for 20 min. and then back titrated with 0·104 N-HCl, using phenolphthalein. (Found: 11·82 ml. 0·1 N-NaOH used. Calc. 11·45 ml.)

Estimation of glucose. The glucoside is so rapidly decomposed by alkali that it reduces Fehling's solution like free glucose. It was titrated with Fehling's solution by the method of Lane & Enyon (1923). (Found 63% glucose. Calc. (structure IV) 70%.)

Formation of glucosazone. Ranunculin was boiled with dilute sodium acetate for 5 min. and then treated at 100° for 20 min. with phenylhydrazine and dilute acetic acid. Glucosazone was produced. No glucosazone was given under similar conditions from the glucoside itself, without previous hydrolysis.

Formation of protoanemonin. The glucoside (0·2 g.) was dissolved in 5 ml. water containing 2 g. sodium acetate, and the solution was steam distilled for 25 min. The distillate was then neutralized using phenolphthalein (1·6 ml. 0·1 N-NaOH were required to neutralize free volatile acid). 8 ml. 0·1 N-NaOH were added and the solution kept at 60° for 45 min. Back titration with 0·1 N-HCl showed that 5·9 ml. 0·1N-NaOH were used.

The distillation was continued for a further 30 min. and the distillate neutralized as before. Back titration: 0·55 ml. Total NaOH: 6·45 ml. Calc. for structure IV: 7·2 ml. 0·1 N-NaOH.

Preparation of protoanemonin. Ranunculin (3 g.) was dissolved in 300 ml. 10% sodium acetate. This solution was distilled at atmospheric pressure for 25–30 min., or until no more protoanemonin came over. Any acetic acid in the distillate (about 150 ml.) was neutralized with $NaHCO_3$, and the solution was saturated with NaCl and extracted eight times with ether. The ether extract (about 120 ml.) was shaken with a little more $NaHCO_3$, dried over Na_2SO_4, and the ether removed *in vacuo*. The residue was a liquid having the vesicant properties described for protoanemonin; it very rapidly polymerized to a solid.

SUMMARY

1. The protoanemonin obtained from species of buttercups (*Ranunculus*) is derived from a glucoside.

2. The isolation and some properties of the glucoside are described; a provisional structure is given.

3. The substance seems likely to be present only in Ranunculaceae, and the name ranunculin is proposed for this glucoside which yields protoanemonin.

4. The pure glucoside is a stable preparation which is a convenient source of protoanemonin.

This work formed part of a programme of research carried out for the Chemical Defence Development Department of the Ministry of Supply by an extramural research team under the direction of Dr M. Dixon, F.R.S., and was reported to the Ministry (Hill & van Heyningen, 1943). We are indebted to the Director General of Scientific Research (Defence) of the Ministry of Supply for permission to publish this work. We would like to thank Dr B. Lythgoe for his suggestion that ranunculin shows analogy with crocetin and for his help in stating the most probable provisional constitution for the former.

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Suppression of Catalase Activity by Peroxidase and its Substrates

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While studying the darkening of doughs made from low-grade flours, the authors wished to ascertain whether the peroxidase of wheat germ could function in the presence of the catalase also present in germ. A vast amount of work has been done on catalase as well as on peroxidase, but little seems to be known of what happens on addition of hydrogen peroxide to a system showing both catalase and peroxidase activities. Zeile (1934) expresses the opinion that peroxidase activity is inhibited in the presence of catalase. Later in the same article he says that, if catalase is present in small amounts only, peroxidase activity is predominant, whereas large amounts of catalase cause purely oxidative effects by providing molecular oxygen for oxidases. This is how Sumner & Somers (1947) sum up the situation: 'It would appear that peroxidase is of value to plants and animals to bring about the oxidation of certain phenols, but there is little or no experimental evidence to support this. Whether enough hydrogen

peroxide can occur in cells which contain catalase is dubious; whether sufficient phenols are likely to be present is also somewhat dubious. Finally, we can see no especial value to the animal in the formation of such products of oxidation as quinone, for example. It is possible that peroxidase serves a purpose by removing hydrogen peroxide, but here again we have no evidence.'

