CIII. THE KINETICS OF PEROXIDASE ACTION.

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This work was started with two objects in view. First to test the suggestion of Haldane [1930], that, at low concentrations of hydrogen peroxide, peroxidase would have a temperature coefficient of unity, so that the reaction velocity would be independent of temperature, and secondly to investigate the effect of changes in the concentrations of the hydrogen peroxide and the reducing substrates on the velocity of reaction.

Willstätter and Weber [1926, 1] have shown that the temperature coefficient of peroxidase acting on leucomalachite green is $Q_{10} = 2{\cdot}0$ approximately, over a range of temperature from 0 to 25°. The concentration of hydrogen peroxide was not stated but was probably 2·5 mg. per litre. Now the primary factor in the velocity of enzyme action is presumably the rate of breakdown of the enzyme-substrate compound, but Haldane suggested from kinetic considerations, that at very low hydrogen peroxide concentrations the rate of peroxidase action might rather depend on the number of collisions of hydrogen peroxide molecules with the enzyme, since most collisions would be fruitful. Since this factor is only slightly influenced by temperature, under such conditions peroxidase should have a Q_{10} of unity.

It has long been known that the velocity of peroxidase action increases with increase of peroxide concentration until an optimum is reached, after which further increase in peroxide concentration causes inhibition of the reaction. Willstätter and Weber [1926, 2] found that the inhibition thus produced by excess hydrogen peroxide was combated by increase in the concentration of the reducing substrate, such as pyrogallol or leucomalachite green. This effect has been studied in the second part of this work, using guaiacol and leucomalachite green as the reducing substrates, and from a consideration of the results obtained a new hypothesis is suggested to account for the phenomenon.

Preparation of the enzyme.

The peroxidase was prepared according to the method of Willstätter and Stoll [1918]. Horseradish was used as the source of the enzyme, owing to the absence of aerobic oxidases other than peroxidase.

2·5 kg. of horseradish was cut into slices about 1 mm. thick, and dialysed in running tap-water for 6 days. The peroxidase was then precipitated on the

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plant tissue by treatment with oxalic acid, and the slices were minced to a porridge-like mass from which the enzyme was liberated with baryta. The active extract of 1¼ litres was freed from barium by passing carbon dioxide into the solution, and a large amount of protein was precipitated by the addition of 0·9 vol. of alcohol. The clear filtrate was evaporated under reduced pressure to a volume of 30 cc. and, after removal of barium carbonate by filtration, the peroxidase was precipitated in the form of yellow flakes by the addition of 5 vols. of alcohol. The precipitate was centrifuged, washed with alcohol and dried. The product was a yellow powder 0·9 g. in weight. In this form the enzyme was quite stable, but in aqueous solution it lost 5–10 % of its activity in 24 hours at room temperature.

The activity of the preparation.

The activity of the preparation was estimated by determining the purpurogallin number, which is defined by Willstätter and Pollinger [1923] as the mg. of purpurogallin which 1 mg. of enzyme preparation will form in 5 minutes from 5 mg. of pyrogallol and 50 mg. of hydrogen peroxide dissolved in 2 litres of water at 20°, when the amount of enzyme is so small that the change in substrate concentration during the reaction time is practically negligible.

The reaction was stopped at the end of 5 minutes by the addition of dilute sulphuric acid, and the purpurogallin was extracted with ether, the ethereal extracts being made up to a total volume of 500 cc. The amount of purpurogallin present was then estimated in a colorimeter, the standard for comparison consisting of a solution of purpurogallin containing 100 mg. of purpurogallin in a litre of ether. The purpurogallin for the standard was prepared by the method of Perkin and Steven [1903], and was recrystallised from alcohol and ether.

As the result of a large number of estimations an average purpurogallin number of 70 was obtained, compared with an average value of 145 obtained by Willstätter and Pollinger for preparations at the same stage in the purification.

The temperature coefficient.

As previously stated, Haldane [1930] suggested that at sufficiently low concentrations of hydrogen peroxide peroxidase would have a temperature coefficient of unity. The hypothesis depends on the fact that peroxidase is capable of activating many times its weight of hydrogen peroxide per second. Thus Willstätter and Pollinger's best peroxidase preparation could activate 1000 times its weight of hydrogen peroxide per second at 20° when used on leucomalachite green, which is oxidised 120 times as fast as pyrogallol. The hydrogen peroxide concentration was 2·5 mg. per litre (Willstätter and Weber, 1926, 1].

