Assessment of Acyl Groups and Reaction Conditions in the Competition between Perhydrolysis and Hydrolysis of Acyl Resorufins for Developing an Indicator Reaction for Fluorometric Analysis of Hydrogen Peroxide

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Perhydrolysis of acetyl resorufin (AR) was reported previously to work as a fluorometric indicator reaction for glucose determination using only glucose oxidase. However, hydrolysis of AR in blank solution rendered the working concentration range of this method less than two orders of magnitude. To exclude or at least significantly reduce this interference, acyl groups and reaction conditions in the competition between perhydrolysis and hydrolysis of various acyl resorufins were assessed. Fluorometric evaluation of reactions in the presence or absence of H_2O_2 in phosphate buffer (pH 7.5, 100 mM)–CH_3CN at 25 °C demonstrated that in *tert*-butylacetyl, isobutyryl, cyclohexanecarbonyl and pivaloyl resorufins (TBAR, IBR, CHR and PVR, respectively) among 10 acyl resorufins examined here, the competitive situation was shifted in a much more favorable way to perhydrolysis than in AR, although fluorometric responses due to their H_2O_2-dependent deacylation were suppressed in comparison with AR. Examination of the effects of pH, components and concentrations of buffers as well as reaction temperature established reaction conditions that not only allowed perhydrolysis of each of these four compounds to prevail over hydrolysis more effectively, but also improved the H_2O_2-based fluorometric responses. Thus, perhydrolysis of TBAR, IBR, CHR and PVR in phosphate buffer (pH 8.0, 20 mM)–CH_3CN at 25 °C worked effectively as fluorometric indicator reactions for H_2O_2 analysis, affording a calibration curve over a concentration range of three orders of magnitude. Taking sensitivity, reproducibility and the response for blank solution into consideration, PVR seemed to be the best choice as a fluorochromogen for H_2O_2 determination under these conditions. For H_2O_2 analysis at lower pH, perhydrolysis of IBR in phosphate buffer (pH 7.5, 20 mM)–CH_3CN was shown to effectively function as an indicator reaction. Applicability of the fluorometric methods with PVR and IBR to blood glucose determination was also discussed, comparing with Trinder's method with phenol, 4-aminoantipyrine and peroxidase (POD).

Key words acyl resorufin; perhydrolysis; hydrolysis; fluorometric analysis; hydrogen peroxide; deacylation

Recently, we found that acetyl resorufin (AR) is transformed to fluorescent resorufin through deacetylation by H_2O_2 (perhydrolysis), and hence perhydrolysis of AR can be used as a fluorometric indicator reaction for glucose determination using only glucose oxidase (GOD).[1] In the fluorometric method with GOD and AR, glucose analysis is performed without interference by ascorbic acid, uric acid or bilirubin. These compounds interfere with colorimetric determination of serum components with indicator reactions based on H_2O_2-dependent oxidative coupling of two chromogens in the presence of peroxidase (POD), which are represented by so called Trinder's reactions of 4-aminoantipyrine with phenolic or anilinic derivatives.[2] Thus, it was demonstrated that the fluorometric method with GOD and AR offers a more reliable and accurate tool for determination of blood glucose than the POD-dependent colorimetric method with 4-aminoantipyrine and phenol.[3] However, there is a problem to be resolved in utilizing perhydrolysis of AR as a general fluorometric indicator reaction for determination of H_2O_2: although AR remains intact in CH_3CN at room temperature for more than 6 months,[3] AR undergoes spontaneous hydrolysis when its CH_3CN solution is mixed with pH 7.4 phosphate buffer (blank solution), which prevents the fluorometric method from being employed for the measurement of glucose over a concentration range of more than two orders of magnitude.

The key to success in improving the measurement range in the fluorometric method seems to be molecular design conferring the ability to resist hydrolysis of AR. The susceptibility of esters to hydrolysis can be controlled by changing steric factors of acyl groups. To our knowledge, there have been no reports of the effects of acyl groups on perhydrolysis of esters, although perhydrolysis of esters or amides has been recognized as a useful tool for generation of peroxyacids in chemical bleaching processes.[4,5] Since the molecular sizes of HOO^- and HO^- are quite similar, steric effects in perhydrolysis and hydrolysis must be in the same order of magnitude. Accordingly, it seemed doubtful whether judicious choice of acyl groups in AR could shift the competition between perhydrolysis and hydrolysis in a manner favorable towards H_2O_2-based deacetylation. However, perhydrolysis of aryl acetates is known to be much faster than their hydrolysis, *i.e.*, the nucleophilic reactivity of HOO^- toward aryl esters is markedly higher than that of HO^-,[6—9] which is referred to as the α-effect: nucleophilicity is enhanced when the atom adjacent to a nucleophilic site bears a lone pair of electrons.[10—12] AR is a type of aryl acetate, and hence the α-effect is likely to be operative in the H_2O_2-dependent deacetylation of AR, which allows the fluorometric determination of glucose with the generation of resorufin as an indicator reaction even though AR undergoes hydrolysis. Taking the α-effect into consideration, it was expected that establishing reaction conditions for the α-effect to be significantly appreciated as well as replacing the acetyl group in AR with acyl groups resistant to hydrolysis will exclude or at least significantly reduce the interference by hydrolysis in fluorometric analysis of H_2O_2. Thus, we assessed acyl groups and reaction conditions in the competition between perhydrolysis and hydrolysis of

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various acyl resorufins. Here, we describe the results of these experiments, demonstrating that perhydrolyses of pivaloyl resorufin (PVR) in phosphate buffer (pH 8.0)–CH_3CN and isobutyryl resorufin (IBR) in phosphate buffer (pH 7.5)–CH_3CN are promising indicator reactions for fluorometric analysis of H_2O_2 over wide working concentration ranges.

