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HORSERADISH PEROXIDASE WITH 2,4-MODIFIED HAEMATINS, INCLUDING VINYL HOMOLOGUES

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SUMMARY

1. Some acyl and vinyl homologue porphyrins have been prepared.

2. Pyridine is superior to alkali as a solvent for haemin to be used for the preparation of haemochrome.

3. The Fe^{3+} and Fe^{2+} spectra of peroxidases with proto-, haemato-, meso-, deutero-, diacetyldeutero-, dipropenyldeutero- and dibutenyldeuterohaematins have been determined. Diacetyldeuterohaematin peroxidase gives a mixed-type spectrum which is sligthly temperature dependent even above 0 °C.

4. Meso-, proto- and diacetyldeuterohaematin peroxidases shift from the neutral to the alkaline form with pK 11.8, 11.1 and 9.0.

5. At pH 4.5, k_1 (H_2O_2, stopped-flow) is essentially equal for the meso-, haemato-, deutero- and diacetyldeuterohaematin peroxidases and about twice as high for the three alkenyl peroxidases. At pH 7.0, k_1 of the first group remains unchanged, whereas k_1 of the second group increases, the first demonstration of a pH effect upon k_1.

6. k_4 (ascorbate) shows a 200-fold difference between the two extremes, dipropenyl- and diacetyldeuterohaematin peroxidase. The dihydroxyfumarate oxidase activity differs some 10 times between the extremes meso- and diacetyldeuterohaematin peroxidase, with only slight correlation to k_4 for ascorbate.

INTRODUCTION

The reversible splitting of horseradish peroxidase (donor: H_2O_2 oxidoreductase,

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Symbols:

Horseradish peroxidase + $H_2O_2 \xrightarrow{k_1}$ Compound I;

Compound I + $AH_2 \xrightarrow{k_7}$ Compound II + AH·;

Compound II + $AH_2 \xrightarrow{k_4}$ horseradish peroxidase + AH·.

2,4-substituents: haemato CH_3CHOH–, meso CH_3CH_2–, deutero H–. RZ absorbance of Soret band/$A_{280\ nm}$.

EC 1.11.1.7) into apoprotein and protohaematin opened the way for studies of this enzyme by means of modifications of the porphyrin[1]. The loss of the 6-propionic acid residue and the introduction of other reactive groups completely abolished the activity[2,3]. The meso- and deuterohaematins with 6,8-propionic acid residues gave some activity, although much less than the 6,7-isomers[4,5]. The esterification of both carboxyl groups prevented the formation of a holoperoxidase[6] and also of Compound I (refs 7 and 8), and the monoester gave a peroxidase of low activity[7]. There may be some difference between the 6- and 7-positions[7].

Most studies, however, have dealt with the 2- and 4-positions. Thus, the combination of apoperoxidase with variously 2,4-substituted haematins has given the activities shown in Table I (recombination with protohaematin = 100).

TABLE I

Substituent				*Hydrogen donor**	*Ref.*
H–	*CH_3CH_2–*	*CH_3CHOH–*	*CH_3CO–*		
62	53	0		Pyrogallol. C	2
	≈ 160	≈ 80		Guaiacol. C	9
56	63			Pyrogallol. C	3
53	137	135	3	Mesidine. C	4
			< 20	Ascorbate. k_4	10
90	92			Guaiacol. k_4	7
68	236	100	6	Ascorbate. k_4	8
132	182	900?	2	Guaiacol. k_4	8

* Activity evaluated from colour formation (C) or k_4 determination.

Different peroxidase preparations and test methods have been used and there are some striking variations between the results. The choice of haematins seems to have been somewhat confined. The 2,4 substitution as a source of information about peroxidase mechanisms has probably not been fully explored. The steric nature of the protein–haematin interactions could be studied by means of protohaematin homologues. The effects of the electron distribution in the prosthetic group on the activity should be analyzed by separate determinations of k_1 and k_4.

MATERIALS AND GENERAL METHODS

Horseradish peroxidase

Horseradish peroxidase, Fraction IIIb, was isolated as described[11].

