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Multiple Components in Horse-Radish Peroxidase

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The horse-radish (*Cochlearia armoracia* L.) is the source of an active peroxidase which has been the subject of many studies. These studies have comprised both accurate work on the kinetics and spectroscopy of the purified enzyme (Keilin & Hartree, 1951; Chance, 1951), and work on the chemical action of relatively crude enzyme extracts. Theorell (1942) first crystallized horse-radish peroxidase, and subsequent workers have used methods of preparation which are substantially modifications of his methods. Although Theorell showed that a substantial portion of the enzyme ('Peroxidase I') was different in electrophoretic and other properties from the material which he finally crystallized ('Peroxidase II'), this finding has been generally neglected. Subsequent workers have tended to assume that their specimens of crystalline peroxidase are a representative sample of the native enzyme of the horse-radish. Keilin & Hartree (1951) go so far as to deny the existence in nature of any component of horse-radish peroxidase other than the major one.

None the less, horse-radish peroxidase is described in textbooks as a typical example of an enzyme with more than one component (see, for example, Sumner & Somers, 1947). In the course of other work it seemed desirable to compare multi-component enzyme systems from *Aspergillus oryzae* (Gillespie, Jermyn & Woods, 1952) with this

apparently well-authenticated example of the natural coexistence of enzymes of the same specificity. A brief account of an investigation which showed the existence of four components in horse-radish peroxidase was published by Jermyn (1952*a*). Kondo & Morita (1953) have recently isolated from the sweet potato two peroxidases, *a* and *b*, by differential adsorption on alumina gel. Their peroxidase *a* was similar in properties to horse-radish peroxidase II; the properties of peroxidase *b*, although sharply differentiated from those of peroxidase *a*, were also quite distinct from those reported for horse-radish peroxidase I by Theorell.

Filter-paper chromatography as used by Jermyn (1952*a*) demanded both partial purification of the enzyme and a rigid control of the conditions of development of the chromatogram. A certain amount of trial-and-error adjustment of enzyme concentration was necessary to show up the various components. There was thus always room for the suspicion that some form of artifact had been produced. We have therefore used the method of filter-paper electrophoresis (Cremer & Tiselius, 1950) which could be applied to untreated horse-radish juice. The enzyme system has thus been investigated in a state as little removed as possible from that existing *in vivo*, and the application of any methods of purification has been deliberately avoided.

EXPERIMENTAL

Horse-radish juice

The peeled but unwashed roots were crushed in a Carver laboratory press and the expressed juice was collected and spun in a centrifuge (30 min. at approximately 1000 g) until the supernatant liquor was clear. The clarified juice was stored at 5°. No attempt was made to sterilize it, since horse-radish juice is strongly bactericidal (Foter, 1940).

Fig-latex serum

Incisions were made in the outer bark of a Moreton Bay fig tree (*Ficus macrophylla* Roxb.). The latex which escaped was collected and centrifuged in a high-speed head (30 min. at 25 000 g). The slightly cloudy serum was separated and centrifuged again (15 min.) until clear.

Filter-paper electrophoresis and chromatography

An outline of methods used has been given by Gillespie *et al.* (1952). The technique of electrophoresis is discussed more fully by Woods & Gillespie (1953) and that of chromatography by Jermyn (1953). Electrophoresis was normally carried out at 5–10° on strips of Whatman 3MM paper (40 × 4 cm.) at a potential gradient of 9 v/cm., the initial spot being formed by the application of a volume of 20 μ l. to the paper.

The technique for producing 'positive' filter-paper strips by oxidizing with HIO_4 and coupling the resulting carbonyl groups with Girard's reagent P has been outlined by Jermyn & Thomas (1953). In order to produce papers with consistent properties, the papers were invariably oxidized by 0.1 M- HIO_4 in the dark at 28°. For the enzymes of horse-radish juice the best patterns were obtained when the paper strips were 6 cm. above the level of the liquid in the electrode vessels.