Existing indicator reactions for spectrophotometric determination of H_2O_2 are generally classified into two categories: oxidative formation of dyes in the presence of POD[2,13—21] and complex formation with metals associated with bathochromic shift.[22—26] Although the former has been found a wide variety of clinical application, the interference mentioned above must be always taken into consideration. The latter was shown to overcome such interference, and yet sensitivity and accuracy of detection methods based on bathochromic shift are inevitably low. As perhydrolysis of AR was found to proceed without the influences of ascorbic acid, uric acid or bilirubin, and also as generation or consumption of resorufin has been shown to be a highly sensitive fluorometric indicator reaction,[27—38] it was expected that fluorometry based on perhydrolysis of acyl resorufins would offer a highly sensitive and accurate method for H_2O_2 determination, provided that the contribution of spontaneous hydrolysis to generation of resorufin can be significantly reduced. Therefore, the present study was performed to develop a totally new class of indicator reactions for fluorometric analysis of H_2O_2.

Experimental

A fluorescence spectrophotometer (FP-750, JASCO) equipped with a Peltier thermostatted single cell holder (ETC-272, JASCO) was used. All melting points were measured on a Yanako MP-S3 micro-melting point apparatus, and are given uncorrected. Infrared (IR) spectra were taken on a JASCO VALOR-III spectrometer. ^{1}H- and ^{13}C-NMR spectra were obtained in $CDCl_3$ at 270 and 67.8 MHz on a JEOL EX-270 spectrometer. ^{13}C-NMR spectral data were obtained under off-resonance conditions. Chemical shifts are given in ppm relative to tetramethylsilane (TMS) as an internal standard (δ 0.00).

Materials GOD from *Aspergillus niger* (EC 1.1.3.4, 238 U/mg) was used as supplied from Wako Pure Chemical Industries, Ltd. All other chemicals were of reagent grade and were used as received. Stock solutions of acyl resorufins were prepared in HPLC-grade CH_3CN (Wako Pure Chemical Industries, Ltd.). Solutions of H_2O_2 were prepared daily in deionized and distilled water, phosphate (Na_2HPO_4+NaH_2PO_4; pH 7.0, 7.5 or 8.0; 100, 50, 20 or 10 mM), HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethansulfonic acid+NaCl adjusted with aqueous 0.1 M NaOH; pH 8.0 or 8.5; 50 mM) or borate (H_3BO_3+KCl adjusted with aqueous 0.1 M NaOH; pH 8.0 or 8.5; 50 mM) buffer. AR was prepared and purified as reported previously.[1] Other acyl resorufins were synthesized as follows: To a suspension of resorufin sodium salt (2.0 g, 8.5 mmol) in pyridine (20 ml) at −40 °C was added the corresponding acid chloride (10.2 mmol) under a nitrogen atmosphere, and the resulting mixture was stirred at −40—−10 °C for 6 h. The mixture was poured into CH_2Cl_2 (400 ml), and the organic layer was washed with 1 M aq. HCl (400 ml), 10% aq. $NaHCO_3$ (400 ml), and brine (400 ml) and dried over $MgSO_4$. After concentration under reduced pressure, the residue was subjected to column chromatography with CH_2Cl_2–acetone (50 : 1—20 : 1) as the eluent, and the obtained product was further purified by recrystallization.

Isovaleryl Resorufin (IVR): Yield 48%; mp 154 °C (from EtOAc); IR (KBr): 1762, 1625 cm^{-1}; ^{1}H-NMR: δ 7.79 (dd, 1H, J=8.66, 0.50 Hz), 7.43 (d, 1H, J=9.73 Hz), 7.14—7.09 (m, 2H), 6.86 (dd, 1H, J=9.89, 1.98 Hz), 6.33 (d, 1H, J=1.98 Hz), 2.49 (d, 2H, J=7.09 Hz), 2.33—2.18 (m, 1H), 1.08 (d, 6H, J=6.60 Hz); ^{13}C-NMR: δ 185.95, 170.35, 153.34, 149.06, 147.97, 144.11, 134.90, 134.55, 130.94, 130.89, 119.11, 109.52, 107.02, 43.18, 25.79, 22.33 (2C); *Anal.* Calcd for $C_{17}H_{15}NO_4$: C 68.68; H 5.09; N 4.71. Found: C 68.62; H 5.14; N 4.68.

tert-Butylacetyl Resorufin (TBAR): Yield 45%; mp 168 °C (from EtOAc); IR (KBr): 1759, 1625 cm^{-1}; ^{1}H-NMR: δ 7.80 (d, 1H, J=8.41 Hz), 7.43 (d, 1H, J=9.89 Hz), 7.14—7.09 (m, 2H), 6.86 (d, 1H, J=9.89 Hz), 6.33 (s, 1H), 2.48 (s, 2H), 1.15 (s, 9H); ^{13}C-NMR: δ 185.98, 169.55, 153.29, 149.08, 147.97, 144.11, 134.91, 134.56, 130.95, 130.89, 119.19, 109.58, 107.02, 47.68, 31.26, 29.60 (3C); *Anal.* Calcd for $C_{18}H_{17}NO_4$: C 69.44; H 5.50; N 4.50. Found: C 69.39; H 5.54; N 4.52.

Isobutyryl Resorufin (IBR): Yield 43%; mp 165 °C (from EtOAc); IR (KBr): 1755, 1623 cm^{-1}; ^{1}H-NMR: δ 7.80 (d, 1H, J=8.41 Hz), 7.43 (d, 1H, J=9.89 Hz), 7.14—7.09 (m, 2H), 6.86 (dd, 1H, J=9.89, 1.98 Hz), 6.33 (d, 1H, J=1.98 Hz), 2.85 (sept, 1H, J=6.93 Hz), 1.34 (d, 6H, J=6.93 Hz); ^{13}C-NMR: δ 185.90, 174.38, 153.61, 149.08, 147.99, 144.14, 134.89, 134.55, 130.95, 130.88, 119.03, 109.44, 107.03, 34.24, 18.78 (2C); *Anal.* Calcd for $C_{16}H_{13}NO_4$: C 67.84; H 4.62; N 4.95. Found: C 68.00; H 4.72; N 4.95.

