A RAPID METHOD OF DETERMINING PEROXIDASE ACTIVITY

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It was reported previously (Srinivasan, 1936) that ascorbic acid oxidase in drumstick is accompanied by peroxidase. In subsequent experiments on the separation of these two enzymes, the need arose for a rapid method of determining peroxidase activity. We found that the method of Willstätter and Stoll (1917), involving, as it does, large volumes of reaction mixture and good quantities of ether, did not quite meet our experimental requirements. Search among other known methods indicated that the one based on the oxidation of benzidine to purpurobenzidine (Zirm, *et al.*, 1932) might prove simple and rapid. On actually trying out this method, however, it was found that the filtration of the dye prior to its dissolution in absolute alcohol, was tedious and time-consuming. It became obvious that the difficulty could be overcome by the use of a partially miscible and high-boiling solvent for extracting out the purpurobenzidine quantitatively. We observed that *n*-butyl alcohol was found to be an ideal solvent for the purpose. Based on this observation, the original method of Zirm, *et al.*, has been modified as described herein.

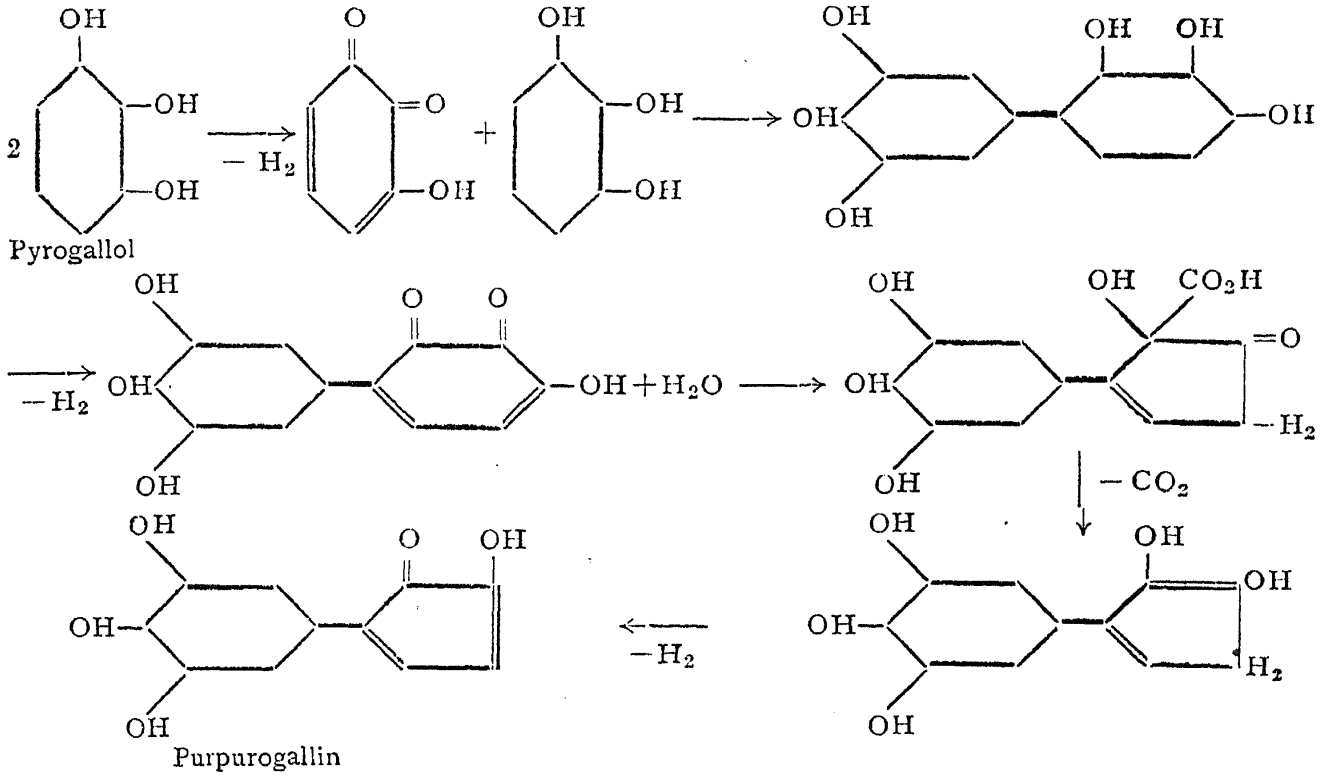
Different methods have been proposed from time to time for the quantitative determination of peroxidase. These methods are all based on the production of coloured compounds from one or the other of the following substrates in presence of H_2O_2 and the enzyme: Quinol (Bertrand, 1894); phenol (Bourquelot, 1896[1]); guaiacol (Bourquelot, 1896[2]); guaiaconic acid or guaiacum (Moore and Whitley, 1909); α-naphthol (Bourquelot, 1896[3]); benzidine (Schreiner, 1909); *p*-phenylene-diamine + α-naphthol (Vernon, 1911). Of these the guaiac reaction is unsatisfactory and unspecific for the determination of peroxidase, for, hæmoglobin, as well as hæmocyanins and many metallic chlorides give the reaction (Alsberg, 1908). One of the earliest methods for the quantitative determination of peroxidase is due to Bach and Chodat (1904). The method makes use of the liberation of iodine by peroxidase from acidified KI in presence of H_2O_2.

