POLYMERISATION OF OESTRADIOL BY POTASSIUM FERRICYANIDE AND BY HORSERADISH PEROXIDASE

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1. Introduction

Reactions of horseradish peroxidase-hydrogen peroxide with oestradiol [1,2] and with oestrone [3] gave products which have remained unrecognised. Transformations of oestradiol catalysed by an uterine peroxidase [4–7] and by a peroxidase from an induced mammary tumour [8] add to the interest in the nature of the products of interactions between oestrogens, peroxidases and hydrogen peroxide. A biomimetic approach to the problem is now reported, based on the well-established ability of one-electron-transfer reagents, notably potassium ferricyanide, to simulate the peroxidase-catalysed metabolic transformations of plant phenols [9,10]. More specifically, the aim was to oxidise oestradiol with potassium ferricyanide and, having established the properties of the isolated product(s), to re-examine the enzyme-catalysed reaction.

2. Experimental

2.1. *General*

Melting point samples were taken in evacuated capillary tubes on a Kofler hot-stage. Optical rotations were measured with a Perkin Elmer Model 141 polarimeter and molecular weights with a Mechrolab Model 301A vapour pressure osmometer. Infrared spectra were taken with a Unicam SP200 spectrophotometer and ultraviolet spectra with a Unicam SP8000 spectrophotometer. Samples for analysis were dried overnight in vacuo at 120°C. Horseradish peroxidase (60 purpurogallin units/mg) was supplied by British Drug Houses, Ltd, Poole, England.

2.2. *Ferricyanide oxidation of oestradiol*

A solution of oestradiol (1.9 g) in hot benzene (400 ml) was cooled to room temperature and then stirred with 0.1 M NaOH (180 ml) under a stream of nitrogen. Freshly prepared 0.1 M $K_3Fe(CN)_6$ (180 ml) was added drop-wise over a period of 5 h. Stirring was continued for 1 h. Ethyl acetate (400 ml) was added and the mixture was then worked-up in the customary manner. The crude product was applied onto an Amberlite MB-1 (25 g) column in methanol. Elution with methanol (200 ml) gave a brownish product (1.4 g). Its solution in dioxan (10 ml) was dropped into stirred ether (100 ml) to give a pale-yellow micro-crystalline material (0.97 g). Crystallisation from dichloromethane–methanol and then from dichloromethane–acetone gave a substance whose physical constants are recorded in table 1 and in figs 1 and 2. Judging by its i.r. spectrum, the material eluted from the Amberlite column was already essentially pure.

2.3. *Enzyme-catalysed oxidation of oestradiol*

Oestradiol (200 mg) in *iso*-propanol (25 ml) was added to a stirred mixture of 0.05 M phosphate buffer (1800 ml, pH 7), Triton X-100 (1.8 ml) and *iso*-propanol (25 ml) at 45°C. Horseradish peroxidase (10 mg) was added and then, drop-wise over a period of 30 min, hydrogen peroxide (25 ml, 0.6%, v/v). After 16 h at room temperature the buff-coloured precipitate was filtered off and washed well with water. A solution of this material in dioxan (2.5 ml) was dropped into stirred ether (25 ml) to give a pale-yellow micro-crystalline precipitate (125 mg). This was quantitatively eluted with methanol from an Amberlite MB-1 column. After two crystallisations

from methanol and one from dichloromethane–acetone the substance had the physical constants recorded in table 1 and in figs 1 and 2. Judging by its infrared spectrum the ether precipitate was already essentially pure.

3. Results and discussion

Table 1 and figs 1 and 2 summarise the evidence for the identity of the ferricyanide oxidation product of oestradiol with the product of the enzyme-catalysed reaction. The slight difference between the ultraviolet spectra (fig.1) of the two samples – a just perceptible maximum at 280 nm (E^1_1 125) versus an inflection at 277 nm (E^1_1 139) – is not significant as similar differences were observed between different batches of the ferricyanide product. Vapour pressure osmometry of the compound in ethanol gave values consistent with the molecular weight of an oestradiol octamer whilst measurements carried out in dioxan gave values consistent with the molecular weight of a tetramer. It seems likely that in ethanolic solution two tetramer molecules associate by forming hydrogen bonds and that the hydrogen-bonding properties of dioxan prevent that association. Elemental analyses indicate that the tetramer contains either two additional oxygen atoms or two molecules of water. On the assumption that the tetramer was formed by the classical mechanism of oxidative coupling of phenols, the absence of an infrared carbonyl band (see fig.2) rules out position 10 as a site of coupling and leaves positions 2 and 4 as the only likely alternatives [9,10]. It is further tentatively assumed that the tetramer is formed by C–O rather than by C–C bonding as its ultraviolet infrared and spectra resemble more closely those of a C–O bonded guaiacol polymer than those of a C–C bonded guaiacol tetramer [11]. The tetramer behaved like a neutral compound on columns of Amberlite MB-1 and of alumina but its ultraviolet spectrum in alkaline solution (fig.1) disclosed a weakly phenolic character. The compound is hygroscopic but insoluble in water.

It is non-volatile and consequently its mass spectrum could not be obtained.

The isolation and partial characterisation of an oestradiol tetramer formed by the horseradish peroxidase-catalysed oxidation of oestradiol provide both motive and means for examining the possibility that other peroxidases transform oestradiol in the same manner. The transformation of oestradiol by uterine peroxidase is of particular interest because of the possible physiological role of the process [5,12,13]. Recent reports of an increase in oestrogen receptors induced by treatment with oestradiol [14] and of a hydrogen peroxide-stimulated increase of oestradiol binding by oestrogen receptors in vitro [15], in conjuction with the stimulation of uterine peroxidase production by oestradiol [4–7], appear to suggest an interdependence between peroxidase and oestrogen receptors in the uterus.

