SPLITTING OF HORSERADISH PEROXIDASE INTO PROSTHETIC GROUP AND PROTEIN AS A MEANS OF STUDYING THE LINKAGES BETWEEN HEMIN AND PROTEIN*

by

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I. INTRODUCTION

The group of pigments and enzymes known as hemoproteins contain protohemin or a closely related hemin as a prosthetic group. They show marked differences in their individual chemical properties and physiological behaviour, in spite of the similarity or identity of the prosthetic moiety. Certain parts of the protein play a dominant role in bringing about the specific mode of reaction displayed by each class of hemoproteins. These decisive groups have been called the heme-linked groups of the protein. THEORELL and his co-workers have done considerable work on the detection and characterization of these groups, mainly in cytochrome c, and hydroperoxidases (catalases and peroxidases).

The methods of approach are manifold (*cf.* 1), including measurement of the magnetic susceptibility, differential titration, spectrophotometric measurements, determination of the redox potential and amino acid analysis[1,2]. In this paper, a kinetic method was used in an attempt to determine both the minimal number and the type of linkages between the heme and the protein part of the peroxidase molecule.

In 1926 HILL AND HOLDEN[3] first succeeded in splitting hemoglobin reversibly into prosthetic group and protein by treating it with HCl-Acetone. THEORELL[4] applied this procedure successfully to horseradish peroxidase. Later, THEORELL AND PAUL[5] followed spectroscopically the course of the splitting reaction in aqueous HCl-solution. In this way they opened up a new method of investigation of the hemin-protein linkages.

The recombination reactions of peroxidase protein with various hemins were measured qualitatively by THEORELL, BERGSTRÖM AND ÅKESON[6] as well as by GJESSING AND SUMNER[7]. Both groups of workers also determined the peroxidatic activity of the partly synthetic peroxidases obtained. In 1950, THEORELL AND MAEHLY[8] took up the study of these reactions again, measuring the kinetics of the recombination by determining the changes of optical density in the Soret region with time.

The present work represents a more detailed study of the reactions leading to the splitting of horseradish peroxidase into hemin and protein in dilute aqueous solution.

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II. EXPERIMENTAL

The crystalline horseradish peroxidase used in these experiments was prepared by Dr K. G. PAUL of Dr Theorell's laboratories. It was carefully dialyzed against dist. water. In the experiments described, the concentration of the enzyme was kept between 0.5 and 1.5 μM.

The p_H was measured with Beckman meters Mod. M and G, using "baby stomach electrodes", which could be inserted into the cuvettes of the spectrophotometer without interfering with the optical density readings.

The spectrophotometric determinations were made in a Beckman Spectrophotometer Mod. DU, the electrical part of which had been replaced by an apparatus specially designed by Dr B. CHANCE for recording small changes in optical density continuously. It consists mainly of

a. a highly stabilized light source
b. a photocurrent amplifier of high stability
c. a paper recorder with variable speeds

The design of the apparatus is described elsewhere[9].

The graphs shown in Fig. 3, 4, 5, 9 and 14 are pictures of paper recordings obtained using this apparatus. The optical density is recorded on the ordinate, the time on the abscissa.

The spectroscopic measurements were limited to the Soret region (about 360–450 mμ), as the extinction in the case of hemoproteins and their derivatives is maximal there, and density changes during chemical reactions thus most pronounced. The apparatus was situated in a room of constant temperature. In all the experiments the temperature inside the cuvettes of the spectrophotometer was found to be 26° ± 2°.

As a rule, the small amounts of reagents required to bring about the desired reaction were added by applying a concentrated solution to a stirring rod which in turn was quickly swirled around in the cuvette to insure rapid mixing (mixing time less than 1 second). Throughout this paper, horseradish peroxidase will be abbreviated as HRP.

III. RESULTS

A. *The course of splitting with HCl*

Upon adding HCl to an aqueous solution of HRP one does not observe a single reaction, but one can distinguish spectrophotometrically between four reactions which take place in sequence and spontaneously.

The spectral changes which occur when the p_H of the enzyme solution is lowered by the addition of HCl shall now be described in some detail. Fig. 1 and 2 illustrate the reactions outlined below. As most of the compounds, the spectra of which are shown in these figures, are unstable, a direct measurement of the spectra in the ordinary way was not possible. Instead a special technique was employed, which will be described below.

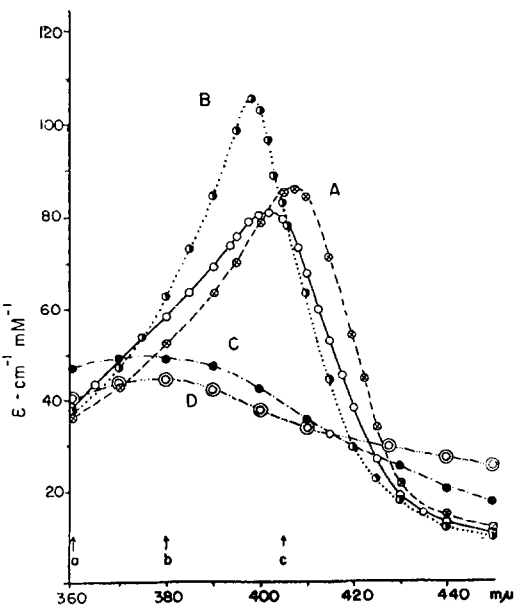

Fig. 1. The spectra of the intermediate compounds found in the stepwise splitting reaction of horse radish peroxidase with HCl
o———o horseradish peroxidase p_H 7
⊕———⊕ horseradish peroxidase chloride p_H 2.2
◑····◑ compound B (with HCl) p_H 2.2
●–·–·–● compound C (protohemin I) p_H 2.2
◉–···–◉ compound D (protohemin II) p_H 2.2
a ⟶ 360 mμ, wavelength of record in Fig. 3
b ⟶ 380 mμ, wavelength of record in Fig. 4
c ⟶ 405 mμ, wavelength of record in Fig. 5
for further details compare text

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When small amounts of HCl are added to the cuvette, no measurable optical changes take place above p_H 3, while the half time of splitting (overall reaction) at p_H 2.5 still exceeds 10^3 seconds. Below p_H 2.5 peroxidase is very rapidly converted to compound A (Fig. 1 and 2). Its spectrum shows a peak at 407.5 mμ in the Soret region. The millimolar extinction coefficient $\varepsilon = 87$ cm$^{-1}\cdot$mM^{-1}. The velocity of formation of this compound is beyond the limits of measurement in the method employed. CHANCE* has already found that no change in the spectrum of HRP occurs down to p_H 2.75 with H_2SO_4, but that chloride can cause the formation of compound A.

The chloride compound is increasingly unstable with decreasing p_H and is spontaneously converted into an intermediate compound (henceforth called compound B). Its spectrum shows a high peak at 398.5 mμ with $\varepsilon = 106$ cm$^{-1}\cdot$mM^{-1} at p_H 2.2.