The method, now almost universally employed, is due to Willstätter and Stoll (1917). A solution of 5 g. of purest pyrogallol in 2 l. H_2O is

mixed with about 10 ml. of 5% H_2O_2 (exactly 50 mg.), regulated to 20° C. in a thermostat and then treated with 1–5 ml. of 5 mg. of the enzyme in 100–500 ml. H_2O. After exactly 5 minutes, the reaction is stopped by adding 50 ml. of dil. H_2SO_4 and the purpurogallin extracted with ether and estimated colorimetrically by comparison with a solution containing 100 mg. of the pure pigment in 1 l. ether. Elliot and Keilin (1934) found the estimation satisfactory and more convenient using one-fourth the quantities. Simply represented the reaction is:

$$2\,C_6H_6O_3 + 3\,O + H_2O = C_{11}H_8O_5 + CO_2 + 3\,H_2O.$$

Willstätter and Heiss (1923), however, found the mechanism of purpurogallin formation to be complex thus:—



Grassman comments on this method: 'Eigentümliche Schwankungen der enzymatischen Wirksamkeit, die bei reineren Peroxydasepräparaten beobachtet werden, lassen trotzdem die Sicherheit der quantitativen Methode zweifelhaft erscheinen. In der Tat scheint die Wahl einer so kompliziert verlaufenden Oxydationsreaktion nicht unbedenklich zu sein. Es ist mit der Möglichkeit zu rechnen', " dass von den wechselnden Begleitstoffen der Peroxidase irgendwelche auf eines der sehr reaktionsfähigen Zwischenprodukte der Purpurogallinbildung einwirken und dadurch den quantitativen Verlauf der Reaktion stören ".

Therefore, Willstätter and Weber (1926) developed a simpler method which involves only one atom of oxygen without the formation of any

intermediate products. This method consists in the oxidation of leuco-base of malachite green in acetate buffer by peroxidase and H_2O_2. Quite inexplicably, this later method, however, does not appear to have found as much favour as the purpurogallin method.

The formation of indophenol from a mixture of *p*-phenylene-diamine and α-naphthol in citrate buffer at pH 4·5, is the basis of yet another quantitative determination of peroxidase, developed by Guthrie (1931) and later modified by Pack (1934).

