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PRODUCTS OF ESTRADIOL/PEROXIDASE INTERACTION; THEIR STRUCTURAL FEATURES AND BIOPOLYMERIC CHARACTER

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Summary—Loss of tritium from [2,4,6α,7α-^{3}H]estradiol and from [2-^{3}H]estradiol during their conversion into polyestradiol (PEL) by horseradish peroxidase/H_2O_2 and the NMR spectrum of PEL permethyl ether suggest that PEL is composed of two or more different subunits, each formed by the joining of four molecules of estradiol with the loss of five hydrogen atoms from positions 2 and 4 and of three phenolic hydrogens leading to the formation of one C—C bond and three C—O bonds. At very low concentrations of estradiol the main reaction products were monomers; this is attributed to the initial formation of transient tetraestradiols which combine with water at high dilution and with themselves at low dilution. Association of the monomeric products to oligomers occurred on a Sephadex G-50 column and was readily reversed in phosphate buffer. In aqueous solution PEL underwent non-covalent changes induced by heat, time and electrolytes, and affecting its solubility, u.v. absorbance, extraction by organic solvents and ability to bind estradiol.

INTRODUCTION

Peroxidases known to transform estradiol include horseradish peroxidase (HRP) [1], lactoperoxidase [2], uterine peroxidase [3] and mammary tumour peroxidase [2]. The last two are of special interest since they are generated by estradiol in its target tissues [4, 5].

Early work by Klebanoff and Segal [1] showed that the presence of a free phenolic group in estradiol is essential for its transformation by HRP/H_2O_2. This made it likely that, in analogy to other phenols, estradiol underwent oxidative coupling, i.e. the joining of two or more molecules by C—C bonds, or by C—O bonds, or by both [6]. Confirmation came from the isolation, in low yield, of 2,2′- and 4,4′-bisestradiol from the reaction of estradiol with HRP/H_2O_2 in a heterogeneous medium [7] and of polyestradiol (PEL), in high yield, when the reaction was performed in a homogeneous medium [8, 9]. The present communication concerns the interrelation between PEL, its subunits and its low-molecular by-products and the unusual properties of these entities. Some of the results were briefly reported in 1980 [10] but since then were neither described in detail nor followed up.

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EXPERIMENTAL

General

[2-^{3}H]Estradiol was donated by Dr M. Gut, Worcester Foundation for Experimental Biology (Worcester, MA, U.S.A.). Other radiochemicals were supplied by the Radiochemical Centre (Amersham, U.K.). HRP was supplied by British Drug Houses (Poole, U.K.). Melting points were taken on a Kofler stage. Aqueous solutions of PEL were prepared as in the following example: a solution of 0.5 mg PEL in 0.5 ml ethanol was diluted with 5 ml water and the mixture was concentrated *in vacuo* below 30°C for 5 min; loss of weight was noted to ensure complete removal of ethanol.

Dialysis

Visking tubings (1–8/32) were washed with water in a Soxhlet; they were then folded whilst still pliable to form V-shaped containers. Each of these was placed in a glass vial of 12 mm dia. The inner compartment was filled with 1.0 ml of solution and the outer compartment with 4.0 ml of solvent. The vial was stoppered and dialysis allowed to proceed. For PEL and vitamin B_{12}, concentration of the solute in the outer compartment was determined from its light absorption at 275 and 360 nm, respectively.

Dialysis of tritiated material was followed by counting tritium in samples taken from the inner and outer compartments (0.125 and 0.5 ml, respectively). The measurements gave a correct estimate of the dialysis rate at the first sampling but an overestimate at successive samplings since, owing to the shape of the inner compartment, the ratio of the effective area of the semipermeable membrane to the volume of the solution in the inner compartment increased after each sampling.

HRP-catalysed oxidation of [4-^{14}C][2,4,6α,7α-^{3}H]estradiol

The starting material was prepared from specimens of [4-^{14}C]estrone and [2,4,6α,7α-^{3}H]estrone previously used for their conversion into polyestrone by potassium ferricyanide [9]; 2 μCi of the former and 15 μCi of the latter in 15 ml ethanol were admixed with 7 mg of potassium borohydride in 1 ml water. After 16 h at room temperature, 3 drops of acetic acid, 94 mg inert estradiol and 15 ml water were successively added. Concentration of this mixture gave crystalline [4-^{14}C][2,4,6α,7α-^{3}H]estradiol which was repeatedly recrystallized from acetone–benzene to a constant $^{3}H/^{14}C$ ratio of 6.4; 60 mg of this material were treated with HRP/H_2O_2 and the formed PEL was purified on an Amberlite MB-1 column as previously reported [8]. Subsequent precipitation from dioxan with ether followed by incipient precipitation from acetone gave a constant $^{3}H/^{14}C$ ratio of 4.5 corresponding to an overall loss of 30% tritium. The loss of tritium from positions 2 and 4 was 61% since the starting material contained 49% of its tritium at these two positions and no loss of tritium could have occurred from positions 6 and 7 [9].