It is extremely desirable when comparing the reaction velocities of peroxidase at different temperatures to take the necessary readings while the

reaction is following a linear course. Hence the change in substrate concentration during the reaction time must be negligible. Now with the purpurogallin method concentrations of the dye of less than 1 mg./100 cc. cannot be measured, and when the hydrogen peroxide concentration is extremely small all the latter may be used up before the necessary amount of purpurogallin is obtained. Thus the method is unsuitable for measurement of the temperature coefficient of peroxidase at low concentrations of hydrogen peroxide. The colour intensity of the triphenylmethane dyes is much greater than that of purpurogallin, and a method using leucomalachite green as acceptor has been found very reliable by Willstätter and Weber [1926, 1]. The procedure is much simpler than that of the purpurogallin method, as it is unnecessary to extract the dye with ether, the amount of malachite green which is formed by the action of the enzyme on the leuco-base being estimated colorimetrically in the reaction mixture itself. The chief disadvantage of the method is the insolubility of the leuco-base, which makes it impossible to work at a reaction much less acid than p_H 4.

The leucomalachite green was prepared by the condensation of benzaldehyde and dimethylaniline in presence of anhydrous zinc chloride, and was repeatedly recrystallised from alcohol and ether, the final product being quite white. A stock solution of reducing substrate was prepared by saturating $N/20$ acetic acid with the leuco-base *in vacuo*. The resulting solution contained approximately 10 mg. of leucomalachite green per 100 cc., and remained colourless for several weeks if kept *in vacuo*. The reaction mixture was made up of 10 cc. of this solution, 0·2 cc. of 0·166 N sodium acetate which adjusted the reaction to p_H 4, the required amount of hydrogen peroxide, 1 or 2 cc. of the enzyme solution prepared by dissolving a few mg. of the powder in 100 cc. of water, and sufficient distilled water to make the volume up to 15 cc. The reaction was carried out in a water-bath at the required temperature for a time varying between 2 and 8 minutes, at the conclusion of which the enzyme was destroyed by the addition of 1 cc. of N sulphuric acid, which after 30 seconds was neutralised with an equivalent amount of sodium carbonate solution. The mixture was shaken to remove carbon dioxide, and the malachite green produced by the reaction estimated in a colorimeter against a standard solution prepared by dissolving 10 mg. of malachite green in a litre of $N/20$ acetic acid. The acetic acid inhibits the hydrolysis of the dye and thus prevents fading of the standard solution.

The range of hydrogen peroxide concentrations which could be used while allowing the experimental readings to be made during the linear course of the reaction was first determined. Fig. 1 illustrates the course of the reaction in presence of different concentrations of hydrogen peroxide at p_H 4. The amount of malachite green formed is plotted against the time. In this case the curves were obtained by placing the reaction solution in the colorimeter cup immediately after the addition of the enzyme, and observing the depth of colour at various times, and thus a complete curve could be drawn for one

solution. The reaction is comparatively slow at a concentration of hydrogen peroxide of 6×10^{-3} M, reaches a maximum at 6×10^{-5} M, and there is little change in velocity between concentrations of 6×10^{-4} M and 6×10^{-6} M, after which the velocity falls off rapidly as the hydrogen peroxide concentration is further decreased. The curves are all linear except in the case of

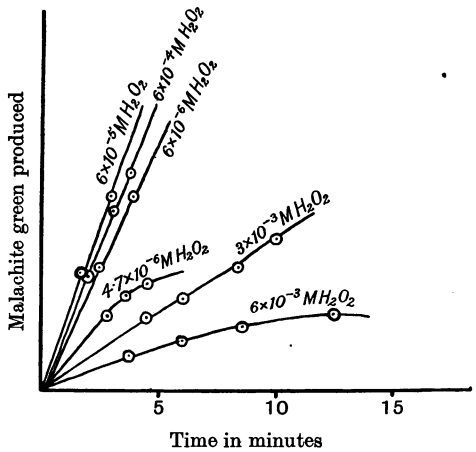


Fig. 1.

the highest concentration where the slight falling off in velocity is probably due to enzyme destruction caused by the high concentration of hydrogen peroxide, and in the lowest concentration where the falling off is due to the using up of a large proportion of the hydrogen peroxide during the course of the reaction.