Porphyrins

Porphyrins were esterified with HCl–saturated methanol and the esters were chromatographed on alumina grade IV (ref. 12) with 0–200 mM methanol in chloroform (acylporphyrins) or 0–700 mM chloroform in benzene. It was necessary, in particular with the acylporphyrins, to chromatograph several times to achieve homogeneity in two-dimensional paper chromatography, and to obtain sharp melting points[13–15]. LH-Sephadex with various eluants brought about some separation, but was on the whole less efficient than alumina. The esters were saponified for 1 h in

boiling methanol containing 4% KOH (w/v) or 24 h at room temperature in concentrated HCl (diacetyldeuteroporphyrin ester).

Crystalline protohaemin[16]

Crystalline protohaemin was analyzed by chromatography[13] and used without further treatment, or converted to mesoporphyrin by refluxing over Pd–H_2 in boiling formic acid[17]. Deuterohaematin[18] for coupling to apoperoxidase was purified *via* the porphyrin ester. The higher dialkenylhaematin homologues were prepared *via* the acylporphyrins. The condensation of deuterohaemin with acetic, propionic or butyric anhydride[17] (0 °C, reaction time 10–25 min, anhydrous $SnCl_4$ as catalyst, spectroscopic control of the haemochromogen spectrum) to give the three 2,4-diacylporphyrins yielded $< 10\%$, not improved by the use of acid chlorides or $AlCl_3$. It was found, however, that the replacement of sodium or potassium salts by ammonium salts in all steps raised the yield by as much as five times. Fischer and Orth[17] have drawn attention to the low solubility of the sodium salts of the diacetyldeuteroporphyrin. The iron content of the crude acylhaematins was removed by stirring for 72 h at 45 °C in HBr-saturated glacial acetic acid, and the porphyrins were precipitated by the addition of excess water and some ammonia. Deuteroporphyrin was removed by 0.3 M HCl and the diacylporphyrin, together with some monoacylporphyrin as well as some ill-defined material, was taken up in 5 M HCl, precipitated, esterified, chromatographed[18] and crystallized. The porphyrin ester was reduced in chloroform by means of an excess of sodium borohydride in an equal volume of peroxide-free diglyme[19], the spectral change being completed in 20 min. The solution was washed exhaustively (> 15 times) with de-ionized, quartz-distilled water to remove diglyme and salts. The porphyrin, presumably all in the 2,4-α-hydroxy form, was not isolated but refluxed in glacial acetic acid–acetic anhydride (3:1, v/v). Spectroscopy revealed complete conversion to the alkenylporphyrin in 60–90 min. The product, homogeneous in paper chromatography, was saponified and converted to the haematin.

Recombination experiments with haematohaemin have given some opposing results[2,8,20–22]. When prepared from protohaemin via the HBr adduct to protoporphyrin, haematohaemin will be a mixture of isomers and the product is likely to be contaminated by poorly defined material. Haematohaematin was therefore obtained by the splitting of cytochrome *c* (ref. 23) (Sigma, Type VI, tested for the absence of protohaemin by means of acid acetone), the product taken up in ether, washed with dilute sulphuric acid and water until flocculation and dried under argon.

The introduction of iron was slightly modified from Morell *et al.*[24]. The porphyrin was dissolved in 1 ml of pyridine and 25 ml of glacial acetic acid, and 1 ml of a water solution of ferrous sulphate was added. After 10 min under argon at 80 °C, the mixture was cooled and allowed to oxidize in air for 15 min. The haematin was taken up in ether, washed with water, 15% HCl and excess water and finally taken to dryness under argon. None of the haematins was crystallized, but they all appeared homogeneous in paper chromatography[16,25] and their pyridine haemochrome spectra were free from a maximum or a plateau at 600–650 nm[26].

Apoperoxidase–haematin coupling

The splitting of horseradish peroxidase followed the method of Teale[27], but a

pH of 1.8 was required. This acidity seems to have no harmful effect for a short time, since recombined protohaematin–horseradish peroxidase and unsplit horseradish peroxidase showed similar kinetics. For coupling, an amount of a haematin in 25% excess over the apoperoxidase was dissolved in 10 equivalents of sodium or ammonium (diacetyldeuterohaematin) hydroxyde and diluted 5-fold with 10 mM Tris–HCl, pH 8.0, before mixing with the apoprotein. After 90 min the solution was dialysed against 10 mM potassium phosphate, pH 6.0, and chromatographed on a 10 cm × 0.8 cm column of DEAE-cellulose in the same buffer. A brown zone, presumably the excess of haematin, was retained at the top, whereas the holoperoxidase travelled as a single band, as judged by ultraviolet and visible light. The subsequent shift to 200 mM phosphate eluted no more material. The peroxidase was dialysed against several changes of distilled water, concentrated in collodion sacs and assayed by dry weight determinations (105 °C to constant weight; no ash in excess of Fe_2O_3). The homogeneities of the peroxidases thus prepared were confirmed by polyacrylamide gel electrophoresis at pH 6.5 (ref. 28) with staining for protein and peroxidase.