HRP-catalysed oxidation of [4-^{14}C][2-^{3}H]-estradiol

A mixture of 0.2 μCi [4-^{14}C]estradiol, 2.6 μCi [2-^{3}H]estradiol and 120 mg of the inert compound was crystallized to a constant $^{3}H/^{14}C$ ratio of 13.4; 65 mg of this material treated with HRP/H_2O_2 as above gave 46 mg PEL from an Amberlite MB-1 column, 23 mg after its precipitation with ether from dioxan and 6 mg after subsequent incipient precipitation from acetone, with respective $^{3}H/^{14}C$ ratios of 5.4, 5.1 and 4.8. The last ratio corresponds to a loss of 64% tritium.

Methylation of PEL

The methylation procedure was that of Stoochnoff and Benoiton [11]. 3 g Of a suspension of sodium hydride in oil (1:1, w/w) were mixed with 5 ml dry benzene; the supernatant was removed and the residue washed with 2 × 5 ml of benzene. The still moist reagent was admixed with 12 ml tetrahydrofuran, 1 g PEL and 2 ml iodomethane. After stirring for 24 h at room temperature methanol was added dropwise until effervescence ceased; 50 ml water were added and the mixture was extracted with 3 × 50 ml carbon tetrachloride. The extract was taken to dryness and the residue chromatographed on a column of 30 g alumina (Merck, alkaline, act. I). Elution with carbon tetrachloride and then with its mixtures with increasing amounts of chloroform gave 6 mg of colourless crystals (fraction A) followed by 53 mg of amorphous material (fraction B) and then by 640 mg of the main product (fraction C). Fraction B re-chromatographed on alumina separated into 8 mg identical with fraction A and 27 mg identical with fraction C.

Incipient precipitation of fraction C from benzene–ethanol gave 400 mg of amorphous dull-yellow material which after drying for 24 h at 90°C and 0.1 mmHg, had: m.p. 268–270°C (dec., *in vacuo*); $[\alpha]_D + 53°$ (dioxan, *c.* 0.19); inflection at 280 nm (E_1^1 167, in ethanol) unchanged by NaOH; V_{max} (in CCl_4) 1107 cm^{-1} (CH_30). Its NMR spectrum is shown in Fig. 1(a). Found: C, 77.65; H, 8.5%; M_w by vapour pressure osmometry in dioxan 1291, in chloroform 1817. A cyclic tetraestradiol pentamethyl ether dihydrate $C_{77}H_{102}O_{10}$ requires: C, 77.87; H, 8.66% M_w 1188. Fraction A was sublimed at 130° and 0.1 mmHg. The sublimate had m.p. 158–160°C, M^+/e 300 and the NMR spectrum shown in Fig. 1(b). It is thus identified as estradiol dimethyl ether.

RESULTS

The nature of PEL subunits

The HRP-catalysed oxidation of [4-^{14}C][2,4,6α,7α-^{3}H]estradiol gave PEL with the loss of 61% tritium from positions 2 and 4 (similar to the loss suffered by tritiated estrone treated with potassium ferricyanide [9]), whilst the same treatment of [4-^{14}C][2-^{3}H]estradiol proceeded with the loss of 64% tritium. Since earlier