Table 1
Some physical constants of oestradiol oxidation products

	Ferricyanide product		Peroxidase product
Melting point (in vacuo)[a]	320–327°C		325–330°C
Mixed melting point		322–330°C	
$[\alpha]_D$ (in dioxan)	+96°C		+96°C
Mol. wt in:			
ethanol, found	2083		2011
dioxan, found	1260		1026
Calc. for A and B[b]		1119 and 1117	
C, H, found	77.0, 8.0%		77.4, 8.0%
Calc. for A[b]		77.2, 8.5%	
Calc. for B[b]		77.4, 8.3%	

[a] Melting point samples of finely ground material
[b] A, $C_{72}H_{90}O_8 \cdot 2H_2O$ (open-chain tetramer)
B, $C_{72}H_{88}O_8 \cdot 2H_2O$ (cyclic tetramer)

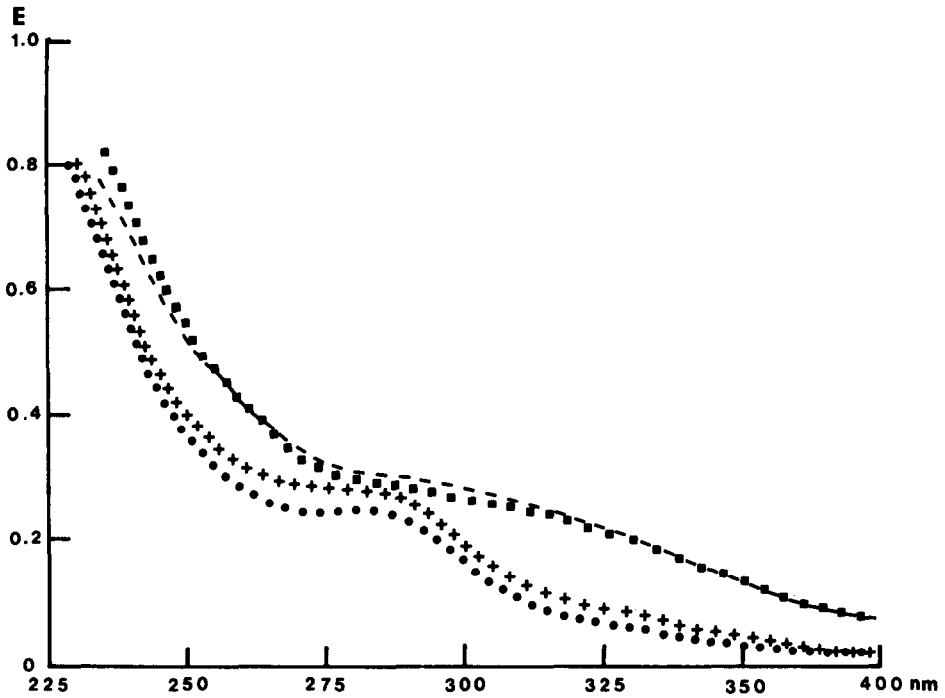

Fig.1. Ultraviolet spectra of oestradiol oxidation products. Ferricyanide product in ethanol (+++) and in ethanolic 0.1 M NaOH (—) (17.8 μg/ml). Horseradish peroxidase product in ethanol (. . .) and in ethanolic 0.1 M NaOH (■ ■ ■) 17.4 μg/ml).

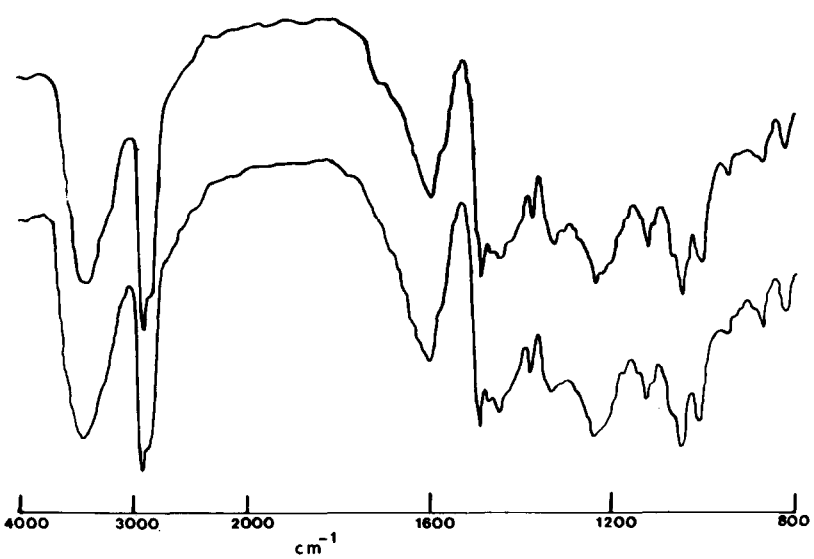

Fig.2. Infrared spectra (in KBr) of oestradiol oxidation products. Ferricyanide product, lower tracing. Horseradish peroxidase, upper tracing.

Note added in proof (22 March 1977)

Microcrystalline character was attributed to the reported oestradiol tetramer because of its deflection of polarised light. Dr N. A. Bailey (Chemistry Department of this University) informs me that an X-ray powder diffraction diagram shows the substance to be essentially amorphous.

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