Derivatives and Kinetics of Lactoperoxidase

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Following the investigations of Elliott (1932*a, b*) and Yakashuji (1939) on the peroxidase occurring in cow's milk, Theorell & Åkeson (1943) showed that this enzyme differs in its prosthetic group from both myeloperoxidase and the known plant peroxidases. Chance (1949, 1950) has studied the kinetics of lactoperoxidase. The present paper describes the absorption spectra of various derivatives of lactoperoxidase and an investigation into some aspects of the kinetics of the enzyme.

For the purpose of the present study a method of obtaining the enzyme in high spectroscopic purity was required. Since the method used by Theorell and co-workers (Theorell & Åkeson, 1943; Theorell & Pedersen, 1944), who obtained homogeneous crystalline preparations of the enzyme, was not published in detail, and moreover required the use of electrophoresis, a suitable method was developed and is briefly described in this paper. However, this method gives a non-crystalline preparation of 40–50% purity. After the completion of this work a paper has appeared by Polis & Shmukler (1953) describing a chromatographic method by which they prepared the pure crystalline enzyme.

EXPERIMENTAL

Measurement of lactoperoxidase activity

Chance (1950) has shown that the modified Willstätter method of measuring lactoperoxidase activity used by Theorell & Åkeson (1943) gave low purpurogallin numbers owing to the low percentage saturation of the enzyme with peroxide. Chance (1949, 1950) has investigated the kinetics of lactoperoxidase action spectrophotometrically using reduced cytochrome *c*, hydroquinone, ascorbic acid, guaiacol and pyrogallol as hydrogen donors. In the present work the peroxidation of pyrogallol has been measured spectrophotometrically under conditions such that the reaction of the enzyme with its substrate (H_2O_2) was limiting. Because it was not desirable to saturate the enzyme with peroxide (see later) it was necessary to use the same initial peroxide concentration for all measurements.

In Table 1 are listed the reagents used in the activity test, together with their concentrations and in the order in which they were added to a 1 cm. silica cell. A stop-watch was started immediately after the addition and mixing of the peroxide. Readings of optical density at 300 μm . were

recorded against time for 1–2 min. using a Beckman Model DU Spectrophotometer. The reference cell contained distilled water. The increase in optical density/min. was determined from the slope of the straight line obtained by plotting the readings against time. The measurements were corrected for a slight 'blank' which was determined separately by substituting water for the enzyme in the test. All reaction rates have been corrected to 20°, assuming the rate of reaction to be increased by a factor of 2.0 for an increase in temperature of 10°.

The method gave good proportionality between enzyme concentration and increase in optical density at 300 μm /min.

Enzyme activity unit. A unit of enzyme activity has been defined so that 1 unit/ml. in the activity test gives an increase in optical density at 300 μm . of 10/min. The specific activity (s.a.) of an enzyme preparation is defined as the number of units of activity per mg. dry wt. or per mg. protein weight of enzyme preparation. The protein content was determined spectrophotometrically at 280 μm . from the expression, concentration of protein (mg./ml.) = $1.43 \times$ optical density at 280 μm . (1 cm. cell). The factor 1.43 was determined experimentally, using an impure enzyme preparation.

Partial purification of lactoperoxidase

Methods of estimating enzyme purity. The purity of the enzyme was measured by two methods: (1) determination of specific activity, and (2) measurement of the haem

Table 1. *Reagents for test of enzyme activity*

For conditions see text.

Reagent	Concn. (M)	Vol. added (ml.)	Final concn. (M)
Pyrogallol*	6×10^{-2}	0.20	4×10^{-3}
Phosphate buffer, pH 6.5	0.1	1.00	3.3×10^{-2}
Enzyme solution†	—	0.10	—
Distilled water	—	1.40	—
H_2O_2 ‡	4.36×10^{-3}	0.30	4.36×10^{-4}

* Pyrogallol—purified by sublimation under reduced pressure—was supplied by Dr E. F. Hartree. A fresh solution was made in 0.05M phosphate buffer, pH 6.5, for each series of determinations. Tests showed that after 5 hr. autoxidation at room temperature and pH 6.5 no enzyme-inhibitory substances were produced.

† Enzyme—diluted at 0° in 0.05M phosphate buffer, pH 6.5, immediately before use so that 0.10 ml. gave an increase in optical density at 300 μm . not greater than 0.2/min.