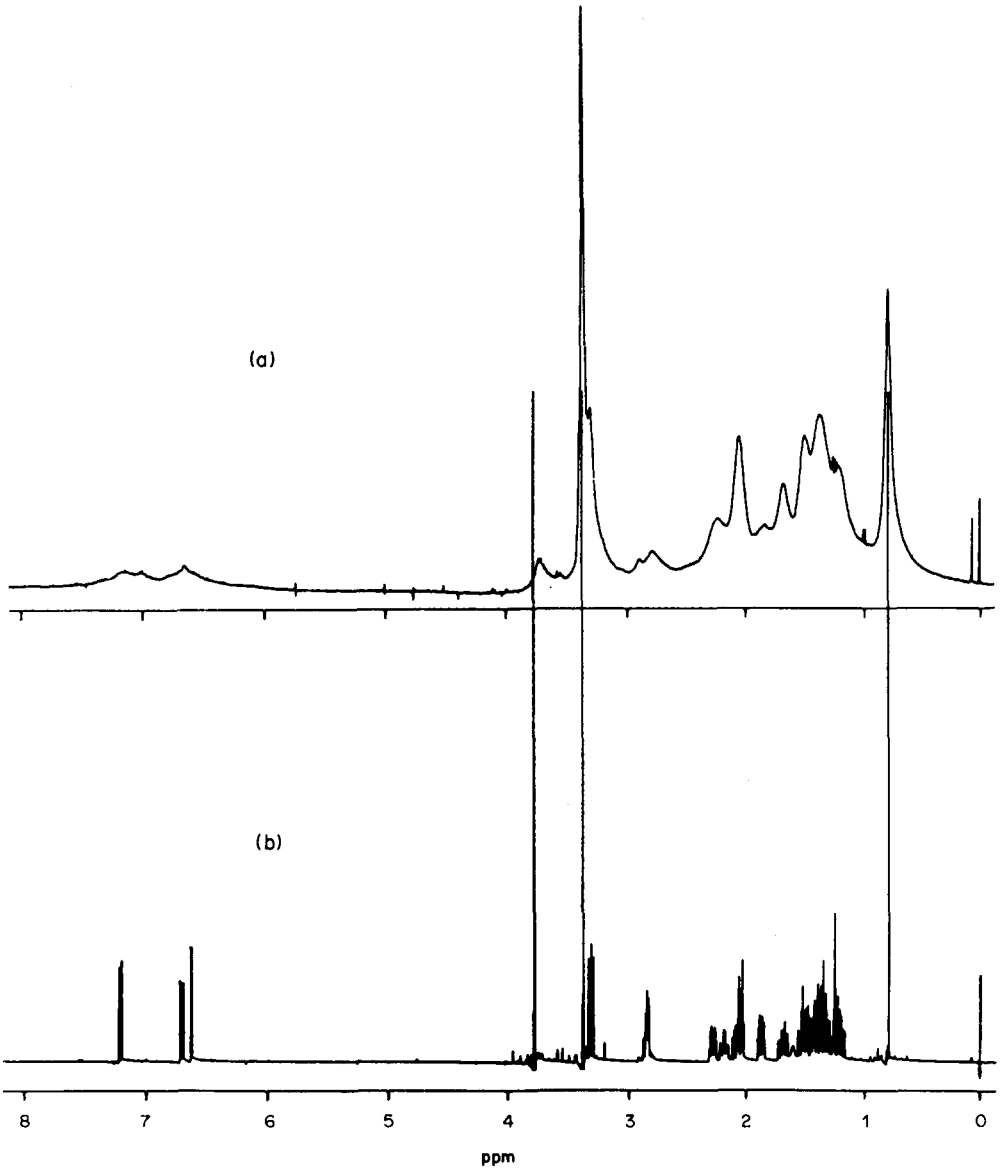
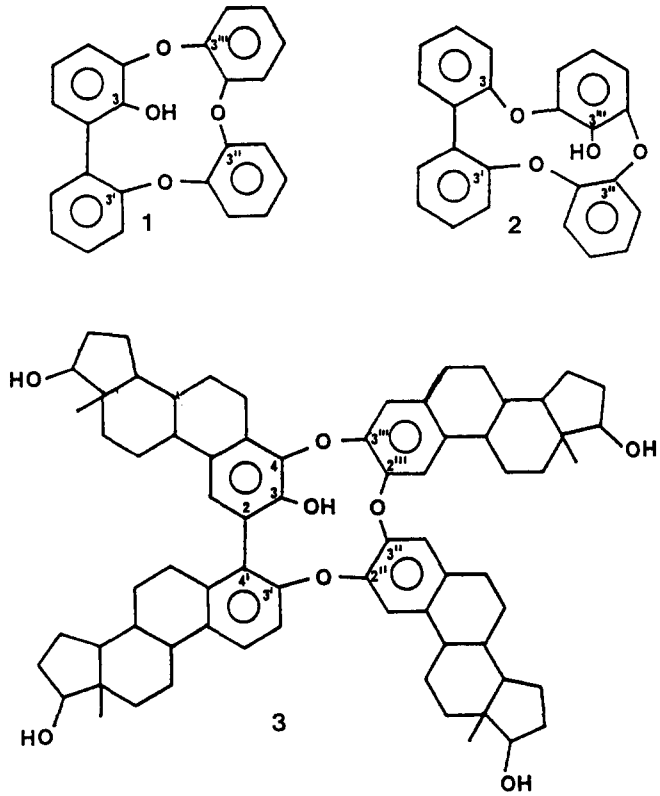


Fig. 1. ^{1}H NMR spectra (400 MHz; $CDCl_3$) of polyestradiol permethyl ether (a) and estradiol dimethyl ether (b).

results [8, 9] and those presented in the following paragraphs suggest that PEL subunits are tetraestradiols, it follows that the reaction is likely to involve the joining of four molecules of estradiol with an average loss of 2.5 hydrogen atoms from the four C-2 atoms and the same loss from the four C-4 atoms and hence that two or more different PEL subunits were formed.