Cyclohexanecarbonyl Resorufin (CHR): Yield 71%; mp 184 °C (from EtOAc); IR (KBr): 1751, 1624 cm^{-1}; ^{1}H-NMR: δ 7.79 (dd, 1H, J=8.41, 0.50 Hz), 7.43 (d, 1H, J=9.89 Hz), 7.14—7.09 (m, 2H), 6.86 (dd, 1H, J=9.89, 1.98 Hz), 6.33 (d, 1H, J=1.98 Hz), 2.61 (tt, 1H, J=11.05, 3.63 Hz), 2.16—2.02 (m, 2H), 1.87—1.29 (m, 8H); ^{13}C-NMR: δ 185.90, 173.31, 153.66, 149.09, 147.95, 144.14, 134.88, 134.55, 130.92, 130.85, 119.09, 109.48, 107.02, 43.15, 28.82 (2C), 25.60, 25.24 (2C); *Anal.* Calcd for $C_{19}H_{17}NO_4$: C 70.58; H 5.30; N 4.33. Found: C 70.72; H 5.37; N 4.28.

Pivaloyl Resorufin (PVR): Yield 86%; mp 211 °C (from EtOAc); IR (KBr): 1756, 1628 cm^{-1}; ^{1}H-NMR: δ 7.80 (d, 1H, J=8.73 Hz), 7.44 (d, 1H, J=9.73 Hz), 7.13—7.08 (m, 2H), 6.86 (dd, 1H, J=9.73, 2.14 Hz), 6.33 (d, 1H, J=2.14 Hz), 1.39 (s, 9H); ^{13}C-NMR: δ 185.88, 175.89, 153.88, 149.07, 147.95, 144.13, 134.87, 134.55, 130.91, 130.84, 119.04, 109.44, 107.01, 39.28, 26.99 (3C); *Anal.* Calcd for $C_{17}H_{15}NO_4$: C 68.68; H 5.09; N 4.71. Found: C 68.81; H 5.19; N 4.73.

(1-Adamantanecarbonyl) Resorufin (ADR): Yield 64%; mp 256 °C (from benzene); IR (KBr): 1752, 1627 cm^{-1}; ^{1}H-NMR: δ 7.79 (d, 1H, J=8.41 Hz), 7.43 (d, 1H, J=9.89 Hz), 7.11—7.06 (m, 2H), 6.86 (dd, 1H, J=9.89, 1.98 Hz), 6.32 (d, 1H, J=1.98 Hz), 2.16—1.98 (m, 9H), 1.84—1.74 (m, 6H); ^{13}C-NMR: δ 185.92, 174.98, 154.00, 149.12, 147.91, 144.15, 134.87, 134.55, 130.89, 130.82, 119.14, 109.51, 107.01, 41.27, 38.64 (3C), 36.32 (3C), 27.81 (3C); *Anal.* Calcd for $C_{23}H_{21}NO_4$: C 73.58; H 5.64; N 3.73. Found: C 73.70; H 5.74; N 3.69.

Benzoyl Resorufin (BR): Yield 72%; mp 261—263 °C (from benzene); IR (KBr): 1735, 1627 cm^{-1}; ^{1}H-NMR: δ 8.21 (d, 2H, J=7.99 Hz), 7.86 (d, 1H, J=8.41 Hz), 7.72—7.66 (m, 1H), 7.58—7.52 (m, 2H), 7.45 (d, 1H, J=9.73 Hz), 7.30—7.25 (m, 2H), 6.87 (dd, 1H, J=9.73, 1.98 Hz), 6.35 (d, 1H, J=1.98 Hz); ^{13}C-NMR: δ 185.98, 164.06, 153.54, 149.08, 148.09, 144.19, 134.95, 134.58, 133.99, 131.10, 130.98, 130.12 (2C), 128.56 (2C), 128.40, 119.24, 109.72, 107.09; *Anal.* Calcd for $C_{19}H_{11}NO_4$: C 71.92; H 3.50; N 4.42. Found: C 71.92; H 3.65; N 4.34.

(4-Methoxybenzoyl) Resorufin (MOBR): Yield 93%; mp 233 °C (from benzene); IR (KBr): 1732, 1628 cm^{-1}; ^{1}H-NMR: δ 8.16 (d, 2H, J=8.90 Hz), 7.84 (d, 1H, J=8.41 Hz), 7.45 (d, 1H, J=9.89 Hz), 7.28—7.23 (m, 2H), 7.01 (d, 2H, J=8.90 Hz), 6.87 (dd, 1H, J=9.89, 2.14 Hz), 6.35 (d, 1H, J=2.14 Hz), 3.92 (s, 3H); ^{13}C-NMR: δ 186.00, 164.12, 163.74, 153.80, 149.13, 147.95, 144.19, 134.90, 134.59, 132.32 (2C), 130.99, 130.92, 120.59, 119.36, 113.89 (2C), 109.74, 107.04, 55.51; *Anal.* Calcd for $C_{20}H_{13}NO_4$: C 69.16; H 3.77; N 4.03. Found: C 69.02; H 3.93; N 3.96.

(2-Furoyl) Resorufin (FUR): Yield 77%; mp 255—258 °C (from benzene); IR (KBr): 1747, 1623 cm^{-1}; ^{1}H-NMR: δ 7.84 (d, 1H, J=8.41 Hz), 7.73—7.72 (m, 1H), 7.46—7.43 (m, 2H), 7.29—7.24 (m, 2H), 6.87 (dd, 1H, J=9.89, 1.32 Hz), 6.64—6.63 (m, 1H), 6.34 (d, 1H, J=1.32 Hz); ^{13}C-NMR: δ 185.97, 155.59, 152.70, 149.03, 148.20, 147.59, 144.16, 142.90, 134.99, 134.58, 131.17, 131.02, 120.32, 119.02, 112.31, 109.58, 107.13; *Anal.* Calcd for $C_{17}H_9NO_5$: C 66.45; H 2.95; N 4.56. Found: C 66.41; H 3.16; N 4.57.

