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The Assay of Peroxidase with Mesidine as the Hydrogen Donor

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A number of procedures have been suggested for the assay of peroxidases [1–3], but none of them is quite satisfactory. Disadvantages are lack of specificity as regards the catalyzing enzyme (*e. g.* in the oxidation of ascorbic acid by crude peroxidase preparations) and the formation of byproducts which influence the result. The purpurogallin test [4–7], which perhaps is most commonly used, may function quite well on one occasion and give duplicates of good agreement, but later the same operator may obtain quite different values with the same specimen of peroxidase. Thus Ettori [7] during the course of seven weeks found variations from 1 160 to 1 780 in the purpurogallin number of one horse-radish peroxidase (HRP) preparation. Such irregularities have been attributed to impurities in the pyrogallol [8] or to metal ions in the water [9]. Consequently the comparison of actual results with data from the literature or from other laboratories may give rise to misleading conclusions. There are also other disadvantages: a considerable blank value, the necessity of several ether extractions, and the occasional formation of byproducts, noticeable as a reddish colour in the aqueous residues.

Chapman and Saunders [10] examined the peroxidative oxidation of mesidine (1-amino-2,4,6-trimethyl-benzene), catalyzed by HRP. Only one condensation product was formed (2,6-dimethylbenzoquinone-4-(2,4,6-trimethyl benzene) anil, "purple compound"). The absence of other condensation products was attributed to the fact that mesidine contains only two free nuclear hydrogen atoms. We have attempted to use mesidine for the assay of HRP and some other peroxidases **.

MATERIAL AND METHODS

Horse-radish peroxidase was crystallized as described by Theorell [11]. Although the material appeared to be homogenous in solubility tests *ad modum* Cohn [12], the first batch of crystals contained 1.33 % haemin and the second, from the mother liquor of the first,

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** Dr. C. B. Saunders suggested mesidine for this purpose upon a question from Professor H. Theorell at the Biochemical Congress in Paris, 1952.

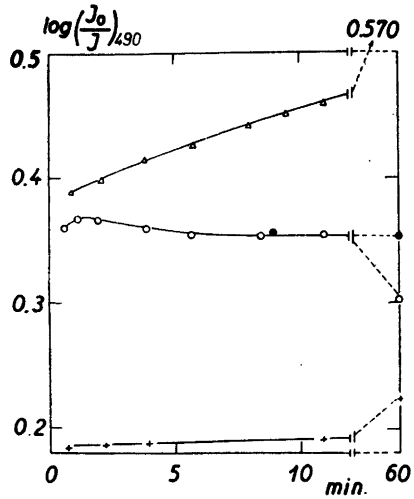

Fig. 1. Interruption of the enzymatic reaction by one volume of ethanol of various acidities. Five min. after the addition of HRP were added 2 ml of ethanol without acid (+), with 6 (△) or 20 (○) ml 3.5 M H_2SO_4 per 1 000 ml ethanol. (●) same as (○) but after further 7 min. were added 0.32 ml 1 M NaOH. Density values recalculated to 4 ml.

1.26 %. Material from the second batch was used for the experiments in this paper (P.N. 1 230, $D_{402}/D_{280} = 2.74$). Haemin was determined as pyridine haemochromogen, the molar absorption coefficient $\beta_{\alpha\text{-band}}$ being 8.0×10^7 cm² × mole $^{-1}$ [13]. [HRP] of the stock solutions were determined in the same way. Haemin contents for HRP earlier reported from this institute (1.36—1.48 %), had been based on the previously accepted value $\beta_{\alpha\text{-band}} = 7.3 \times 10^7$ cm² × mole^{-1} and should thus be multiplied with 0.915 to become correct.

A HRP preparation from the Molteno Institute, Cambridge, contained, according to the submitted analyses, 1.61 % haemin, P.N. 1 220 (1948), 983 (1953). The preparation had been kept at −15° since 1948. We found the haemin content of the Molteno preparation to be 1.36 % (calculated on $\beta = 8.0 \times 10^7$ cm² × mole^{-1}), a value close to those of our preparations. D_{402}/D_{280} of the Cambridge preparation was 2.79.