A preliminary qualitative test showed that a wheat-germ suspension, on addition of hydrogen peroxide, gave a strong catalase reaction. When a small quantity of a peroxidase substrate, e.g. catechol, was added, the oxygen liberation stopped, darkening began immediately and proceeded to a maximum, after which oxygen liberation resumed. Alternating additions of hydrogen peroxide and substrate resulted in alternating induction and suppression of oxygen evolution, suppression being accompanied by oxidation of the substrate. As a result of this observation a series of quantitative experiments was carried out. The results were considered of sufficient general interest to warrant extending the study to other materials, including an artificial catalase-peroxidase system of purified horse-liver catalase and horse-radish extract. The results have a bearing on certain observations made by other workers and on the accepted methods of catalase determination.

EXPERIMENTAL

Materials

Suspensions of wheat germ (Kat.f. 0·44, P.Z. 0·4) and horseradish (P.Z. 2·92) were used as sources of peroxidase and catalase. Purified catalase (Kat.f. 7670) was prepared from horse liver by the method of Keilin & Hartree (1945*a*). For the definition of catalase activity (Kat.f.) and peroxidase activity (P.Z.) see Euler & Josephson (1927) and Willstätter & Pollinger (1923) respectively. Catechol, quinol, pyrogallol, phloroglucinol and *p*-phenylenediamine were used as substrates. The H_2O_2 solution was made by diluting a commercial 130 vol. preparation.

Methods

The quantitative method used was based on the following considerations. If a suspension or solution showing catalase and peroxidase activity receives successive additions of hydrogen peroxide, each of them small enough to be completely decomposed by the catalase within a short time, say 5 min., then the volumes of oxygen recorded after each addition should depend only on the quantity of hydrogen peroxide added. If peroxidase, in the presence of a substrate, is able to compete with catalase for the limited amount of hydrogen peroxide added, then the volume of oxygen evolved after each addition of hydrogen peroxide should be reduced. These conditions were taken to be similar to those existing in the living cell which is assumed to decompose small amounts of hydrogen peroxide rapidly and completely.

The apparatus used in carrying out the experiments consisted of a 200 ml. wide-necked conical flask fitted with a rubber stopper carrying the tip of a microburette and a capillary-tube T-piece opening via a glass stopcock on one side to the air and connected on the other side to a U-tube water manometer fitted with a graduated tube and levelling bulb for volume measurement. The other side of the manometer was connected by means of a similar T-piece to a 200 ml. temperature-compensating flask. Both flasks were immersed in a water bath maintained at 26·5° and the whole assembly was shaken in a horizontal plane at the rate of 180 oscillations per min. (amplitude 40 mm.).

The catalase-peroxidase enzyme material, suspended or dissolved in 20 ml. 0·2 M-phosphate buffer, pH 6·8, was placed in the reaction flask. When temperature equilibrium had been reached, 0·2 ml. of 0·64 N-H_2O_2 was added from the microburette and shaking commenced. After 5 min. the volume of liberated O_2 at atmospheric pressure was measured. The initial condition of the system was then restored by expulsion of this gas and the procedure repeated by adding a further 0·2 ml. H_2O_2. Several such additions were made and O_2 volumes taken to establish that part of the graph which is due to total decomposition of H_2O_2 by catalase.

Peroxidase substrate, usually in an amount to give a 0·01 M solution in the reaction mixture, was then added and when temperature equilibrium had been restored the stepwise addition of H_2O_2 was continued.

RESULTS

In all experiments, whatever the source of catalase and peroxidase, the addition of one of the familiar

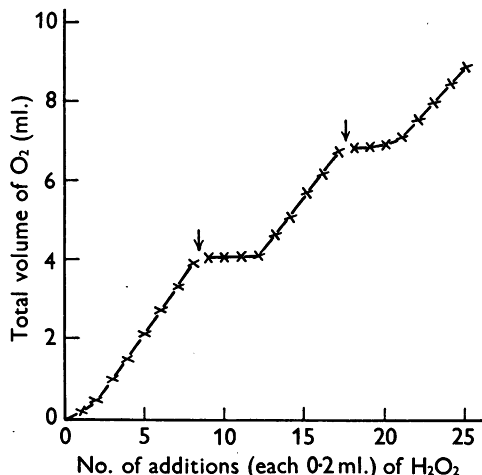

Fig. 1. Effect of quinol on the catalase activity of wheat-germ suspensions. Arrows indicate addition of 22 mg. quinol to suspension of 2 g. finely divided wheat germ (Kat.f. 0·44, P.Z. 0·4) in 20 ml. 0·2 M-phosphate, pH 6·8.