Assessment of Acyl Groups and Reaction Conditions All fluorometric measurements were carried out at excitation and emission wavelengths of 572 and 589 nm, respectively. A CH_3CN solution of acyl resorufin (0.1 mM, 1.0 ml), blank buffer (1.0 ml) and H_2O_2 solution (1.2 or 0 mM, 1.0 ml) in the same buffer were added in this order to a cuvette (10×10×45 mm) in the cell holder with stirring at 500 rpm and the specified temperature. The fluorometric measurement of the mixture was started 30 s after addition of H_2O_2 solution, and the reaction was followed for 300 s.

Calibration Curves The same procedure as described above was used except that a CH_3CN solution of acyl resorufin (0.1 mM, 0.8 ml), blank buffer (2.0 ml) and aqueous H_2O_2 solution (0.2 ml) were added to the cuvette in this order.

Glucose Determination In a microtube (1.5 ml), H_2O (150 μl), aqueous $ZnSO_4$ (62 mM, 600 μl), aqueous $Ba(OH)_2$ (30 mM, 600 μl) and aqueous glucose solution (0—300 mg/dl, 150 μl) or plasma (150 μl) were added, and the resulting mixture was centrifuged at 4 °C and 10000 rpm for 10 min. The obtained supernatants were used as glucose standard solution or plasma sam-

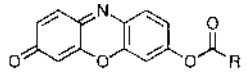
ple. The fluorometric detection of glucose was performed as follows. A CH_3CN solution of PVR or IBR (0.1 mM, 0.8 ml), a GOD (0.25 mg/ml) solution in phosphate buffer (20 mM, pH 8.0 or 7.5, 2.0 ml) and glucose standard solution or plasma sample (0.2 ml) were added in this order to the cuvette in the cell holder with stirring at 500 rpm and 25 °C. The fluorometric response of the mixture was measured 300 s after addition of glucose standard solution or plasma sample. Glucose determination by Trinder's method was carried out with the same glucose standard solution or plasma in the previously reported manner.[3)]

Results and Discussion

The acyl resorufins examined in this study are shown in Fig. 1. Generation of resorufin by deacylation in the presence or absence of H_2O_2 was fluorometrically followed from 0.5 to 5.5 min after starting the reactions. Typical traces for AR and PVR are shown in Fig. 2. The effects of acyl groups and reaction conditions on the competition between perhydrolysis and hydrolysis were assessed with the following measures obtained from the fluorometric traces: increments in fluorescence intensity during the 5 min measurements of perhydrolysis and hydrolysis (ΔI and ΔI_0, respectively) and their ratio ($\Delta I/\Delta I_0$). Values of ΔI and ΔI_0 were informative for estimating the extent of perhydrolysis and hydrolysis of acyl resorufins. Especially, ΔI and $\Delta I/\Delta I_0$ values served to assess acyl groups and reaction conditions in the competition.

Acyl groups were first screened. Deacylation of acyl resorufins (0.1 μmol) were carried out in phosphate buffer (pH 7.5, 100 mM, 2.0 ml)–CH_3CN (1.0 ml) in the presence or absence of H_2O_2 (1.2 μmol) at 25 °C. The results are summarized in Table 1. Judging from ΔI_0 values, FUR was hydrolyzed as easily as AR and the other acyl resorufins were less susceptible to hydrolysis than AR. Retardation of hydrolysis of these acyl resorufins was always accompanied by decreases in fluorometric responses by H_2O_2-dependent deacylation (*cf.* ΔI values). However, comparison of $\Delta I/\Delta I_0$ values suggested that replacing the acetyl group with sterically hindered acyl groups such as *tert*-butylacetyl, cyclohexanecarbonyl and pivaloyl exerted suppressive effects on hydrolysis much more strongly than on perhydrolysis. Thus, TBAR, CHR and PVR were chosen for further examination to develop a perhydrolysis-based indicator reaction for H_2O_2 analysis. In addition, IBR was also chosen as a candidate for our purpose because IBR exhibited much larger ΔI than TBAR, CHR and PVR, although its $\Delta I/\Delta I_0$ was lower than those for other three compounds.

The dependence of the competition between perhydrolysis and hydrolysis of TBAR, IBR, CHR and PVR on pH and buffer components was examined, where phosphate (pH 7.0, 7.5 and 8.0, 100 mM), HEPES (pH 8.0 and 8.5, 50 mM) and borate (pH 8.0 and 8.5, 50 mM) buffers were used. Deacylation of these acyl resorufins (0.1 μmol) in the presence or absence of H_2O_2 (1.2 μmol) was carried out at 25 °C in buffer (2.0 ml)–CH_3CN (1.0 ml). Figure 3 compares $\Delta I/\Delta I_0$ and ΔI obtained in deacylation of these acyl resorufins. Similar trends in these measures were recognized regardless of acyl groups. When the reactions were performed in phosphate or HEPES buffer–CH_3CN, larger values of ΔI as well as $\Delta I/\Delta I_0$ were obtained with increasing pH of the buffer. The pH-dependent increments in $\Delta I/\Delta I_0$ were due to greater increase in ΔI rather than decrease in ΔI_0 by more basic media. For borate buffer, larger values of $\Delta I/\Delta I_0$ were obtained at pH 8.0 rather than pH 8.5, although ΔI was larger at pH 8.5 than 8.0.