Having thus found the range of hydrogen peroxide concentrations which it was practicable to use, a number of reaction solutions were made up containing different amounts of peroxide, placed in a water-bath at 20°, and after a definite time the malachite green produced was estimated according to the method already described. The p_s curve was obtained by plotting the reaction velocity against the logarithm of the substrate concentration. The result is shown in Fig. 2. It will be observed that the curve is approximately symmetrical. A possible explanation of its form is suggested in the second part of this work. An advantage of plotting the substrate concentration logarithmically is that the points corresponding to low concentrations which are important in determining the Michaelis constant K_m, that is, the substrate concentration at which half the maximum velocity is reached, are not crowded together. From the curve the value of the apparent affinity of the enzyme for hydrogen peroxide may be obtained, the Michaelis constant, which is the reciprocal of the affinity, being 5×10^{-6} M, which is in agreement with the value of 6×10^{-6} M, calculated by Haldane from the data of Willstätter and Weber [1926, 1].

Willstätter and Weber showed that the Q_{10} of peroxidase at a concentration of peroxide of 25 mg. per litre ($7{\cdot}4 \times 10^{-4}$ *M*) was approximately 2. In order to test the hypothesis of Haldane it was decided to see if this was still the case at a concentration of approximately 10^{-6} *M*. At this concentration it was found impossible to obtain readings while the reaction was following

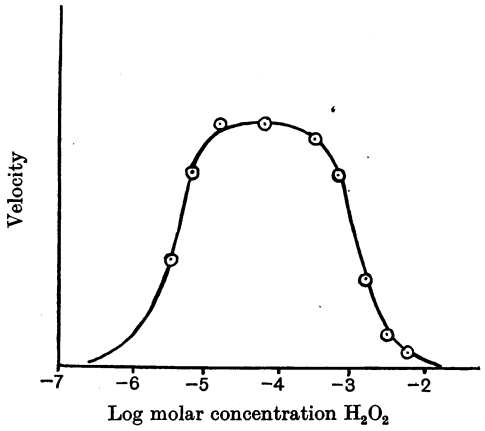


Fig. 2.

a linear course, using the original method of estimation, and to make this possible the method of estimation was altered. 100 cc. of the leucomalachite green solution were used instead of 10 cc., and the malachite green was extracted twice with amyl alcohol, the combined extracts being made up to a total volume of 10 or 15 cc. By this means the dye intensity could be increased 10-fold. The standard solution for comparison in the colorimeter was obtained by extracting the original standard with an equal volume of amyl alcohol.

By means of this method curves were obtained at a concentration of hydrogen peroxide of $1{\cdot}3 \times 10^{-6}$ *M* showing the relative velocities of the

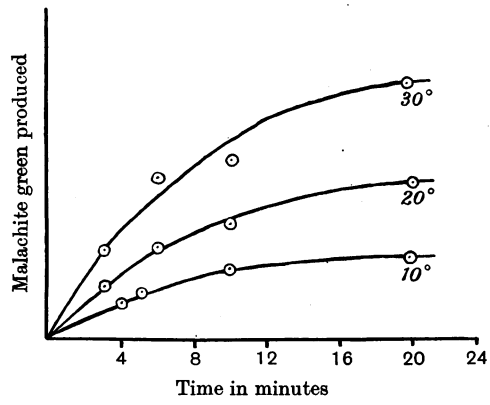


Fig. 3.

reaction at temperatures of 10°, 20° and 30°. It will be observed (Fig. 3) that the reaction follows a linear course for a considerable period, and thus it is possible to obtain the initial velocities at the three temperatures without drawing the tangents to the curves. The curves give Q_{10} 1·9 between 10° and 20°, and 1·8 between 20° and 30°, and thus, contrary to the suggestion of Haldane, the temperature coefficient appears to have been little affected by the change in hydrogen peroxide concentration.