‡ Hydrogen peroxide—freshly diluted from stock solution of approx. $4 \times 10^{-2}\text{M}$, kept at 4°, and titrated against standardized permanganate before the appropriate dilution.

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Table 2. *Enzyme purification*

Stage	...	...	Milk	1	2A	2B	3	4	5A	5B	5C
Activity units			31 700	20 600	5450	2000	6000	2850	520	960	99
Dry weight (D.W.) or protein wt. (P.W.) (mg.)			6.3×10^6 (D.W.)	2.6×10^6 (P.W.)	—	—	1.3×10^4 (P.W.)	2000 (P.W.)	344 (P.W.)	228 (P.W.)	31 (P.W.)
Specific activity			0.005	0.08	—	—	0.47	1.42	1.51	4.2	3.18
Original activity units (%)			100	65	17.3	6.3	19	9.1	1.6	3.0	—
Enzyme (mg.)*			3740	2430	—	—	708	336	61	113	12
Purity (%)†			—	—	—	—	5.5	14.6	15.4	49.6	60.1
$E_{412} m\mu.$ ‡			—	—	—	—	7.7	—	8.8	10.0	8.6
$E_{480} m\mu.$			—	—	—	—	—	—	—	—	—
Purity (%)†			—	—	—	—	11.7	10.3	10.2	11.8	18.9
Specific activity			—	—	—	—	—	—	—	—	—

* Calculated as activity units/8.47, see text.

† Spectrophotometric measurement, method (2), see text.

‡ Measure of contamination by red pigment. For pure enzyme, ratio = 10.7, see text.

absorption (Soret band at 412 $m\mu.$) relative to that of the protein (280 $m\mu.$). In this method the percentage purity of the enzyme is defined by the expression, $1.11 \times \text{optical density at } 412 m\mu. \times 100 / \text{optical density at } 280 m\mu.$, where 1.11 is the ratio of the optical density at 280 $m\mu.$ to that at 412 $m\mu.$ calculated for the pure enzyme from the data of Polis & Shmukler (1953). The method has been used to detect the formation of soluble, inactivated enzyme. The presence of inactivated enzyme is shown by an increase in the ratio, percentage purity (method 2)/specific activity above the value $100/8.47 = 11.8$ (see fraction 5C, Table 2).

The specific activity for the pure enzyme was calculated by extrapolation using a preparation in which the specific activity was proportional to the percentage purity (method 2) over a wide range of purification. This value was found to be 8.47. The number of activity units divided by 8.47 thus gives the approximate weight (mg.) of enzyme in any preparation.

The separation of the enzyme from a red pigment having a broad band at 480 $m\mu.$ (Theorell & Pedersen, 1944) and no Soret band (Polis & Shmukler, 1953) was followed by comparing the ratio, optical density at 412 $m\mu.$ /optical density at 480 $m\mu.$, found for the lactoperoxidase preparation, with that calculated for the pure enzyme from the data of Polis & Shmukler (1953).

Method of purification. In view of the detailed publication by Polis & Shmukler (1953) of a method by which the crystalline enzyme can be prepared, only a brief description of the method used in the present work is given. The results of this method are tabulated in Table 2.

Stage 1. Separated milk (70 l.) was fractionated in two lots approximately according to the procedure of Elliott (1932a) and the resultant enzyme solutions were combined (vol., 2 l.).

Stage 2A. Further procedures were carried out in the cold where possible. Thoroughly dialysed stage-1 enzyme solution was treated at pH 5.5 with 3 l. of tricalcium phosphate gel (50 mg./ml. prepared according to Keilin & Hartree, 1951) to adsorb protein impurities. More protein impurities were removed after precipitation at pH 7.7. The enzyme was concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation.

Stage 2B. Enzyme was recovered from the phosphate gel centrifugate of the previous step by elution with 3 l. of 1.5% (w/v) $(\text{NH}_4)_2\text{SO}_4$ soln. (pH 5.5–6.0) and collected after precipitation with $(\text{NH}_4)_2\text{SO}_4$.

Stage 3. Dialysed stages-2B and -2A enzyme solutions were combined and the enzyme was precipitated by $(\text{NH}_4)_2\text{SO}_4$ (70% saturation). Vol., 410 ml.