Treatment of PEL with iodomethane and sodium hydride gave a fully methylated product (PEL permethyl ether) as shown by the absence of a hydroxyl band from its i.r. spectrum. Its NMR spectrum is shown together with that of estradiol dimethyl ether in Fig. 1. The signals at 0.77, 3.30 and 3.35 ppm are assigned to C-18H_3, C-17H and C-17OCH_3, respectively. Integration of the PEL permethyl ether signals in the 6–8 ppm region accounts for the presence of seven aromatic protons in a subunit, which is in agreement with the loss of tritium from the *ortho*-positions of [2,4,6α,7α-^{3}H]estradiol during its conversion into PEL. The 3.72 ppm signal accounts for one 3-methoxy group for every four 17-methoxy groups and thus for the formation of three C—O bonds in the coupling reaction. The loss of eight hydrogen atoms (three from phenolic hydroxyls and five from positions 2 and 4) indicates that the reaction involves a cyclization step.



Scheme 1

These results are accommodated by formulating the reaction products as cyclic tetraestradiols containing a structural fragment of the type **1** or **2** (Scheme 1), each type representing 16 possible structures. The simplest case would be that of PEL being made up of only two different tetraestradiol subunits, one of them with either one or no hydrogen atoms at its four C-2 positions and either two or three hydrogens at its four C-4 positions, respectively, and the other with the inverse distribution of the three hydrogens. A specific example of a subunit of type **1** is given by formula **3** which has one hydrogen at a C-2 position and two hydrogens at C-4 positions.

Non-covalent transformations of PEL in water

Aqueous solutions of PEL were prepared by dilution of its ethanolic solutions with water followed by azeotropic removal of ethanol. Dialysis showed that this process is accompanied by increasing aggregation of PEL subunits. Thus, the time for half of the theoretical amount to dialyse at 0°C was 42 h in ethanol, more than 700 h in 50% (v/v) ethanol, whilst no dialysis occurred in 20% ethanol or in water. The polymeric character of PEL in water was confirmed by its elution before blue dextran (average M_w $2 \cdot 10^6$) from a Sephadex G-200 column.

As reported [12], the fraction of PEL extracted from water with ether is independent of the volume ratio of the solvents and slowly increases with the time of mixing. The same was now found for the extraction with dichloromethane. The material extracted with either solvent was not re-extractable with water. Whilst the time dependence of extraction was repeatedly confirmed, the rate of extraction was found to vary considerably. Thus, a freshly prepared solution of PEL in water (43 μg/ml) shaken with an equal volume of dichloromethane retained 88% of solute after 6 h of shaking and 77% after 48 h (from E_{275} of the aqueous solution), whilst for a 1-year-old solution the corresponding values were 57 and 40%, respectively. The discrepancy is attributed to the ageing of PEL in water. When a fresh solution of PEL was boiled at reflux for 30 min and then shaken with dichloromethane for 48 h, 92% of the solute remained in water. More pronounced was the effect of heat on the binding of estradiol by PEL [12].

The presence of sodium chloride in an aqueous solution of PEL (34 μg/ml; 4°C) led to

Table 1. Changes of PEL in water induced by sodium chloride

Sodium chloride (mM)	Relative light absorption at 275 nm after: Days at 4°C 0	1	2	15	Extracted immediately: Water	Dichloromethane	Extracted after 15 days: Water	Dichloromethane
0	=100	101	99	103	99	<5	96	<5
1	102	101	99	102	93	<5	90	11
2.5	102	103	100	102	89	8	70	22
3.75	102	106	99	93	87	12	56	33
5	106	95	65[a]	43[a]	82	20	35	43
10	118	51[a]	8[a]	7[a]	15[a]	33	6[a]	57
100	118	50[a]	<5[a]	<5[a]	8[a]	33	<5[a]	57
500	116	18[a]	<5[a]	<5[a]	<5[a]	46	<5[a]	68

A freshly prepared solution of PEL in water (68 μg/ml) was admixed with an equal volume of aqueous sodium chloride (0.002–1 M) or of water. Measurements at day 0 were taken immediately after mixing. Samples were briefly centrifuged before each measurement. Extraction with dichloromethane (1 vol) was by shaking for 1 min.