The question was further studied by comparing the p_s curves obtained at different temperatures. If the temperature coefficient is different for different hydrogen peroxide concentrations, one would expect an alteration in the shape of the p_s curve on the horizontal as well as the vertical scale, but, on the other hand, if the temperature coefficient is independent of hydrogen peroxide concentration, the only effect of increase of temperature should be to increase the vertical without altering the horizontal scale. p_s curves were therefore obtained at temperatures of 0°, 20° and 30°, and are shown in Fig. 4. The

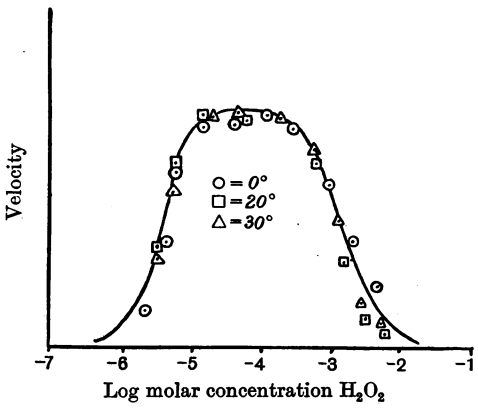


Fig. 4.

curves have all been reduced to the same vertical scale the better to illustrate the small change in affinity. It is obvious that there is little change in the horizontal range of the curves and thus one would expect the temperature coefficient to remain at almost the same figure even at still lower peroxide concentrations than those employed.

Dann [1931], working with citric acid dehydrogenase, has successfully used the temperature coefficient of the affinity as a means of estimating the heat of formation of the enzyme substrate compound. From the curves of Fig. 4 it is obvious that the temperature coefficient of the affinity of peroxidase for hydrogen peroxide is too small to be accurately measured, and thus the heat of formation of the enzyme-substrate compound must be very small.

Haldane's prediction was based upon the assumption that the gas laws could be applied to a substance dissolved in a liquid, so that the frequency of collisions could be approximately deduced by regarding the substance as

in the gaseous state under a pressure equal to its osmotic pressure in solution. The results of this work do not bear out the assumption and may be regarded as evidence that the frequency of collisions in the liquid state is at least several hundred times as great as that indicated by an application of the ordinary gas laws.

The object of the second part of this work was to investigate the relations between the hydrogen peroxide and the reducing substrates at the enzyme. Many years ago Bach [1904], working on the oxidation of hydriodic acid by peroxidase, showed that excess of hydrogen peroxide inhibited the reaction. He attributed the inhibition to irreversible destruction of the enzyme. Willstätter and Weber [1926, 2] investigated this inhibition, and showed that while irreversible destruction of the enzyme may be caused by high concentrations of hydrogen peroxide, reversible inactivation is also produced. In such cases the velocity may be increased either by increasing the concentration of reducing substrate (pyrogallol or leucomalachite green), or by removing some of the hydrogen peroxide by means of catalase. They suggest as an explanation of the reversible inhibition that the peroxide is able to combine with the enzyme in either of two ways, giving an active and an inactive enzyme-substrate compound respectively. Thus in presence of low concentrations of hydrogen peroxide the peroxide combines with the enzyme in the form $\begin{matrix}H\\H\end{matrix}\!\!>\!O = O$ to give an unstable compound which is catalytically active. In high concentrations, however, it combines as H—O—O—H, giving a relatively stable enzyme-substrate compound which is catalytically inactive.

This explanation is unsatisfactory, since by the law of mass action the ratio of the two forms of the enzyme-substrate compound should be independent of the substrate concentration. On the basis of the results obtained by the author an alternative hypothesis is put forward. This was suggested by the addition compound theory of enzyme action [Woolf, 1931], according to which for an enzyme reaction to occur all the substrates must be combined at the enzyme. The explanation depends on the assumption that the reducing substrate combines with the enzyme, a view which has hitherto not been accepted. Haldane [1930] summing up the evidence on this question writes "The reducing substrates of peroxidase appear to act, not by combining with it as a preliminary to oxidation, but by combating the inhibition of its activity by the relatively high hydrogen peroxide concentrations used." The results obtained, however, indicate that the reducing substrate does combine with the enzyme. It is further postulated that the hydrogen peroxide combines with the enzyme at a group which is moderately specific, as it can only be replaced by compounds of related structure such as ethyl hydrogen peroxide [Wieland and Sutter, 1930]. The group with which the reducing substrate combines has a far wider range of specificity, as shown by the variety of substances which can be oxidised by the peroxidase system. It is suggested that hydrogen peroxide is also capable of combining at the latter group. When

both peroxide and reducing substrate are combined at the enzyme at their respective groups, an active enzyme-substrate compound is produced, but in presence of excess of hydrogen peroxide the latter may combine with the enzyme at the non-specific group, thus keeping the reducing substrate from the enzyme and causing inhibition. Such inhibition would be reversible and could be combated by either of the methods described by Willstätter and Weber, *i.e.* by raising the concentration of reducing substrate or by removing some of the hydrogen peroxide with catalase. In both these cases the reducing substrate would be in a better position to compete with the peroxide for the enzyme and thus the reaction velocity would be increased.