substrates of peroxidase resulted in oxidation of the substrate and severe suppression of oxygen liberation. Fig. 1 shows the typical effect on the catalase

activity when two successive additions of quinol were made to a wheat-germ suspension. After the bulk of the substrate had been oxidized the volumes of oxygen set free for successive additions of hydrogen peroxide began to increase, eventually reaching the level found before addition of the substrate. A second addition of substrate had the same effect in suppressing catalase activity. When the amount of substrate was increased the flat portion of the graph, representing suppression of catalase activity, was lengthened proportionately, but when the substrate concentration in the reaction mixture exceeded a certain limit, for example 0·02 M, the final slope of the graph did not quite revert to the initial angle.

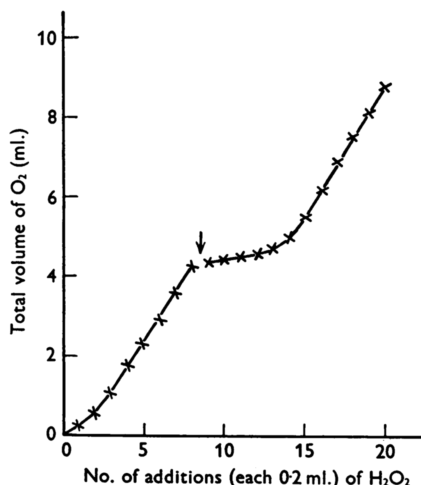

Fig. 2. Effect of *p*-phenylenediamine on the catalase activity of wheat-germ suspensions. Arrow indicates addition of 21·6 mg. *p*-phenylenediamine to suspension of 2 g. finely divided wheat germ (Kat.f. 0·44, P.Z. 0·4) in 20 ml. 0·2 M-phosphate, pH 6·8.

With other substrates the trend was the same, although the return to the initial slope was somewhat more gradual. This is shown in Fig. 2, which represents the effect of *p*-phenylenediamine.

Experiments showed that the initial slight bend seen in the graphs (Figs. 1 and 2) was due mainly to a substrate naturally occurring in wheat germ; the presence of reducing matter such as glutathione (GSH) was a minor contributory factor. Randall's (1946) finding that thiol compounds act as peroxidase substrates was confirmed, but the amount of GSH in the germ suspension used was insufficient to account for more than a small part of this initial suppression of oxygen liberation. The addition of hydrogen peroxide to a germ suspension was accompanied by a colour change from yellow to pink, probably due to oxidation of the natural substrate.

The ratio of catalase to peroxidase was varied by addition of horse-liver catalase to germ suspensions. With such systems it was found that suppression of oxygen liberation depended not only on the ratio of catalase to peroxidase activities, but also on the absolute concentration of the enzymes. Suppression of oxygen liberation was negligible in a system containing 0·086 mg. of horse-liver catalase and 2000 mg. of wheat germ, but was almost complete in an experiment where 100 mg. of germ were used (Fig. 3). The divergence of the initial part of the curves is due to the different concentrations of natural substrates.

An artificial system containing 0·086 mg. of horse-liver catalase (Kat.f. 7670) and 57·4 mg. of horse-radish (P.Z. 2·92) was also used to study the effect of a catalase: peroxidase ratio different from that of wheat germ. Fig. 4 illustrates the behaviour of this system when quinol was added.

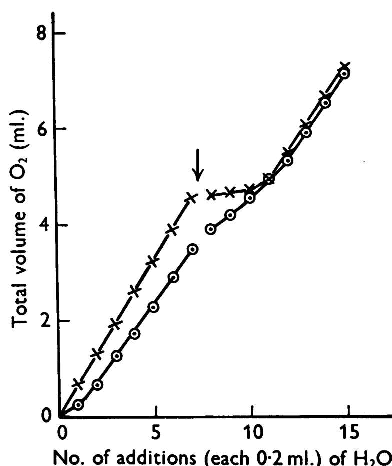

Fig. 3. Effect of quinol on systems containing 0·086 mg. horse-liver catalase (Kat.f. 7670) and 2000 mg. (⊙—⊙) or 100 mg. (×—×) of wheat germ (Kat.f. 0·44, P.Z. 0·4) suspended in 20 ml. 0·2 M-phosphate, pH 6·8. Arrow indicates addition of 22 mg. quinol.