Stage 4. The product of stage 3 was further purified by treatment with tricalcium phosphate gel (400 ml., 50 mg./ml.) as in step 2A, the enzyme collected from the supernatant by $(\text{NH}_4)_2\text{SO}_4$ precipitation and dialysed. Enzyme was recovered from the phosphate gel as in step 2B, further treated with gel to give approximately the same purity (14.5%) as the first supernatant of this step and concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation. The two products were then combined.

Stage 5. Stage-4 enzyme was fractionated with $(\text{NH}_4)_2\text{SO}_4$ at pH 7.7 and the fraction collected between the limits 48–55% (w/v) was dialysed and again treated with tricalcium phosphate gel. Fractionation of the gel supernatant with $(\text{NH}_4)_2\text{SO}_4$ (pH 7.7) between the limits 45–50, 50–56, 56–64% (w/v) gave three fractions which were labelled stages 5A, 5B and 5C, respectively. Because of the satisfactory spectroscopic purity of the stage-5B preparation and because of the low yields, further purification was not sought. Under the conditions of purification employed the enzyme was found to be very labile above the level 40–50% purity and in further purification the ratio, percentage purity/specific activity, rose (see Table 2, fraction 5C).

RESULTS

Theorell & Åkeson (1943) found that lactoperoxidase was inactivated by high concentrations of hydrogen peroxide. The concentration of hydrogen peroxide in the activity test described in the present paper is below that which causes significant inactivation of the enzyme under the conditions of the test. In Fig. 1 is shown the effect of a range of peroxide concentration on a purified enzyme pre-

Fig. 1. Effect of peroxide concentration on lactoperoxidase activity using 60% pure enzyme (O) and crude (●) (<1% pure) enzyme fraction. At high concentrations of peroxide where density/time plot is not a straight line, the rate of increase of optical density at 300 mμ. was calculated from the first two readings made within 30 sec.

paration and a crude enzyme preparation. Using a spectrophotometric method of measuring the oxidation of dihydroxyphenylalanine (dopa), Polis & Shmukler (1953) found that lactoperoxidase is inhibited by concentrations of hydrogen peroxide above about 1×10^{-2} M. It is shown by these authors that peroxide competes with dopa for a 'reactive site' on the enzyme. This competitive inhibition was not evident in the present study with pyrogallol as hydrogen donor.

Michaelis constant

The inactivation of the enzyme by peroxide shown in Fig. 1 shows that it is not possible for the purpose of the activity test to saturate the enzyme with peroxide. Such an activity test is only valid therefore for the purpose of following the purification of the enzyme if the Michaelis constant (K_m) remains constant throughout the purification. The K_m for the enzyme-hydrogen peroxide reaction was determined at three stages in the purification of the enzyme (less than 1, 50 and 60% purity) and the values found were 1.08×10^{-3} , 0.96×10^{-3} and 1.2×10^{-3} M, respectively, when the concentration of pyrogallol was 4×10^{-3} M. Thus, purification of the enzyme had no significant effect upon K_m . The average value for K_m (1.08×10^{-3} M) is used later in this paper as the experimentally determined value. It is shown that this value is in good agreement with the approximate value calculated by Chance. In Fig. 2 the graphic evaluation of K_m is given for the enzyme of 60% purity.

Fig. 2. Graphic evaluation of Michaelis constant for lactoperoxidase (reaction with H_2O_2) using an enzyme preparation of 60% purity. c = millimolar concentration of H_2O_2 and v = velocity in activity test. Results plotted according to method of Lineweaver & Burk (1934).

Absorption spectra of lactoperoxidase and derivatives

The positions of the absorption bands of lactoperoxidase and various derivatives have been given by Yakashuji (1939), Theorell & Åkeson (1943), Theorell & Pedersen (1944), Polis & Shmukler (1953) and Chance (1950). Theorell & Pedersen have given values for the absorption of the electrophoretically homogeneous enzyme at various wavelengths. Chance has given absorption curves for the Soret bands of lactoperoxidase and its compounds formed by hydrogen peroxide. Polis & Shmukler have given absorption curves for the free enzyme and for its compound with excess of hydrogen peroxide.