The relations between the hydrogen peroxide and the reducing substrate at the enzyme were first studied by obtaining a series of p_s curves each at a different concentration of leuco-malachite green. Now at p_H 4 a saturated solution of leucomalachite green contains only 10 mg. of the base in 100 cc., and thus it is impossible to obtain the wide range of concentrations of reducing substrate which is desirable. At more acid reactions, however, the solubility is considerably increased, being about 10 times as great at p_H 3·2, at which reaction the work to be described was carried out. The leucomalachite green was dissolved in *N* acetic acid, and the reaction was adjusted to p_H 3·2 with sodium acetate.

Fig. 5 shows the series of peroxide p_s curves obtained in presence of

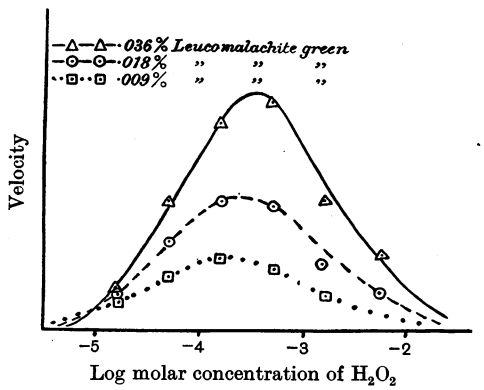


Fig. 5.

different concentrations of leucomalachite green. In the case of a simple enzymic reaction, the curve obtained by plotting the substrate concentration is a rectangular hyperbola or Michaelis curve, or if the substrate concentration is plotted logarithmically an S-shaped curve results which is of the same form as when the amount of dissociation of a weak acid is plotted against p_H. In the case of peroxidase a similar curve would be expected if there were no competition between the two substrates. It will be observed that the p_s curves obtained with peroxidase, shown in Fig. 5, do not resemble the typical form of p_s curve just described. They are symmetrical curves similar to those

obtained when the dissociation residues of ampholytes are plotted against p_H. As the peroxide concentration is increased the velocity rises rapidly until an optimum is reached, after which further increase in concentration causes a rapid fall in reaction velocity. It is suggested that this fall in velocity is due to the competitive combination of hydrogen peroxide at the non-specific grouping of the enzyme. This view is further supported by consideration of the effect of increasing the concentration of reducing substrate. As the concentration of reducing substrate is increased the range of peroxide concentration over which activity is obtained is extended, the system being catalytically active in higher concentrations of peroxide. The curves obtained, however, are still approximately symmetrical, as the position of the optimum is correspondingly shifted. Besides the extension of the horizontal range of the curves, the vertical range is also extended by increase in the leucomalachite green concentration. The increase in reducing substrate concentration has a marked activating effect on the velocity when the peroxide is present in such concentration as formerly produced inhibition. These facts are in complete accordance with the hypothesis outlined above.

Leucomalachite green was used as the reducing substrate in the work on the temperature coefficient because, owing to its colour intensity, the dye produced could be estimated in very low concentrations. For the present work, however, its insolubility rendered it unsuitable. It was therefore decided that further work on the subject should be done with another more soluble substrate. Nitrite, which is oxidised by the peroxidase system to nitrate, and quinol, which is oxidised to quinone, were both tried, but had to be discarded owing to the difficulties of accurately estimating, in presence of hydrogen peroxide, the small amounts of nitrate and quinone produced.