[a] Visible precipitate.

the changes recorded in Table 1. At low concentrations of the salt (1 mM) PEL was slowly converted into products extractable with dichloromethane and the rate of this conversion increased with salt concentration. Higher salt concentration ($\geqslant 5$ mM) caused an instant intensification of u.v. absorption followed by precipitation. At a salt concentration of $\geqslant 10$ mM, instant precipitation occurred on shaking the aqueous solution with dichloromethane. Raised temperature accelerated precipitation: in 0.1 M sodium chloride at 23°C the amount of PEL remaining in solution (initial concentration 36 μg/ml) was 25% after 1 h, 15% after 2 h and 7% after 24 h. Essentially the same results were obtained with 0.1 M solutions of calcium chloride, magnesium sulphate, sodium sulphate and hydrochloric acid. No precipitation occurred in the presence of sodium hydroxide (0.1 M), sodium dodecyl sulphate (0.1 M) or urea (4 M).

Effect of estradiol concentration on the outcome of its reaction with HRP

When [6α,7α-^{3}H]estradiol and H_2O_2 were dialysed into a compartment containing HRP it was found that the yield of non-dialysable product(s) increased from 3 to 67% with increasing concentration of estradiol (Fig. 2). This suggested the formation of low-molecular product(s) at low estradiol concentrations, which was confirmed by chromatography of the reaction mixture on a G-50 Sephadex column (Fig. 3): of the two main chromatographic fractions, one (A) was eluted in the void volume

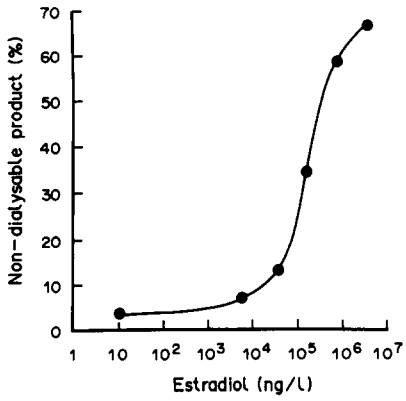


Fig. 2. Effect of the concentration of estradiol on the outcome of its transformation by HRP. Reaction-dialysis was carried out for 4 days at room temperature. The inner compartment contained 0.2 μg HRP in 1.0 ml 2.5 mM phosphate buffer, pH 7; the outer compartment contained [6α,7α-^{3}H]estradiol (19,000 dpm; 52 pg) with or without added inert material, 30 μg H_2O_2 and 0.05 ml ethanol in 4.5 ml of the same buffer. Radioactivity was counted in 0.7 ml samples taken from each compartment. Estradiol concentrations are those that would have been obtained had its dialysis been completed before the reaction started.

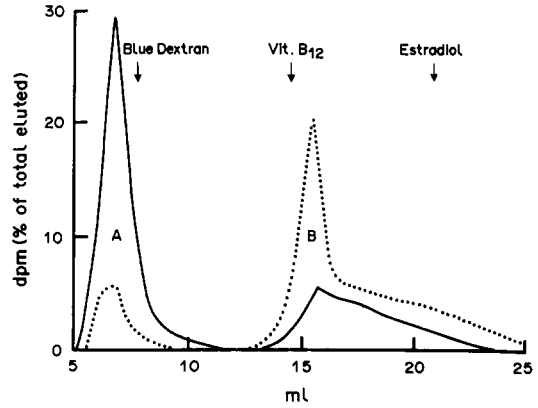


Fig. 3. HRP-catalysed transformation of estradiol at its low and high concentration; separation of products on a G-50 Sephadex column. A mixture containing 0.14 ng [6α,7α-^{3}H]estradiol (50,000 dpm), 2 μg or 4 ng inert estradiol, 0.16 μg HRP, 3 μg H_2O_2 and 0.025 ml ethanol in 2.5 mM phosphate buffer of pH 7 (total volume 0.53 ml) was kept 1 h at 45°C, then cooled in ice-water and applied onto a G-50 Sephadex column (7.5 × 450 mm). Elution was with water at 15–20 ml/h. Fractions of 0.7 ml were collected and 0.5 ml of each was taken for counting radioactivity. ——, 2 μg inert estradiol; ·····, 4 ng inert estradiol.