Spraying reagents

A spraying reagent to detect peroxidase on paper must give a strong colour over a wide range of enzyme concentrations under conditions where an excess of H_2O_2 is present, since very dilute H_2O_2 solutions are too readily decomposed on the paper to be effective. Benzidine and guaiacol were found to be the most satisfactory for this purpose. Saturated, aqueous solutions of benzidine acetate or guaiacol were mixed with 0.01 vol. of 30% (w/v) H_2O_2 soln. Under favourable conditions the guaiacol red was stable for some hours; the benzidine blue faded within a few minutes at high enzyme concentrations. The benzidine action is inhibited on the paper at pH values above 7.5. Other, somewhat less satisfactory, reagents were *p*-aminodimethylaniline (reddish violet), *o*-anisidine (blue-violet) and hydroquinone (brown).

In general there was a critical threshold concentration of enzyme for the appearance of a component on spraying; components visible after electrophoresis as discrete spots when the horse-radish juice was applied directly were often invisible when it was applied after dilution.

Determination of peroxidase

After electrophoresis the paper strip was cut transversely into pieces of 0.5 cm. width which were placed serially in 6 × $\frac{1}{8}$ in. test tubes and covered with 2 ml. of 0.1 M sodium acetate buffer (pH 5.0) for an hour at room temperature. To each tube were added 3 ml. of a mixture of equal volumes of saturated guaiacol solution, 0.1 M- H_2O_2 solution and water.

After incubation for 10 min. at 37°, 2 ml. of N-NaOH were added and the optical density at 400 μ was measured in a Coleman Universal Spectrophotometer. The greenish-yellow colour was stable for at least 24 hr. There is a linear relationship between optical density and enzyme concentration up to an optical density of about 0.8. Optical density at 400 μ may therefore be taken as a measure of the enzyme extracted from the paper pieces; in certain cases, however, the enzyme was found to be tenaciously adsorbed by the paper. Incubation with guaiacol and H_2O_2 then led to strong development of colour on the paper, little or none developing in the solution. Addition of alkali extracted the red colour from the paper to give a very faintly yellow solution. Paper pieces carrying adsorbed enzyme thus give a falsely low or zero reading for their enzyme content. Direct spraying of the entire strip is therefore the only valid measure of enzyme distribution. Determination of this distribution by extraction methods is no more than a rough quantitative guide to the relations between those components that can be readily extracted from the paper.

Precipitation with picric acid

In experiments where precipitation with picric acid was studied, horse-radish juice, water and saturated picric acid solution were mixed in a centrifuge tube in the required proportions and centrifuged till the supernatant was clear. The clarified supernatant was then investigated electrophoretically.

RESULTS

The existence of multiple components

We have found that the most satisfactory ionic environment for the separation of horse-radish peroxidase into distinct components is 0.1 M sodium acetate buffer at pH 5.0. The enzyme is quite stable on the paper in the pH range 5–8, but outside these limits considerable inactivation takes place. This inactivation is most marked on the acid side and at pH 3, where rapid splitting of peroxidase is known to take place (Maehly, 1951); the survival time of the enzyme is very short. As the pH of the buffer is raised above 5.0, or its strength is decreased below 0.1 M, electro-osmotic flow towards the cathode increases and the resulting displacement of the pattern produces a much smaller separation of the anodic components from the adsorbed cathodic material; nevertheless, for certain experiments where rapid migration was important, we have used 0.01 M sodium acetate buffer.

Using the standard conditions and untreated paper strips, electrophoresis of freshly expressed horse-radish juice (for a period of about 24 hr.) gives a pattern which is shown diagrammatically in Fig. 1. The anodic components (A_1 and A_2) appear as discrete spots, but the cathodic components (C_1 and C_2) appear as well-defined streaks of complicated structure over which the neutral (N) component is superimposed by electro-osmotic flow. The cathodic appearances are similar to the phenomena which Weber (1953) called 'comets', for which he has given a theoretical explanation.