A third HRP preparation, obtained from Philadelphia, gave $D_{402}/D_{280} = 2.9$.

Hydrogen peroxide 30 % ("Perhydrol" Merck) was titrated with $KMnO_4$ and suitably diluted with water. The dilute solution was kept in icewater and made up twice daily.

Mesidine. Nitromesitylene [14] was refluxed with tin and 4 *M* HCl (molar ratios 1 : 10 : 10) for two hours on a sandbath, and the metal ions subsequently removed by H_2S. Mesidine hydrochloride was crystallized twice from water and kept in the solid state. An 0.2 *M* solution in water is stable for one week in the icebox.

Water. Ordinary distilled water (tin pipes) was generally used. No difference was noticed between this and glassdistilled water or, in a few experiments, tap water.

Spectrophotometric determinations were made with a Beckman DU spectrophotometer, 1-cm cells being consistently used.

EXPERIMENTS

General procedure. Buffer, mesidine, and peroxide are pipetted into a test tube. HRP, in a volume of 0.02—0.06 ml is added as a droplet on a hook-shaped glass rod, and simultaneously a stop-watch is started. The reaction is interrupted after a suitable time by blowing down one volume of acid ethanol, which immediately develops a blue colour, within a few minutes changing to orange-red. This colour in acid is not quite stable (Fig. 1) and therefore alkali sufficient to give a slight excess is added 6—9 min after the alcohol. The colour

then becomes purple red and stable for at least 18 h. It can also be seen from Fig. 1 that alcohol alone does not cause any colour change, and that one volume of neutral or insufficiently acid alcohol does not stop the enzymatic reaction. The optical density of the purple red solution is determined at 490 mμ. A solution of the anil in neutral or faintly alkaline aqueous alcohol follows Beer's law to an optical density of at least 1.2. The molar absorption coefficient β_{490} is 2.92×10^6 cm^2 $\times$ mole^{-1}.

The purpose of the alcohol is to keep the purple compound in solution; it is not necessary in itself for the reactions. It is sometimes preferable to substitute the acid ethanol by acid butanol, *e. g.* when turbid tissue extracts are examined.

The influences of some factors on the formation of the purple compound were studied.

pH. Aliquots of an 0.2 *M* solution of mesidine hydrochloride in 0.1 *M* acetate buffer were adjusted with 4 *M* NaOH or acetic acid to desired pH-values. At pH $>$ 6 mesidine separated to give an emulsion and therefore no higher pH than 6 was tried. The other reagents and HRP were added as described above. The largest amount of purple compound was formed when pH was kept at 4.2 during the enzymatic reaction. Since, however, only slightly slower formation took place at pH 4.9 this value was preferred because of the instability of the anil towards acids. At pH 6 no purple compound was formed; at pH 3 less than half of the quantity at pH 4.2.

Temperature. The formation of the purple compound was twice as rapid at 18° as at ice-water temperature. At 28° the rate was the same as for 18°. The latter fact depends probably upon the cancelling of a higher velocity at 28° by an increased rate of inactivation of the enzyme by the peroxide (Table 1). The determinations should therefore be made at a low temperature. It was found to be essential that the temperature was kept constant and at an easily reproducible level. The numerical values of the constants given below were obtained with finely crushed ice as cooling medium. With ice cubes significantly different numerical values and larger dispersion were noted.

[H_2O_2]. The colour density of the solution, after the addition of acid alcohol and alkali, is proportional to the available amount of peroxide, provided that an excess of mesidine and a sufficiently long reaction time have been employed. An initial peroxide concentration of 5 m*M* was found to give D_{490} around 1 under these conditions. The linear part of a curve, giving D_{490} *versus* time (*cf.* below) will then range from $D_{490} = 0.15$ to 0.8, which is suitable for spectrophotometric determinations without further manipulations.