Absorption curves are given in the present paper for lactoperoxidase (Figs. 3, 4), its cyanide and fluoride derivatives (Fig. 3), and carbon monoxide and pyridine haemochromogen derivatives (Fig. 4). The measurements were made in a Hilger 'Uvispec' Spectrophotometer, the wavelength scale of which was checked using the band of 550 mμ. of reduced cytochrome *c*. The solutions were prepared as shown in Table 3. The absorption curve shown for the free enzyme in Figs. 3 and 4 is similar to that given by Theorell & Pedersen (1944) and Polis & Shmukler (1953). The absorption maxima of the free lactoperoxidase, like those of horse-radish peroxidase, are considerably less sharp than those of the derivatives. Thus lactoperoxidase is also more easily characterized in crude preparations by the preparation of one of these derivatives.

An alkaline form of lactoperoxidase, analogous to that formed from horse-radish peroxidase (Keilin & Hartree, 1951) could not be demonstrated in the present study. With increasing pH the acid

Fig. 3. Absorption spectra of lactoperoxidase and of its cyanide (CN^-) and fluoride (F^-) compounds. For details of solutions used see Table 3. $\epsilon = (\log_{10} I_0/I)/cl$, where c = molar concentration of haematin and l = optical depth in cm.

Fig. 4. Absorption spectra of lactoperoxidase, carbon monoxide-reduced lactoperoxidase and the pyridine haemochromogen formed from lactoperoxidase. For details of solutions used see Table 3. For definition of units see Fig. 3.

Table 3. *Preparation of solutions for spectrophotometric measurements on lactoperoxidase and various derivatives*

The mixtures were measured in 1 cm. silica cells. Reference cells contained all reagents except lactoperoxidase.

Enzyme* (ml.)	Additional reagents	Compound formed
1.0	0.2M Phosphate buffer (2.0 ml.) pH 5.6	Lactoperoxidase
1.0	As above, saturated with CO + 20 mg. $\text{Na}_2\text{S}_2\text{O}_4$ †	CO-reduced lactoperoxidase
1.0	0.2M Phosphate buffer (2.0 ml.) pH 5.6, containing 0.096M-KCN	Cyanide lactoperoxidase
1.0	0.2M Phosphate buffer (2.0 ml.) pH 5.6, containing 0.63M-NaF	Fluoride lactoperoxidase
1.0	1.25 ml. H_2O + 0.6 ml. pyridine + 0.15 ml. 2.5N-NaOH + 20 mg. $\text{Na}_2\text{S}_2\text{O}_4$ †	Pyridine haemochromogen

* 49.6% pure lactoperoxidase—stage-5B preparation, $3.78 \times 10^{-5}\text{M}$. The molarity was calculated from the optical density at 630 $\text{m}\mu$. and the data of Theorell & Pedersen (1944).

† Solutions were covered by a layer of paraffin during measurements.

form of lactoperoxidase persisted until, at pH 11.4, a further increase in pH caused the intensity of the Soret band to drop considerably.

DISCUSSION

It is of interest to compare the spectra of lactoperoxidase and its derivatives with those of other haem compounds such as horse-radish peroxidase, catalase and methaemoglobin, absorption curves for which have been given and compared by Keilin & Hartree (1951), and myeloperoxidase, absorption bands for which have been published by Agner (1941). The absorption curves for the first four compounds are of the same general pattern, but there are three important respects in which their spectral properties differ. (1) Of the free haemo-proteins only lactoperoxidase has a faint band in the vicinity of 600 $\text{m}\mu$. Methaemoglobin has a small maximum at 580 $\text{m}\mu$. (2) The general level of absorption/mole haemin varies considerably. In general, over the visible range of the spectrum, the order of intensity is catalase > lactoperoxidase > horse-radish peroxidase and methaemoglobin. (3) Despite the similarity in the general shape of spectra there are significant variations in the positions of absorption bands.

In Table 4, absorption bands for all the above haematin compounds are given for comparison. The absorption bands of myeloperoxidase are quite atypical and, moreover, this enzyme does not react with carbon monoxide or fluoride. According to Foulkes, Lemberg & Purdom (1951), when myeloperoxidase was reduced with dithionite and made alkaline, the type of spectrum did not change. On boiling, however, the bands of protohaemochromogen were obtained. For the other haemoproteins the bands of the cyanide and fluoride derivatives are of particular interest. The α and β bands of catalase and lactoperoxidase cyanide compounds are very similar, and are 12 and 20 $\text{m}\mu$. farther to the

red compared with the cyanide compounds of methaemoglobin and horse-radish peroxidase, respectively. In the fluoride compounds, however, the bands of the catalase derivative lie farther to the blue whereas the strong band of lactoperoxidase fluoride is approximately 10 $\text{m}\mu$. farther to the red compared with the bands for the fluoride compounds of methaemoglobin and horse-radish peroxidase. This comparison of the band positions of the cyanide and fluoride derivatives of catalase with those of the same derivatives of horse-radish peroxidase and methaemoglobin is an outstanding example of the effect of specific proteins on the spectrum of a common prosthetic group.