It has been possible to resolve the confused pattern on the cathodic side by the use of 'positive' papers. We have previously shown (Jermyn & Thomas, 1953) that, as the time of periodic acid oxidation is lengthened and the number of substituted pyridinium groups correspondingly increased, the normal cathodic electro-osmotic flow at pH 5 slows down, becomes zero ('neutral' paper), and reverses direction, becoming increasingly anodic ('positive' papers). Polyelectrolytes with a net positive charge, which are absorbed by the untreated papers (weakly 'negative' under the conditions of these experiments at pH 5), become free to move in the electric field on 'positive' papers; negatively charged polyelectrolytes however will show exactly opposite behaviour.

The results of experiments using 'positive' papers are shown in Fig. 2. All the main features of these photographs can be explained in terms of the principles just stated. Thus it is to be expected that the cathodic components will show the observed maximum migration towards the cathode in view of the opposite effects of decreased adsorption and increasingly anodic liquid flow.

We have also carried out some experiments using bromine-oxidized 'negative' (Jermyn & Thomas, 1953) papers at pH 5.0. *N* here moves rapidly towards the cathode quite clear of C_1 and C_2 , which are very strongly adsorbed. By using sufficiently negative papers where the cathodic liquid flow is several cm./hr. under our conditions, A_1 and A_2 can be brought to lie cathodically to C_1 and C_2 , although still on the anodic side of the position occupied by a neutral indicator.

Fig. 1. Diagrammatic representation of the distribution of peroxidase components in freshly expressed horse-radish juice after 24 hr. electrophoresis on untreated paper at pH 5.0 in 0.1 M sodium acetate buffer.

Fig. 2. Filter-paper electrophoresis of freshly expressed horse-radish juice in 0.1 M sodium acetate buffer at pH 5.0. *A* = anodic end of strip; the numbers (10, 20, 45, 90, 180) indicate the time in minutes for which the paper strips were oxidized in 0.1 M-HIO₄ at 28° before treatment with the Girard reagent *P*. *A*, six hr. electrophoresis; sprayed with the guaiacol reagent. Broken lines indicate the position to which neutral substances are carried by liquid flow. *B*, the same after further spraying with the benzidine reagent. *C*, a different sample after 15 hr. electrophoresis; sprayed with the guaiacol reagent. *D*, the same after further spraying with the benzidine reagent. Photographic reproduction does not do justice to the immediate visual appearances, since considerable diffusion and blurring is inherent in the double spraying of wet papers, besides the fogging that occurs as traces of enzyme show up with the lapse of time.

No colour was developed in the positions of any of the components on spraying with a reagent containing benzidine or guaiacol alone in the absence of H_2O_2 . The peroxidase activity was totally and irreversibly destroyed by heating. Hence none of the components was an oxidase or an inorganic catalyst being revealed as an apparent peroxidase by our technique of detection.

Anodic components

The two anodic components have very distinct properties. These are set out in Table 1. The concentration of A_2 is rarely high enough for it to react with guaiacol on the paper; guaiacol-sprayed papers therefore reveal A_1 alone. A subsequent spraying with the benzidine reagent brings up A_2 and we have regularly used this method to differentiate between the two components (cf. Fig. 2D). A_1 is negatively charged at pH 5.0, as shown by its migration towards the anode; rapidly increasing inactivation in experiments at pH values much below this has prevented us from determining the isoelectric point. In keeping with this negative charge, A_1 shows an increasing tendency to trail as more positive papers are used. Under certain conditions A_1 shows a tendency to split into two components, the slower moving of which reacts less intensely with guaiacol. However, we have not been able to establish definite conditions for bringing about this separation.