Table 1. Effect of heat. Reactants denoted by (+), and buffer were pipetted into the tubes and warmed for 15 min. at 59°. After rapid cooling lacking reactants (−) were added, and after further 5 min. acid ethanol. Reactions of tubes 1 and 2 were made at 18° and 59°. Initial concentrations: Mesidine 13 mM, H_2O_2 7.5 mM, HRP 2.9 mμM.

Mesidine	H_2O_2	HRP	D_{490}	% of tube 1
+	+	+	0.370	—
+	+	+	0.105	28
+	—	—	0.370	100
—	+	—	0.340	92
—	—	+	0.105	28
+	+	—	0.380	103
+	—	+	0.140	38
—	+	+	0.000	0

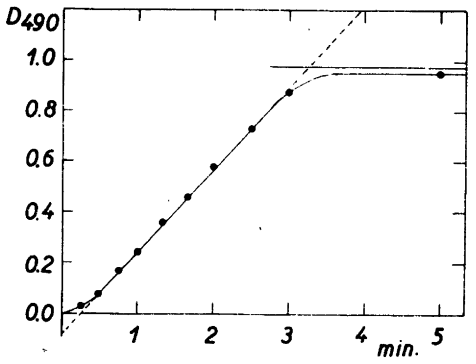

Fig. 2. Increase in amount of purple compound with time. [HRP] = 30.5 mμM. HRP added at t = 0. D_{490} = 0.98 calculated for 3 H_2O_2/1 purple compound. Dashed line: $D_{490} = D_{t_0} + k \times t$; 0°, H_2O_2 5 mM, mesidine 15 mM.

[*Mesidine*]. The overall formation of the purple compound per unit time was found to be proportional to the mesidine concentration to a value of around 13 mM; 20 mM gave only 35 % instead of 54 % higher overall velocity than 13 mM. Since the peroxide concentration should be 5 mM and two moles of mesidine correspond to three moles of peroxide in the net formula, nothing can be gained stoichiometrically by using [mesidine] > 13 mM, when a pure peroxidase is being examined. On the other hand a high mesidine concentration will counteract undesired side-reactions between the oxidation product of mesidine and amino groups in impure peroxidase preparations. The appearrance of a turbidity due to unreacted mesidine will eventually be seen upon the addition of alcohol if the initial mesidine concentration has been 20 mM or higher. We have used 15 mM mesidine.

Reaction time and [*HRP*]. In a number of experiments at one peroxide concentration the addition of acid ethanol *etc.* was made at different times. Plots of D_{490} of the final solution against time showed that for a certain time the enzymatic reaction proceeds as a zero order reaction (Fig. 2). The rectilinear part is limited upwards by the exhaustion of the peroxide, downwards by a "lag" phase. The duration of the latter varies slightly with the concentration of HRP, but for all tested concentrations (4.0—96.6 mμM HRP) the linear part extended at least from 0.150 to 0.750 density units. The results are collected in Table 2. The slope of the linear part (k in Table 2) is proportional to [HRP]. Thus it should be possible to calculate [HRP] from two determinations (D_1, t_1 and D_2, t_2) where $0.150 < D_1 < D_2 < 0.750$. Because of the lag phase at least two points are necessary. However, it seems to be more accurate to calculate [HRP] from the two experimental points in the following way. Let $t_{0.450}$ be the time which, at a certain HRP concentration, is needed to give $D_{490} = 0.450$ = mean of 0.150 and 0.750) of the final, alkaline aqueous solution. Table 2 shows that $1/t_{0.450}$ is proportional to [HRP]; the relation is

$$[\text{HRP}] = (49.8 \pm 1.00) \times \frac{1}{t_{0.450}} - 0.09 \qquad (1)$$

where HRP is obtained in millimicromolarity ($M \times 10^{-9}$) in the reaction mixture when t is given in min. The term — 0.09 can be neglected when $t_{0.450} >$ 5.3 min. (see below). The intensity of the violet colour, which develops during the reaction prior to the addition of alcohol, can be estimated by the eye.