Test for peroxidase activity using benzidine as substrate is due to Schreiner (1909). This qualitative reaction was developed into a quantitative method by Zirm, *et al.* (1932). Their method in brief is as follows:— A 1% solution of benzidine in acetate buffer is treated with an optimum concentration of H_2O_2 and the test solution of peroxidase. After an interval of 5 minutes, strong alkali (33%) is added, the precipitate consisting of purpurobenzidine and unreacted benzidine is dissovled in absolute alcohol and the colour of the resulting solution compared with a standard solution of the same pigment obtained by oxidation of benzidine by permanganate. The use of absolute alcohol, which the method enjoins, makes its imperative that the precipitate of the dye should be filtered off from the aqueous solution. This operation is by no means easy or quick, mainly due to the strong alkali that the reaction mixture contains. On the other hand, it was found that with the use of butyl alcohol, filtration could be avoided, the dye being quantitatively taken up from the reaction mixture by butyl alcohol. This constitutes the main improvement of the present method.

It should be emphasised that no attempt has been made here to evolve a method for the determination of the absolute activity of peroxidase in a given preparation; from that point of view, Willstätter's method is perhaps the best. Rather, the aim has been to evolve a method which would be simple and rapid and therefore useful for a series of comparative determinations of peroxidase activity of a preparation at various stages of its purification. From this standpoint, we found that the method of Zirm, *et al.*, but using butyl alcohol, was found eminently suitable.

We also thought it beside our present purpose to go into the question of the mechanism of the formation of purpurobenzidine. For the same reason, the correlation of purpurobenzidine number with that of purpurogallin has been omitted. We are aware of the more interesting question whether butyl alcohol cannot be employed with equal facility for extracting purpurogallin in Willstätter's method or the other dyes in the various

methods. The advantages of using butyl alcohol in the colorimetric methods are obvious.

Experimental

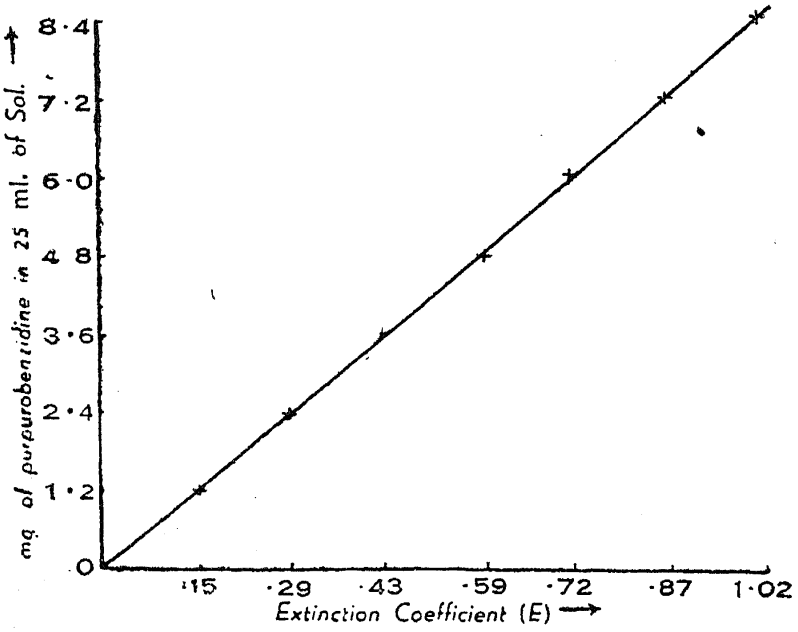
Preparation of the enzyme solution.—As source of enzyme, the fruit of *Sechium edule* (*cucurbitacæ* family) was chosen. The fresh vegetable is minced and pressed. The *press* juice, on centrifuging, yields a clear liquid. On half saturation with ammonium sulphate, a precipitate separates which is recovered by centrifuging. The precipitate is dissolved in the minimum amount of water and the resulting solution formed our stock solution of enzyme.