Cathodic components

Three components may be distinguished in the material with an apparent cathodic migration on untreated paper. One is nearly at its isoelectric point, at pH 5.0 and occupies almost exactly the same position as the neutral indicator substances (trinitrobenzene and *o*-nitroaniline) used to determine the direction and amount of liquid flow. At pH 7.5 it is negatively charged and migrates

anodically behind A_1 . On highly positive paper both this component N and the anodic components migrate much faster than would be expected from the apparent extent of anodic liquid flow (Fig. 2, 180 min.). Thus the simple model which we have used to interpret our observations does not account for the mobility of proteins on highly charged papers. N is almost always the major single component present; only during the autumn of 1952 was there more of A_1 than of N .

Of the two cathodic components, Fig. 2 shows that the individuality of C_1 is most readily demonstrated. It is very readily inactivated on storing the expressed juice, or on very positive papers as shown by the disappearance of this component on the 90 and 180 min. papers in Fig. 2, A and B, where the sample of juice used was low in C_1 . C_1 shows an almost specific reactivity with *o*-anisidine, none of the other components giving more than a faint reaction with this reagent under our conditions. The absorption of C_1 is very slight and it moves relatively freely on 'neutral' papers.

It is doubtful what significance is to be attached to C_2 , which includes the rest of the cathodic material. The plate shows that even on quite positive papers there are multiple fronts and obvious adsorption in the region which C_2 occupies. None the less, on the most positive papers (180 min.), this cathodic trailing has disappeared and a fairly distinct spot can be seen immediately on the cathodic side of N .

In contrast to A_1 , A_2 and N which are quite unaffected in mobility by the ageing of a given sample of juice, or by variations between different samples, C_1 and C_2 give patterns which are strongly affected by these factors. Whole series of overlapping fronts appear and disappear and the apparent mobility on untreated paper alters markedly. These phenomena always disappear as increasingly positive papers are used.

Table 1. *Comparison of the properties of the two anodic components of horse-radish peroxidase*

	Slow anodic component (A_1)	Fast anodic component (A_2)
Reaction with various spray reagents:		
Benzidine	+++	++
Guaiacol	++	±
<i>o</i> -Anisidine	-	-
Hydroquinone	+	-
<i>p</i> -Aminodimethylaniline	+++	++
Adsorption on untreated paper	Slight	Nil
Approximate mobility at pH 5.0 in 0.1M sodium acetate buffer (cm. ² /v/sec.)	1.0×10^{-5}	1.5×10^{-5}
Seasonal variation	Absent in summer	Invariably present
Concentration	Major component in winter	Always a minor component
Homogeneity	Sometimes shows incipient splitting into two spots	Always a single spot

Immobile material

A certain amount of immobile material is always apparent occupying an area corresponding exactly to that of the original spot of juice. The phenomenon seems to be due to the precipitation of non-enzyme material on the paper followed by secondary adsorption of the enzyme, since the adsorbed material gives all the colour reactions of the mobile components. It is interesting to note that esterase, which is detected by a diacetylfluorescein spray reagent and is the only other enzyme discovered in horse-radish juice, forms a single immobile spot under all conditions of electrophoresis.

Seasonal variations

Since the peroxidase content of horse-radish roots varied according to soil, weather and season, arrangements were made to obtain regular small samples of roots from a permanent bed maintained in a market garden. The following variations were noted: (a) Peak peroxidase activity occurs in autumn–winter (May 1952; June 1953). After this the activity declines to a minimum in early summer (December). (b) The cathodic components (N , C_1 and C_2) are always present, but show a minimum in summer. (c) A_2 is less affected by seasonal variation than any other component. (d) A_1 is completely absent at the peroxidase minimum (summer) and is present in maximum amount at peroxidase maximum (winter). (e) The concentration of peroxidase present at the maximum varies from season to season. Thus in 1952 the juice had to be diluted with water (1:2, v/v) to obtain reasonable patterns; in 1953 it could be run undiluted (1952 was a very wet autumn, 1953 a very dry one).

