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PHENOL RED: A SUBSTRATE FOR BROMOPEROXIDATION BY MYELOPEROXIDASE AND ITS APPLICATIONS

PATCHAREE BOONSIRI

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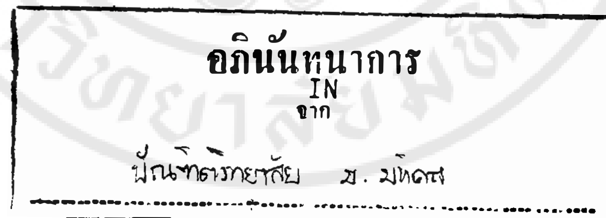
(BIOCHEMISTRY)



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Thesis

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ชื่อวิทยานิพนธ์ พีนอล เรด: สับสเตรทสำหรับโบรมิเพอร์ออกซิเดชั่น โดยไมอีโลเพอร์ออกซิเดสและการประยุกต์ใช้

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บทคัดย่อ

พีนอล เรด ได้ถูกนำมาใช้เป็นสับสเตรทชนิดใหม่สำหรับปฏิกิริยาโบรมิเพอร์ออกซิเดชั่นที่ถูกเร่งด้วยเอนไซม์ไมอีโลเพอร์ออกซิเดส และเอนไซม์เฮโลเพอร์ออกซิเดสอื่นๆ ได้ศึกษาสารผลิตขั้นสุดท้ายโดยการเปรียบเทียบค่า Rf กับสารมาตรฐานด้วย Thin layer chromatography บน Silica gel GF_{254} และใช้ตัวทำลาย amyl alcohol-ethanol-conc.NH_4OH (50:45:5) รวมทั้งเปรียบเทียบค่า absorption maxima, molar extinction coefficient, pH-dependent isobestic points และ pK_a ของสารผลิตผลสุดท้ายด้วย ส่วนหมู่ฟังก์ชันนัลนั้นได้ศึกษาโดยใช้อินฟราเรดสเปคโตรสโคบี โครงสร้างและตำแหน่งที่ถูกโบรมิเนชันหาได้จากการศึกษาโปรตอน นิวเคลียร์ แมกเนติก เรโซแนนซ์ และแมสสเปคโตรเมตรี นอกจากนี้ยังพบสารตัวกลางของปฏิกิริยาจากการทำ high pressure liquid chromatography on reverse phase system ปฏิกิริยาโบรมิเพอร์ออกซิเดชันของพีนอล เรด ทำให้การ assay เฮโลเพอร์ออกซิเดสในหลอดทดลองทำได้ง่ายขึ้น ในการย้อมเม็ดเลือดขาวนั้นพีนอล เรด สามารถใช้บอกความแตกต่างระหว่าง granulocytic series และ lymphocytic series ได้ และยังแยกตัวอ่อนของ myeloblasts จาก monoblasts ได้ด้วย สีนี้ยังใช้ย้อม bromoperoxidatic และ chloroperoxidatic enzymes ใน polyacrylamide gel ได้

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Thesis Title Phenol red: A substrate for bromoperoxidation by myeloperoxidase and its application.

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ABSTRACT

Phenol red was used as a new colored substrate for bromoperoxidatic reaction catalyzed by myeloperoxidase (MPO) and other haloperoxidases. It was bromoperoxidized to bromophenol blue (purple). The final product was identified by comparison of its R_f with standard sulfonphthalein dyes by thin layer chromatography on Silica gel GF_{254} using amyl alcohol - ethanol - conc. NH_4OH (50:45:5) as solvent. The absorption maxima, molar extinction coefficeints, pH-dependent isobestic points and pK_a values of the final products and these sulfonphthalein dyes were also compared. Functional groups were studied by infrared spectroscopy. structures and sites of bromination were identified by proton magnetic resonance and mass spectrometry respectively. The reaction intermediates were detected by high pressure liquid chromatography on reverse phase system.