The reducing substrate finally chosen was guaiacol, which, according to Bertrand [1903], is oxidised to a complex red compound, tetraguaiacol.

$$4C_6H_4OH.OCH_3 + 2O_2 = 4H_2O + \begin{matrix} C_6H_3(OCH_3)—O—O—C_6H_3(OCH_3) \\ | \qquad\qquad\qquad | \\ C_6H_3(OCH_3)—O—O—C_6H_3(OCH_3) \end{matrix}$$

The method of estimation employed was that of Bansi and Ucko [1926]. The method is a colorimetric one, essentially similar to the leucomalachite green method, but the standard solution for comparison in the colorimeter is an artificial one, made by mixing cobalt nitrate solution and potassium dichromate solution until the correct tint is obtained. The reaction is stopped by the addition not of acid, which causes the colour to fade rapidly, but of mercuric chloride solution.

Employing this method, the series of peroxide p_s curves shown in Fig. 6 was obtained at p_H 4·7, using four different concentrations of guaiacol. The curves are similar to those obtained with leucomalachite green (Fig. 5), being symmetrical and resembling in form the dissociation residue curves of ampholytes. Increase in guaiacol concentration has the same effect as increase in leucomalachite green concentration, the curves being extended in range both

horizontally and vertically. The increase of guaiacol concentration shows an activating effect on the velocity when the peroxide is present in concentration so high as formerly to have produced an inhibition. This was also the case with leucomalachite green. These facts afford additional support for the

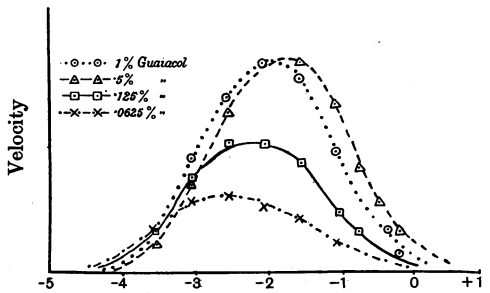


Fig. 6.

theory that the peroxide and reducing substrate compete for the relatively non-specific group of the enzyme. It will be observed that with the highest concentration of guaiacol the velocity at low concentrations of peroxide is less than that when the concentration of guaiacol is 0·5 %, though in higher concentrations of peroxide an activation is produced. This was not observed with leuco-malachite green, possibly because, owing to the insolubility of the latter compound, the necessary concentration to produce the effect could not be obtained.

This phenomenon was further studied in a different way. A series of curves was obtained at p_H 4·7 showing the relation between the concentration of guaiacol and the initial reaction velocity, in presence of several constant concentrations of peroxide. These are shown in Fig. 7. A true Michaelis curve

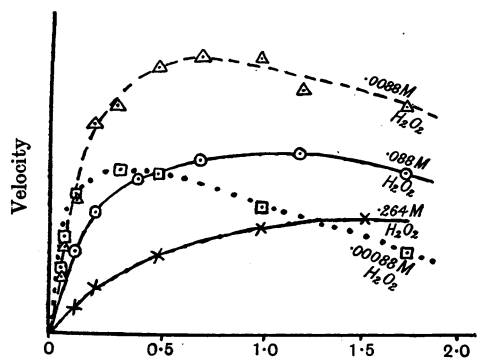


Fig. 7.

is not obtained. At the highest peroxide concentration employed, 0·264 *M*, the optimum concentration of guaiacol has not been reached, the reaction

velocity still increasing with increase in guaiacol concentration. At a concentration of 0·088 *M* peroxide, an optimum guaiacol concentration can be reached, but as the guaiacol is further increased, the reaction velocity, instead of remaining constant, begins to decrease. This fall in velocity is better seen in the other two curves, in presence of still lower peroxide concentrations. As the concentration of peroxide is decreased the optimum velocity is obtained at progressively lower guaiacol concentrations. These curves are shown plotted logarithmically in Fig. 8. They are approximately symmetrical, and again resemble dissociation residue curves of ampholytes.

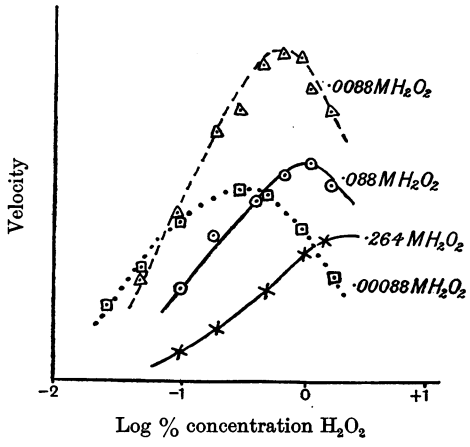
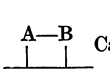


Fig. 8.