R		R	
AR:	CH_3–	PVR:	$(CH_3)_3C$–
IVR:	$(CH_3)_2CHCH_2$–	ADR:	1-adamantyl
TBAR:	$(CH_3)_3CCH_2$–	BR:	phenyl
IBR:	$(CH_3)_2CH$–	MOBR:	*p*-$CH_3OC_6H_4$–
CHR:	cyclo-C_6H_{11}–	FUR:	1-furyl

Fig. 1. Chemical Structures and Abbreviations of Acyl Resorufins Used in This Study

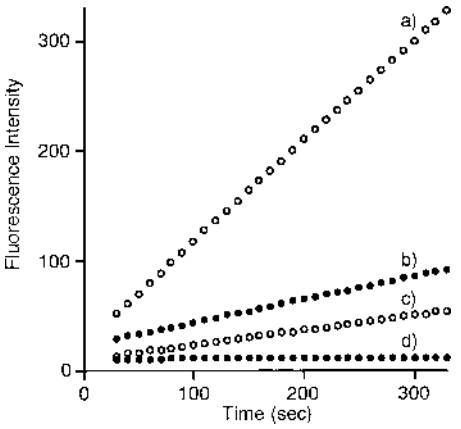


Fig. 2. Fluorometric Traces Obtained for Generation of Resorufin from AR (a, b) and PVR (c, d) (0.1 μmol Each) in the Presence (a, c) or Absence (b, d) of H_2O_2 (1.2 μmol) in Phosphate Buffer (pH 7.5, 100 mM, 2.0 ml)–CH_3CN (1.0 ml) at 25 °C

Table 1. Increments in Fluorescence Intensity during Perhydrolysis (ΔI) or Hydrolysis (ΔI_0) of Acyl Resorufin (0.1 μmol) in the Presence or Absence of H_2O_2 (1.2 μmol), Respectively, in Phosphate Buffer (pH 7.5, 100 mM, 2.0 ml)–CH_3CN (1.0 ml) at 25 °C[a)]

Acyl resorufin	ΔI	ΔI_0	$\Delta I/\Delta I_0$
AR	261.5	63.1	4.1
IVR	43.8	4.5	9.8
TBAR	18.0	0.9	20.0
IBR	116.8	13.6	8.6
CHR	55.4	5.4	10.3
PVR	38.1	2.8	13.6
ADR	10.1	4.2	2.4
BR	40.4	5.0	8.1
MOBR	13.2	3.2	4.1
FUR	140.8	54.8	2.6

a) The values of ΔI or ΔI_0 were obtained as changes between the fluorometric responses observed 0.5 and 5.5 min after starting the reactions in the presence or absence of H_2O_2, respectively.

In borate buffer (pH 8.5)–CH_3CN, ΔI_0 values were increased to a greater extent than ΔI, probably because the interaction[9)] of H_2O_2 with $B(OH)_3$ inhibits perhydrolysis of acyl resorufins. Examination of $\Delta I/\Delta I_0$ values demonstrated that perhydrolysis of these acyl resorufins was favored over hydrolysis when pH 8.0 phosphate or pH 8.5 HEPES buffer was used. However, as can be seen in Fig. 3, the effectiveness of buffer components to increase ΔI values in buffer (pH 8.0)–CH_3CN was borate>phosphate>HEPES. The values of ΔI observed in HEPES buffer (pH 8.5)–CH_3CN were less than 70% of those in phosphate buffer (pH 8.0)–CH_3CN. Thus, from the standpoint of sensitivity, it was concluded

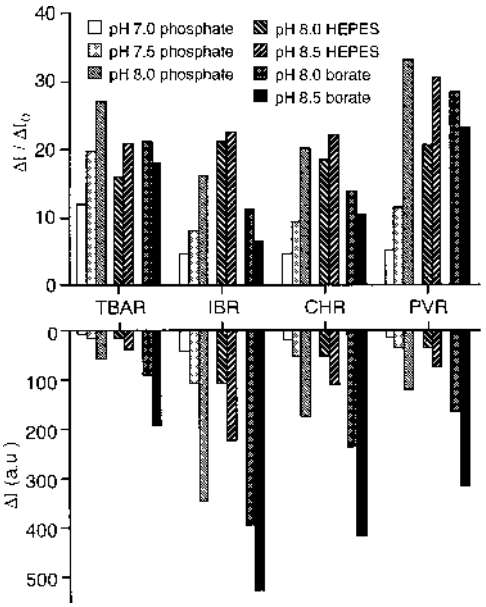


Fig. 3. Effects of pH and Components of Buffer on $\Delta I/\Delta I_0$ and ΔI Obtained in Deacylation of TBAR, IBR, CHR and PVR (0.1 μmol Each) in the Presence or Absence of H_2O_2 (1.2 μmol) in Buffer (2.0 ml)–CH_3CN (1.0 ml) at 25 °C

The concentrations of phosphate, HEPES and borate buffers were 100, 50 and 50 mM, respectively.

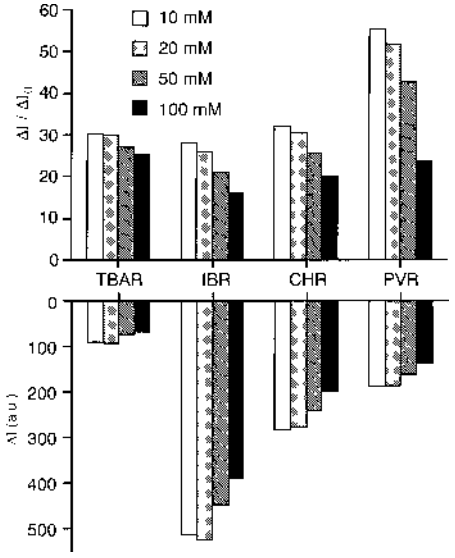


Fig. 4. Effects of the Concentration of pH 8.0 Phosphate Buffer on $\Delta I/\Delta I_0$ and ΔI Obtained in Deacylation of TBAR, IBR, CHR and PVR (0.1 μmol Each) in the Presence or Absence of H_2O_2 (1.2 μmol) in the Buffer (2.0 ml)–CH_3CN (1.0 ml) at 25 °C

that phosphate buffer (pH 8.0)–CH_3CN is the solvent of choice for perhydrolysis of acyl resorufins.