Compound B is finally spontaneously transformed into compound C, with a spectrum very similar to that of protohemin; this compound will also be referred to as protohemin I. It shows an indistinct maximum around 375 mμ ($\varepsilon = 50$ cm$^{-1}\cdot$mM^{-1}).

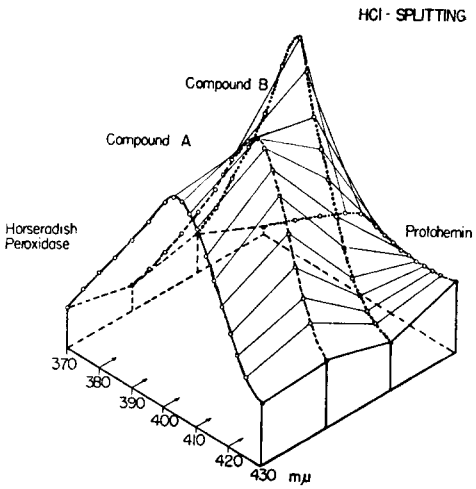

Fig. 2. The same spectra as in Fig. 1, with the exception of protohemin II, drawn in a system of 3 coordinates:
x-axis: wavelength
y-axis: molecular extinction
z-axis: time sequence
It would have been technically very difficult to plot the actual time relations of the reactions. The first step is completed in an immeasurably short time, whereas the reaction time of the 2nd step is about 10 times shorter than that of the 3rd step.

In the course of several hours (at p_H 2 $\pm$ 0.5) a further spectroscopic change can be observed, as compound C is converted into compound D with a slightly, but significantly different spectrum. Compound D will also be designated as protohemin II. It is apparently this last optical change which was observed by THEORELL AND PAUL[5] upon acidifying aqueous peroxidase solutions. They measured the change in optical density at somewhat longer wavelengths (490–550 mμ).

TABLE I

Compounds	HRP	A	B	C	D
HRP	—	401.5	374 and 405.5	371 and 425	363 and 423
A	401.5	—	360 and 404	377.5 and 428	371 and 426
B	374 and 405.5	360 and 404	—	372 and 420	366 and 419.5
C	371 and 425	377.5 and 428	372 and 420	—	416

The wavelength scale of the spectrophotometer was standardized by the position of the Soret band of HRP which lies at 402 mμ.

* Unpublished data

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The isosbestic points between the 5 compounds of the HCl-splitting reaction were observed as shown in Table I (all wavelengths in mμ):

The abbreviations used refer to:

HRP horseradish peroxidase
A the chloride of HRP
B compound B
C compound C or "protohemin I"
D compound D or "protohemin II"

By chosing appropriate wavelengths, all the transformations designated by HRP $\rightarrow$A$\rightarrow$B$\rightarrow$C$\rightarrow$D could be demonstrated and the kinetics of some of the steps could be measured. In Fig. 1, three of the wavelengths employed for these studies are indicated by lettered arrows.

1. The changes in optical densities shown in Fig. 3 were obtained at 360 mμ (indicated by arrow a in Fig. 1). At this wavelength, the reactions HRP$\rightarrow$A and B$\rightarrow$C$\rightarrow$D appear as changes in optical density. After the first addition of HCl, a decrease of

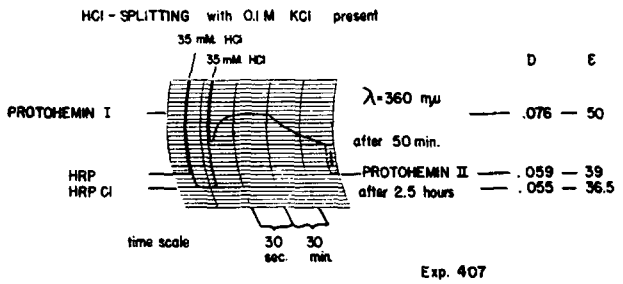

Fig. 3. Spectrophotometric recording of the changes in optical density at 360 mμ upon acidification of HRP with HCl. The reaction proceeds from left to right. p_H 1.6, 0.1 *M* KCl, D = optical density, ε = extinction coefficient $mM^{-1} \cdot cm^{-1}$

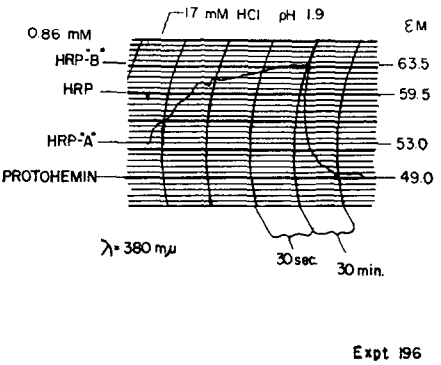

Fig. 4. Spectrophotometric recording of the changes in optical density at 380 mμ upon acidification of HRP with HCl. p_H 1.9, no KCl. The reaction proceeds from left to right

extinction is observed which results from the formation of compound A. Upon a second addition of a very small amount of HCl, A is very rapidly converted to B. This reaction is not visible as a density change at this particular wavelength, because A and B have an isosbestic point here. The next optical change recorded is a rise of extinction caused by the formation of protohemin I (C), followed by a slow decrease in extinction as C is finally converted to D (protohemin II).

2. At 380 mμ (arrow b in Fig. 1), the reactions HRP$\rightarrow$A$\rightarrow$B$\rightarrow$C can be followed (Fig. 4). On addition of HCl, the extinction drops to the level of A, rises to B, and decreases finally as compound C is formed. The transformation from C to D is too slow to be seen on this record.

3. The wavelength of 405 mμ (arrow c) lies very near an isosbestic point of A and B, so that only the reactions (HRP$\rightarrow$A) and (B$\rightarrow$C) are recorded as changes in optical density (Fig. 5).

Within the p_H range employed for measuring this reaction the transformation

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A⟶B is completed in less than 1 second and is therefore not detectable in this experiment. A change in optical density does not occur during the reaction A⟶B. The final conversion of C into D is again too slow to be seen in Fig. 5. The transformation B⟶C is the most pronounced reaction as judged by spectral changes. It leads from a compound still closely related to the intact enzyme, to a substance whose spectrum shows a definite resemblance to free protohemin. This transformation is a first order reaction as judged from the analysis of the spectroscopic trace in Fig. 5.

