and steric specificity. *Recent Prog. Horm. Res.* **23** (1967) 349.
23. Prelog V.: Specification of the stereospecificity of some oxido-reductases by diamond lattice sections. *Pure appl. Chem.* **9** (1964) 119–130.
24. Michel F., Nicolas J. C. and Crastes de Paulet A.: 17β-hydroxysteroid dehydrogenase of the sheep ovary: Purification, properties and substrate binding site. *Biochimie* **57** (1975) 1131–1140.
25. Penasse L. and Nominé G.: Le centre actif de la 3-oxostéroide Δ-1 déshydrogénase d'Arthrobacter Simplex. *Eur. J. Biochem.* **47** (1974) 555–559.
26. Buzas A., Canac F., Egnell C. and Freon P.: Sur la réactivité des carbodiimides: leur application à la préparation des amides. *C.r. hebd. Séanc. Acad. Sci., Paris* **260** (1965) 2249–2251.
27. Buzas A., Egnell C. and Freon P.: Sur la réactivité des carbodiimides. Leur application à la préparation des esters *C.r. hebd. Séanc. Acad Sci., Paris* **255** (1962) 945–947.
28. Pons M., Marchand J. and Crastes de Paulet A.: Synthèse de quelques dérivés alcoylants de la série du 1.3.5.(10) estratriène. *C.r. hebd. Séanc. Acad. Sci., Sér. C Sci. Nat., Paris* **283** (1976) 507–510.
29. Boussioux A. M., Pons M., Nicolas J. C., Descomps B. and Crastes de Paulet A.: Enhancement of the catalytic activity of the human placental 17β-estradiol dehydrogenase by carboxymethylation of an histidyl residue of the active site. *FEBS Lett.* **36** (1973) 27–31.
30. Pons M., Nicolas J. C., Boussioux A. M., Descomps B. and Crastes de Paulet A.: Affinity labelling of the estradiol-17β dehydrogenase from human placenta with substrate analogs. *Eur. J. Biochem.* **68** (1976) 385–394.
31. Groman E. V., Shultz R. M. and Engel L. L.: Catalytic competence, a new criterion for affinity labelling: Demonstration of the reversible enzymatic interconversion of estrone and estradiol-17β covalently bound to human placental estradiol-17β-dehydrogenase. *J. biol. Chem.* **50** (1975) 5450–5454.
32. Mani J. C., Dornand J., Mousseron-Canet M. and Vial-Reveillon F.: Etude de la complexation du TNS avec la 17β-hydroxystéroïde déshydrogénase placentaire. *Biochimie* **55** (1973) 851–857.
33. Biellman J. F., Branland G., Nicolas J. C., Pons M., Descomps B. and Crastes de Paulet A.: Alkylation of estradiol-17β-dehydrogenase from human placenta with 3-chloroacetyl pyridine-adénine dinucléotide. *Eur. J. Biochem.* **63** (1976) 477–481.
34. Branlant G.: Thèse Sciences "Etude de site actif de deshydrogénases à NAD^+" December, the 13th 1974, Strasbourg.
35. Jonscher G., Woenckhaus J. G. C., Nicolas J. C., Pons M., Descomps B. and Crastes de Paulet A.: Specific modification of 17β estradiol dehydrogenase from human placenta by nicotinamide -(5 bromoacetyl-4-methyl imidazole) dinucleotide. *FEBS Lett.* **61** (1976) 176–179.
36. Ganguly M. and Warren J. C.: Affinity labelling of steroid binding sites: Synthesis of cortisone 21-iodoacetate and study of 20β-hydroxy-steroid dehydrogenase. *J. biol. Chem.* **246** (1971) 3646–3652.
37. Sweet F., Arias F. and Warren J. C.: Affinity labelling of steroid binding sites: Synthesis of 16α-bromoacetoxy progesterone and its use for affinity labelling of 20β-hydroxy-steroid dehydrogenase. *J. biol. Chem.* **247** (1972) 3424–3433.
38. Arias F., Sweet F. and Warren J. C.: Affinity labelling of steroid binding sites: Study of the active site of 20β-hydroxysteroid dehydrogenase, with 6β and 11α-bromo-acetoxy-progesterone. *J. biol. Chem.* **248** (1973) 5641–5647.
39. Chin C. C., Dence J. B. and Warren J. C.: Crystallization of human placental estradiol 17β-dehydrogenase: A new method for crystallizing labile enzymes. *J. biol. Chem.* **251** (1976) 3700–3705.
40. Menini E. and Engel L. L.: Kinetic study of the aromatization of testosterone and androstene-dione by human placental microsomes. suppl. 119 *Acta endocr., Copenh.* (1967) **76** abstract no. 60.
41. Diczfalusy E., Cassmer O., Alonso C. and Montserrat de Miquel: Estrogen metabolism in the human fetus and newborn. *Recent Prog. Horm. Res.* **17** (1961) 147–206.
42. Diczfalusy E. and Troen P.: On Endocrine functions of the human placenta. *Vitams Horm.* **19** (1961) 229–331.