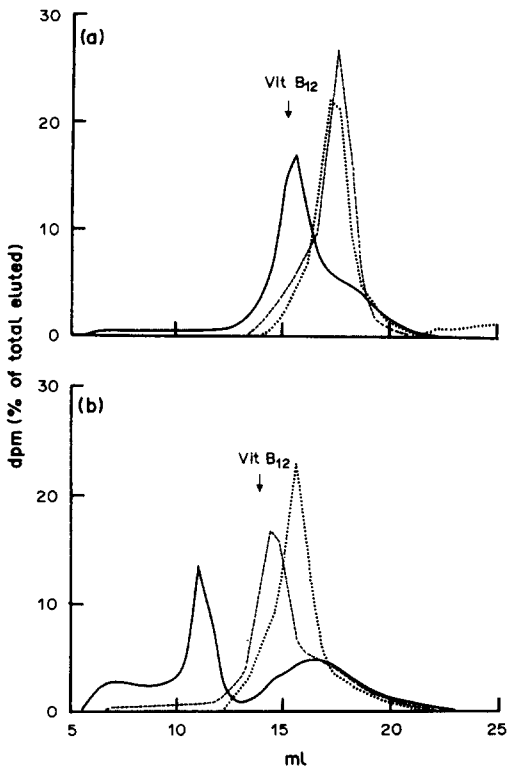


Fig. 4. Fraction B re-chromatographed on two different batches of Sephadex G-50; effect of pre-treatment with buffer. (a) Sephadex G-50/1: product B (5.5 ng, 15,000 dpm) in 0.15 ml water (——); in 0.6 ml 50 mM Na_2HPO_4 after 72 h at room temperature (-----); in 0.6 ml 50 mM KH_2PO_4 after 72 h at room temperature (·····). Recovery of tritium was 95–100%. (b) Sephadex G-50/2: product B (0.7 ng, 24,000 dpm) in 0.1 ml water (——); in 0.13 ml 2.5 mM phosphate buffer (pH 7) after 1 h at 45°C (-----); in 0.4 ml phosphate buffer (pH 7) after 24 h at room temperature (·····). Recovery of tritium was 85–87%. Chromatography was as in the legend to Fig. 3.

(V_e = 6.5 to 7 ml) before blue dextran (average M_w $2 \cdot 10^6$), the other (B) had V_e = 15.5 to 16 ml (vitamin B_{12}, M_w 1335, had V_e = 14.5 to 15 ml). Fraction B was not fully resolved from some less mobile material. At a high concentration of estradiol (3.8 μg/ml) A was the main reaction product and at its low concentration (7.8 ng/ml) B was the main product. It is noted that Mucchielli *et al.* [13] reported the isolation of a product of the HRP-catalysed oxidation of estradiol with gel-chromatographic properties similar to those of product B.

Product B re-chromatographed on the same batch of Sephadex gel (G-50/1) was accompanied by a less mobile component [product B1; see Fig. 4(a), ——]. Since B was obtained from its chromatographic peak fractions and so separated from all or most of the less mobile reaction products, its subsequent partial conversion into B1 must have occurred either in water or on the second Sephadex column. The elution pattern shown in Fig. 4(a) (——) was not changed by leaving product B in 2.5 mM phosphate buffer (pH 7) for 1 h at 45°C before chromatography, but pre-treatment with 50 mM Na_2HPO_4 or KH_2PO_4 transformed B to B1 [Fig. 4(a), --- and ····]. Chromatography of B on a different batch of Sephadex G-50 (G-50/2) gave a surprisingly different elution pattern [Fig. 4(b)]: in addition to a broad band (V_e = 16.5 ml) with a shoulder (V_e = 14.5 ml) attributed to B1 and B, respectively, two components of V_e = 11 ml (B2) and V_e = 7 ml (B3) were detected, but when B was pre-treated with 2.5 mM phosphate buffer (pH 7) the elution pattern [Fig. 4(b), ---] was as from a G-50/1 column. Pre-treatment of B with 50 mM phosphate buffer (pH 7) gave the same elution pattern on G-50/2 [Fig. 4(b), ····] as that on G-50/1 after treatment of B with 50 mM Na_2HPO_4 or with 50 mM KH_2PO_4. It follows that B2 and B3 dissociate to B at low electrolyte concentration and to B1 (presumably via B) at high electrolyte concentration. As the dissociation of B2 and B3 to B in 2.5 mM buffer was complete and as the enzymic transformation of estradiol took place in the same buffer, it also follows that B2 and B3 were not products of that reaction but that they formed from B on the G-50/2 column or in water after its separation from the reaction mixture on the first G-50/1 column and followed by the dissociation of B2 and B3 on the second G-50/1 column. B and B1, because of the closeness of their elution volumes to that of vitamin B_{12} (M_w 1335), are likely to be tetraestradiols which possibly differ in the extent of their hydration or in their conformation. B2 and B3 are clearly aggregates of B.