On repeating this series of splitting reactions over the whole region of the wavelengths studied in this paper, it was possible to obtain the spectra of all intermediate compounds. The experimental conditions were chosen so that rapid reactions were slowed down and slow reactions speeded up in order to be able to measure the endpoint of each reaction step accurately. As the spectrum of the starting material (pure HRP)

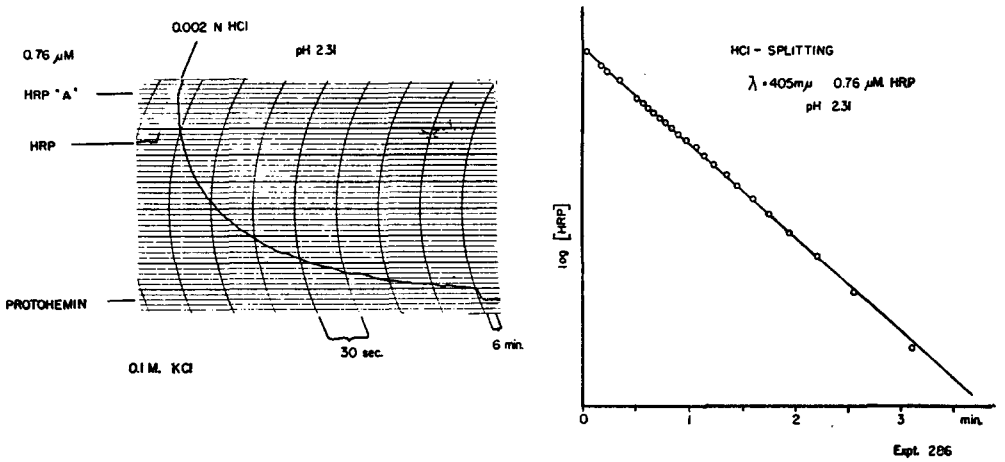

Fig. 5. Spectrophotometric recording (left) and semilogarithmical plot (right) of the change in optical density with time, measured at 405 mμ upon acidification of HRP with HCl. Direction of reaction from left to right. p_H 2.31, 0.1 *M* KCl

was known and was carefully checked for this particular preparation, all differences in extinction obtained on the recording spectrophotometer could be related to this standard spectrum. The spectra thus obtained are represented in Fig. 1 and 2.

On the basis of the determination of the separate intermediate reactions and the measurement of their kinetics, a more detailed study of the influence of p_H and of the presence and concentration of halogen ions (henceforth designated as Hal') on the kinetics of the main splitting reaction (B⟶C) was undertaken.

The rate of the reaction $B \xrightarrow{k} C$ was measured at 405 mμ at different p_H values. Fig. 6 shows the plot of log $1/t_{½}$ ($\propto \log k$) against the p_H of the solution. Within the limits covered by these experiments all obtained values lie on a straight line (Fig. 6, curve 1).

In the presence of 0.1 *M* Cl' as KCl this curve is shifted towards higher p_H values as shown in curve 2. F' (added as NaF to a final concentration of 0.1 *M*) has a still greater effect (curve 3), the displacement of the line towards lower H' concentration reaching 1.5 p_H units. This last reaction was measured at 417 mμ. A measurement at 405 mμ would have given unreliable results, as the acid splitting in the presence of F' takes a spectroscopically different course from the splitting with HCl alone, as will be explained

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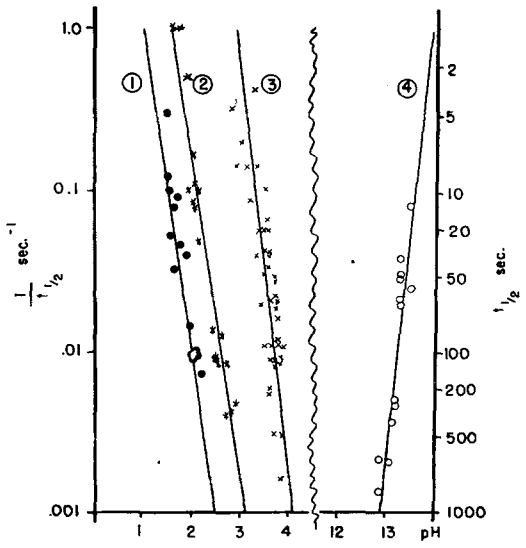

Fig. 6. Rate of splitting of horseradish peroxidase as a function of pH. The rates were measured at 402 mμ on splitting with HCl, at 417 mμ in the presence of F', and at 416 mμ in the alkaline range. Logarithmic scale used on the ordinate.
A) Straight lines:
● 1 splitting with HCl, no addition, slope —2.0 ±.2
✱ 2 splitting with HCl, 0.1 *M* KCl added, slope —2.0 ±.2
× 3 splitting with HCl, 0.1 *M* NaF added, slope ~ —2.5
○ 4 splitting with NaOH, no additions*

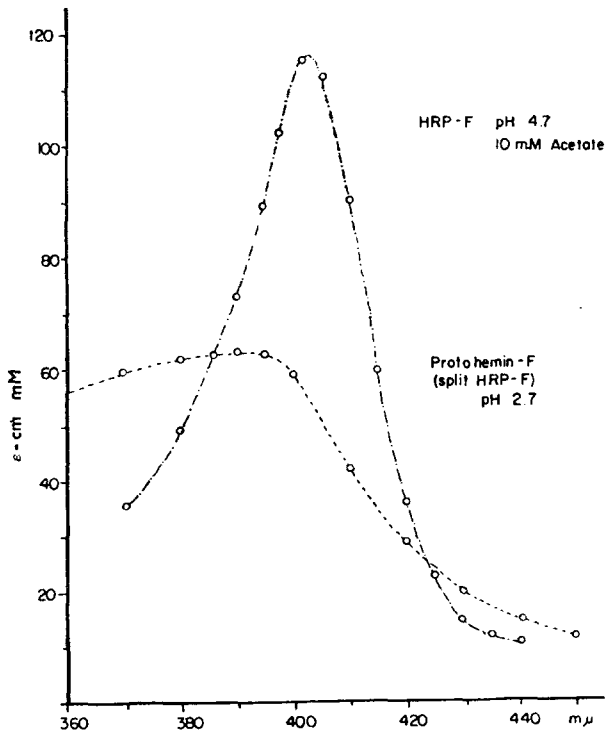

Fig. 7. The spectra of the fluoride of HRP and the protohemin derivative obtained by splitting this fluoride compound by acidifying it with H_2SO_4

below. The presence of 30 m*M* NaCN (reaction measured at 422 mμ) or of 200 m*M* NaN_3 (measured at 406 mμ) didnot increase the splitting rate by HCl to a measurable amount. In all these experiments the temperature in the cuvettes of the spectrophotometer was found to have been 26 ± 2°. The apparatus was situated in an air-conditioned room.

On neutralizing the HCl-split solution with NaOH in the presence of a suitable buffer, recombination of protohemin and protein to active peroxidase takes place. This reversible reaction could be repeated several times with the same solution of the enzyme without appreciable losses in activity.

B. *The acid splitting in presence of F'*

Peroxidase forms a compound with F' in acid solutions as KEILIN AND MANN[10] had observed in 1937. The fluoride compound has been studied intensively since, especially by THEORELL and his co-workers. However, the absorption in the Soret region was measured only in 1949 by CHANCE[11]. In Fig 7 the F'-compound (max $\varepsilon_{403} = 116$ cm^{-1} ·mM^{-1}) is plotted together with the spectrum of the compound formed by the splitting with HCl in an excess of NaF. This latter compound is assumed to be protohemin-F, as the spectrum of protohemin + HF was found to resemble it very closely. On acidification of HRP in

* More detailed data on alkaline splitting will appear in a forthcoming paper.

the presence of F', the fluoride compound is formed (pK 3.45, as found by THEORELL AND PAUL[5]). As the p_H is further lowered (see Fig. 6, curve 3), this compound undergoes splitting and is finally converted into protohemin-F. The splitting reaction was measured at 417 mμ where HRP and HRP-F' have an isosbestic point. On measuring the changes of optical density with time, the transformation HRP⟶HRP-F' is not seen at this wavelength; the splitting reaction that follows produces a decrease in optical density.