Molar absorption coefficients were based on dry weight determinations, and peroxidase concentrations were assayed spectrophotometrically.

It was deemed unsatisfactory to base concentrations on pyridine haemochrome measurements, because of occasional variations in the molar absorption coefficients found for the pyridine protohaemochrome. The variations could be traced back to the choice of NaOH as the primary solvent for the sample of crystalline haemin. Table II recommends pyridine as solvent for solid haemin, in agreement with earlier results[29] and the structure proposed for pyridine parahaematin[30,31]; moreover, the time for completely dissolving crystalline haemin in alkali is long enough to overlap the onset of the irreversible changes in disodium protohaematin solutions (Elliot, W. B., personal communication). Pyridine, as solvent for metalloporphyrins in coupling experiments[32], however, gave holoperoxidases which did not differ in spectra from those obtained with alkali as solvent. The use of amorphous haematin preparations in the present studies may have been fortuitously advantageous.

Infrared spectra

Infrared spectra at 1500–2000 cm^{-1} were registered on a Perkin–Elmer Type 257 instrument with 0.5-mm NaCl cells with dry chloroform (using $CaCl_2$) as solvent and reference. The ratio of the areas (height · half-height width) for acyl and ester C = O were used to compare the chromatographic fractions.

pH

pH was determined by means of a Radiometer PHM 25, the combination electrode GK 2321 C being used for the experiment in Fig. 4 with calibration at pH 9 and correction for salt effects.

Spectra

Spectra in ultraviolet and visible light were registered on Beckman DU or Acta III instruments, calibrated against an Ho filter.

Melting points

Melting points were determined on a Reichert melting point microscope,

calibrated against phenacetin (135 °C), istizin (194 °C) and dicyandiamide (210 °C), with temperature increments of 0.5–1 degree per min above 150 °C.

Solvents

Solvents were purified, dried and tested for peroxides[33], care being taken to avoid light during the porphyrin work.

Kinetic measurements

k_1 was determined in a Durrum–Gibson stopped-flow spectrophotometer with 2-cm cuvettes, giving a dead-time of about 5 ms. For k_4 the procedure given by Chance[34] was preferred to the indirect method[35], because of the inhibition by guaiacol[36]. Under the conditions of the experiments, the formation of Compound III is negligible. A Beckman DB spectrophotometer with a lin-log converter and a Servogor RE 511 recorder gave a good signal-to-noise ratio (Fig. 1). The standard difference between two determinations of k_1 or k_4 was less than 4% for a given set of solutions.

The dihydroxyfumarate oxidase activity was arbitrarily expressed as $t_{0.2}^{-1}$, where $t_{0.2}$ is the time required for $-\Delta A_{292\text{nm}} = 0.2$ in 10 mM sodium acetate, pH 4.8.

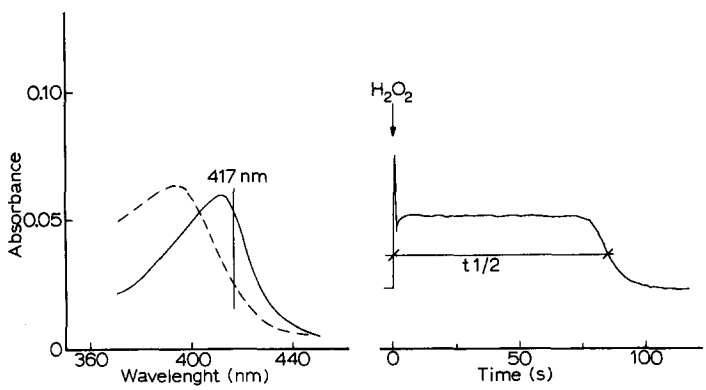

Fig. 1. Determination of k_4 for mesohaematin peroxidase with ascorbic acid (208 μM) as hydrogen donor. 0.75 μM peroxidase, 17.5 μM H_2O_2. 10 mM sodium phosphate, pH 7.0. – – –, peroxidase; ——, Compound II.