The fact that the bands of lactoperoxidase derivatives are found consistently at the longer wavelengths is evidence in addition to that supplied by the haemochromogen, that the prosthetic group of lactoperoxidase is not protohaematin. The similarity in the general pattern of the absorption spectra of derivatives of lactoperoxidase and protohaematin enzymes suggests, on the other hand, that lactoperoxidase haematin is not greatly different from protohaematin. The reversion of heated, alkaline, reduced myeloperoxidase to protohaemochromogen strongly suggests that the arrangement of the porphyrin side-chains of this enzyme is similar to, or identical with, that of protoporphyrin. Moreover, myeloperoxidase porphyrin must still contain its four methene carbon atoms one of which, according to Foulkes *et al.* (1951), is probably in an oxidized state.

Kinetics

Theorell & Åkeson (1943) have shown that lactoperoxidase forms two spectroscopically observable compounds with hydrogen peroxide which are analogous to complexes II and III of horse-radish peroxidase. Using his rapid-flow technique, Chance (1950) was able to demonstrate the formation of

Table 4. Comparison of absorption bands of horse-radish peroxidase (H.-R.P.), methaemoglobin (MetHb), catalase, lactoperoxidase (L.P.) and myeloperoxidase (Myel.P.)*

Compound	Enzyme	Absorption maxima (mμ.)					
Free enzyme	H.-R.P.	403	497	545			641
	MetHb	405	505	540	580		632
	Catalase	407	500	540			627
	L.P.	412	500	550		600	635
	Myel.P.		430 495		570		625 690
Reduced enzyme	H.-R.P.		437		556	590	
	Catalase	Not reducible by Na ₂ S ₂ O ₄					
	L.P.		442		565	600	
	Myel.P.			475		590	637
CO-reduced enzyme	H.-R.P.		423		542 572		
	L.P.		425		545 582		
	Myel.P.	No reaction					
Cyanide enzyme	H.-R.P.		423		538 570		
	MetHb		415		543 578		
	Catalase		425		558	590	
	L.P.		432		558	595	
	Myel.P.		438				634
Fluoride enzyme	H.-R.P.	404		487	560		612
	MetHb	405		488	550		608
	Catalase	408			532	600	
	L.P.	413		500		590	620
	Myel.P.	No reaction					
Pyridine haemochromogen	H.-R.P.	Pyridine protohaemochromogen					
	MetHb						
	Catalase						
	L.P.						
	Myel.P.	Heating with alkaline Na ₂ S ₂ O ₄ gives protohaemochromogen					

* Data for first three enzymes derived from absorption curves of Keilin & Hartree (1951), of myeloperoxidase from Agner (1941).

a primary compound which could not be observed by Theorell & Åkeson because of its short life. Chance (1950) has suggested the following mechanism for the action of lactoperoxidase. In these reactions, as rewritten below, the symbol 'FeOH' represents the free enzyme in which the OH group is bound to the iron atom.

Chance (1949, 1950) found that the experimentally determined value of k_7 varied with the concentration of the hydrogen donor, $[\text{AH}_2]$. George (1952a, b) and Chance (1952) have recently produced evidence suggesting that reactions (B) and (C) should be written,

Thus, in reaction (B) we may write $k_7 = k_3/[\text{AH}_2]$.

For the mechanism described in reactions (A), (B) and (C), Chance (1950) found the following

experimentally determined values for the reaction constants at 25–30° and pH = 7.0.

$$k_1 = 2.2 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}; \quad k_7 = 4 \text{ sec}^{-1}$$

(in the absence of added hydrogen donor, AH_2) and k_4 (pyrogallol) = $6.6 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$. The fact that k_7 does not equal zero in the absence of added donor demonstrates the presence of a donor in the enzyme preparation. In the following derivation it is assumed that the pyrogallol concentration is very much greater than the concentration of endogenous donor and that k_3 is of the same order as k'_3 , where the latter is the reaction constant for the endogenous donor.