A reaction step corresponding to either the formation or decomposition of a substance similar to compound B in HCl splitting (Fig. 1) could not be observed in the presence of F' in spite of a careful search at all wavelengths from 360 to 450 mμ. The reason for this became apparent only later and will be discussed below.

Recombination of the split components to HRP sets in upon neutralization with alkali in a similar way as in the case of HCl splitting.

C. *The acid splitting with HBr*

When HBr is added to a HRP solution, no bromide compound is optically detectable. On lowering the p_H sufficiently, a compound very similar to the above mentioned compound B is formed with a peak at 396.5 mμ. This in turn is unstable and is split into a protohemin derivative + protein. This reaction has not been studied in great detail, as little additional information could be expected.

The rates of the main splitting reaction B⟶C using acids of the type HHal*, with and without addition of salts of the type MeHal* are given in Table II.

TABLE II

Acid	Added MeHal*	p_H at which $1/t_{1/2}$ = 100 seconds
HF	—	2.55
HCl	—	2.05
HBr	—	0.70
HCl	0.1 *M* KCl	2.50
HCl	0.1 *M* NaF	3.70

D. *Splitting of HRP with H_2SO_4*

Sulfuric acid reacts with HRP in still a different manner. Until the p_H is decreased to 4.0 no spectroscopic changes are observed. On increasing the acidity a new spectral band with a maximum at 396.5 mμ replaces the original Soret band of HRP (maximum at 402 mμ) (see Fig. 10). This compound is unstable, the rate of decomposition increasing with increase in acidity. The products formed on decomposition have no well defined absorption spectrum. When the solution is neutralized after treatment with H_2SO_4 no recombination occurs. This is in contrast to the splitting with HHal acids.

The formation of the intermediate compound with a peak at 396.5 mμ (incidentally the same wavelength as the intermediate compound in HBr splitting) was studied in some detail. As the p_H of the solution is decreased below 1.2 the extinction coefficient reaches a maximal value of $\varepsilon_{396.5} = 130\ cm^{-1} \cdot mM^{-1}$. The spectrum at that p_H is shown in Fig. 8 and labeled "compound $B_{H_2SO_4}$". The formation of $B_{H_2SO_4}$ could theoretically result from a reaction with H^+, SO_4^{--} or possibly the H_2SO_4 molecule. A spectrophoto-

* Hal designates halogen ions; Me designates alkali metal ions.

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metric titration was carried out in order to decide whether the formation of this substance is dependent upon the SO_4'' concentration, and also to determine at which p_H the formation is half completed (pK of the group titrated). A typical experiment, carried out at a wavelength of 396.5 mμ, is shown in Fig. 9.

Due to the instability of the compound the value of maximal optical density is maintained only for a short period of time, a time interval which decreases with decreasing p_H. In order to obtain the correct value for the extinction coefficient at a given p_H, the maximal changes in optical density must be obtained from the recording trace and were used in the computation of the extinction coefficients. The type of experiment shown in Fig. 9 was repeated at the same wavelength with varying amounts of H_2SO_4 added to the neutral HRP solution. The results are shown in Fig. 10, together with the

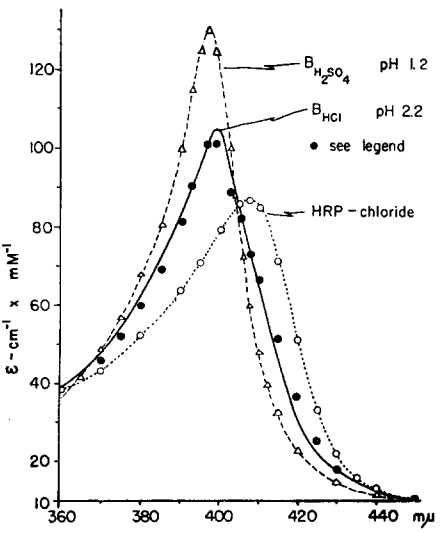

Fig. 8. The spectra of compound B_{HCl} formed by the addition of HCl to HRP, as it was measured in an actual experiment, together with values obtained by computing a 50% complete transformation of HRP-Cl into compound $B_{H_2SO_4}$. The spectrum of the latter was obtained by splitting HRP with H_2SO_4 at p_H 1.2

——— spectrum of compound B as obtained experimentally by adding HCl to HRP at a p_H of 2.3

• • • points calculated by adding the densities of HRP-Cl and of compound B at p_H 1.2 and dividing the result by 2

····· spectrum of HRP-Cl, measured at p_H 2.2 in a series of splitting experiments with HCl, each performed at a given wavelength

– – – – – spectrum of compound B, measured at p_H 1.2 in a series of splitting experiments with H_2SO_4, each performed at a given wavelength

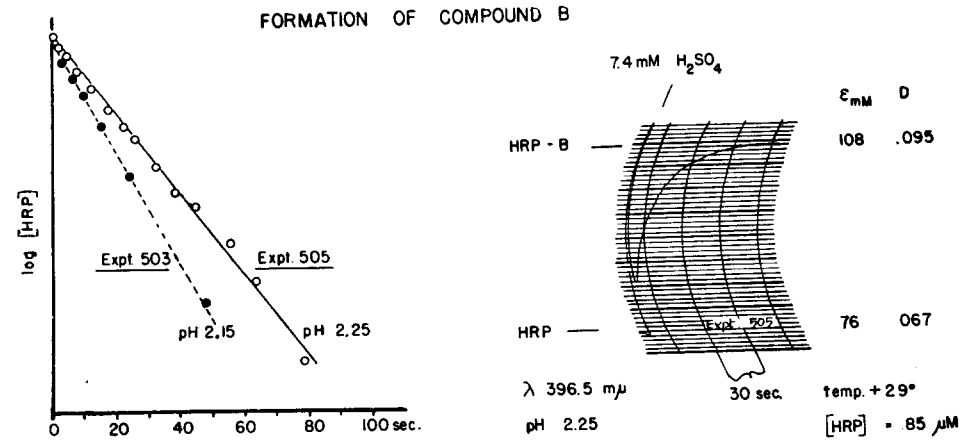

Fig. 9 The kinetics of the formation of compound B, obtained by adding H_2SO_4 to HRP. The reaction proceeds from left to right. On the right hand side, the actual record of an experiment at p_H 2.25 is shown. On the left hand side, the logarithm of the HRP concentration is plotted against time, using the data obtained in the experiment shown on the right and of an analogous experiment performed at p_H 2.15

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dissociation curve computed for p_H 2.30. The agreement is excellent, with the exception of the values below p_H 1.0, where the decay of the compound is very rapid.