Reaction with picric acid

One of the points of difference between peroxidases I and II noted by Theorell was that peroxidase I was precipitated by picric acid and peroxidase II was not. We have observed that very little peroxidase activity is precipitated from absolutely fresh horse-radish juice by picric acid, unless the juice was diluted. This is true even when the picric acid concentration is high, e.g. for mixtures of one part of juice with two parts of saturated picric acid solution. Neither of the two major components (A_1 and N) is at all readily precipitated by picric acid and this property is almost entirely confined to the cathodic components C_1 and C_2 . We have found that these minor components are usually precipitated by an equal volume of saturated picric acid, although dilution with water is necessary with samples taken at the period of maximum activity.

Ageing of horse-radish juice

It has been found that intact horse-radish roots can be stored in the refrigerator at 5° for some weeks without any change in the electrophoretic pattern of the juice when expressed. The changes observed in successive samples of fresh material appear therefore to be associated with the continuance of normal growth processes. When undiluted horse-radish juice was stored at 5° there was little or no apparent change in any of the components for about 3 weeks. After this time A_1 began to lose activity and become increasingly precipitable by picric acid. The changes on storage are illustrated by the histograms of Fig. 3.

The juice used in these experiments was expressed during the 1952 maximum of peroxidase activity, when it had to be diluted (1 in 3) to obtain patterns with discrete spots. The standard mixture for test of

Fig. 3. Histograms of the distribution of horse-radish juice peroxidase on filter-paper electrophoresis (pH 5.0, 24 hr. at 6 v/cm., 0.1 M sodium acetate buffer) after various times of storage. Activities measured by the guaiacol: alkali method. Left-hand side, sample diluted to 1 in 3 with water; right-hand side dilution; sample: sat. picric acid solution: water (1:1:1, v/v). (a) Sample 2, fresh juice; (b) sample 2, 4 days storage; (c) sample 2, 11 days storage; (d) sample 2, 25 days storage; (e) sample 1, 40 days' storage.

picric acid precipitability was, therefore, equal volumes of juice, saturated picric acid solution and water. The diluted juice and the supernatant from the picric acid precipitation could thus be compared directly.

C_1 and C_2 do not show up as significant peaks on histograms of fresh material, and observation shows them to be adsorbed strongly on the paper. The appearance of more than one peak on the cathodic side of the histograms of aged material is due to less firm adsorption on the paper, and the enzyme reaction can now be seen to take place not only on the surface of the paper but in the reagent solution as well.

Re-running of isolated components

Objection is commonly made to the reality of multiple components such as those we have observed, on the grounds that they are artifacts of the process of separation, and such artifacts are in fact well-known from paper chromatography (Smith, 1952; Boman, 1952; Moore & Stein, 1952). Such objection can usually be met by isolating and purifying individual components and subjecting them to the original experimental conditions; if these isolated components show no signs of splitting into the multiple components of the original experiment they are adjudged to have real existence. In order to meet objections that have been strongly urged against the idea of multiple peroxidase components on other grounds (Keilin & Hartree, 1951) we were anxious to avoid any isolation or purification of individual components that involved the usual chemical and physical manipulations. We have therefore resorted to the following expedient.

A peroxidase component which has moved into an area of paper which is entirely separate from the

areas occupied by other components is effectively 'isolated'. It is impracticable to elute this component and use the resulting solution of the 'pure' component to provide the initial spot for a new electrophoretic run, since quite a high minimum concentration of enzyme is necessary to leave sufficient activity to be detected by our method. We have therefore removed the area of paper containing the desired component (located by comparison with a second control strip) and placed it at the exact centre of the glass plate. Fresh paper strips saturated with buffer were placed in contact with it and a new electrophoresis was begun. The process is illustrated for A_2 in Fig. 4. We have been able to demonstrate that A_1 , A_2 and N each continue their normal migration into the fresh strips with approximately the same mobility as before. There is no tendency to give rise to any of the other components. A_2 , for instance, continued to migrate rapidly into the anodic strip, leaving the original area it occupied devoid of enzyme activity, and giving rise at no stage to guaiacol-sensitive material.