Derivation of expressions describing Chance's mechanism of action of lactoperoxidase. The concentrations of the components are given in brackets in equations (A), (B) and (C). The total enzyme concentration is e .

In the steady state,

$$dp/dt = k_1(e-p-q)s - k_2p - k_7p = 0, \quad (1)$$

$$dq/dt = k_7p - k_4aq = 0. \quad (2)$$

From (2), $q = k_7p/k_4a$. Substituting this value in equation (1),

$$p = \frac{k_1 es}{k_2 + k_7 + k_1s + k_1k_7s/k_4a}.$$

The velocity of the reaction, $v = k_7 p$, is

$$\frac{k_1 k_7 e s}{k_2 + k_7 + k_1 s + k_1 k_7 s / k_4 a}.$$

Since, according to Chance, k_2 is insignificant compared with k_7 , this becomes

$$v = \frac{k_1 k_7 e s}{k_7 + k_1 s (1 + k_7 / k_4 a)}.$$

Dividing numerator and denominator by

$$\begin{aligned} & \frac{k_1 s (1 + k_7 / k_4 a)}{k_7 e} \\ v &= \frac{1}{1 + \frac{1}{s} \left(\frac{k_7}{k_1 (1 + k_7 / k_4 a)} \right)} \\ &= \frac{V}{1 + K_m / s}, \end{aligned} \quad (3)$$

where V , the velocity at infinite substrate concentration, equals $\frac{k_7 e}{1 + k_7 / k_4 a}$, and K_m , the Michaelis

constant, equals $\frac{k_7}{k_1 (1 + k_7 / k_4 a)}$.

Substituting $k_7 = k_8 a$, we find

$$K_m = \frac{k_8 a}{k_1 (1 + k_8 / k_4 a)}. \quad (4)$$

Michaelis constant. Chance (1950) calculated the K_m for lactoperoxidase ($s = [\text{H}_2\text{O}_2]$) to be approximately $7 \times 10^{-3} \text{ M}$ for a pyrogallol concentration of $20 \times 10^{-3} \text{ M}$. The experimentally determined value of $1.08 \times 10^{-3} \text{ M}$ given in this paper was obtained using a pyrogallol concentration of $4 \times 10^{-3} \text{ M}$. Since, from equation (4), K_m is proportional to the pyrogallol concentration, the experimentally determined value can be multiplied by 5 for comparison with Chance's calculated K_m . The value thus obtained, $5.4 \times 10^{-3} \text{ M}$, is in reasonable agreement with the approximate value calculated by Chance.

Evaluation of k_8 and k_7 . The value of k_8 can be calculated from equation (4) by substitution of numerical values for the other terms as follows: $K_m = 1.08 \times 10^{-3} \text{ M}$ (experimental); $a = 4 \times 10^{-3} \text{ M}$ (the concentration used in the present study); k_1 and $k_4 = 2.2 \times 10^7$ and $6.6 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$, respectively (Chance, 1950). Thus

$$k_8 = 5.85 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$$

and

$$k_7 = 2.37 \times 10^6 \text{ sec}^{-1}.$$

Chance (1952) found that k_8 for horse-radish peroxidase was greater than k_4 , as was to be expected from the earlier finding that compound II was the rate-limiting intermediate. The value of the ratio, k_8/k_4 varied from about 40 for *p*-amino-benzoic acid as donor to about 80 for nitrite. It is now found that k_8/k_4 for lactoperoxidase is approximately 9, confirming that compound II is the rate-limiting intermediate when pyrogallol is the donor.

Different forms of lactoperoxidase

After the present work was completed, a paper by Polis & Shmukler (1953) described the purification of the enzyme largely by chromatographic methods. Examination of their preparation by electrophoresis and by spectrophotometry revealed the presence of two forms of lactoperoxidase distinguished by their different mobility in acetate buffer, by their absorption ratios at 412 and 280 $\text{m}\mu$. and by their different turnover numbers in the reaction with hydrogen peroxide and dihydroxyphenylalanine. In the present study it was found that when the enzyme was approximately 40–50% pure, further purification inevitably led to inactivation of the enzyme. The value of the comparison of specific activity with the spectrophotometrically determined enzyme-haem concentration is thus emphasized (see Table 2).