Table 2. Formation of the purple compound at various peroxidase concentrations. The expression $D_{490} = D_{t_0} + k \times t$ *is used for the rectilinear part of a plot of density against time. For every [HRP] the coefficients* D_{t_0} *and k were calculated by means of the method of least squares.* $t_{0.450}$ *was obtained for every [HRP] by putting* $D_{490} = 0.450$.

HRP points mμM	Number of $0.150 < D_{490} < 0.750$	$-D_{t_0}$ density	k density × min^{-1}	$1/t_{0.450}$ min^{-1}
Our HRP				
4.0	5	0.062	0.035	0.068
8.7	7	0.005	0.072	0.158
15.1	9	0.056	0.148	0.292
20.2	4	0.055	0.207	0.410
30.5	6	0.079	0.326	0.616
40.5	8	0.095	0.465	0.853
50.7	7	0.026	0.529	1.111
60.7	5	0.123	0.676	1.180
72.8	5	0.125	0.840	1.461
86.7	6	0.164	1.057	1.721
96.6	6	0.129	1.111	1.919
Cambridge HRP				
18.9	4	0.068	0.215	0.415
37.9	8	0.090	0.422	0.781
68.1	10	0.170	0.798	1.287
94.0	7	0.150	1.065	1.775
Philadelphia HRP				
52.7	8	0.140	0.644	1.092

With a little practice it is easily possible to stop the reaction at such a time that the final solutions give $0.150<D_1<450<D_2<0.750$. From equiform triangles in the density-time diagram it follows that

$$t_{0.450} = \frac{\Delta t(0.450 - D_1)}{\Delta D} + t_1 \qquad (2)$$

[HRP] is then obtained from (1) and (2).

$$[\text{HRP}] = (49.8 \pm 1.00) \times \frac{\Delta D}{\Delta t(0.450 - D_1) + \Delta D \times t_1} \qquad (3)$$

Table 2 also gives the results with two other HRP preparations. Although a statistical comparison is not possible on these few points, there does not seem to be any appreciable difference in activity per haemin between the three preparations.

The values in Table 2 cannot be used to determine the error of the method, since the error in the dilutions of the stock solutions is included. A number of determinations were therefore made with one HRP concentration (adjusted to 20.2 mμM in the reaction mixture). The experimental values were divided into two groups ($0.150<D_1<0.450$ and $0.450<D_2<0.750$). Two values, one

from each group, were randomly combined, and [HRP] was calculated from every pair according to (3). The concentration was found to be 20.5 ± 0.11 * mμ*M* (19.9—21.1, $n = = 9, \sigma_{\%} \pm 1.6$).

The upper limit for the peroxidase concentrations which can be assayed is probably dependent only upon the minimum time, necessary for the manipulations. Our highest concentrations, [HRP] = 96.6 mμ*M*, corresponds to $\Delta t = 31$ sec. for $\Delta D = 0.60$. This concentration is close to the highest one which can practically be assayed, since we have found the error in the determination to be roughly proportional to $1/\Delta t$ (or $1/\Delta D$). Our lowest value is [HRP] = 4.0 mμ*M*. It is, however, doubtful if the departure of that point from the rectilinear regression line is random. We think that [HRP] in the reaction mixture should be > 10 mμ*M*, corresponding to $t_{0.450} < 5.3$ min.

The "*Standard procedure*" is summarized as follows. Into each of two test tubes are pipetted 1.75 ml 0.1 *M* acetate buffer pH 4.9 + 0.15 ml 0.2 *M* mesidine hydrochloride solution in water + 0.10 ml 0.10 *M* hydrogen peroxide. The tubes are carefully cooled in a water bath containing crushed ice, and 0.02—0.06 (= *a*) ml of the HRP solution to be examined is added to one of them. When the violet colour has reached an intensity which can be estimated as corresponding to a density value of the final solution between 0.150 and 0.450, 2.0 ml acid ethanol (20 ml 3.5 *M* H_2SO_4/1 000 ml alcohol) are added. After further 6—9 min in air at room temperature 0.32 ml 1 *M* NaOH are added. A similar operation is performed in the second tube but here the final density should reach between 0.450 and 0.750. The solutions are measured at 490 mμ, *d* = 1 cm. [HRP] in the reaction mixture is then calculated according to (3). If the time is given in minutes [HRP] in the reaction mixture (1.75 + 0.15 + 0.10 + *a* ml) will be obtained in mμmolarity. [HRP] of the unknown solution is $(2 + a)/a$ times higher.