RESULTS AND DISCUSSION

The results are shown in Tables II–VII and Figs 2–6.

The difficulties in preparing homogeneous diacylporphyrin esters increased with the length of the side chains. Monoacylporphyrins were clearly distinguished by their rhodo-type spectra, and their acyl/carboxyl carbonyl infrared absorbance ratio (0.48; ref. 18) is well below the ratios in Fig. 2 and Table III.

Some properties of the porphyrin esters are collected in Table III. No literature reference has been found to the butyryl-, propenyl- and butenylporphyrins. The propionyl- and butyrylporphyrin esters melted partially at the lower temperature given in Table III, solid crystalline and liquid material existing in apparently constant proportions until the upper temperature was reached. The crystals showed no sign of isomorphy while floating in the form of confluent droplets. Both changes

TABLE II

THE EFFECT OF THE DISSOLVING MEDIUM ON THE PYRIDINE HAEMOCHROME SPECTRUM

The qualities of the spectra are expressed as the ratios between the absorbance in red and at the minimum between the α- and β-bands (A_{650}/A_{min}) and between the absorbances of the α-band and the minimum.
(Mean $\pm$ S.D.; $n = 10$)

Primary solvent for protohaemin	*ε (mM, α-band)*	A_{650}/A_{min}	$A_{\alpha\text{-band}}/A_{min}$
0.1 M NaOH	30.4 ± 2.2	0.070 ± 0.010	3.6 ± 0.1
Pyridine	33.9 ± 0.9	0.076 ± 0.007	3.6 ± 0.1

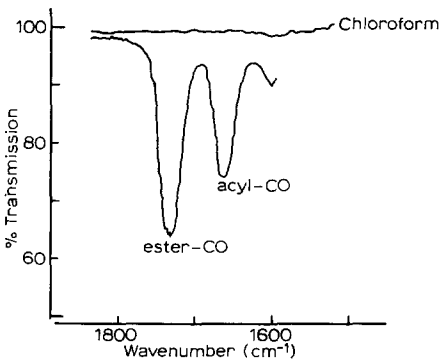

Fig. 2. Infrared transmission spectrum of 2,4-diacetyldeuteroporphyrin ester.

TABLE III

SOME PROPERTIES OF 2,4-DISUBSTITUTED PORPHYRIN DIMETHYL ESTERS

Solvent for spectrophotometry was dioxane. Crystals from (a) chloroform–methanol or (b) benzene–light petroleum. (c) Caughey *et al.* o 80 (ref. 18).

Substituent	*Spectrum Wavelengths and relative absorbances*				*Mp. °C*	*Acyl CO/Ester CO*
Vinyl	505	541	575	629	226[a]	
(Proto)	1.00	0.81	0.47	0.37		
1-Propenyl	504	539	576	631	222[b]	
	1.00	0.91	0.48	0.44		
1-Butenyl	504	539	576	631	194.5[b]	
	1.00	0.88	0.47	0.44		
Ethyl	499	534	528	621	214–15.5[a]	
(Meso)	1.00	0.71	0.48	0.35		
Acetyl	513	547	584	637	?/245[a]	0.80
	1.00	0.55	0.45	0.24		
Propionyl	513	547	584	637	186/234–5[a]	0.82[c]
	1.00	0.55	0.45	0.24		
Butyryl	513	547	584	637	194–5/204–6[b]	0.84
	1.00	0.54	0.45	0.24		

occurred very distinctly. The diacetylporphyrin ester gave no such double melting point.

The close spectral similarity between the three alkenylporphyrins confirmed the close structural relationship of the two higher homologues to protoporphyrin, *i.e.* 1-alkenyl substitution at the 2,4-positions of the porphyrin. Trans configuration was presumed.

Unspecific apoperoxidase–haematin interactions have been suspected[8,21], admittedly with haematohaematin. However, the specific binding of all haematins to the apoperoxidase to give genuine holoperoxidases is proven by the homogeneities of the artificial peroxidases on DEAE-cellulose, in gel electrophoresis, and by the appearance of external Cotton effects (Ohlsson, P.-I., Paul, K.-G. and Sjöholm, I., unpublished). Most important, and evidence in itself for a specific binding, is the ability of the haemoproteins prepared to form Compound I in a quantitative manner at high reaction rates ($\approx 10^7\ M^{-1}\ s^{-1}$, *cf.* Table VI), a property unique to hydroperoxidases.