SUMMARY

1. A spectrophotometric method of measuring the activity of lactoperoxidase using pyrogallol as hydrogen donor is described. The conditions of measurement are such that, with high donor concentration and low peroxide concentration, the reaction of enzyme with peroxide is measured.

2. A brief description is given of the preparation of lactoperoxidase of high spectroscopic purity in the visible region of the spectrum and of 40–50% absolute purity.

3. The absorption curves in the visible and Soret regions are given for lactoperoxidase and its carbon monoxide-, cyanide-, fluoride-, and pyridine-haemochromogen derivatives.

4. The absorption spectra are very similar in pattern to those for the protohaematin compounds, horse-radish peroxidase, catalase and methaemoglobin. The prosthetic group of lactoperoxidase is probably a haematin closely related to protohaematin. The absorption bands of lactoperoxidase and of its derivatives lie further to the red than the protohaematin enzymes indicating that the prosthetic group is not identical with protohaematin.

5. The kinetics of lactoperoxidase have been examined mathematically using the mechanism of peroxidase action described by Chance and modified by George.

6. The Michaelis constant ($1.08 \times 10^{-3} \text{ M}$) was determined experimentally and found to be in good agreement with the value calculated by Chance.

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The Isolation and Estimation of the Steroid Oestrogens in Placental Tissue

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Work on the biosynthesis of the natural oestrogens has been rendered very difficult in the past by the lack of quantitative methods of purification of the microgram quantities of these hormones which exist in tissue. Though there were several methods of estimating the pure oestrogens, they were not directly applicable to accurate estimations in urinary or tissue extracts because of the presence of impurities.

The rigorous purification of oestrone, oestradiol and oestriol has been the aim of several recent investigations (Engel, Slaunwhite, Carter & Nathanson, 1950; Boscott, 1951; Swyer & Braunsberg, 1951; Bauld, 1952) all of which deal with urinary extracts, whilst Diczfalusy (1953) and Ryan & Engel (1953*a, b*) have recently applied the counter-current distribution method of Engel *et al.* (1950) to tissue extracts. In this technique, however, non-specific fluorogenic material present, particularly in the early fractions, makes difficult the estimation of minute quantities of oestrogen by fluorimetry.

The methods to be described were developed in order to make possible further work on the *in vitro* synthesis of oestrogens in tissue slices. Initially this had been investigated by using a bio-assay. The normal method of injecting the oestrogen into spayed, adult female mice had not proved to be sufficiently sensitive, but the intravaginal insertion technique of Emmens (1941) produced 50% vaginal cornification with 1.0 μ mg. oestrone, and by this means it had been shown that full-term human placental tissue contained 30 Emmens mouse units/g. When this placental tissue was incubated in Warburg flasks for 3 hr., the oestrogenic potency

increased by approximately 100%. However, the large number of mice required for assay, and the lack of specificity, made it clear that chemical estimations were required.

After a study of existing chromatographic techniques, a method was developed which involved a preliminary chemical separation followed by paper partition chromatography and a special spotting technique, with elution of the spots and estimation by fluorimetry.

A preliminary account of part of this work has already been published (Mitchell, 1952).

EXPERIMENTAL AND RESULTS

Reagents

Ether (May and Baker Ltd., Dagenham), A.R., peroxide-free, was refractionated through a 100 cm. column packed with single-turn glass helices, and used the same day. Benzene, A.R., thiophen-free, was refluxed for 30 min. with conc. H_2SO_4 and refractionated through a similar 60 cm. column. This column was also used to refractionate the AnalaR carbon tetrachloride and methanol. Ethanol was B.P. quality (James Burrough Ltd., London). Diazotized *p*-nitrobenzeneazodimethoxyaniline (Fast Black Salt K) was from the Clayton Aniline Co. Ltd., Manchester. All other reagents were of AnalaR quality.

Extraction procedure

The procedure developed in the course of this work is described in Fig. 1. Fresh placenta was minced and mixed with an equal volume of 80% (v/v) ethanol within 30 min. of delivery. The method of extraction separated the oestrogens into 'free' (1), 'conjugated' (2), and 'protein-bound' (3) fractions (Diczfalusy, 1953). These were each further