Both perhydrolysis and hydrolysis of TBAR, IBR, CHR and PVR in phosphate buffer (pH 8.0)–CH_3CN at 25 °C were affected by the buffer concentration. As shown in Fig. 4, $\Delta I/\Delta I_0$ values became larger as a lower concentration buffer was employed. The concentration effects were smaller when acyl groups bore less substitutions at α-carbons as in the case of TBAR. These observations stemmed from the following: [from 100 to 20 mM] ΔI informative for the extent of

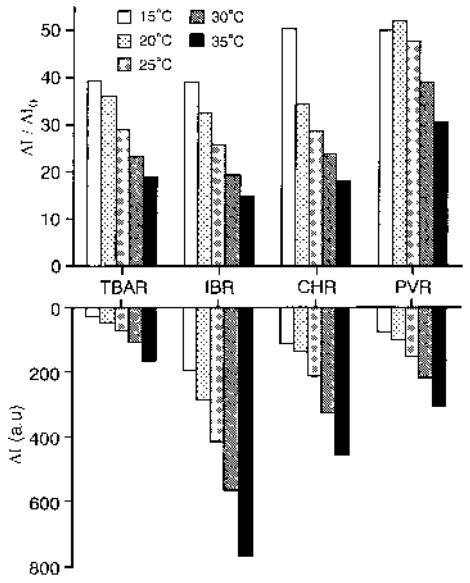


Fig. 5. Effects of Reaction Temperature on $\Delta I/\Delta I_0$ and ΔI Obtained in Deacylation of TBAR, IBR, CHR and PVR (0.1 μmol Each) in the Presence or Absence of H_2O_2 (1.2 μmol) in Phosphate Buffer (pH 8.0, 20 mM, 2.0 ml)–CH_3CN (1.0 ml)

perhydrolysis became larger, while hydrolysis was suppressed to a greater extent, leading to a larger $\Delta I/\Delta I_0$; and [from 20 to 10 mM] ΔI was almost constant, while ΔI_0 decreased further. Thus, when the concentration of phosphate buffer was changed from 100 to 10 mM, ΔI reached almost the maximum value in phosphate buffer (20 mM)–CH_3CN, while ΔI_0 continued to decrease. The competition between perhydrolysis and hydrolysis was shifted in the most favorable manner for perhydrolysis of these four acyl resorufins as an indicator reaction for H_2O_2 analysis, when the fluorometric measurements were conducted in phosphate buffer (pH 8.0, 20 mM)–CH_3CN. It should be emphasized here that employing these conditions considerably restored the loss of H_2O_2-dependent fluorometric responses induced by replacing the acetyl group in AR with *tert*-butylacetyl, isobutyryl, cyclohexanecarbonyl and pivaloyl groups when perhydrolysis was carried out in phosphate buffer (pH 7.5, 100 mM)–CH_3CN.

Reaction temperature also exerted an influence on both perhydrolysis and hydrolysis of TBAR, IBR, CHR and PVR. As deacylation of these acyl resorufins was carried out at a lower reaction temperature, both ΔI and ΔI_0 became smaller. However, the suppressive effects on hydrolysis were about twofold stronger than on perhydrolysis, leading to larger values of $\Delta I/\Delta I_0$ at lower reaction temperature as shown in Fig. 5. With regard to $\Delta I/\Delta I_0$, the best reaction temperature was 15 °C. However, consideration of ΔI values as well as experimental convenience for analytical application indicated that fluorometric analysis of H_2O_2 would be effectively achieved with perhydrolysis of these acyl resorufins at 25 °C as an indicator reaction.

The relationships between H_2O_2 concentration and fluorescence intensity of resorufin generated through perhydrolysis of TBAR, IBR, CHR or PVR were examined. Perhydrolysis was performed by adding aqueous H_2O_2 solution (0.2 ml) to a mixture of a CH_3CN solution of each acyl resorufin (0.1 mM, 0.8 ml) and phosphate buffer (pH 8.0, 20 mM,

Table 2. Calibration Curves for H_2O_2 Obtained by Fluorometry with Perhydrolysis of TBAR, IBR, CHR or PVR as an Indicator Reaction

Conditions[a)]	Data for calibration curves		
	Slope (a.u./nmol)	Interception (a.u.)	Linear range (nmol)
TBAR/pH 8.0	0.070	5.80	1200—1.2
IBR/pH 8.0	0.356	21.12	1200—0.6
CHR/pH 8.0	0.184	10.88	1200—1.2
PVR/pH 8.0	0.128	4.99	1200—0.6
IBR/pH 7.5	0.129	9.21	1200—1.2

a) Fluorometric measurements were carried out in 20 mM phosphate buffer at 25 °C.

2.0 ml) at 25 °C. Calibration curves were obtained by plotting H_2O_2 concentrations *vs.* absolute fluorescence intensity observed 5.5 min after commencement of perhydrolysis. The results are summarized in Table 2. The lowest detection limit was determined as the H_2O_2 concentration allowing its fluorescence intensity to always exceed that of blank solution when the measurements were repeated three times.

Perhydrolysis of each of TBAR, IBR, CHR and PVR exhibited a good linear relationship with H_2O_2 concentration over the specified range with three orders of magnitude, with correlation coefficients (r) being more than 0.999. Relative standard deviations (RSDs) ($n=3$) of fluorescence responses obtained with these acyl resorufins were less than 2.5% over the specified concentration ranges: using perhydrolysis of TBAR, IBR, CHR and PVR, RSD values of fluorescence intensity for the lowest H_2O_2 concentrations were 2.2, 1.7, 1.3 and 1.1%, and those for blank solution were 2.0, 1.1, 1.8 and 0.2%, respectively. Although perhydrolysis of these four acyl resorufins will work as fluorometric indicator reactions, the method with PVR under these conditions seemed to provide the best tool for H_2O_2 analysis, taking sensitivity, reproducibility and the response for blank solution into consideration.

