The Study of Horseradish Peroxidase Catalyzed Oxidations by Means of Chemiluminescence

GUNNAR AHNSTRÖM,
GÜNTHER v. EHRENSTEIN and
ROBERT NILSSON

Institute of Organic Chemistry and Biochemistry, University of Stockholm, Sweden

In the oxidation of pyrogallol coloured endproducts are formed, the main component of which is presumably purpurogallin [1]. This process is also accompanied by chemiluminescence [2].

A standard method for the determination of peroxidase activity has been the measurement of the amount of purpurogallin formed in the enzymic oxidation of pyrogallol by hydrogen peroxide. In the present investigation we have studied the above mentioned reaction by means of light emission measurements.

Experimental. The equipment used for the luminescence measurements is described in detail in a previous paper [3]. A Sigma HRP preparation of a purity corresponding to 21 purpurogallin units per mg (20 sec.) was used [1]. All other chemicals were analytically pure. To prevent autoxidation, the freshly prepared pyrogallol solutions were immediately acidified.

Phosphate buffer, pyrogallol and enzyme solutions were mixed in a Beckman standard cuvette, and the volume of the reaction mixture adjusted to 1 ml by the addition of distilled water. The reaction was initiated by injection of the peroxide. The luminescence intensity was then recorded as a function of time under varying peroxide, pyrogallol and enzyme concentrations.

Results. The effect of varying HRP-concentrations on the light intensity-time dependance curve of constant peroxide and pyrogallol concentrations is shown in Fig. 1.

It may be seen from this figure that after a certain induction period the light intensity reaches a fairly constant maximum value and then slowly decreases. This probably implies that in the enzymic oxidation of the hydrogen donor the concentration of an active, intermediate molecular species is built up which then undergoes a secondary chemiluminescent elimination without necessarily requiring the participation of the enzyme protein. This is supported by Chance's observation [4] that the formation of reaction products continues long after the active enzyme-peroxide, usually rate determinating, complex (complex II) has disappeared. However, at present no method is available for the determination of the actual concentrations of the enzyme peroxide complexes at these low HRP concentrations. The rate of production of the intermediate directly involved in the luminescent reaction is dependent upon the enzyme concentration and, in consequence, the light intensity during the steady state, when the rate of production of this species is balanced by its chemiluminescent elimination, is also a function of the same.

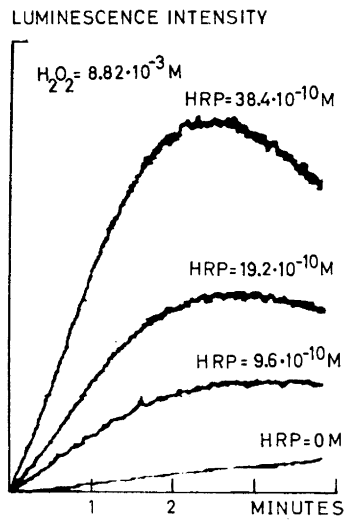

Fig. 1. Luminescence intensity as a function of time at different HRP concentrations. H_2O_2 and pyrogallol concentrations constant.

Fig. 2 shows the light intensity at the plateau as a function of the HRP concentration at four H_2O_2 levels. In the presence of a larger excess of peroxide the inhibition previously noted by Mann [5] is observed. This inhibition is illustrated in more detail in Fig. 3 which shows the variations in the light intensity at steady state for increasing peroxide concentrations at five different enzyme levels.

The effect of varying pyrogallol concentrations at constant peroxide and enzyme

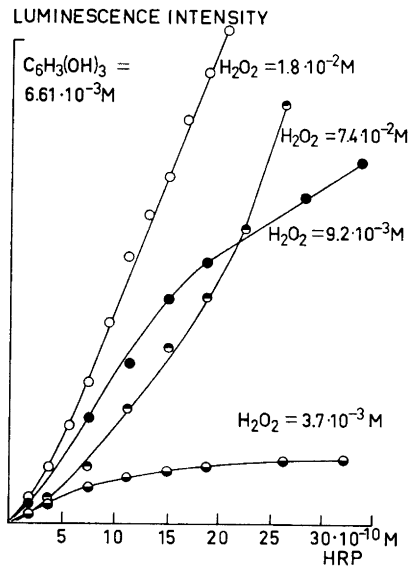

Fig. 2. Luminescence intensity at steady state as a function of HRP concentration at different H_2O_2 levels. Pyrogallol concentration constant.