Since the p_H is a function of the H_2SO_4 concentration, the result remains ambigious, *i.e.*, the formation of an anion compound $(HRP)_n - (SO_4)_m$ would give a similar dissociation curve.

Therefore, the above experiments were repeated in the presence of 0.1 *M* K_2SO_4 between p_H values of 2–3. In this p_H range, the concentration of H_2SO_4 is 0.01–0.001 *N*,

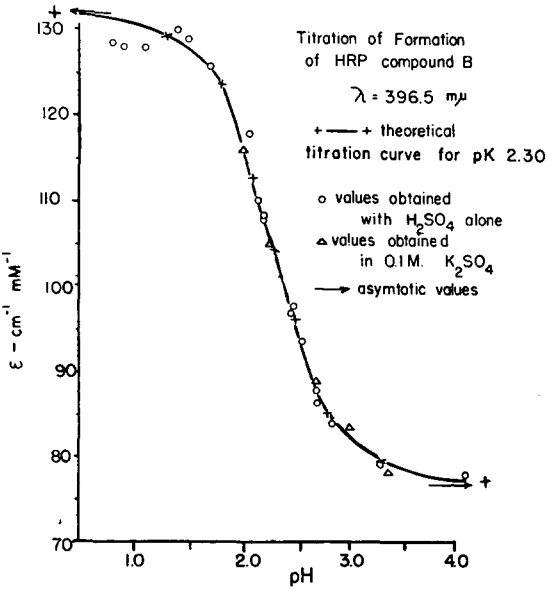

Fig. 10. Titration of HRP with various amounts of H_2SO_4, each point representing the maximal value in $\varepsilon_{369.5}$ obtained in experiments like the one represented in Fig. 9. The compound is half formed at p_H 2.30

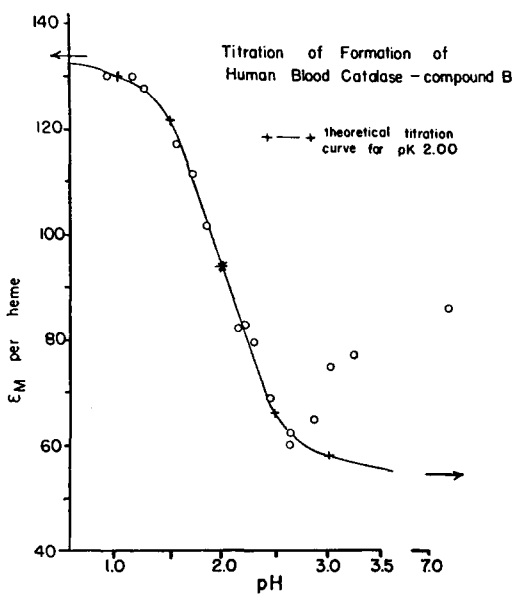

Fig. 11. Titration of human blood catalase with various amounts of H_2SO_4, analogous to the procedure used in the experiments with HRP (Fig. 9 and 10). The compound formation is half complete at p_H 2.00. A content of 4 hemes per molecule is assumed. The extinction coefficients are based on the value of $\varepsilon_{405} = 378\ cm^{-1} \cdot mM^{-1}$, given by Bonnichsen[21] for blood catalases

so that the ratio $\frac{[SO_4'']}{H^+}$ is reasonably high. The values obtained in these series were found to lie exactly on the same curve as the ones measured in the absence of K_2SO_4, demonstrating the independence of this reaction of the SO_4'' concentration.

Replotting the trace of the spectrophotometer curve on a semilogarithmic scale demonstrates that the reaction is of first order. From the experimental data at two slightly different p_H values it is apparent that the rate of this reaction is dependent on the concentration of H^+, SO_4'' or H_2SO_4. This relationship was not further investigated.

By way of comparison, a similar titration was carried out with crystallized and carefully dialyzed human blood catalase, prepared according to HERBERT AND PINSENT[12]. Fig. 11 shows the result.

The pK of the group titrated in catalase lies at 2.00. The values above p_H 2.5 no longer follow the theoretical curve, owing to the titration of another group probably

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related to the formation of an anion compound of catalase and SO_4". Such compounds were observed by MICHAELIS AND PECHSTEIN[13] in 1913, by AGNER AND THEORELL[14] and others (*cf.* 1). Measurements at other wavelengths would be necessary to get reliable values of the dissociation curve of this group.

It is of interest to note, that the peak of the catalase-compound formed on addition of H_2SO_4 lies at the same wavelength (396.5 mμ) as that of HRP, in spite of the different wavelengths of the Soret bands of the free enzymes at neutral p_H (405 and 402 mμ respectively), and that the maximal extinction coefficients per heme are also identical.

E. *Reactions of HRP with acids other than HHal and H_2SO_4*

In addition to HCl, HF, HBr, and H_2SO_4, the reactions of the following acids with aqueous solutions of HRP were studied in less detail: HNO_3, H_3PO_4, $HClO_4$, HCN, (KCN added and HCl used for acidification), CH_3COOH, C_2H_5COOH, aspartic, succinic and glutamic acids.

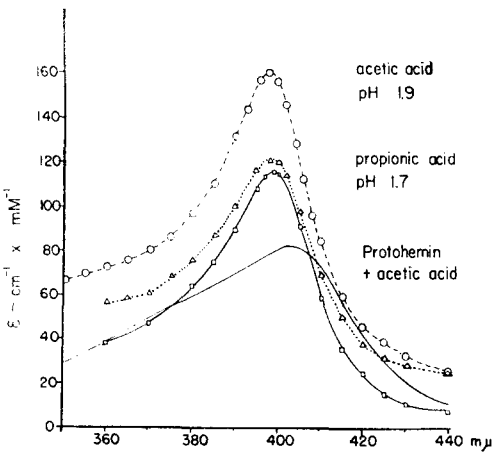

Fig. 12. The spectra of the compounds of HRP with acetic and propionic acids and the spectrum of the protohemin-acetic acid compound. Note the scale of the abscissa, which is different from the scale of the other spectra shown in this paper
o——o HRP + acetic acid
△····△ HRP + propionic acid
□——□ propohemin + acetic acid
——— unmarked curve, HRP, for comparison

The two fatty acids form HRP-compounds with very high and broad Soret bands, indicating a pronounced change in the configuration of the hemin part. In accordance with this assumption, it was observed that the spectrum of free protohemin (recrystallized according to FISCHER AND ORTH[15], and dissolved in 0.1 *N* NaOH) acidified with a large excess of concentrated acetic acid shows a peak of even higher intensity (ε = 115 $cm^{-1} \cdot mM^{-1}$) than that of the pyridinhemochromogen (ε = 75 $cm^{-1} \cdot mM^{-1}$, *cf.* LEMBERG[16]), see Fig. 12.

Aspartic, succinic, and glutamic acids produced no observable change of the spectrum in the observed range. It is, however, not possible to obtain high concentrations of these acids due to their low solubility; 10 m*M* solutions were used.

These latter observations are in accordance with a statement by THEORELL[17] that compounds of HRP with these three acids could not be demonstrated. In addition, it was found that acetic acid does react with HRP, as stated above.