Fig. 3 shows that the suppression of catalase activity in the artificial system was definite though somewhat less marked than in wheat-germ suspensions. The other substrates produced similar suppressions of catalase activity, although, as with wheat-germ suspensions, the return to the initial slope was more gradual.

Keilin & Hartree (1945*b*) found that catalase is able to oxidize certain alcohols by using the oxygen evolved in the decomposition of hydrogen peroxide, and suggested that it might be able to oxidize other biologically important compounds in the same way.

In order to make sure that the observations reported above were not due to purely catalatic oxidation, the authors used the method described in this paper in an attempt to oxidize typical peroxidase substrates by means of purified catalase. The degree of oxidation did not exceed that obtained in a control

experiment where catalase was absent, but the volume of oxygen evolved in the usual 5 min. reaction time showed that the peroxidase substrates listed under the Materials section, when used in concentrations of 0·01–0·02 M at pH 6·8, caused severe reduction of catalase activity. This decrease in reaction rate made it necessary to lengthen the reaction time between successive additions of hydrogen peroxide so as to ensure full decomposition of each addition before making the next one.

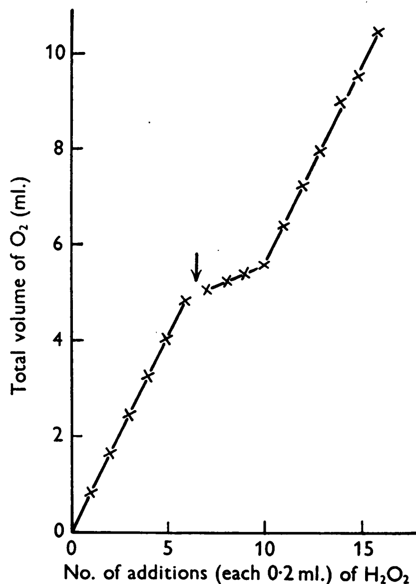

Fig. 4. Effect of quinol on a system containing 0·086 mg. purified horse-liver catalase (Kat.f. 7670) and 57·4 mg. horse-radish extract (P.Z. 2·92) in 20 ml. 0·2 M-phosphate, pH 6·8. Arrow indicates addition of 22 mg. quinol.

DISCUSSION

The results reported above show that the addition of hydrogen peroxide to a wheat-germ suspension containing catalase, peroxidase and a substrate of the latter caused preferential oxidation of the peroxidase substrate. Catalase activity was largely suppressed until oxidation was complete. In the other systems studied, the extent of this suppression was found to depend on the concentration of the enzymes as well as on the ratio of their activities. Altschul & Karon (1947), referring to Kasanski's (1912) finding that catalase activity can be selectively destroyed by incubating the enzyme solutions with pyrogallol, used this method to destroy the catalase activity of cotton seed. The observations reported in this paper bear out the conclusion that pyrogallol, as well as other peroxidase substrates, damages catalase activity. The damage, however, did not occur in presence of peroxidase and substrates in concentrations up to 0·02 M.

Methods for catalase determination are based mainly on the establishment of the velocity constant for hydrogen peroxide decomposition. In systems containing any peroxidase substrates, including thiol compounds, the suppression of catalase activity by peroxidase might vitiate these methods. A similar complication affecting catalase activity might arise in presence of ascorbic acid and a phenolic substrate. In such a system the quinone formed by peroxidase would catalyse the oxidation of ascorbic acid. Under these conditions ascorbic acid would play the part of an additional substrate of peroxidase.

The method described in this paper offers possibilities for the detection of naturally occurring or added substrates of peroxidase.

SUMMARY

1. Hydrogen peroxide added to a wheat-germ suspension containing catalase, peroxidase and a substrate of the latter was preferentially used by peroxidase in the oxidation of the substrate. Catalase activity was largely suppressed while the oxidation of the substrate proceeded.

2. In the other systems studied, the extent of this suppression was found to depend on the concentration of the enzymes as well as on the ratio of their activities.

3. The peroxidase substrates tested damaged catalase activity, but were much less injurious in presence of peroxidase.

Our thanks are due to Mr D. G. Holliss for his assistance in the experimental part of this work.

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