From the pronounced effect of haematin esterifications and the tiny effect of 2,4-modifications on the cytochrome *c* peroxidase activity, Yonetani *et al.*[37] found it conceivable that the 2,4-positions were directed, not inwards towards a hydrophobic cleft as in haemoglobin and myoglobin, but outwards, protruding from the surface. The specific binding, even of the higher homologues to protohaematin, reveals that the propenyl and butenyl groups constitute no steric hindrance to the haematin–protein coupling. This gives experimental support to the concept of a position of the haematin in peroxidases with protruding vinyl groups.

TABLE IV

POSITION AND MILLIMOLAR ABSORPTION COEFFICIENTS OF THE ARTIFICIAL PEROXIDASES

Experiments were done in 10 mM sodium phosphate pH 7.0. Reduction by dithionite.

Peroxidase	*Oxidized*						*R Z*
	I (nm)	*II (nm)*	*Soret (nm)*	*ε(mM) I*	*ε(mM) II*	*ε(mM) Soret*	
Native	641	498	402	3.23	10.95	100.0	3.40
Proto	641	499	402	2.89	10.46	94.3	3.48
Dipropenyldeuterohaematin	635	500	402	2.87	11.22	102.2	3.40
Dibutenyldeuterohaematin	636	502	404	2.98	11.23	111.8	3.29
Deuterohaematin	627	497	393	2.72	7.08	112.6	4.50
Meso	634	492	395	2.75	9.42	88.0	3.25
Diacetyldeuterohaematin	642	508	412	2.26	7.32	80.1	3.17
Haemato	633	497	397	2.99	8.50	102.7	3.70

Peroxidase	*Reduced*			
	I (nm)	*Soret (nm)*	*ε(mM) I*	*ε(mM) Soret*
Native	556	436	12.60	88.6
Proto	555	433	12.55	75.3
Dipropenyldeuterohaematin	555	433	12.13	84.5
Dibutenyldeuterohaematin	556	433	12.51	84.9
Deuterohaematin	546	427	10.00	79.0
Meso	549	428	11.56	88.0
Diacetyldeuterohaematin	562	447	10.37	78.2
Haemato	549	431	10.93	89.1

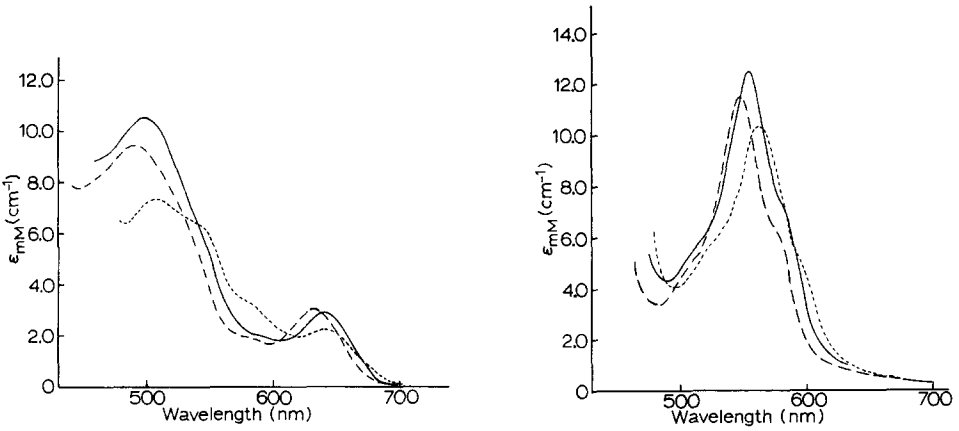

Fig. 3. Spectra of oxidized (a) and reduced (b) proto (——), meso (- - - -) and diacetyldeuteroheamatin (· · · · ·) peroxidases in 10 mM sodium phosphate, pH 7.0. Dithionite as reducing agent.