Since perhaps only 0.1% of the material in the original horse-radish juice was peroxidase it was possible that the isolated components might run differently in the absence of most of the material with which they had been originally associated. The areas containing isolated components were therefore dipped into horse-radish juice that had been boiled till peroxidase activity was just destroyed and a second electrophoresis was carried out as before. There was no perceptible difference in the results. A certain amount of material was thrown out of the horse-radish juice on boiling and had to be removed by centrifuging.

Fig peroxidase

Fresh fig-latex serum (diluted 1 in 3) was run at pH 5.0 under the same conditions as horse-radish juice. The electrophoretic pattern is shown by the histogram of Fig. 5. This was confirmed by spraying, which showed an intense anodic spot with a decreasing trail back to a faint spot of absorbed material at the starting-point.

The fig peroxidase was completely removed by picric acid at a ratio of two parts of serum to one part of saturated picric acid solution; under parallel conditions very little of the fresh horse-radish peroxidase was precipitated. It is thus apparent that fig peroxidase is a protein of different properties from those of the horse-radish enzymes. Its amount is subject to very little seasonal variation; the only other component that we have been able to detect is a very small quantity of strongly adsorbed cathodic material, in early spring. It should be possible to obtain a single peroxidase from fig latex which is much more representative of the total

Fig. 4. Diagrammatic illustration of experiment demonstrating that A_2 is a pure component. Electrophoresis carried out in 0.01 M sodium acetate at pH 5.0. Duration of first run, 2 hr.; of second run, 4 hr.

Fig. 5. Histogram showing the distribution of the peroxidase of fig-latex serum (diluted 1 in 3) after filter-paper electrophoresis (pH 5.0, 24 hr. at 6 v/cm. 0.1 M sodium acetate).

peroxidase of the source than that usually obtained from the horse-radish.

When fig-latex serum and horse-radish juice were mixed (2:1) and run at pH 5.0, the anodic spot of the fig peroxidase segregated quite independently of the pattern due to the horse-radish enzyme. Its position was exactly the same as that from a serum diluted 2:1 with water and run in parallel.

Other plant peroxidases

A single experiment on the peroxidases of the expressed juice of the sweet potato (*Ipomoea batatas* Poir.) and the swede turnip (*Brassica napobrassica* Miller) carried in early spring (September) showed in each case the presence of major anodic components of mobility similar to that of A_2 , which were reactive with both guaiacol and benzidine. The sweet potato further showed a strongly adsorbed, slower, anodic component and a benzidine-sensitive component which was approximately neutral. The turnip, in addition to the major component, showed only a single, slowly moving, strongly adsorbed cathodic component, sensitive to both benzidine and guaiacol.

DISCUSSION

Nature of the components

The evidence presented in this paper and that of Jermyn (1952a) seems enough to justify the conclusion that multiple components are present in the native peroxidase of the horse-radish. However, since the existence of multiple components in naturally occurring enzymes is far from being generally accepted, it is essential to consider other possible explanations of the observed phenomena. The following hypotheses may be examined:

(a) The observed components are artifacts of the technique of electrophoresis. Our experiments in

re-running isolated components seem to rule out the occurrence of 'double fronting' of the type found by Boman (1952) and Smith (1952) in paper chromatography. In any case the small amount of ions applied in the spot seems insufficient to produce lasting 'fronts' of different ionic concentrations during a long enough period to affect the electrophoresis, and to the best of our knowledge such effects have never been observed in filter-paper electrophoresis.