Purpurobenzidine.—Pure benzidine (2 g.) is heated with a mixture of 100 ml. N sodium acetate solution and 50 ml. of glacial acetic acid. The solution is filtered and the filtrate made up to 200 ml. The final solution is dark yellow in colour and has a pH of 3·5 to 3·65. To 60 ml. of this stock solution is added 90 ml. of N/200 $KMnO_4$ in a separating funnel (500 ml.). The mixture is vigorously shaken for 20 minutes when the solution attains a purple colour. Now strong alkali (30% NaOH) is added to make the solution distinctly alkaline to thymol blue (*i.e.*, pH $>$ 10·0). The red dye which is precipitated is extracted with 100 ml. butyl alcohol. The extraction is complete and quantitative, as the aqueous layer becomes colourless after the process. The butyl alcohol layer, which is coloured orange red, is washed free of alkali, dried with anhydrous sodium sulphate and filtered. The concentration of purpurobenzidine in this stock solution is found by the determination of the total solids in a measured volume of the solution. The stock solution we prepared had a concentration of 2·4 mg. of purpurobenzidine per ml.

Standard graph.—Different aliquots of the dye solution standardised as above are diluted to 25 ml., with ethyl alcohol. The extinction coefficients of these different solutions are determined in a Pulfrich Photometer (Filter, S_{53} and Cell, 20·06 mm.). The control consists of butyl alcohol diluted with ethyl alcohol in concentrations corresponding to the experimental solution.

From a reference to this standard graph (Graph I), the quantity of dye formed by enzymatic oxidation in the subsequent experiments is computed.

Determination of peroxidase activity.—After repeated trials, the following conditions were found to be most suitable for the determination of the peroxidase activity: 2 ml. of benzidine acetate solution (pH 3·5–3·65), prepared as above, are mixed with 48 ml. distilled water and aliquots of the test enzyme solution (suitably diluted). To this mixture contained in a separating funnel 1 ml. H_2O_2 (1·8 mg.), which was found to be the optimum



GRAPH I

Purpurobenzidine vs. E

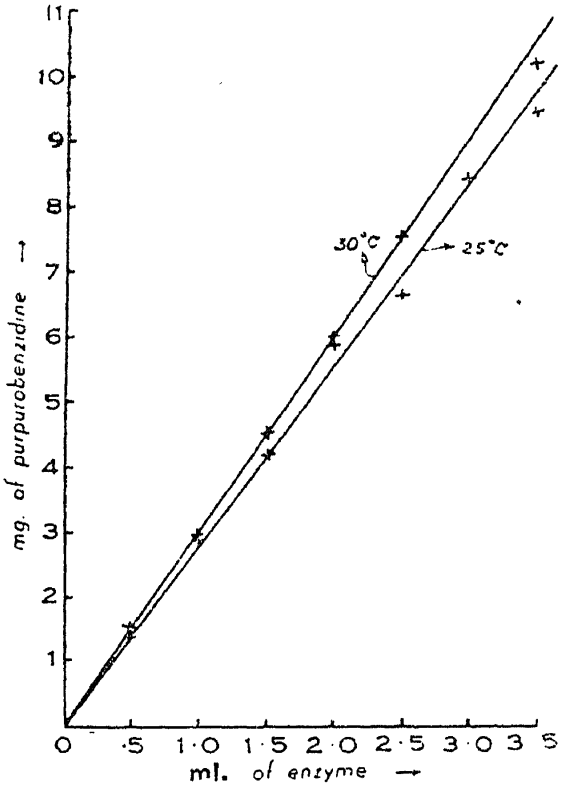
quantity (see Fig. 3), is added and well shaken. Exactly 5 minutes after the addition of H_2O_2, the reaction is arrested by the addition of 2 ml. of 25% NaOH. The solution is saturated with NaCl and shaken with 5 ml. butyl alcohol. After the separation of layers, the aqueous layer is drawn off and discarded. The butyl alcohol layer is run into a 25 ml. flask through a filter of cotton wool to retain insoluble impurities, if any. The separator is washed with ethyl alcohol and the washings passed through the original

