The simplest way of recording the pattern is to photograph the horizontal sweeps on a vertically moving film running continuously at slow speed, with the external (vertical) time-base circuit disconnected.

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1 Elmore, W. C., and Sands, M., "Electronics", 249 (McGraw-Hill Book Co., New York, 1949).
2 Kolmodin, G. M., and Skoglund, C. R., *Experientia*, 10, 505 (1954).
3 Andrew, A. M., and Roberts, T. D. M., *Electronic Eng.*, 26, 469 and 543 (1954).

A Sensitive Method for the Estimation of Hydrogen Peroxide in Biological Materials

During recent years a number of methods for the estimation of hydrogen peroxide which utilize the principle of coupled oxidation have been published. Catalase or peroxidase combines with hydrogen peroxide as it is liberated, and the complex thus formed then brings about the oxidation of such substances as nitrite[1], ethanol[2], cytochrome *c*[3], or manganous ions in presence of *p*-cresol[4]. The extent of these oxidations may be measured by manometric, colorimetric or spectrophotometric means. The method of detecting hydrogen peroxide to be described is based on the same principle but has the advantage of greater sensitivity. In this case the disappearance of the fluorescent peroxidase substrate scopoletin (6-methyl-7-hydroxy-1 : 2-benzopyrone) is observed with the aid of a suitable fluorometer. The Beckman spectrophotometer model *DU* used as for the estimation of fluorescence in solution (Beckman Bull. 149-*G*) was found satisfactory, a concentration of 2×10^{-10} mole per ml. giving a reading of 100 divisions on the intensity scale with the instrument adjusted to its highest sensitivity. Up to $2{\cdot}5 \times 10^{-9}$ mole per ml., the intensity of fluorescence is proportional to the concentration of scopoletin (Fig. 1).

An aqueous solution of scopoletin is reasonably stable in diffused light and is not oxidized by either peroxidase or hydrogen peroxide alone. However, if peroxidase and hydrogen peroxide are both added, oxidation with loss of fluorescence proceeds very rapidly. Fig. 2 shows the quantitative relationship between the amount of hydrogen peroxide added and disappearance of scopoletin. In this experiment each reaction mixture was made up to 1 ml. and contained an aqueous extract of commercial dehydrated horse radish at *p*H 4·5, $2{\cdot}5 \times 10^{-8}$ mole of scopoletin and varying amounts of hydrogen peroxide. After a few minutes, 9·0 ml. of borate buffer (*p*H 10) was added and the residual scopoletin fluorescence measured. Precisely one mole of hydrogen peroxide was required for the oxidation of one mole of scopoletin. This simple stoichiometry applied so long as the scopoletin was present in about 20 per cent excess.

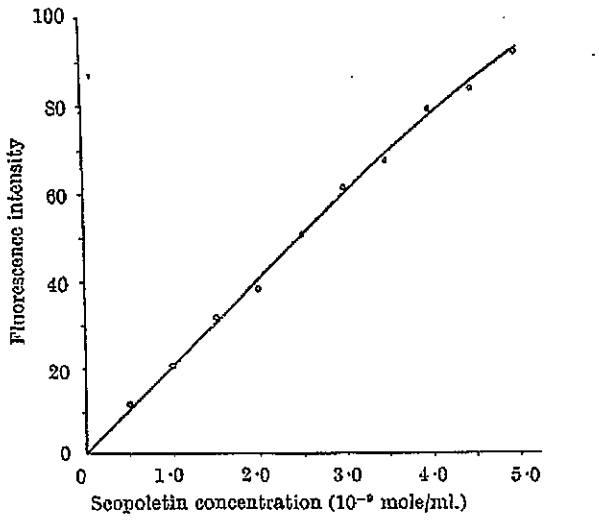

Fig. 1. Fluorescent intensity of scopoletin solutions at concentrations up to 5×10^{-9} mole per ml. in borate buffer *p*H 10

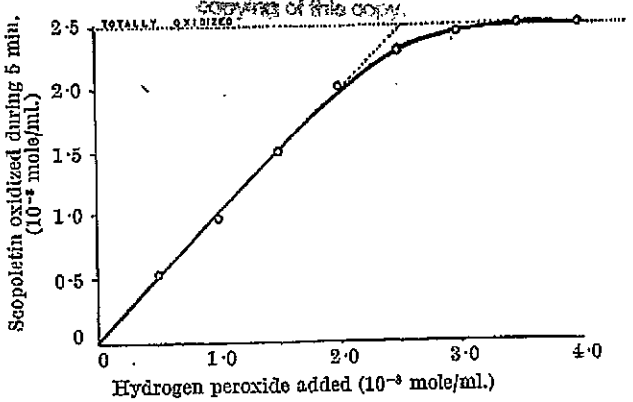

Fig. 2. Relationship between the quantity of hydrogen peroxide and the amount of peroxidative oxidation of scopoletin. Reaction mixtures contain per ml. $2{\cdot}5 \times 10^{-8}$ mole scopoletin, hydrogen peroxide as indicated in the figure and aqueous extracts of dehydrated horse radish at *p*H 4·5