Comparison of the slope values indicated that the method with IBR exhibited the highest sensitivity, although its intercept indicated that the stability of IBR in blank solution was the worst. The possibility of perhydrolysis of IBR as an indicator reaction at pH lower than 8.0 was examined. Perhydrolysis was conducted under essentially the same conditions except that pH 7.5, 20 mM phosphate buffer was used. Under these conditions, the sensitivity was decreased by more than 40% of that with pH 8.0 buffer, and yet the method with IBR provided a satisfactory calibration curve with a correlation coefficient of 1.000 where RSDs of fluorescence responses for the lowest H_2O_2 concentration and for blank solution were 0.3 and 0.8%, respectively. Thus, perhydrolysis of IBR may function well as an indicator reaction for H_2O_2 analysis at pH 7.5.

The present fluorometric methods with PVR and IBR feature that the indicator reactions proceed at a neutral pH region without recourse of POD, which seemed to eliminate interference by reducing biological compounds such as ascorbic acid as described below. Under similar neutral and POD-independent conditions, peroxyoxalate chemiluminescence (PO-CL) methods are known to be useful for determination of H_2O_2.[39—47] Detection limits of PO-CL methods were reported to range from 10^{-10} to 10^{-12} mol levels, depending on oxalate esters, fluorophores and reaction systems used. Accordingly, the sensitivity in the present fluorometry was lower than in PO-CL methods. However, highly sensitive and reproducible detection of H_2O_2 by the CL methods seem to depend on how an oxalate ester and a fluorophore in organic solvent such as ethyl acetate, chloroform or CH_3CN are effectively mixed with H_2O_2 in water. This is at least in part why PO-CL methods have been used mainly in a flow system. In addition, the solubility and stability of generally used oxalates esters such as bis(2,4,6-trichlorophenyl) oxalate in a water-rich system seem much poorer than those of PVR and IBR. Thus, the present fluorometry is believed to work as a complementary tool useful for determination of H_2O_2 in a static and water-rich system, although the sensitivity must be improved somehow.

Table 3. Comparison between Fluorometry with Perhydrolysis of PVR at pH 8.0 (Method I) or IBR at pH 7.5 (Method II) and Colorimetry with Phenol, 4-Aminoantipyrine and POD (Trinder's Method) as a Tool for Glucose Determination

	Method I	Method II	Trinder's Method
Calibration curve	300—50[a,b)] mg/dl	300—50[a,b)] mg/dl	300—50[a,b)] mg/dl
(r)	(1.000)	(1.000)	(1.000)
Detection limit[b)]	10 mg/dl	5 mg/dl	20 mg/dl
(RSD, $n=3$)	(11.5%)	(18.5%)	(19.6%)
Glucose level obtained for plasma	87.5 mg/dl	91.2 mg/dl	83.2 mg/dl
(RSD, $n=6$)	(1.2%)	(1.9%)	(1.2%)
Effects[c)] of ascorbic acid			
0.2 mM	99.0%	97.2%	101.1%
1.0 mM	102.6%	99.4%	93.5%

a) Determination limit. *b*) The limits of determination and detection were determined as the glucose concentrations giving spectrophotometric responses with RSD not exceeding 10 and 30%, respectively. *c*) Shown as relative values between responses observed for 100 mg/dl glucose in the presence and absence of ascorbic acid.

Possibility of the fluorometry with perhydrolysis of PVR at pH 8.0 (Method I) and that of IBR at pH 7.5 (Method II) as a method of glucose determination with GOD was evaluated by comparison with colorimetry using phenol, 4-aminoantipyrine and POD as an indicator reaction system (Trinder's method). The results are summarized in Table 3. Determination and detection limits were determined as the glucose concentrations giving spectrophotometric responses with RSD values not exceeding 10 and 30%, respectively. The determination limits were the same in these three methods, providing linear calibration graphs up to 300 mg/dl. Both Methods I and II gave slightly lower detection limits than Trinder's method. Although a deproteinization procedure was required as in the case of AR,[3] the present methods were successfully applied for blood glucose determination with satisfactory reproducibility. The determined glucose levels for the same plasma sample were comparable to each other, but slightly higher than that obtained by Trinder's method. It was expected that the methods with PVR and IBR were not adversely affected by ascorbic acid, uric acid or bilirubin similarly to the fluorometry with AR.[1,3] To confirm this anticipation, the effects of ascorbic acid were examined. As also shown in Table 3, ascorbic acid even at 1.0 mM much higher than in normal plasma had only negligible influence on the fluorometric responses observed for a 100 mg/dl glucose solution by Methods I and II. In Trinder's method, glucose at the same concentration was successfully detected in the presence of ascorbic acid at 0.2 mM, but was underesti-

mated by more than 5% for the glucose solution containing 1.0 mM ascorbic acid.

In conclusion, the results described here demonstrated that judicious choice of acyl groups and reaction conditions can shift the competition between perhydrolysis and hydrolysis of acyl resorufins in a manner favorable for perhydrolysis to work as a fluorometric indicator reaction for H_2O_2 analysis with a desirable working range. Furthermore, the fluorometry with perhydrolysis of PVR and IBR chosen as the best indicator reactions for determination of H_2O_2 at pH 8.0 and 7.5, respectively, were shown to be applied for determination of blood glucose with only GOD. Thus, acyl resorufins such as PVR and IBR are believed to work not only as novel fluorochromogens useful for clinical chemistry but also as new types of fluorescent probes for detecting H_2O_2 released from or generated in cells. However, for clinical or cell-biological uses, H_2O or non-toxic dimethylsulfoxide is generally preferred to toxic CH_3CN as a solvent for a reagent solution. Accordingly, water-soluble acyl resorufins must be designed or a detection system with acyl resorufins in dimethylsulfoxide at as small volumes as possible should be developed. Further studies along these lines are currently underway in our laboratory.