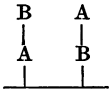
The curves of Figs. 7 and 8 cannot be completely explained by the simple theory that the peroxide competes with the reducing substrate at the enzyme, and some extension of the theory is accordingly necessitated. The form of the curves does not seem to support the possibility that the reducing substrate competes with the peroxide at what has been assumed to be the specific combining group of the enzyme. A more adequate explanation which fits the curves, at any rate qualitatively, is to be found from a study of the following considerations.

Simple enzyme p_s curves are S-shaped, *e.g.* that of saccharase, further increase in substrate concentration after the maximum velocity has been reached having little effect on the velocity until, in the case of saccharase, the substrate concentration is so high that water becomes a limiting factor [Nelson and Schubert, 1928]. In the case of other enzymes however, and in particular of lipase, after the optimum velocity is reached, further increase in substrate concentration causes a rapid fall in the velocity of the reaction, approximately symmetrical curves being obtained, as was observed, *e.g.* by Bamann [1929], for the velocity of hydrolysis of ethyl *d*-mandelate by dog-liver lipase. Haldane [1930] has suggested that such a phenomenon may be explained by the assumption that the substrate is capable of combining

twice with the enzyme, giving an inactive compound. Whereas in the case of the normal enzyme-substrate compound ES, the enzyme is united to the ester by two linkages, one at the alcohol, the other at the acid residue, in the case of the ES_2 compound each substrate molecule is only once united, and hence the strain producing hydrolysis does not arise. This is illustrated by the diagrams shown below which are taken from a paper on lipase by Murray [1930].



Capable of breakdown

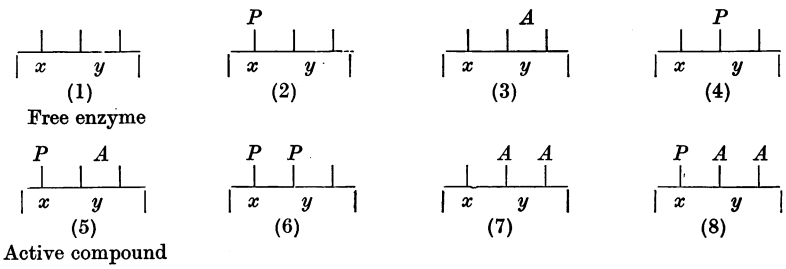


Incapable of breakdown

Murray tested the suggestion of Haldane using the esterase of sheep-liver, and found that it provided a satisfactory explanation of his experimental results.

Such a hypothesis affords an adequate explanation of the action of excess of reducing substrate in producing inhibition of peroxidase action. It is therefore suggested that each molecule of reducing substrate normally combines at two linkages with the enzyme, forming a compound which, when combined with peroxide at the requisite group, gives a catalytically active enzyme-substrate compound. The reducing substrate is, however, also capable of combining in a different way with the enzyme, forming an inactive compound ES_2, a molecule combining with each of the two linkages across which one molecule normally combines. In presence of excess of reducing substrate this inactive compound is naturally produced to a large extent and thus inhibition of the reaction is caused.

On this hypothesis, in a solution of peroxidase, peroxide, and reducing substrate at least 8 forms of the enzyme may be present. These are shown in the scheme below, in which the combining groups of the enzyme are denoted by x (the group specific for peroxide), and y, the peroxide by P, and the reducing substrate or acceptor by A.



The active enzyme-substrate compound is denoted by 5. In presence of excess of hydrogen peroxide, a large amount of the inactive compounds 4 and 6 will be produced, and thus inhibition will be caused. In presence of excess of reducing substrate a large proportion of the enzyme will be combined to form the compounds 7 and 8, which are also inactive and produce inhibition. This appears to be the simplest explanation of the results obtained.

SUMMARY.

The suggestion of Haldane [1930] that the temperature coefficient of peroxidase action would be approximately unity at very low concentrations of hydrogen peroxide has been tested and found to be erroneous. The temperature coefficient is just under 2 at all hydrogen peroxide concentrations.

The effect has been studied of variation of the concentrations of hydrogen peroxide and the reducing substrate on the velocity of peroxidase action, the reducing substrates used being leucomalachite green and guaiacol. The results suggest that both peroxide and reducing substrate must be combined at the enzyme before catalysis can take place.

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