

Short Communications

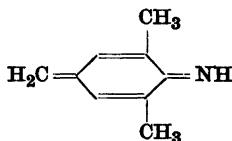
The Assay of Horse Radish Peroxidase (HRP) with Mesidine

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Several procedures have been suggested for the assay of HRP (for references see ¹), of which the purpurogallin test is the most commonly used. There is a common experience of this method that it frequently gives erratic results, which have been attributed to impurities in the pyrogallol or in the distilled water. Byproducts can also sometimes appear in the reaction.

Chapman and Saunders ², studying the HRP-catalyzed oxidation of mesidine (2,4,6-trimethylaniline) by hydrogen peroxide, found that mesidine gave rise only to one compound (2,6-dimethyl-benzoquinone-4-(2',4',6')anil, "purple compound"), which could be isolated in 95% yield. One mole of ammonia is liberated during the reaction. They also suggested as a hypothesis that an intermediary compound could be formed with the subsequent



release of the methylene group as formaldehyde.

We have confirmed their hypothesis and found the over-all reaction formula to be
 $2 \text{ mesidine} + 3 \text{ H}_2\text{O}_2 = 1 \text{ purple compound} + 1 \text{ NH}_3 + 1 \text{ HCHO} + 4 \text{ H}_2\text{O}$;

The formaldehyde was identified as methylene dimedone (80% yield, m.p. 189–192° after 3 crystallizations).

When the reaction is carried out at 0° and pH 4.7–4.8 (5 mM H₂O₂, 15 mM mesidine, total volume 2 ml) and stopped by the addition of one volume of acid alcohol, the formation of the purple compounds is linearly proportional to the time after a certain "lag" phase. This permits the calculation of the amount of HRP from the slope of $\log \frac{I_0}{I}$ at 490 m μ vs. time according to

$$[\text{HRP}] = k \times \frac{\log \left(\frac{I_0}{I} \right)_2 - \log \left(\frac{I_0}{I} \right)_1}{t_2 - t_1};$$

[HRP] and reaction time must be chosen so that $\log \left(\frac{I_0}{I} \right)_1 > 0.175$, and the time to reach or pass this value less than 1.2 min. Practically this corresponds to [HRP] = 15–75 m μ M. For the specimen of HRP (twice crystallized) employed in our determinations, HRP is obtained in m μ M when $k_1 = 89.3$. Experiments with other preparations of HRP are being done to test the reproducibility of the numerical values of k .

The reaction is very little, if at all, sensitive to ferric and cupric ions.

We express our thanks to Dr. E. B. Saunders for suggesting mesidine as a suitable hydrogen donor for the method.

1. Zeile, K. in Bamann, E., and Myrbäck, K. *Die Methoden der Fermentforschung*, Georg Thieme, Leipzig, 1941, p. 2598. Theorell, H. in Sumner, J. B., and Myrbäck, K. *The Enzymes*, Vol. II, Academic Press, New York, 1951, p. 397.
2. Chapman, N. B., and Saunders, B. C. J. *Chem. Soc.* 1941 496.

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