2. The data show that one mole of orthophosphate is required to degrade an equivalent of citrulline to ornithine, carbon dioxide and ammonia. An equivalent of ATP is synthesized.

3. Extracts of *Strep. lactis* release orthophosphate from ATP but not from ADP. The presence of an adenylate kinase system is indicated.

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A Simple Method for the Preparation of Horseradish Peroxidase

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Of the two available methods for the preparation of highly purified horseradish peroxidase, that of Theorell (1942) involves the use of electrophoresis apparatus, and there are many steps in that of Keilin & Hartree (1951). A simpler method has now been devised which avoids the use of electrophoresis and considerably reduces the number of fractionation procedures. The method depends on the removal of much inactive material from the crude extract by precipitation with a mixture of chloroform and ethanol (the reagent of Tsuchihashi, 1923). Following this treatment, highly purified peroxidase preparations have been obtained by fractional precipitations with ammonium sulphate and ethanol. The product obtained is a stable dry preparation which can be crystallized from ammonium sulphate solution.

EXPERIMENTAL

Methods

Activity of the peroxidase preparations. The 'Purpurogallinzahl' (P.Z.) (Willstätter & Stoll, 1918) of the preparations, i.e. mg. of purpurogallin formed by 1 mg. of the enzyme preparation in 5 min. from pyrogallol and H_2O_2 under fixed conditions, was estimated by the method of Keilin & Hartree (1951) using an EEL (Evans Electroselenium Ltd.) colorimeter with Ilford Bright Spectrum Blue filter 622.

Peroxidase units. The number of peroxidase units (E.U.) in the various fractions and final preparations is given by the total weight of purpurogallin (g.) which these would give under the conditions of the above method.

Dry weights. Samples were dried to constant weight at 105°.

Absorption data. These were obtained using the Unicam Model S.P. 500 quartz spectrophotometer with 1 cm. cells.

Haemin. The haemin content of the peroxidase preparations was estimated at a concentration of 0·4 mg. preparation/ml. by measuring the light absorption at 557 mμ. after conversion into pyridine haemochromogen (Paul, Theorell & Åkeson, 1953). The value for the molar absorption of the α-band of the pyridine ferroprotoporphyrin ($8{\cdot}0 \times 10^7$ cm.^{2} mol.$^{-1}$) given by Paul *et al.* (1953) was used for calculation of the haemin content.

Isolation of peroxidase from horseradish root

Preparation of crude extract. Roots of wild horseradish (*Cochlearia armoracia* L.) were gathered in spring when interference by root polysaccharides during the fractionation is at a minimum (Keilin & Hartree, 1951). Washed root (5 kg.) was cut into small pieces and put through a large domestic meat mincer. The mince was mixed thoroughly with 7 l. of water and then macerated in batches in a Waring Blendor. Each batch was macerated for 2 min. The macerate was squeezed by hand in strong cotton cloth and the extract (9 l.) was stored overnight at 0–2° (P.Z. 1·1; E.U. 598).

Treatment with ethanol:chloroform. A 2:1 (v/v) ethanol:chloroform mixture (2·7 l.) was cooled to 0° and added to the extract at 0° with brisk mechanical stirring. The stirring was continued for 20 min. The mixture was then centrifuged at 3000 rev./min. for 15 min. The clear yellow supernatant (10·42 l.; E.U. 537) was adjusted to pH 5·5 with 2 N-NaOH and concentrated by distillation under reduced pressure to 2·18 l. (E.U. 488). The temperature of the solution was kept below 30° and the distillation rate was about 0·9 l./hr.

Fractional precipitation with ammonium sulphate. The relatively inactive precipitate obtained by adding $(NH_4)_2SO_4$ (250 g./l. concentrate) was centrifuged off and discarded (E.U. 29). A further 250 g. of $(NH_4)_2SO_4$ for each litre of original concentrate was added to the supernatant. The suspension was kept overnight in the cold room (0–2°) and then the precipitate, which contained most of the

peroxidase, was centrifuged off. The precipitate was suspended in 50 ml. of 0·2 M orthophosphate buffer (pH 7) and dialysed for 3 hr. against running tap water and finally against 5 l. of distilled water at 0–2° overnight. The dialysed suspension was centrifuged and the precipitate washed twice on the centrifuge with a little water. The combined supernatants had a total volume of 83·5 ml. (E.U. 395).