The mineral acids HNO_3, $HClO_4$ and H_3PO_4 gave similar reactions with HRP leading to an intermediate compound B with a Soret band peak at the same wavelengths as in the case of H_2SO_4 and HBr (396.5 mμ). HCN leads to the formation of the cyanide of HRP (main band at 423 mμ), which, when acidified with H_2SO_4, is transformed into compound B as well.

F. *Loss of enzymatic activity on acid splitting*

The chloride of horseradish peroxidase as well as the compound obtained by addition of H_2SO_4 to HRP, were tested for their activity towards H_2O_2. To this end the spectro-

scopic change upon adding 4–6 μM H_2O_2 to the 1 μM solution of the HRP derivative was measured. At the wavelength of 390 mμ, all 3 complexes of peroxidase with H_2O_2 have the same molecular extinction coefficient ($\varepsilon_{390} = 36$ cm$^{-1}\cdot$mM^{-1}) as shown by CHANCE[11], whereas the coefficient is $\varepsilon_{390} = 64$ cm$^{-1}\cdot$mM^{-1} for the chloride of HRP, and $\varepsilon_{390} = 100$ cm$^{-1}\cdot$mM^{-1} for compound B (H_2SO_4). The experiment showed, that the chloride of HRP still reacts with H_2O_2, giving the expected decrease in extinction of $\Delta\varepsilon = \varepsilon_{HRP\cdot Cl} - \varepsilon_{HRP-H_2O_2} = 38$ cm$^{-1}\cdot$mM^{-1}. Compound B, on the other hand, does not show any decrease in optical density at all upon adding H_2O_2 and must therefore be regarded as unable to react with this substrate. This means that the activity of horseradish peroxidase is surely lost when compound B is formed, although no proof is offered that the A $\rightarrow$ B reaction is the sole reaction causing loss of enzymatic activity.

G. *Alkaline splitting*

The stability of horseradish peroxidase in the alkaline range is remarkable for a protein molecule. The color of HRP changes from brown to red upon addition of alkali. This change was first observed by KEILIN AND MANN[10]. THEORELL[18] found the pK of this change to be 10.9 as measured spectroscopically, and 11.27 by measurement of magnetic susceptability. The peak of the Soret band is shifted from 402 mμ to 416 mμ ($\varepsilon = 93$ cm$^{-1}\cdot$mM^{-1} at p_H 12.6). Up to a p_H of about 12.0 no measurable unstability is observed and undamaged HRP can be obtained by lowering the p_H again. At p_H 12.5 splitting sets in, leading to alkaline protohemin (hematin). The spectrum of the latter has a flat maximum at about 395 mμ. Fig. 13 shows the spectra of the alkaline HRP compounds, together with compounds A and C formed during HCl-splitting. Intermediate compounds could not be observed spectroscopically. The measurements of the rate of alkaline splitting were carried out at the wavelength of the largest change in optical density, which is 416 mμ. Fig. 14 shows the kinetics of a typical alkaline splitting reaction, which is of first order.

The values of 13 measurements of log $1/t_{½}$ *vs.* p_H for the alkaline splitting lie all on a straight line, as seen in curve 4 of Fig. 6. The slope of this line has a similar absolute value as the slope of the line obtained with the HCl splitting, though its sign is naturally opposite. Experiments with added halogen-ions in the alkaline region were not undertaken, as it proved difficult to reach high enough p_H values in the presence of Hal'. The products of the alkaline splitting reaction could not be recombined to the original enzyme by lowering the p_H again under the conditions of these experiments.

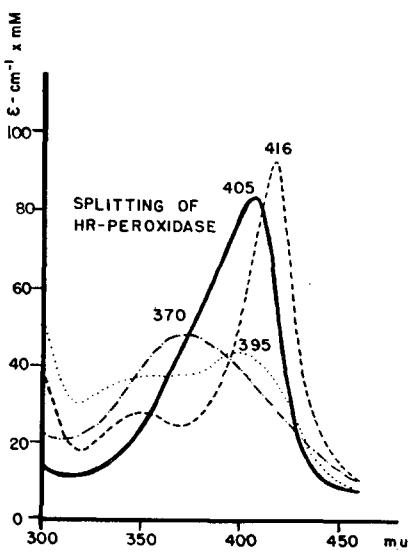

Fig. 13. The spectra of the acid and the alkaline forms of protohemin (as obtained by splitting HRP), as well as of the HRP chloride and the alkaline form of HRP. The numbers on the top of the curves indicate the wavelength of their maxima in mμ. The peak of the chloride lies at 407 mμ at lower pH
———— HRP chloride, peak at 405 mμ, p_H 2.5
– – – – – – alkaline HRP, peak at 416 mμ, p_H 12.5
–·–·–·–·– acid protohemin, peak at 370 mμ, p_H 2.0
····· alkaline protohemin at 395 mμ, p_H 13.0

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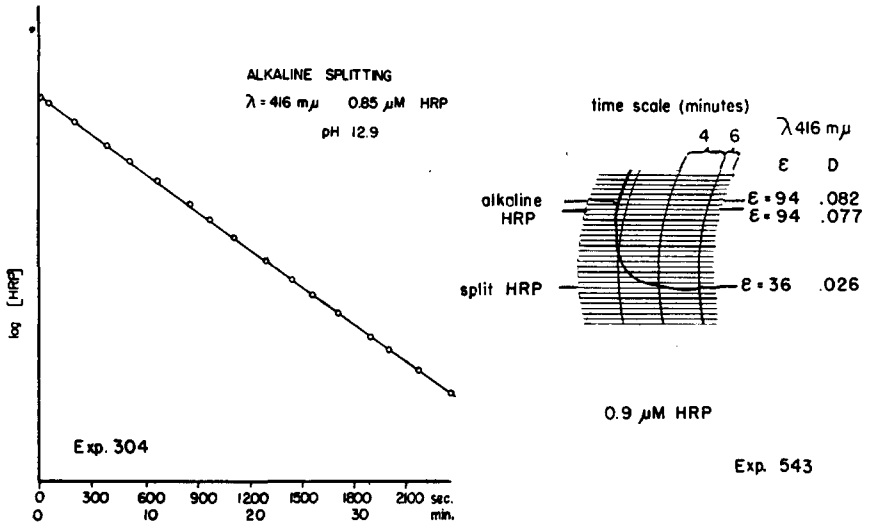

Fig. 14. A typical experiment of splitting HRP by addition of alkali. On the right the trace of the recording spectrophotometer is shown. On the left the data of a similar experiment are plotted on a semi-logarithmic scale. The total decrease of optical density was .060, 1 hour after the start of the experiment

H. *The recombination reactions*

After splitting HRP with HCl, HBr and HF, recombination of the protein part with the hemin part to yield active HRP again sets in upon neutralizing the solution in the presence of a suitable buffer system. The recombination occurs stepwise, as shown by spectroscopic determinations, but the pathway is different from the course of the splitting reactions. The details of the recombination reactions are the subject of further investigation.