DISCUSSIONS

Osawa. Your slides show that the highest S.A. of the pure enzyme is 4 units per milligram. According to Drs. Chin and Warren they obtained the crystallized enzyme with 7 units per milligram. Could you explain the reason for such a large difference?

Crastes de Paulet. First of all I would like to say that it is very difficult to give a precise number for the S.A. It depends on the accuracy of the determinations of the protein concentrations. Secondly, the S.A. of an enzyme, even if found homogenous in the usual tests (ultracentrifugation, electrophoresis, *N*-terminal amino acid) can be altered by the presence of variable amounts of pure but inactivated protein. We have also obtained crystallized estradiol dehydrogenase but our crystals were very small and I know that the ones obtained by Chin *et al.* were also very small and unfortunately not suitable for X ray analysis.

Westphal. First of all I would like to say that I am much impressed by the very thorough analysis of the molecular basis of the interaction of this steroid hormone with the enzyme protein. Commenting on your results: the attachment of estradiol to the histidine residue is of particular interest since Talalay found that also the histidine of the 5-ene-3-keto-isomerase reacts with the A-ring area of the steroid.

I would like to ask one question, and I apologize if

I am asking something that goes too far into the future. You have mentioned a number of other sites which you call recognition sites, hydrophobic sites which are not involved in the enzymatic reaction. We are interested in these sites for various other reasons. I would like to ask you, do you have any information on which amino acids are involved in the interactions at these recognition sites?

Crastes de Paulet. Actually, I have not mentioned other sites than the substrate and the coenzyme recognition sites, and what I would like to say is that in both of them, there are large hydrophobic areas. But the only kind of amino acids we have been able to localize using this kind of affinity labels are the nucleophilics. We have the idea that we can find the hydrophobic amino acid residues by photoaffinity labelling, but I think this method is very difficult to use because even if we can reach some amino acids, their identification would be problematic because of the type of reaction of carbenes and nitrenes: It is very difficult to get the corresponding "reference" derivatives of amino acids because of the multiplicity of the reaction products for a single amino acid residue.

Tamaoki. My first question is, how did you confirm the purity of your enzyme? Have you ever tried to do disc electrophoretic analysis of your placental dehydrogenase? According to our experience with disc electrophoresis of testicular 17β-hydroxysteroid dehydrogenase which has been solubilized from the microsomal fraction, the presence of several isozymes in the preparation was demonstrated. Each isoenzyme has identical molecular weight of 34,000 dalton, but still showed different isoelectric points. Therefore I would like to know how you confirmed the purity of your enzyme isolated from placentae.

Crastes de Paulet. First I have mentioned that it is possible that this enzyme is isozymic as reported by Engel, but, as I have said, it is probable that whatever the isozymic structure is, the structure is the same for the active site: the fact that we find for instance only one heptadecapeptide bearing the essential cystein C5 is in accordance with this hypothesis.

To test the purity we have not performed isoelectrofocusing but we have done acrylamide gel electrophoresis in standard conditions and in dissociative conditions: a single band is present with pure enzyme. Moreover, we have a single *N*-terminal amino acid residue and the same chymotryptic peptide map owing to the cysteine-containing peptides.

Tamaoki. Have you ever tried to do active enzyme centrifugation, in order to find the active form of the placental dehydrogenase? The method of active enzyme centrifugation is a centrifugation method of the enzyme over the sucrose density gradient which contains the substrate and cofactor, if required. Then, the temperature during the centrifugation is controlled at 10° or 20°C to permit the enzyme reaction. By this method, we can demonstrate that the active form of the testicular 17β-hydroxysteroid dehydrogenase which we purified is monomer in the enzymic reaction. This is my additional comment.

Crastes de Paulet. I agree with Dr Tamaoki. We have not performed centrifugation experiments to determine the molecular weight of the active form, but we have performed electrophoresis in gels of different concentrations of acrylamide which lead to the same results and which allow the measurement of the molecular weight of the active form: with estradiol dehydrogenase of human placenta, only the dimer is active. We never found activity of the monomer and sometimes there were some forms, maybe trimeric or tetrameric, which had a small activity but the main active form is the dimer.