Other catalysts. A number of other possible catalysts were examined (Tables 3, 4 and 5). In the calculations of moles of purple compound per mole of haemin per min. it was assumed that the reaction was of zero order from the beginning.

Reaction formula. Chapman and Saunders established the structure of the purple compound. They also found that two moles of mesidine produced one mole of ammonia. As a hypothesis for the reaction mechanism they suggested that 2,6-dimethyl-4-methylene-quinone imine was formed as the primary oxidation product, the labile imino group being spontaneously liberated as ammonia by hydrolysis. The methylene group, they suggested, could be liberated as formaldehyde or possibly methane, giving 2,6-dimethyl benzoquinone. We have been able to confirm their hypothesis and to determine the formaldehyde as methylene dimedon.

HRP and peroxide, in slight excess, were added in five portions to 0.69 mg (4 μmoles) of mesidine hydrochloride in 3 ml acetate buffer of pH 4.9. The red precipitate was removed by centrifugation after every addition. The supernatant liquid was decanted, and 0.05 ml of a 10 % dimedon solution in alcohol was added. A white turbidity appeared

* $\bar{X} \pm \sqrt{\frac{\Sigma(X-\bar{X})^2}{n(n-1)}}$

Table 3. Catalytic effects of some haem enzymes. Standard conditions.

Enzyme	[Enzyme]	Reaction time min.	D_{490}
Cytochrome oxidase (as heart muscle prep.)	1 mg/ml	5 *	0
Cytochrome oxidase + cytochrome c	1 mg/ml 4.3 μM	5 *	0
Ditto	Ditto	5	0.148
Cytochrome c	4.3 μM	5	0.143
HRP	3.8 mμM	10	0.678
HRP + + catalase	3.8 mμM 3.8 mμM	10	0.662
HRP + + catalase	3.8 mμM 68.0 mμM	10	0.238
Catalase	68.0 mμM		
After 5 min. HRP	3.8 mμM	5 + 10	0.028
Catalase	1.2 μM	10	0

* No peroxide added.

Table 4. Catalytic effect of some haem-proteids. Standard conditions. Haemin determined as pyridine haemochromogen.

Catalyst	[Catalyst] in terms of haemin	Reaction time min.	D_{490}	Moles of purple compound per mole of haemin per min.
HRP	20 mμM	2	0.485	21 000
Methaemoglobin	38 μM	4	0.645	7
Metmyoglobin	22 »	4	0.500	10
Haemalbumin	18 »	4	0.205	4
Cytochrome c	4.5 »	5	0.143	11

Table 5. Effect of cupric and ferric ions. Standard conditions. Reaction time 4 min. The tubes were centrifuged 6 h after the addition of alkali. A very slight turbidity remained in the tube with only $CuSO_4$.

Catalyst	[Catalyst]	D
HRP	13.2 mμM	0.506
HRP $CuSO_4$	13.2 mμM 925 »	0.540
$CuSO_4$	15.4 mM	0.030
HRP $FeCl_3$	13.2 muM 0.9 mM	0.508
$FeCl_3$	2.5 mM	0.005
Blank		0

in thirty seconds and grew to a crystalline precipitate in three min. The rapid appearance of the turbidity indicated that formaldehyde was present [15]. It was recrystallized three times from ethanol-water and dried over calcium chloride. Yield 4.36 mg (3.2 μmoles of methylene dimedone), m.p. 181—190° (189° [16]). In another experiment the same amounts of reactants were used, but the formaldehyde was separated by steam distillation, and dimedon added to the distillate. Yield 3.70 mg (2.8 μmoles), m.p. 189—190°.