TABLE I

Volume of enzyme solution (original stock solution diluted 50 times)	E (Observed)		Mg. of dye produced (calculated for the standard graph)	
	At 30° C. (thermostat)	At 25° C. (room)	At 30° C. (thermostat)	At 25° C. (room)
ml.				
0·5	0·18	0·17	1·49	1·40
1·0	0·36	0·35	2·98	2·88
1·5	0·54	0·51	4·48	4·21
2·0	0·73	0·71	6·00	5·89
2·5	0·90	0·81	7·46	6·60
3·0	1·03	1·02	8·54	8·41
3·5	1·23	1·15	10·20	9·46

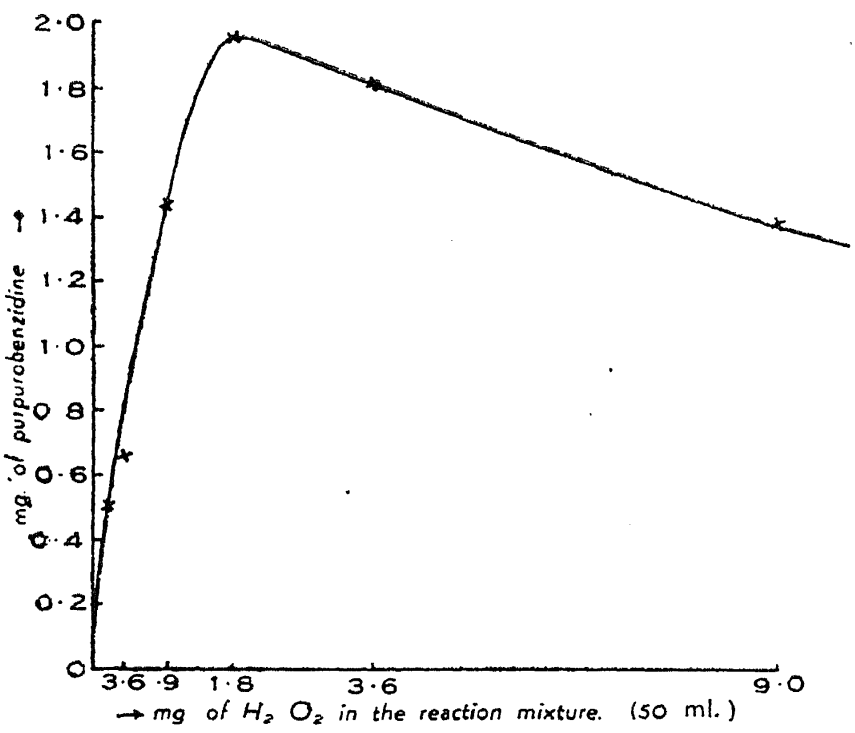
filter into the receiver. The contents are made up to 25 ml. with ethyl alcohol. A control is simultaneously run, but with the boiled enzyme. The extinction coefficients of the experimental and control solutions are read as before in the Pulfrich photometer. From these readings, the amount of dye is calculated from the standard graph.

The above table and Graph II represent the relation between enzyme action and dye formation. Under the conditions of the experiment, it is found, therefore, that the dye formed is strictly proportional to enzyme



GRAPH II

Enzyme Concentration vs. Purpuro-Benzidine Formation



GRAPH III

H_2O_2 Concentration vs. Enzyme Action (in terms of Purpurobenzidine)

concentration. Consequently, for the range of enzyme concentration studied, the quantity of purpurobenzidine which is determined, gives us an exact measure of peroxidase activity.

Summary

1. Butyl alcohol has been found to extract quantitatively purpurobenzidine formed from benzidine in acetic acid sodium acetate buffer (pH 3·5–3·65) by peroxidase and optimum concentration of H_2O_2.

2. This property has been made the basis of a quantitative determination (colorimetric) of peroxidase.

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