(b) The observed components are actually a series of complexes between a single peroxidase and a number of complexing agents present in horse-radish juice. Our observations show that these complexes must be sufficiently tightly bound to migrate as discrete spots, to undergo a second electrophoresis without regenerating any other 'peroxidases' and to exhibit distinctly different specificity for a range of substrates. Even flooding the area of paper occupied by a single component with boiled horse-radish juice does not cause these components to break up into multiple components. Hence the postulated complexes must be formed irreversibly and non-competitively and it is difficult to see how they are to be operationally distinguished from separate enzymes. It is possible that the complexing substances occur in the material coagulated by heat. There is evidence against this from the independent development of the characteristic peroxidase patterns of horse-radish juice and fig serum when a mixture of the two is subjected to electrophoresis. Since all the heat-coagulable substances in both materials are still present, it appears that there can be no free complexing agent in either which is capable of reacting with the peroxidase of the other. It is common experience that the electrophoretic mobility of proteins on paper is affected by the presence of other proteins, and this point has been shown specifically for interactions with polyglutamyl peptides by Strange & Harkness (1953). None the less, the effect of such loose interactions is to blur true separations rather than to create new ones (E. F. Woods, private communication). If our components are complexes, then their behaviour is exactly parallel to that of mixtures of chemically distinct, simple substances, rather than to that of the vague and shifting complexes studied by Hall & Wewalka (1952) and Franklin, Quastel & Van Straten (1952) using paper chromatography. Fišer-Herman & Davorin (1953) have shown the sharp segregation of individual haematin-protein complexes on filter-paper electrophoresis even in such a complicated mixture as animal serum.

(c) If the observed components are actually tightly bound complexes, it is possible that they are in fact one and the same haemoprotein with the sixth co-ordination position of iron filled by different ligands. This is the explanation advanced by Keilin

& Hartree (1951) to account for the difference between 'peroxidase' and 'paraperoxidase' which they identify with Theorell's peroxidase I. We observe changes in the colour of horse-radish juice on standing, the original brownish yellow turning to red; such changes are characteristic of alterations in the co-ordination sphere of the iron atom and are found by Keilin & Hartree in the conversion of peroxidase solutions into paraperoxidase solutions on standing. We do not find that colour change in the horse-radish juice is accompanied by any changes in the electrophoretic mobility and it seems most unlikely that such alterations at a single active point in the enzyme could have such a striking effect on the isoelectric point and electrophoretic mobility of the molecule. Furthermore, we have attempted to carry out our experiments in such a way that changes in the haem portion of the molecule are minimized.

(d) The same haemoprotein may be modified by conjugation with other proteins or the addition of non-protein acidic and basic materials to the molecule. The presence of an acidic polysaccharide as part of the molecule of peroxidase II was invoked by Theorell & Åkeson (1942) to explain the differences between peroxidases I and II. The activity of such conjugated enzyme proteins against a given substrate should be nearly equal; Theorell (1940), in fact, found nearly equal activities for peroxidases I and II. There is no evidence available at present by which it could be decided whether the range of specificities we have encountered in our components is compatible with this hypothesis.

(e) The observed components may be separate enzymes, unrelated except that they are all haemoproteins. Kondo & Morita (1953) have shown the existence of electrophoretically distinct peroxidases in the sweet potato, one of them with properties different from any peroxidase described for the horse-radish. Different enzymes of *A. oryzae* occurring in the same mixture (Gillespie *et al.* 1952) show patterns of single or multiple components which are completely independent of each other on electrophoresis under identical conditions. The fig and horse-radish peroxidases and so far as our limited experiments go, turnip and sweet-potato peroxidases also, give patterns at least as different as those of distinct enzymes in *A. oryzae*. Such differences in the electrophoretic mobility of the same enzyme from botanically distinct species would normally be unhesitatingly ascribed to the existence in these species of enzyme proteins of specific properties carrying a universal prosthetic group. There is no reason to suppose that the horse-radish itself could not produce a number of different peroxidase proteins, possibly from separate tissues of the plant. Van Fleet (1947) has demonstrated that peroxidase is differently distributed in the

tissues at succeeding stages in the development of the horse-radish and claims that the peroxidase of different tissues is not of identical properties.

The independent seasonal variation of our components is a point in favour of their being separate enzymes. Gillespie *et al.* (1952) and Jermy (1952b) have demonstrated the separate nature of the β -glucosidase components of *A. oryzae* by showing that one or more of them may be absent from independently prepared samples. Gillespie & Woods (1953) have made a similar deduction for the proteases of *A. oryzae*.