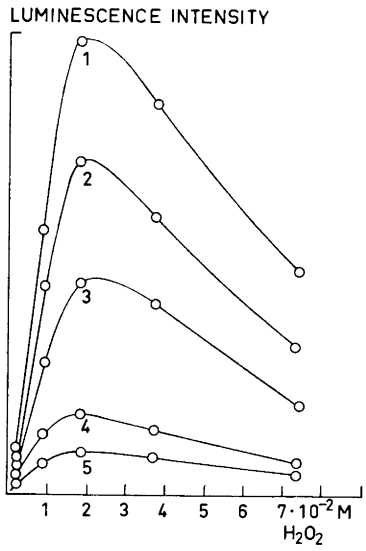

Fig. 3. Luminescence intensity at steady state as a function of H_2O_2 concentration at five different enzyme levels. Pyrogallol concentration constant = $6.61 \cdot 10^{-3}$ M. HRP = 1) $19 \cdot 10^{-10}$ M; 2) $14 \cdot 10^{-10}$ M; 3) $9.5 \cdot 10^{-10}$ M; 4) $4.7 \cdot 10^{-10}$ M; 5) $2.8 \cdot 10^{-10}$ M.

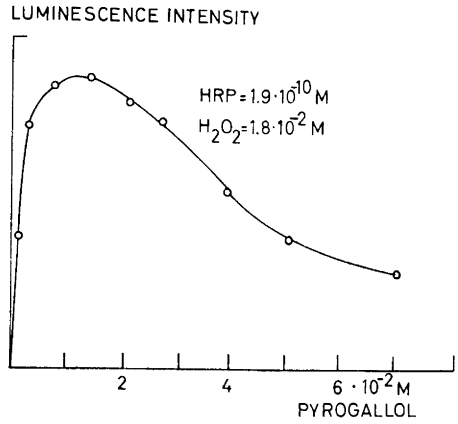

Fig. 4. Luminescence intensity at steady state as a function of pyrogallol concentration. HRP and H_2O_2 concentrations constant.

concentrations is illustrated in Fig. 4. The previously observed inhibition effect is marked at the highest donor concentrations [5].

The classical purpurogallin method has the disadvantage of being very sensitive to factors such as the presence of impurities in the reaction mixture, concentration of buffer etc.[6] We have not found these factors to influence the reproducibility of the results obtained by the luminescence measurements to any appreciable extent. Thus, ordinary tap water could be used.

In dealing with media of high optical density, the emission measurements are definitely preferable to absorption measurements, as the surface of the reaction mixture, in the former case, can be increased, resulting in an appreciable decrease in the internal absorption while retaining the photon counting efficiency.

Furthermore, this method is not restricted to the utilization of pyrogallol as reducing substrate. By the use of very low concentrations of pyrogallol in the presence of an excess of another hydrogen donor to be studied, the pyrogallol is rapidly oxidized in a nonenzymic luminescent reaction by the reaction products formed from the reducing substrate present in excess. Due to this "scavenging" property of the pyrogallol, the light emission under these conditions provides a means of investigating the oxidation of various other substances such as *o*-phenylenediamine, *p*-phenylenediamine and others.

Acknowledgement. The present investigation was supported by grants from *Kungl. Universitetets i Stockholm matematisk-naturvetenskapliga fakultets anslag för främjande av ograduerade forskares verksamhet.*

1. Willstätter, R. and Stoll, A. *Ann.* **416** (1918) 21.
2. Lenard, P. and Wolf, M. *Ann. Phys. und Chem.* 1888, xxxiv, 918.
3. Ahnström, G. *Acta Chem. Scand.* **15** (1961) 463.
4. Chance, B. *Arch. Biochem.* **24** (1949) 410.
5. Mann, P. J. G. *Biochem. J.* **24** (1931) 921.
6. Ettori, J. *Biochem. J.* **44** (1949) 35.