Treatment of a mixture of product B (1.3 ng; 24,000 dpm) and inert estradiol (1.6 μg) with HRP/H_2O_2 under the conditions given in the legend to Fig. 3 followed by chromatography on a Sephadex G-50/2 column gave no tritiated product A; it is therefore concluded that B is an endproduct of the reaction. Identical treatment of [2-^{3}H]estradiol (15,000 dpm) followed by addition of saturated ammonium sulphate (0.5 ml) and extraction of the products with ethyl acetate (1 ml) resulted in essentially the same loss of tritium (61 and 62%) at low and high concentrations of inert estradiol (8 ng/ml and 4 μg/ml, respectively). This shows that A and B are formed with the removal of the same number of hydrogen atoms from position 2 of

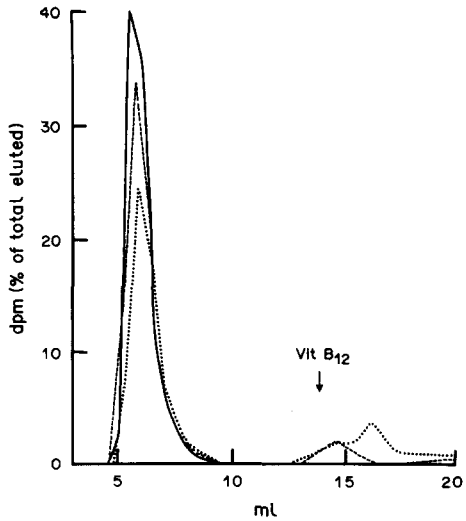


Fig. 5. Fraction A re-chromatographed on Sephadex G-50/2; effect of pre-treatment with buffer. Product A (0.3 ng, 10,500 dpm) in 0.1 ml water (——); product A (0.6 ng, 21,000 dpm) in 0.26 ml 2.5 mM phosphate buffer (pH 7) for 24 h at room temperature (-----); product A (0.6 ng, 21,000 dpm) in 0.8 ml 50 mM phosphate buffer (pH 7) for 24 h at room temperature (·····). Recoveries of tritium were 87, 63 and 45%, respectively. Chromatography was as in the legend to Fig. 3.

estradiol. As the loss of tritium was in reasonable agreement with that (64%) found on the preparative scale, it may be inferred that removal of an equal number of hydrogen atoms from position 4 occurred also in the present experiment.

Product A was completely excluded from a Sephadex G-50 column but after treatment with 2.5 mM phosphate buffer (pH 7) it gave a small fraction with the mobility of product B and after treatment with 50 mM phosphate buffer (pH 7) an additional fraction with the mobility of B1 (Fig. 5). The identity of the former with B is doubtful since, unlike B, it remained unchanged in the 50 mM buffer. On a G-200 column product A behaved like PEL, giving a major fraction in the void volume and a very small fraction in the bed volume.

Table 2. Dialysis of PEL and of Sephadex fractions A and B at 0°C

Substance	Solvent	Time to dialyse (h) 25%	50%	75%
PEL[a]	50% Ethanol	140	700	
	Ethanol	15	42	97
Fraction A[b]	Ethanol	15	38[f]	76[f]
Fraction B[c]	Ethanol	2	4.5[f]	13[f]
Fraction B[d]	Water	2.5	11[f]	32[f]
Vitamin B_{12}[e]	Water	2.5	7	13

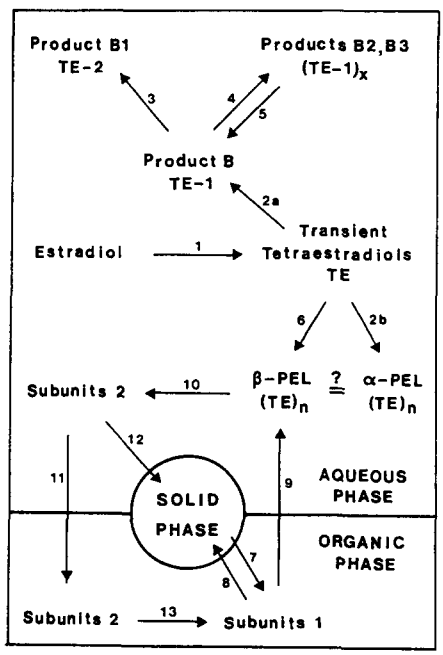
[a]250 μg/ml. [b]0.3 ng/ml; 10,500 dpm. [c]0.7 ng/ml; 24,000 dpm. [d]4 ng/ml; 10,000 dpm. [e]275 μg/ml; M_w 1335. [f]Values underestimated (see Experimental).