The identity of the components

One piece of evidence allows the probable identification of one of our components. A sample of highly purified crystalline peroxidase (peroxidase II) was obtained through the courtesy of Prof. H. Theorell and a suitably diluted solution was subjected to electrophoresis under our standard conditions. The electrophoretic pattern consisted of a single intense spot identical in position and behaviour with component N_1 plus a little adsorbed material appearing as a faint diffuse trail and a trace of anodic material. Peroxidase II, negatively charged at pH 7.5 (Theorell, 1940), is thus apparently nearly isoelectric at pH 5.0; peroxidase I, already positively charged at pH 7.5, must have a still greater tendency to move to the cathode at pH 5.0. If we identify N with peroxidase II then, of the components present in our experiments, peroxidase I could only be represented by either or both of C_1 and C_2 . This possibility is confirmed by the relative ease of precipitation of these components with picric acid, but it would be most unwise, in view of the doubts that have been cast on the real existence of peroxidase I, to make a positive identification on the present evidence. Components A_1 and A_2 do not appear to correspond with anything that has hitherto been reported. It is noteworthy that we have observed major anodic components, carrying a strong negative charge at pH 5, in the fig, turnip and sweet potato.

Peroxidases I and II were originally thought to be of equal importance in the horse-radish and the turnip (Theorell, 1940). The reported seasonal variation in peroxidase I and the difficulty of purifying it have led to its being little studied in comparison with peroxidase II which has usurped the name 'peroxidase'. An agreed terminology appears to be necessary for discussion of the subject—the supposed identity of the 'paraperoxidase' of Theorell (1947) and that of Keilin & Hartree (1951) appears to consist of little more than an identity of labels. The following is suggested. The term 'peroxidase' is to be equally applicable to all native plant enzymes with peroxidatic activity.

The absorption spectrum of the haemoprotein or the nature of the ligands co-ordinated with it would then be irrelevant. All the components revealed by this study would then be 'peroxidases' if they are indeed separate, naturally occurring species. The term 'paraperoxidase' with its implication of the modification of an original enzyme could be applied in the sense of Keilin & Hartree (1951) to the insoluble or less-soluble derived materials formed from peroxidases on storage. Component A_1 may thus have formed a 'paraperoxidase' in our experiments on the ageing of horse-radish juice.

SUMMARY

1. The peroxidases of horse-radish juice and the latex serum of *Ficus macrophylla* have been examined by filter-paper electrophoresis.

2. Horse-radish contains possibly five components with peroxidase activity; *Ficus macrophylla*, one.

3. There is great seasonal variation in the relative amounts of the peroxidase components present in horse-radish juice; one of the two major components is absent in the late spring.

4. A major component is always present in horse-radish juice which corresponds to the peroxidase II of Theorell; other material probably corresponding to his peroxidase I is also present.

5. Two components have been discovered which are negatively charged at pH 5.0 and cannot be equated with either peroxidase I or II.

6. One of these components has a positive peroxidatic activity against benzidine and little or none against guaiacol; all the components show some differences in reactivity towards various substrates.

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A Colorimetric Micro-method for the Determination of Glucose

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When a dilute solution of glucose is heated with concentrated sulphuric acid, a bluish pink colour develops, the intensity of which is proportional to the concentration of glucose (Mendel & Bauch, 1926). This reaction has served as the basis for simple methods of determining glucose in blood (Mendel & Bauch, 1926; Mendel & Hoogland, 1950) and glycogen in tissues (Kemp & Kits van Heijningen,

1954). Both methods depend on the formation of 5-hydroxymethylfurfural (HMF) from glucose in hot acid, a reaction which has been studied by Holzman, MacAllister & Niemann (1947), Singh, Dean & Cantor (1948), Wolfrom, Schuetz & Cavalieri (1948) and Ikawa & Niemann (1949). The coloured compound seems to be produced in a subsequent reaction between HMF and one of its pre-