The spectra of most ferriperoxidases (Table IV a, b, Fig. 3 a, b) are dominated by the absorption bands at about 500 and about 630 nm. The pK for the shift to the alkaline red form (the structural cause of which is being discussed[38]) varies with the electron attraction of the 2,4-substituents, the diacetyl, vinyl and ethyl groups giving pK 9.0 (9.1 (ref. 8)), 11.1 (10.9 (ref. 39), 11.06 (ref. 40), 10.5 (ref. 7), 11.1 (ref. 8)) and 11.8 (11.1 (ref. 7), 11.8 (ref. 8)) for this peroxidase (Table V, Fig. 4). Some older values, were obtained on peroxidase preparations, not identifiable by the present methods of isolation.

TABLE V

THE TRANSITIONS BETWEEN NEUTRAL AND ALKALINE PEROXIDASES FROM MEASUREMENTS AT FOUR WAVELENGTHS

The mean values ± S.D. of pK_a, slope (n), and correlation coefficient (r) from plots of log $(A_{alk}-A_{obs})/(A_{obs}-A_{neutr})$ against pH are given

Apoperoxidase recombined with	pK_a	n	r
Diacetyldeuterohaematin	9.0 ± 0.07	1.00 ± 0.09	1.00 ± 0.00
Protohaemin	11.1 ± 0.16	0.98 ± 0.05	0.99 ± 0.01
Mesohaemin	11.8 ± 0.07	0.94 ± 0.03	1.00 ± 0.00

At pH 6 the diacetyldeuterohaematin peroxidase gives a four-banded spectrum of a mixed type. The maxima at about 500 and about 630 nm are common to all peroxidases, whereas two shoulders, at about 550 and about 580 nm, appear close to the wavelengths where the alkaline form of this peroxidase has its maximum (Fig. 5). However, the transfer to the alkaline peroxidase closely follows an ordinary $n = 1$ dissociation curve with pK 9, and the four-banded spectrum at pH 6, therefore, cannot represent a mixture of two forms in proton equilibrium. This is confirmed by the lack of spectral changes when pH is lowered. Only at pH < 4 is the spectrum gradually replaced by that of acid haematin. Thus, the diacetylperoxidase itself, being a homogeneous substance, produces this four-banded spectrum, attributable

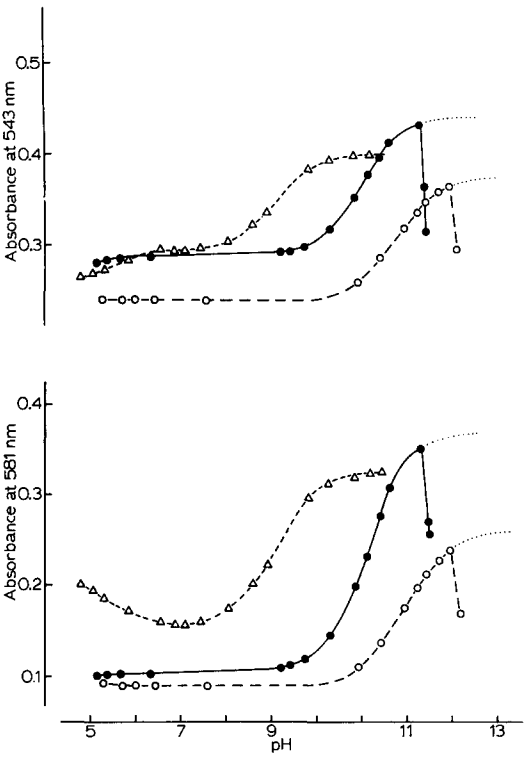

Fig. 4. Transition from neutral to alkaline form of the proto (——), meso (- - - -) and diacetyldeuterohaematin (· · · · ·) Fe^{3+} peroxidases (46 μM). Initial pH 4.7, 30 mM sodium acetate, adjusted by means of 1 mM or 1 M NaOH. Top 543 nm, bottom 581 nm.

to the effect of the strong carbonyl group. The proportion between the two spectral components at pH 7 is temperature dependent (Fig. 5), which may permit studies of the thermal equilibrium between high and low spin forms, without effects from phase changes of the solvent.

The k_1 values (Table VI) fall markedly into two groups, the three alkenylperoxidases and the other four. It is striking that such divergent substituents as H, CH_3CH_2, CH_3CO and CH_3CHOH give peroxidases with essentially the same k_1. The three alkenylperoxidases give about twice as high k_1 values, with slight but significant differences between the substituent homologues. They are also sensitive to changes in pH. Except for an observation on diacetylperoxidase[10], the k_1 values of the artificial horseradish peroxidases have previously not been determined, nor has an effect of pH upon k_1 been noticed before.