From Fig. 2 (and similar experiments with varying amounts of peroxide) it is obvious that three moles of peroxide are consumed to give one mole of purple compound. The over-all reaction formula is thus

2 mesidine + $3H_2O_2$ = 1 "purple compound" + 1 HCHO + 1 NH_3 + 3 H_2O.

When the reaction is carried out under the standard conditions and at a moderate HRP concentration, the solution remains colourless for about five seconds. This supports the assumption that 2,6-dimethyl-4-methylene quinone imine is the primary oxidation product, since quinone imine is colourless [17]. Then a yellow colour appears, which after another five-ten seconds changes to violet. The yellow substance, probably dimethyl benzoquinone, could not be isolated in pure form from the reaction mixture, although it accumulated as a precipitate when the reaction was made at pH 7 and 20°.

COMMENTS

It is essential for a determination method, specific for peroxidases, that the hydrogen donor should not be oxidizable by oxygen (eventually *via* an enzyme) but only by hydrogen peroxide. This criterion, examplified by a comparison between (dimethyl methylene quinone imine/mesidine) and (benzoquinone/hydroquinone), means that substances like ascorbic acid [18], cysteine, leucodyes and ferrocytochrome *c* cannot be used. The assay should also be designed to minimize sidereactions of the primary oxidation product, achieved in the present case by a high mesidine concentration. Moreover it is necessary that the structure of the substance to be oxidized (AH_2) should be such that only one compound (A-AH_2) is formed. In these respects the present method is satisfactory.

Since HRP and, to an even higher extent, some other peroxidases, are inactivated or destroyed by high peroxide concentrations, the ratio of three moles of hydrogen peroxide / one mole of coloured compound is unfavourable. The same ratio is found in the purpurogallin reaction. The initial peroxide concentration 5 *mM* is rather high, about three times higher than the one suggested by Ettori for the purpurogallin test. A reduction of the peroxide concentration to 1.6 *mM* reduces the above-mentioned linear part (Fig. 2) by more than three quarters. The disadvantage is strengthened by the relatively low molar absorption of the purple compound, $\beta_{490} = 2.9 \times 10^6$ cm^2 × × mole^{-1}. The corresponding value for purpurogallin is $\beta_{430} = 5.8 \times 10^6$. Both values are low, however, as compared to the absorption coefficients of some of the commonly used redox-dyes ($\beta_{596,\ \text{thionine}} = 13.1 \times 10^7$, $\beta_{665,\ \text{methylene blue}} = 19.6 \times 10^7$ cm^2 × mole^{-1}).

Until now the procedure described here has been applied mainly to HRP. With the purpurogallin method "yields" of up to 150 % have occasionally been noticed in the initial steps of HRP isolation. The new method gives good reproducibility with the crude extracts from horse-radish roots when the amount of extract and reaction time were varied in the determinations. After the initial ammonium salt fractionations [11] agreement was also found between

activity and haemin content of the preparations. Unpasteurized milk, as well as a ten years old preparation of crystalline milk peroxidase gave no colour, whereas orange peel extract gave a strongly positive reaction.

SUMMARY

1. A procedure has been designed for the assay of horseradish peroxidase, in which mesidine (sym. trimethylaniline) is used as hydrogen donor.

2. The peroxidase concentration of an unknown solution is obtained directly in molarity.

3. No significant differences in activity, referred to haemin, have been found between three horse-radish peroxidase preparations, obtained by different methods in three laboratories.

4. Catalase, the cytochrome system, haemoglobin, and myoglobin are inactive as catalysts, if a weak, unspecific haemin effect is disregarded. Cupric and ferric ions have no influence. The blank value (peroxide + mesidine only) is zero.

5. The method employs a rather high peroxide concentration. Therefore it cannot be used as a general test for peroxidases in biological material.

6. The net reaction formula is given.

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