III. DISCUSSION

The acid splitting of an aqueous solution of horseradish peroxidase proceeds with the formation of a number of intermediate components, formed and decomposed spontaneously once the acid is added. The overall reaction is reversible (although the reverse reaction follows a different pathway) if the splitting is performed in solutions containing an excess of halogen ions. This type of splitting is of special interest, since the chemical changes do not involve measurable protein denaturation or other observable irreversible reactions. These splitting reactions form a new tool for investigating the bonds between hemin and protein and thus will lead to a better understanding of the chemical and eventually enzymatic behaviour of hyroperoxidases and other hemoproteins. Horseradish peroxidase was used as a first example because of its relatively high stability to extreme temperatures[8] and p_H values.

The data given above show that the acid splitting involves several chemical changes, indicating the breaking of more than one type of linkage. The results of these experiments do not yet permit a full understanding of the linkages involved in the reversible splitting of HRP. However, the properties of some of the intermediate compounds are sufficiently evident from the data described to warrant tentative characterization.

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The various steps of splitting are discussed in the order of their occurence. The first step of splitting with HCl consists of the formation of the chloride of HRP. On comparing this chloride compound with the other halogen compounds of HRP, it is interesting to find qualitative differences in spectra and in enzymatic activities, as demonstrated in Table III.

TABLE III

Ion	λ	ε	Dissociation constant	Activity 0.1 *M* Hal'	Ionic radius
F'	403	118	$10^{-3.45}$ (5)	—	1.36 A
Cl'	407	86.5	—	+	1.81 A
Br'	(402)	(81.5)	—	+	1.95 A

The values for the ionic radii are those given by PAULING[19]

The fluoride compound of HRP is greenish in color. However, the color alone does not permit one to draw a conclusion concerning the type of binding. The peak of the Soret band shows a slight shift from that of HRP (1 mμ towards higher λ), but a large increase in molecular extinction ($\Delta\varepsilon = 36.5$). HRP-F is enzymatically inactive, owing to F'-inhibition (*cf.* 10). The Soret band of the chloride of HRP is displaced to 407 mμ, with but a small increase in molecular extinction over HRP itself. With the Cl' concentration used in these studies (= 0.1 *M*), no inhibition of the enzyme activity could be observed. The chloride is able to react enzymatically with H_2O_2.

The bromide shows neither a spectral difference from HRP in the investigated p_H range, nor does Br' inhibit the enzymatic activity up to 0.1 *M* to any easily measurable degree. In fact, there is no evidence for the existence of a Br compound, aside from the observation that Br' facilitates the splitting by HCl to about the same extent as Cl' does.

The data of Table III show a clearly decreasing affinity of Hal' for HRP in the direction F'→Cl'→Br', as could be expected on the basis of the respective ionic radii given in the last column. The lack of observable influence of anions other than Hal' on spectrum and activity may indicate that these are bound in a different way to the hemin.

The second step of the acid splitting reactions studied in this paper leads to the formation of an intermediate compound (referred to as compound B above) having the same spectrum (peak at 396.5 mμ) irrespective of the acid used for acidification, with the exception of HCl and of HF + F'. If HCl is used for the splitting, the peak of "B" lies at 398.5 mμ, a displacement towards the red of 2 mμ. If HF + F' is used for the splitting, no intermediate compound of this type could be found.

The rate of splitting with HF in the presence of F' occurs at p_H 4 with measurable speed and has a half-time of about 1 second at p_H 3. At this p_H value the formation of compound B is still very small, as judged from the dissociation curve of its formation (Fig. 9). This explains why the formation of compound B was not observed spectrophotometrically.

In the case of the splitting with HCl, the chloride compound is formed previous to a "compound B" and on measuring the formation of the latter, we have to consider the reaction HRP–Cl→B rather than HRP→B as with the other acids used in these experiments. The same situation applies to the reaction steps of HF-splitting (HRP →HRP–F→B) but "B" can not be observed as explained above.

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We shall now postulate, that compound B formed when using HCl for the splitting, is identical to compound B formed on splitting with H_2SO_4. Owing to the rapidity of its breakdown it is not possible to measure the spectrum of B formed by HCl at p_H 1.2 as in the case of splitting with H_2SO_4. Therefore a different technique was used to correlate the spectra of B obtained by HCl and H_2SO_4 respectively.

The reaction leads from the chloride of HRP to compound B. If we neglect the subsequent breakdown of compound B, we have to expect intermediate spectra at each given p_H, all lying between the initial spectrum (HRP–Cl) and final spectrum (B). We know from the dissociation curve of Fig. 9 that the formation of compound B is half complete at p_H 2.3. The intermediate spectrum at that p_H should accordingly lie half way in between the initial and final spectra. When the mean values of the extinction coefficients of HRP–Cl and compound B are calculated for different wavelengths the points plotted in Fig. 8 are obtained. Upon splitting HRP with HCl at p_H 2.2, and measuring the extinction change, the drawn-out curve of Fig. 8 is obtained. Theory and experiment match within the limits of experimental error.

Thus the spectral deviations of the compound B obtained by splitting with HCl from the compound B observed during the splitting with other acids can easily be explained.

Summarizing the data on compound B, the following observations can be listed:

1. The Soret band at 396.5 mμ shows a very high extinction, depending on the p_H of the solution and approaching $\varepsilon = 130\ cm^{-1} \cdot mM^{-1}$ below p_H 1.2.

2. The titration curve with H_2SO_4 shows a pK of 2.3.

3. The SO_4" concentration does not influence the titration curve.

4. All acids investigated lead to the same compound B (according to spectrophotometric evidence), though the rate of breakdown may approach (HCl), or surpass (HF+F') the rate of formation.

5. Compound B is unable to form any known H_2O_2 complexes.

Since the titration curve is uninfluenced by the anion concentration and is a function of p_H only, it seems reasonable to conclude that either a proton enters a heme-linked group of the HRP-molecule or that a OH'-group leaves the Fe-atom when compound B is formed. In other words, compound B is an acid form of HRP.

Spectroscopic investigations in the strongly acid region from 3 to 1 have not been made before, mainly owing to the lack of rapid recording equipment. The only exception was the measurement of the splitting of a ca. 60 μM solution of HRP with 2 N HCl by THEORELL AND PAUL[5]. The experimental evidence for groups titrated in that region was hitherto gathered from differential titration studies[17]. Based on these and earlier experiments, THEORELL AND PAUL[5] propose three linkages between the hemin and protein parts of horseradish peroxidase:

a. the iron atom of the hemin linked to a carboxyl group of the protein

b. one propionyl group of the hemin bound with an ester bond to the protein

c. the other propionyl group bound to the protein electrostatically or in some other, still unknown way.

The spectral changes during the splitting reactions with HCl could then be explained as follows to fit into this theoretical scheme. The chloride is formed by an exchange of the OH-group bound to the Fe for a Cl ion. The formation of compound B and the final splitting reaction leading from B to protohemin probably represent the splitting of two linkages. One of the observed spectral changes would then correspond to a breaking of

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the proposed carboxyl-iron bond, the other to the splitting of one or both of the propionyl-protein linkages. The experimental data are still insufficient to correlate a specific reaction as observed spectrophotometrically to a specific linkage.