The effect of the alkenyl groups can be explained in either of two ways. Some Compound I-forming mechanism is inherent in aetiohaematin and its derivatives (provided that they are properly attached to the apoperoxidase) and the alkenyl groups may enhance the mechanism. Alternatively, the alkenyl groups introduce another mechanism. Fig. 6 shows that it is easily possible to fit a straight line to plots of k_1 at the two acidities. Hence the first alternative represents the operating mechanism as far as can be deduced from the parameter pH. Kinetically this means that

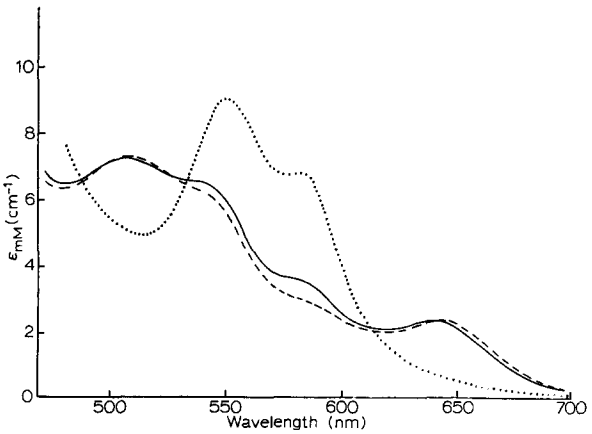

Fig. 5. Spectra of diacetyldeuterohaematin peroxidase (57 μM) in 50 mM sodium phosphate, pH 6.0 (10 °C, ——; 40 °C – – –) and pH 11.5 (25 °C, · · · ·).

TABLE VI

k_1 AND k_4 FOR THE REACTIONS BETWEEN SOME ARTIFICIAL PEROXIDASES, H_2O_2, AND ASCORBIC ACID IN 10mM SODIUM ACETATE OR PHOSPHATE

H_2O_2 2.6 (k_1) or 8–20 (k_4) μM. Peroxidase 0.4–0.6 (k_1) or 0.4–1.0 (k_4) μM. Ascorbic acid 208 μM. Mean ± S.D. for a given set of solutions.

Peroxidase	$k_1 \cdot 10^{-7}$ $M \cdot s$			$k_4 \cdot 10^{-4}$ $M \cdot s$		
	pH 4.5	*pH 7.0*	*wavelength (nm)*	*pH 4.5*	*pH 7.0*	*wavelength (nm)*
Native	1.10***	1.49	397	1.41** ± 0.00	0.05 ± 0.005	427
Proto	1.19	1.37	397	1.25 ± 0.02	0.04 ± 0.005	427
Dipropenyldeuterohaematin	1.47	1.86	397	4.28 ± 0.31	0.10 ± 0.003	427
Dibutenyldeuterohaematin	1.34	1.52	397	2.96 ± 0.23	0.09 ± 0.005	427
Deuterohaematin	0.71	0.71	385	0.60 ± 0.01	0.06 ± 0.005	415
Meso	0.60	0.53	388	3.70 ± 0.04	0.11 ± 0.012	417
Diacetyldeuterohaematin	0.61	0.64	407	0.02 ± 0.00	0.01 ± 0.000	395*
Haemato	0.56	0.57	390	1.98 ± 0.05	0.11 ± 0.004	419

* Decrease in absorbance
** Another horseradish peroxidase preparation 1.36
*** Another horseradish peroxidase preparation 1.25

the function of the alkenyl groups, presumably the double bonds, would be to increase the proportion of collisions leading to the spectrophotometrically operable formation of Compound I.