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References

1) Maeda H., Matsu-ura S., Senba T., Yamauchi Y., Ohmori H., *Chem. Pharm. Bull*., **49**, 294—298 (2001).
2) Michal G., Möllering H., Siedel J., "Methods of Enzymatic Analysis," ed. by Bergmeyer H. U., 3rd ed., VCH Publishers, Weinheim, 1983, Vol. 1, pp. 197—232.
3) Matsu-ura S., Yamauchi Y., Ohmori H., Maeda H., *Bunseki Kagaku*, **50**, 475—479 (2001).
4) James A. P., Mackirdy I. S., *Chem. Ind.* (London), **1990**, 641—645.
5) Grime K., Clauss A., *Chem. Ind.* (London), **1990**, 647—653.
6) Jencks W. P., Carriuolo J., *J. Am. Chem. Soc*., **82**, 1778—1786 (1960).
7) Jencks W. P., Gilchrist M., *J. Am. Chem. Soc*., **90**, 2622—2637 (1968).
8) Gilbert B. C., Holmes R. G. G., Marshall R. D. R., Norman R. O. C., *J. Chem. Res. (M)*, **1977**, 1949—1973.
9) Davis D. M., Deary M., *J. Chem. Res. (M)*, **1988**, 2720—2736.
10) Klopman G., Tsuda K., Louis J. B., Davis R. E., *Tetrahedron*, **26**, 4549—4554 (1970).
11) Grekov A. P., Veselov Y. Ya., *Rus. Chem. Rev. (Engl. Transl.)*, **47**, 631—648 (1978).
12) Hoz S., Nuncel E., *Isr. J. Chem*., **26**, 313—319 (1985).
13) Capaldi D. J., Taylor K. E., *Anal. Biochem*., **129**, 329—336 (1983).
14) Madsen B. C., Kromis M. K., *Anal. Chem*., **56**, 2849—2850 (1984).
15) Reljíc R., Ries M., Aníc N., Ries B., *Clin. Chem*., **38**, 522—525 (1992).
16) Mizoguchi M., Shiga M., Sasamoto K., *Chem. Pharm. Bull*., **41**, 620—623 (1993).
17) Schubert F., Wang F., Rinneberg H., *Mikrochim. Acta*, **121**, 237—247 (1995).
18) Mizoguchi M., Ishiyama M., Shiga M., Sasamoto K., *Bunseki Kagaku*, **45**, 111—124 (1996).
19) Zhu Q.-Z., Zheng X.-Y., Xu J.-G., Liu F.-H., Li Q.-G., *Microchem. J*., **57**, 332—338 (1997).
20) Mizoguchi M., Ishiyama M., Shiga M., Sasamoto K., *Anal. Commun*., **35**, 71—73 (1998).
21) Kakuragawa A., Taniai T., Okutani T., *Anal. Chim. Acta*, **374**, 191—200 (1998).
22) Matsubara C., Ishii K., Takamura K., *Microchem. J*., **26**, 242—249 (1981).
23) Matsubara C., Kudo K., Kawashita T., Takamura K., *Anal. Chem*., **57**, 1107—1109 (1985).
24) Warren L. B., *J. Inst. Brew*., **97**, 457—462 (1991).
25) Harms D., Than R., Pinkernell U., Schmidt M., Krebs B., Karst U., *Analyst*, **123**, 2323—2327 (1998).
26) Harms D., Meyer J., Westerheide L., Krebs B., Karst U., *Anal. Chim. Acta*, **401**, 83—90 (1999).
27) Herrmann R., *Chimia*, **45**, 317—318 (1991).
28) Prough R. A., Burke M. D., Mayer R. T., "Methods in Enzymology," ed. by Wood W. A., Academic Press, New York, 1978, Vol. 52, pp. 372—376.
29) Brotea F. P., Thibert R. J., *Microchem. J*., **37**, 368—376 (1988).
30) Brotea G. P., Draisey T. F., Thibert R. J., *Microchem. J*., **39**, 1—9 (1989).
31) Tokutake S., Kasai K., Tomikura T., Yamaji N., Kato M., *Chem. Pharm. Bull*., **38**, 3466—3470 (1990).
32) Simpson D. J., Unkefer C. J., Whaley T. W., Marrone B. L., *J. Org. Chem*., **56**, 5391—5396 (1991).
33) Kasai K., Yamaji N., *Anal. Sci*., **8**, 161—164 (1992).
34) Hadd A. G., Raymond D. E., Halliwell J. W., Jacobson S. C., Ramsey J. M., *Anal. Chem*., **69**, 3407—3412 (1997).
35) Tortorella M. D., Arner E. C., *Inflamm. Res*., **46**, S122—S123 (1997).
36) Zhou M., Diwu Z., Panchuk-Voloshina N., Haugland R. P., *Anal. Biochem*., **253**, 162—168 (1997).
37) O'neill R. B., Dillon S. A., Morgan P. M., *Biochem. Soc. Trans*., **26**, S84 (1998).
38) Candeias L. P., MacFarlane D. P. S., McWhinnie S. L. W., Maidwell N. L., Roeschlaub C. A., Sammes P. G., Whittlesy R., *J. Chem. Soc. Perkin Trans. 2*, **1998**, 2333—2334.
39) Williams III, D. C., Huff G. F., Seitz W. R., *Anal. Chem*., **48**, 1003—1006 (1976).
40) Sherman P. A., Holzbecher J., Ryan D. E., *Anal. Chim. Acta*, **97**, 21—27 (1978).
41) Scott G., Seitz W. R., Ambrose J., *Anal. Chim. Acta*, **115**, 221—228 (1980).
42) Gübitz G., van Zoonen P., Gooijer C., Velthorst N. H., Frei R. W., *Anal. Chem*., **57**, 2071—2074 (1985).
43) Abdel-Latif M. S., Guilbault G. G., *Anal. Chem*., **60**, 2671—2674 (1988).
44) Nakashima K., Maki K., Kawaguchi S., Akiyama S., Tsukamoto Y., Imai K., *Anal. Sci*., **7**, 709—713 (1991).
45) Nakashima K., Hayashida N., Kawaguchi S., Akiyama S., Tsukamoto Y., Imai K., *Anal. Sci*., **7**, 715—718 (1991).
46) Katayama M., Takeuchi H., Taniguchi H., *Anal. Lett*., **24**, 1005—1015 (1991).
47) Hasebe T., Hasegawa E., Kawashima T., *Anal. Sci*., **12**, 881—885 (1996).