Like PEL, product A did not dialyse in water; both dialysed in ethanol at similar rates and much slower than product B (Table 2), suggesting that both polymers dissociated in ethanol to subunits of the same size but larger than the constituents of fraction B. This is consistent with the presence of dimers in the ethanolic solutions of PEL and A, as inferred for PEL from the osmometric measurement of its molecular weight in ethanol [8].

DISCUSSION

The HRP-catalysed oxidation of estradiol performed on a preparative scale in 50 mM phosphate buffer resulted in the precipitation of PEL from the reaction mixture [8]. The same reaction performed on a microscale in 2.5 mM buffer and followed by gel-chromatography of the reaction mixture gave a high-molecular product A and a low-molecular product B. In order to indicate the polymeric character of product A and its possible difference from PEL, the former will be referred to as α-polyestradiol (α-PEL) and the latter as β-polyestradiol (β-PEL) whilst PEL will be retained as a generic name. Scheme 2 aims to account for the formation of these entities and for some of their properties:

It is proposed that the endproducts of the oxidative coupling of estradiol (step 1)



Scheme 2

are two or more different transient tetraestradiols (TE) which combine with water (step 2a) at low concentration to give TE-1 (product B) and with themselves (step 2b) at high concentration to give $(TE)_n$, α-PEL, probably with hydration at some stage of the latter process. In 50 mM phosphate buffer, monomers TE-1 are transformed (step 3), possibly with partial loss of water, to monomers TE-2 (product B1). TE-1 associate (step 4), either in water or on one of the two batches of Sephadex G-50 used (G-50/2), to oligomers B2 (probably dimers) and B3, the reaction being reversed in 2.5 mM phosphate buffer (step 5). β-PEL, if different from α-PEL, could derive from a different mode of assembly of monomers TE (step 6). Solid PEL dissolves in organic solvents with dissociation to subunits 1 (step 7) composed of monomers and dimers, the former being predominant in dioxan, the latter in ethanol. The reverse process (step 8) is brought about by dilution of either solution with ether. Dilution of the ethanolic solution with water followed by removal of ethanol gives an aqueous solution of β-PEL (step 9). Slow dissociation of β-PEL in water to subunits 2 (step 10) would account for the time-dependence of the extraction with ether or with dichloromethane (step 11) and its acceleration by sodium chloride would account for the coincident increase of extractable material and the precipitation of PEL (step 12). The presumed dissociation of β-PEL to subunits 2 in the presence of electrolytes parallels that of α-PEL to monomers (Fig. 5 and accompanying text). It seems likely that following their extraction from water the composition of subunits 2 changes to that of subunits 1 in the same solvent (step 13).

The non-covalent reactions shown above in Scheme 2 were brought about by solvents, electrolytes and by contact with Sephadex G-50. Other such changes were those induced by heat and by ageing and possibly those involved in the binding of estradiol [12]. These properties resemble the non-covalent changes of proteins which are considered to be essential for the latter's biological functions [14]. Of particular interest is the effect of the concentration of estradiol on the outcome of its HRP-catalysed oxidation (Fig. 2), i.e. on the yields of the low-molecular product B and the polymeric α-PEL, the ratio of which varies most noticeably at estradiol concentrations of $< 1\ \mu g/ml$, i.e. at its physiological levels.

The reaction of estradiol with lactoperoxidase/H_2O_2 gave PEL [9] and proceeded with essentially the same loss of hydrogen atoms from position 2 [15, 16] as the reaction with HRP/H_2O_2. Treatment with uterine peroxidase and H_2O_2 released 22–43% tritium from [2-^{3}H]estradiol (3 experiments) and 25% from [4-^{3}H]estradiol (1 experiment) [15]. The inconsistent results of replicate experiments suggest that the reaction was incomplete. It seems, therefore, worth considering that uterine peroxidase reacts with estradiol in the same manner as lactoperoxidase and HRP and, more speculatively, that the reaction takes place also *in vivo* and that the regulation of its outcome by the concentration of estradiol (or by the rate of its conversion into TE), the generation of a peroxidase by estradiol in its target tissues [4, 17] and the binding of estradiol by PEL [12] are physiologically interconnected phenomena.

The present results establish the sites of the reaction and the types of bonds formed during the HRP-catalysed conversion of estradiol into PEL subunits. They suggest that the subunits are two or more different cyclic tetraestradiols, tentatively assigned the structural fragments **1** and **2**. They also suggest that at low concentration of estradiol its main reaction products are tetraestradiols which do not aggregate to PEL. However, the most striking result is that the *in vitro* reaction of estradiol with HRP/H_2O_2 gives products exhibiting properties usually associated with biopolymers and their subunits.

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