The final slow spectral change (protohemin I→protohemin II) is possibly a rearrangement of the hemin molecules after the loss of the protein. It has become known by the work of SHACK AND CLARK[20] and others, that protohemin forms aggregates in aqueous solutions. These authors even demonstrated spectral changes during this polymerisation process. An aggregation reaction could thus very well account for the reaction C→D. The density change observed by THEORELL AND PAUL[5] during the splitting of HRP with 2 *N* HCl probably corresponds to this final reaction. The reaction rate observed by these authors is of the same order of magnitude as in the experiments described above. It is quite understandable that the first rapid reaction steps escaped the notice of THEORELL, who used a spectrophotometer of high accuracy but relatively low speed of operation.

THEORELL[17] found in his differential titration experiments a difference of 2 equivalents per mole between the free protein and recombined HRP in the range of p_H 5.5–9. This finding would be compatible with these experiments, as we would expect the two groups in the protein bound to the propionyl sidechains to be titrable in free protein within this p_H range. However, THEORELL AND PAUL[5] later proposed, based on their experimental evidence, that the neutral form of HRP contains a OH-group. This finding changes our theoretical concept. Some implications are discussed in detail[5], but further work will be needed to solve the dilemma.

Several observations described in this paper could not be evaluated theoretically. Thus, it is unknown why the halogen compounds of HRP are more susceptible to acid splitting. It is further not known, why only the splitting with acids of the type HHal allows a fast and nearly complete recombination to the active enzyme.

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SUMMARY

1. A study was made of the reactions leading to the splitting of HRP in dilute aqueous solutions at strongly acid and alkaline p_H.

2. The acid splitting leads to an acid form of HRP with a maximal extinction at 396.5 mμ (ε = 130 $cm^{-1} \cdot mM^{-1}$ below p_H 1.2). The spectrum of this compound is not changed by the addition of H_2O_2. The acid HRP compound is formed in a first order reaction. Its pK of formation lies at 2.3.

3. HCN, HF, and HCl lead in acid solutions to spectroscopically well defined compounds. These are formed previously to the acid form of HRP. They are much more susceptible to splitting than HRP itself.

4. The acid form of HRP is decomposed spontaneously in a first order reaction to compound C, which has a spectrum extremely similar to that of protohemin. This step presumably represents the splitting of the last bonds between hemin and protein.

5. Compound C is finally slowly converted into the similar compound D, which is still closely related to protohemin.

6. The acid overall splitting reaction was reversible only when halogen ions were present.

7. The relation between p_H and rate of HCl-splitting was computed.

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8. Alkaline splitting sets in above p_H 12.5. Only one reaction step was observed. The reaction is of apparent first order. Under the experimental conditions of this work, it was not possible to recombine the alkaline-split HRP.

9. The possible chemical reactions upon acid splitting are briefly discussed.

RÉSUMÉ

1. Nous avons étudié les réactions de décomposition de la peroxydase de raifort (HRP) en solution aqueuse diluée à un p_H fortement acide et à un p_H fortement alcalin.

2. En milieu acide, l'on obtient une forme acide d'HRP ayant une extinction maximale à 396.5 mμ (ε = 130 $cm^{-1} \cdot mM^{-1}$ à des valeurs du p_H inférieures à 1.2). Le spectre de ce composé n'est pas modifié par adjonction de H_2O_2. La réaction de formation du composé HRP acide est du premier ordre. Son pK de formation est de 2.3.

3. Sous l'action de HCN, HF et HCl l'on obtient, en milieu acide, des composés de spectres bien définis. Ces composés prennent naissance préalablement à la forme acide de HRP, ils se décomposent beaucoup plus facilement que l'HRP lui-même.

4. La forme acide de HRP se décompose spontanément au cours d'une réaction du premier ordre, donnant naissance au composé C qui possède un spectre extrêmement semblable à celui de la protohémine. Cette étappe représente probablement la rupture des dernières liaisons entre hémine et protéine.

5. Finalement, le composé C est transformé lentement en le composé semblable D qui ressemble encore la protohémine.

6. La réaction totale de décomposition acide est réversible seulement en présence d'ions halogène.

7. Nous avons calculé la relation qui existe entre le p_H et la vitesse de décomposition par HCl.

8. La décomposition sous l'influence de l'alcali commence à des valeurs du p_H supérieures à 12.5. Nous n'avons observé qu'une seule phase de la réaction. Cette réaction est apparemment du premier ordre. Il n'a pas été possible, dans les conditions expérimentales de ce travail, de reconstituer l'enzyme originale à partir des produits résultants de sa décomposition par l'alcali.

9. Nous avons discuté brièvement les réactions qui pourraient avoir lieu au cours de la décomposition du HRP par les acides.

ZUSAMMENFASSUNG

1. Die Reaktionen wurden untersucht, welche zu der Spaltung von Meerrettich-Peroxydase (HRP) in verdünnter wässriger Lösung bei stark saurem und bei stark alkalischem p_H führen.

2. Die Säurespaltung führt zu einer sauren Form von HRP mit einer Maximal-Extinktion bei 396.5 mμ (ε = 130 $cm^{-1} \cdot mM^{-1}$ unterhalb p_H 1.2). Das Spektrum dieser Substanz wird durch Zugabe von H_2O_2 nicht verändert. Die Bildungsreaktion der sauren Form von HRP ist eine Reaktion erster Ordnung. Das pK bei welchem diese Verbindung entsteht, beträgt 2.3.

3. HCN, HF und HCl führen in saurer Lösung zu spektroskopisch gut definierten Verbindungen. Diese bilden sich vor der sauren Form von HRP. Sie werden viel leichter gespalten als HRP selbst.

4. Die saure Form von HRP zersetzt sich spontan und gibt, in einer Reaktion erster Ordnung, die Verbindung C, deren Spektrum demjenigen von Protohämin auserordentlich ähnlich ist. In dieser Stufe werden wahrscheinlich die letzten Bindungen zwischen Hämin und Protein aufgespalten.

5. Schliesslich wird Verbindung C langsam in eine ähnliche Verbindung D verwandelt, welche dem Protohämin noch immer nahe steht.

6. Die gesamte Säurespaltungs-Reaktion war nur in Gegenwart von Halogenionen reversibel.

7. Das Verhältnis zwischen p_H und Geschwindigkeit der HCl-Spaltung wurde berechnet.

8. Die alkalische Spaltung beginnt oberhalb p_H 12.5. Nur eine einzige Reaktionsstufe wurde beobachtet. Es scheint sich um eine Reaktion erster Ordnung zu handeln. Unter den eingehaltenen Versuchsbedingungen war es nicht möglich, die Spaltstücke der Alkali-Spaltung von HRP wieder zu dem ursprünglichen Enzym zu verbinden.

9. Die chemischen Reaktionen, welche möglicherweise während der Säurespaltung stattfinden, wurden kurz erörtert.

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