Received September 8, 1961.

3-Substituted Furans

SALO GRONOWITZ and
GUNNAR SÖRLIN

Chemical Institute, University of Uppsala, Uppsala, Sweden

In connection with work on the substituent effects in heterocyclic systems, a number of 3-substituted furans was needed in order to compare the chemical shifts in their NMR-spectra with those of the corresponding thiophenes [1]. Although some 3-substituted furans occur in nature [2] most compounds of this type are only obtained with difficulty. Some compounds have been prepared by modifying the carboxyl group of 3-furancarboxylic acid [3,4], which is obtained by partial decarboxylation of polycarboxyfurans [3-5]. Recently 3-hydroxytetrahydrofuran has been used for the preparation of 3-phenylfuran [6] and new methods for the synthesis of 3-methylfuran from commercially available aliphatic compounds have been worked out [7,8]. These methods give a carbon-carbon bond in the 3-position.

In the thiophene series it has been shown that 3-thienyllithium, obtained through halogen-metal interconversion between 3-bromothiophene and *n*-butyllithium at $-70°$, is a very useful intermediate both for the formation of a new carbon-carbon bond in the 3-position and also for the introduction of other types of substituents (for review *cf.* Ref.[9]). Of the two 3-halofurans which are suitable for an analogous investigation we found that 3-iodofuran [10] could be more easily and reproducibly synthesised than 3-bromofuran [11] owing to difficulties in the reduction of 4,5-dibromo-2-furancarboxylic acid to 4-bromo-2-furancarboxylic acid. Through minor changes in the Gilman process, which consists of the pyrolysis of the mercury salt of 2-furancarboxylic acid followed by reaction of the resulting chloromercury furan with iodine, the yield of 3-iodofuran was increased from 4.0 % to 5.1 %, calculated on the weight of the 2-furancarboxylic acid used. The reactions of 3-iodofuran have been investigated very briefly by Gilman and Wright only. They found that it did not give a Grignard reagent, but that with a sodium-potassium alloy it formed a metalorganic compound, which upon carbonation gave 3-furancarboxylic acid in low yield [12]. We have now found that 3-iodofuran undergoes halogen-metal interconversion with *n*-butyllithium at $-70°$ to 3-furyllithium. Upon carbonation this compound gives 3-furancarboxylic acid in good yield, and with N,N-dimethyl formamide it reacts to give 3-furaldehyde. By contrast with 2-bromo- and 2-iodofuran [13], 3-iodofuran reacts with sodium methylate in methanol in the presence of cupric oxide, to give 3-methoxyfuran. It is not considered that the yields obtained in these preliminary experiments are the best obtainable.

Thus, although 3-iodofuran is not so readily available as 3-bromothiophene [14], its halogen metal interconversion at $-70°$ with *n*-butyllithium to form 3-furyllithium (a compound sufficiently stable at this temperature) opens up the route to many 3-substituted furans. Further investigation into its potentialities as an intermediate for such compounds is in progress.

Experimental. 3-Furancarboxylic acid. A solution of 8.8 g (0.045 mole) of 3-iodofuran in 25 ml of dry ether was added to a stirred solution of 47 ml of 1.20 N *n*-butyllithium at $-70°$ in the conventional four-necked, nitrogen-swept flask. After 5 min, the mixture was poured onto solid carbon dioxide covered with ether. The reaction mixture was hydrolyzed with water and the ether phase extracted with sodium carbonate solution. The 3-furancarboxylic acid was precipitated out on acidification with dilute hydrochloric acid. One recrystallization from water yielded 3.2 g (63 %) of pure 3-furancarboxylic acid, m.p. 122–123°. (Literature value:[5] m.p. 122–123°.) The characteristic peaks