There are two similarities between the diacetyldeuterohaematin peroxidase and the acidic horseradish peroxidase I (Marklund, S., Ohlsson, P.-I., Opara, A. E. and Paul, K.-G., unpublished). Both shift from neutral to alkaline form with pK values about two units less (9.0, Table V and 9.2 (Marklund, S., Ohlsson, P.-I., Opara, A. E. and Paul, K.-G., unpublished)) than the corresponding pK for horseradish peroxidase III (11.1, Table V). An acidic peroxidase, of the same type as horseradish peroxidase I with $pK_{OH^-} \approx 9$, showed a mixed-type spectrum at 77 °K (ref. 41), very similar to that of the diacetylperoxidase at room temperature. This formulates the question as

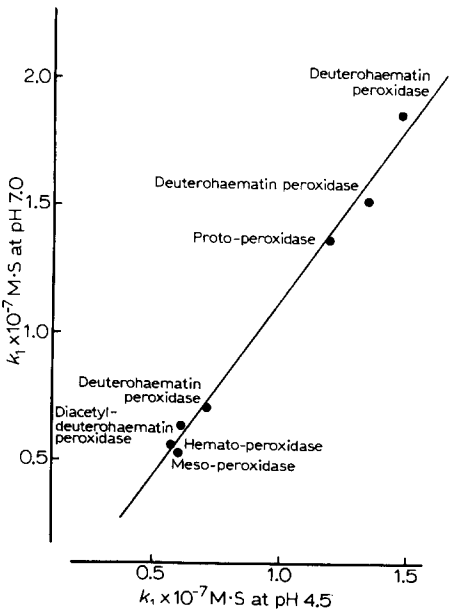

Fig. 6. Plots of k_1, pH 7.0, against k_1, pH 4.5.

to which haematin environments can cause effects of the same kind as the electron-attracting acetyl groups. A third similarity, the lower overall activity of horseradish peroxidase I and the diacetyl enzyme as compared to horseradish peroxidase III towards some donors, is only apparent. For the modified enzyme, the low k_4 limits the activity, k_1 being reduced only by half from horseradish peroxidase III. For horseradish peroxidase I, k_1 is of an order of magnitude lower than for horseradish peroxidase III, p_e being about 0.6 with ascorbic acid (ref. 34 and Table VI). The reacting site(s) in horseradish peroxidase I may be more hidden than in horseradish peroxidase III with some preference for hydrophilic compounds (Marklund, S., Ohlsson, P.-I., Opara, A. E. and Paul, K.-G., unpublished).

Table VI gives the variations in k_4 with a single hydrogen donor. There is a marked effect of the 2,4-substituents, contrary to the conclusion in other reports[7]. However, a comparison of the donor preferences of horseradish peroxidase III and I (Marklund, S., Ohlsson, P.-I., Opara, A. E. and Paul, K.-G., unpublished) disclosed that the donors differed widely in relative reactivity and a classification was attempted. Ascorbic acid and guaiacol belonged to different groups and this may account for the divergent conclusions.

For artificial peroxidases, the wavelength for the Soret band maximum of Compound II is bathochromically shifted 15 nm from the Soret band maximum of the Fe^{3+} peroxidase[7], suggesting that the apparent redox potential of Compound II/Fe^{3+} varies in parallel with that of Fe^{3+}/Fe^{2+}. Hence it should be possible to predict the relative k_4 value from the spectrum of the Fe^{3+} peroxidase. This approximately fits with some selection[4,8] (proto, meso, haemato, diacetyl), but the results with the vinyl homologues and deuterohaematin reveal the existence of other effects. The nature of the structure and redox system $AH^{\cdot}/AH_2$ of the donor may be highly influential.

TABLE VII

RELATIVE RATES OF OXIDATION OF DIHYDROXYFUMARATE (65 μM) BY SOME ARTIFICIAL PEROXIDASES (0.25 μM)

10 mM sodium acetate, pH 4.80. Mean ± S.D. for given peroxidase solution and fresh solutions of dihydroxyfumarate.

Peroxidase	$\frac{1}{t_{0.2}}$ (min^{-1})
Native	0.64 ± 0.07
Proto	0.56 ± 0.02
Dipropenyldeuterohaematin	0.71 ± 0.02
Dibutenyldeuterohaematin	0.54 ± 0.03
Meso	0.87 ± 0.03
Diacetyldeuterohaematin	0.10 ± 0.01

The low k_4 of deuterohaematin peroxidase (Table VI) is interesting, since two lower homologues with three pyrrolic 2-positions occupied by H were even less active[5].

The dihydroxyfumarate oxidase activities of the artificial peroxidases were compared because this reaction involves another mechanism, a reaction with O_2. There was a low correlation between this activity and k_4, not unexpected since the step involving O_2 is faster at pH 4.5 than the adjacent peroxidation. The differences between oxidase activities of the peroxidases were surprisingly small (Table VII).

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