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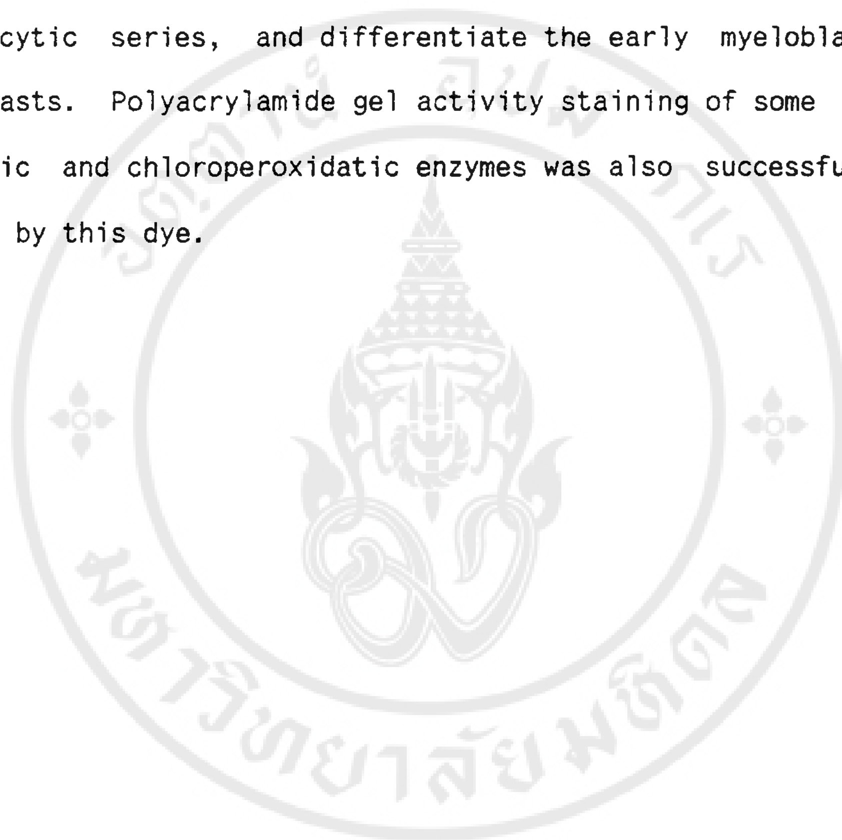
The bromoperoxidatic reaction of phenol red provided a convenient assay for some haloperoxidases *in vitro*. In staining white blood cells, phenol red could distinguish between granulocytic series and lymphocytic series, and differentiate the early myeloblasts from monoblasts. Polyacrylamide gel activity staining of some bromoperoxidatic and chloroperoxidatic enzymes was also successfully performed by this dye.



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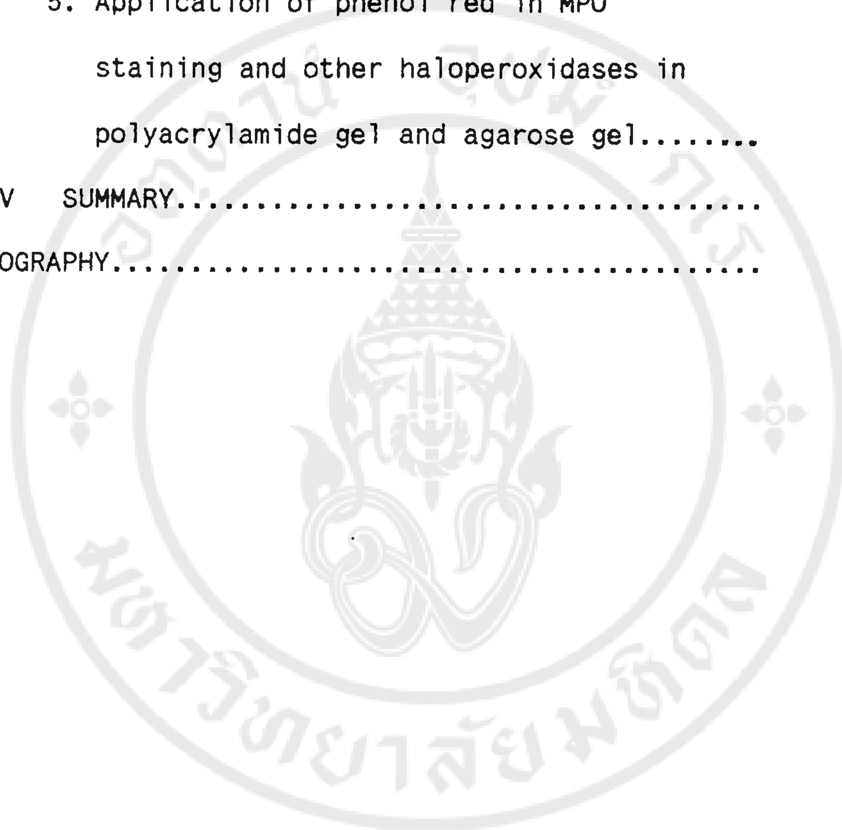

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ABBREVIATION

app	=	apparent
BPO	=	bromoperoxidase
CETAB	=	cetyltrimethylammonium bromide
cm	=	centimetre
CPO	=	chloroperoxidase
DAB	=	3,3'-diaminobenzidine
HPLC	=	high pressure liquid chromatography
hr	=	hour
IR	=	infrared spectroscopy
LPO	=	lactoperoxidase
M	=	molar
MCD	=	monochlorodimedone
mg	=	milligram
min	=	minute
ml	=	millilitre
MPO	=	myeloperoxidase
MS	=	mass spectrometry
MW	=	molecular weight
nm	=	nanometre
NMR	=	nuclear magnetic resonance
pI	=	isoelectric point
rpm	=	revolution per minute
sec	=	second
TEMED	=	N,N,N',N'-tetramethylethylenediamine
TLC	=	thin layer chromatography

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CHAPTER I

INTRODUCTION

1. Haloperoxidases

To date, only one type of enzymes which can catalyze halogenating reactions in biological systems has been isolated. The activity of these halogenating enzymes depends on the presence of hydrogen peroxide (H_2O_2), halide ion and a halogen receptor. They belong to the H_2O_2 - oxidoreductase group. These enzymes are generally called haloperoxidases.