The experiment was repeated using metabolically produced hydrogen peroxide. Pea epicotyl juice contains a diamine oxidase which produces one mole of hydrogen peroxide per mole of putrescine oxidized[4]. It also contains a peroxidase. Fig. 3 illustrates the effect of time on the oxidation of scopoletin by pea epicotyl juice (*p*H 6·8) when enzymatic oxidation of putrescine contributes the necessary hydrogen peroxide. In this experiment $1{\cdot}2 \times 10^{-8}$ mole of putrescine was added to a ten-fold excess of scopoletin. The reaction commenced immediately but proceeded slowly, coming to completion within 11 min. About 80 per cent of the predicted amount of scopoletin was oxidized. On a second addition of $1{\cdot}2 \times 10^{-8}$

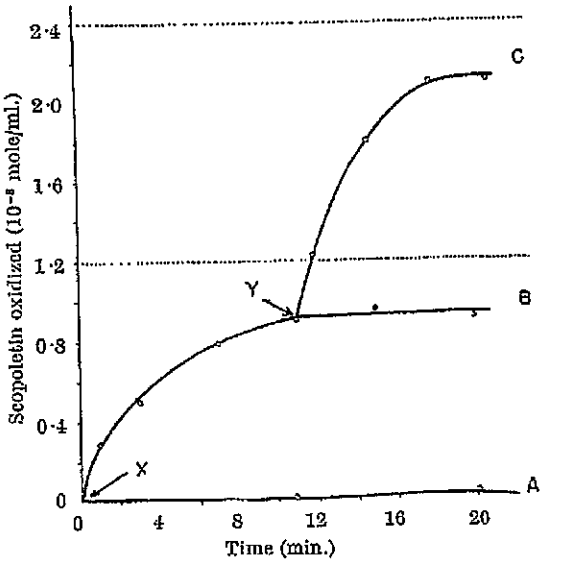

Fig. 3. Rate of oxidation of scopoletin ($1{\cdot}2 \times 10^{-8}$ mole per ml.) by pea juice *p*H 6·8 with putrescine as source of endogenous hydrogen peroxide (curve *A*). Additions: curve *B*, $1{\cdot}2 \times 10^{-8}$ mole putrescine (at *X*); curve *C*, a second addition of $1{\cdot}2 \times 10^{-8}$ mole putrescine 11 min. later (at *Y*)

mole of putrescine, the reaction was faster and oxidation of the theoretical amount of scopoletin took place. When equivalent amounts of hydrogen peroxide were added to the reaction mixture instead of putrescine, it was found that only 66 per cent of the theoretical amount of scopoletin oxidation occurred. Similarly, a second addition of hydrogen peroxide resulted in the theoretical amount of oxidation. Apparently there are present in pea juice alternative peroxidase substrates which may interfere, especially in the more alkaline *p*H ranges. For this reason it sometimes becomes necessary to remove these interfering substances by a preliminary oxidation before proceeding with the quantitative estimation of hydrogen peroxide production.

A number of reducing substances are capable of competitively inhibiting the oxidation of scopoletin by peroxidase; for example, ascorbic acid, glutathione and manganous ions. Consequently other fluorescent phenolic coumarin derivatives were investigated. Esculetin is more readily oxidized by peroxidase than is scopoletin, and ascorbic acid does not interfere, but unfortunately its fluorescence is relatively weak and, moreover, its oxidation by molecular oxygen is catalysed by polyphenol oxidase (scopoletin is not oxidized by polyphenol oxidase over a period of four hours). On the other hand, umbelliferone is resistant to peroxidative oxidation. Scopoletin therefore appears to be the best fluorescent indicator so far found for the peroxidative estimation of hydrogen peroxide. After interfering substances have been removed by a preliminary oxidation, amounts of endogenously produced hydrogen peroxide in the range of 10^{-10} mole can be determined quantitatively. This represents a hundred-fold increase in sensitivity over previous methods. The limitations of available analytical procedures are in large part responsible for the paucity of information of hydrogen peroxide metabolism in plant and animal tissues. This new method incorporates features which make it particularly suitable for biological investigations.

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Dec. 23.