Fractional precipitation with ethanol. The supernatant was cooled to 0° and ethanol (1·5 vol., 125 ml.), previously cooled to −5°, was added slowly with stirring while the temperature of the mixture was kept below 0°. The suspension was left at 0° for 30 min. and then centrifuged clear. The precipitate was dried *in vacuo* over H_2SO_4 (476 mg.; P.Z. 60; E.U. 29). To the supernatant at 0° further chilled ethanol (2·5 vol., 209 ml.) was added, and after standing at 0° for 30 min. the suspension was centrifuged. The precipitate was dried *in vacuo* over H_2SO_4 (470 mg.; P.Z. 610; E.U. 287). This precipitate was suspended in 25 ml. of water, centrifuged clear, and the pellet washed once. The combined supernatants were fractionally precipitated with ethanol under the conditions above except that the fractions precipitating between 0 and 1·2 vol. (*A*) and 1·2 and 2 vol. ethanol (*B*) were collected. The precipitates were dried *in vacuo* over H_2SO_4 (*A*, 60 mg.; P.Z. 130; E.U. 8: *B*, 270 mg.; P.Z. 940; E.U. 254). Precipitate *B* was dissolved in water and again fractionated with ethanol, the precipitates with 1·3 vol. ethanol (*C*) and 1·3–1·9 vol. (*D*) being collected. (*C*, 58 mg.; P.Z. 460; E.U. 27: *D*, 149 mg.; P.Z. 1080; E.U. 161; haemin 1·14 %; $E_{402\,m\mu}/E_{280\,m\mu}$. 2·3.) One such preparation (*D*) showed little loss of activity after storage for a year in a dry atmosphere at room temperature.

Crystallization of horseradish peroxidase. The main purpose of this work was to prepare a stable dry preparation of highly purified peroxidase for use in studies of peroxidase action. For this purpose preparations such as *D* were suitable. Attempts were made to obtain crystalline peroxidase from such preparations by the method of Theorell (1942), but these were unsuccessful possibly because no crystals of peroxidase were available for seeding. On one occasion a solution of preparation *D* was fractionated with $(NH_4)_2SO_4$ and five fractions precipitating between 50 and 64 % saturation were collected. Each precipitate was dissolved in a small volume of water and saturated $(NH_4)_2SO_4$ added dropwise until a faint turbidity appeared. The solutions were left at 0° and examined periodically. After about 3 weeks the fraction originally precipitated at 61–64 % saturation showed the characteristic shimmer of crystalline suspensions. The suspension was centrifuged and the precipitate suspended in 70 % saturated $(NH_4)_2SO_4$; microscopical examination showed that the suspended material consisted of fine, needle-shaped crystals 3–10 μ. long. Solutions of preparation *D* could be readily crystallized by using these crystals for seeding. The following procedure was found suitable. That fraction of preparation *D* which precipitated between 56 and 70 % saturation with $(NH_4)_2SO_4$ was collected. The precipitate was dissolved in a small volume of water in the centrifuge tube and saturated $(NH_4)_2SO_4$ was added dropwise until a faint turbidity appeared. On chilling in ice water the turbidity disappeared and the solution was then inoculated with a drop of the suspension of crystals in 70 % saturated $(NH_4)_2SO_4$. The mouth of the centrifuge tube was covered with a filter paper and the solution was left at room temperature to crystallize. Crystals generally appeared within a few hours. The solution was centrifuged at intervals over the next few days until the absence of colour in the supernatant showed that the bulk of the peroxidase had crystallized. The crystals were suspended and stored in 70 % saturated $(NH_4)_2SO_4$ at 0°.

DISCUSSION

The assessment of the exact degree of purity of peroxidase preparations is, at present, a matter of some difficulty. The purpurogallin method is of doubtful value in view of the varying results it has given with different workers. Thus, Keilin & Hartree (1951) found P.Z. 1220 for their purest preparation while Theorell (1942) and Theorell & Maehly (1950) found P.Z. 930 and 1020, respectively, for two crystalline peroxidase preparations. A similar discrepancy existed until recently between the reported values for the haemin content of these preparations. Thus, Keilin & Hartree (1951) found a haemin content of 1·61 %, while Theorell (1942) and Theorell & Maehly (1950) found 1·48 and 1·36 %, respectively. Recently, however, Paul *et al.* (1953) have redetermined the molar absorption of the α-band of pyridine ferroprotoporphyrin and find a higher value than that previously used in calculating the haemin content of the crystalline preparations obtained in Theorell's laboratory. Using this new value, Paul *et al.* (1953) found values of 1·24–1·36 % for these crystalline preparations and 1·36 % for the preparation of Keilin & Hartree (1951). The ratio of the optical densities $E_{402\,m\mu}/E_{280\,m\mu}$. ('Reinheitzahl', R.Z.) has also been used by Theorell & Maehly (1950) as a criterion of peroxidase purity, but while these authors find R.Z. 3·04 for one crystalline preparation Maehly (1953) finds R.Z. 2·3 and 2·6 for two other crystalline preparations. The final dry preparations obtained in the present work had P.Z. 1040–1120; R.Z. 2·3–2·5; and 1·1–1·2 % haemin. The haemin content as estimated by the method of Paul *et al.* (1953) at present appears to give a better measure of agreement than the P.Z. or R.Z. Using the haemin content as the standard the final dry preparations obtained by the method described in the present work have a purity of about 90 %.

SUMMARY

1. A simple method is described for the preparation of highly purified peroxidase from horseradish roots.

2. Stable, dry preparations are obtained with P.Z. 1040–1120 and haemin contents of 1·1–1·2 %.

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