[1] Thurlow, S., *Biochem. J.*, 19, 175 (1925).
[2] Kellin, D., and Hartree, E. F., *Proc. Roy. Soc.*, B, 119, 141 (1936); *Biochem. J.*, 39, 293 (1945).
[3] Altschul, A. M., Abrams, R., and Hagness, T. R., *J. Biol. Chem.*, 136, 777 (1940); 142, 303 (1942).
[4] Konten, R. H., and Mann, P. J. G., *Biochem. J.*, 45, 255 (1949); 46, 67 (1950); 50, 300 (1951).

The Primary Photochemical Process in Bromine

The absorption spectrum of bromine exhibits a continuum which commences at about 5107 A., and it is known that the absorption of light of wave-length less than this value causes the dissociation of bromine into an atom in its normal $^2P_{3/2}$ state and one in the excited $^2P_{1/2}$ state. Photochemical studies have shown that bromine molecules are also dissociated when irradiated with light of wave-lengths greater than 5107 A. Thus the quantum yield of the hydrogen–bromine reaction[1] is approximately the same at 5460 A. as for light of wave-length in the continuum, that is, 5107 A. Further, it has been demonstrated that the quantum yield[2,3] for the photobromination of ethylene is independent of wave-length between the limits 4800 and 6800 A.

Two theories have been proposed to explain this constant quantum yield for light with wave-length in the discrete region of the bromine spectrum. Jost[1] suggested that the excited bromine molecules formed by the absorption of radiation with wave-length in the banded region of the spectrum dissociate with high yield into normal ($^2P_{3/2}$) atoms on collision with other molecules. Kistiakowsky and Sternberg[3] have controverted this point of view and instead suggest that the absorption of light at the longer wave-lengths is largely due to bromine molecules possessing some vibrational energy due to thermal excitation. The main evidence in support of this new theory comes from measurements of the temperature variation of the coefficients of absorption of light for bromine. In this communication it is shown that theoretical calculations of the temperature variation of the absorption of light by vibrationally excited bromine molecules yield results in good agreement with the experimental data of Kistiakowsky and Sternberg.

The intensity of absorption of light of frequency ν is given by the formula:

$$I^{v'v''}_{\text{abs.}} = \frac{8\pi^3}{3hc} I_0 \, \Delta x \, N_{v''} \, \nu \, |R^{v'v''}|^2 \qquad (1)$$

where I_0 is the intensity of the incident radiation, Δx is the thickness of the absorbing layer, $|R^{v'v''}|$ is the matrix element for the transition between the electronic states with vibrational levels v' and v'', and $N_{v''}$ is the number of molecules in the vibrational level v'' of the ground-state. $N_{v''} = N \dfrac{\exp\{-G_0(v'')hc/kT\}}{f}$, where f is the partition function, and $G_0(v'') = \omega_e v'' - \omega_e x_e v''^2 + \omega_e y_e v''^3 \ldots$, $\omega_0 = \omega_e - x_e\omega_e + 3\omega_e y_e/4 \ldots$, and $\omega_e y_0 = \omega_e y_e \ldots$. Since with Kistiakowsky and Sternberg[3] we suppose that the absorption of radiation by the vibrationally excited bromine molecules leads to dissociation, it is easy to see that the ratios of the absorption coefficients at two different temperatures T_1 and T_2, for light of a given frequency, are:

$$I(T_2)/I(T_1) = N_{v''}(T_2)/N_{v''}(T_1) = \exp\{-G_0(v'')hc(T_1 - T_2)/kT_1T_2\} \qquad (2)$$

For the ground $^1\Sigma^+_g$ state of bromine[4], $\omega_e = 323{\cdot}2$ cm.$^{-1}$ and $\omega_e x_e = 1{\cdot}07$ cm.$^{-1}$; $\omega_e y_e$ is unknown but can be neglected. Using these spectroscopic constants, the ratio $I(422{\cdot}1°\text{ K.})/I(308{\cdot}1°\text{ K.})$ has been calculated for bromine molecules excited in various vibrational levels of the ground-state. Table 1 gives the results of these calculations and shows also the values calculated from the experimental data of Kistiakowsky and Sternberg.

The agreement between the two sets of values is particularly good in the case of the $v'' = 3$ level, and the general concordance between the theoretical and the experimental values for these three levels must

Table 1. Absorption Coefficients for Vibrationally Excited Bromine Molecules

v''	$G_0(v'')$ cm.$^{-1}$	$I(422{\cdot}1)/I(308{\cdot}1)$ Calculated	Experimental
1	321·06	1·49	1·11
2	639·98	2·23	1·7
3	956·76	3·32	3·46