1.1 Haloperoxidase classification

Haloperoxidases are classified according to the range of halide ions that they can use.

Table 1 Classification of haloperoxidases

Name of haloperoxidase	Halide ion range
Chloroperoxidase	Cl^-, Br^-, I^-
Bromoperoxidase	Br^-, I^-
Iodoperoxidase	I^-

These enzymes have been found in many sources, for example, chloroperoxidase from the fungus *Caldariomyces fumago* (1), myeloperoxidase from white blood cells (2), lactoperoxidase from bovine milk (3), bromoperoxidases from marine red and green algae (4) and iodoperoxidase in marine brown algae (5).

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Table 2 Source of haloperoxidases

Halide oxidation	Enzyme source	Common enzyme name
Cl^-, Br^-, I^-	Human/animal Leukocytes	Myeloperoxidase (MPO)
Br^-, I^-	Human/animal Milk,saliva,tears	Lactoperoxidase (LPO)
	Algae *Rhodomela latix* *Penicillus capitatus* and more than 50 others	Bromoperoxidase (BPO)
I^-	Human/animal Thyroid	Thyroid peroxidase(TPO)
	Plant Horseradish	Horseradish peroxidase(HRP)

1.2 Haloperoxidase reaction

Over three decades, this group of enzymes has been extensively studied in its catalytic activity. The search for new substrates and product identification are still hot research topics.

In general, the substrate for the haloperoxidase can be divided into 3 groups. Group 1 substrates react with the enzyme in the presence of peroxide and halide ion to form stable halogenated products. Group 2 substrates are oxidized in a halide ion - dependent reaction. Group 3 substrates are oxidized in the absence of halide ion which is classical peroxidation.

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Table 3 Reaction of haloperoxidases with various types of substrates

Type of substrate	Reaction	
alkene	$CH_2=CH_2$ (ethylene) $\xrightarrow[Cl^-, H_2O_2]{CPO}$ $\underset{OH}{CH_2}-\underset{Cl}{CH_2}$	(8)
alkyne	$C_6H_5-C\equiv CH$ (phenyl acetylene) $\xrightarrow[Br^-, H_2O_2]{LPO}$ $C_6H_5-\overset{O}{\overset{\parallel}{C}}-CH_2-Br$ + $C_6H_5-\overset{O}{\overset{\parallel}{C}}-CH-Br_2$	(10)
cyclopropane	methyl cyclopropane $\xrightarrow[Cl^-, H_2O_2]{CPO}$ HO–…–Cl	(10)
phenols, anilines and other aromatics	$C_6H_5OCH_3$ (anisole) $\xrightarrow[Cl^-, H_2O_2]{CPO}$ o-Cl-$C_6H_4OCH_3$ + p-Cl-$C_6H_4OCH_3$	(10)
β -diketones and β -ketoacids	monochlorodimedone (Cl) $\xrightarrow[Cl^-, H_2O_2]{CPO}$ dichlorodimedone (Cl Cl)	(21)
nitrogen containing compounds	$\underset{NH_2}{CH_2}CH_2SO_3H$ $\xrightarrow[Cl^-, H_2O_2]{MPO}$ $\underset{NHCl}{CH_2}CH_2SO_3H$	(22)
sulfur containing compounds	$CH_3-\overset{O}{\overset{\uparrow}{S}}-CH_3$ (dimethyl sulfoxide) $\xrightarrow[H_2O_2]{CPO}$ $CH_3-\underset{O}{\underset{\downarrow}{\overset{O}{\overset{\uparrow}{S}}}}-CH_3$	(19)
inorganic halogens other than halides	I_2 $\xrightarrow[H_2O_2]{CPO}$ IO_3^-	(23)

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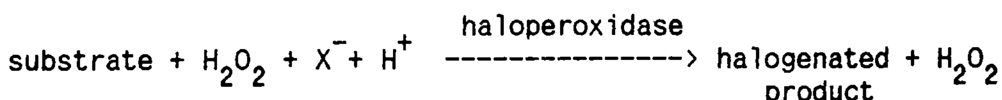
Haloperoxidases can catalyzed the peroxidative formation of a carbon-halogen bond in the presence of H_2O_2 , halide ion and group 1 substrate (6). The general reaction can be written as



Haloperoxidases have been shown to catalyze the halogenation reaction with a wide range of substrates. They can halogenate alkenes such as ethylene, propylene, butadiene, allene to form α,β -halohydrin compound (7) and also α,β -dihalides compounds (8, 9). They can halogenate alkynes such as methyl acetylene, phenyl acetylene to yield β-haloketones (10) and β-diketones (6,11,12,13). Other substrates of haloperoxidase are trans-cinnamic acids (14), aromatic compounds, heterocyclic aromatics (15,16,17) and phenolic dyes (18).

Haloperoxidases not only catalyze halide dependent reaction, but also act as a peroxidase in the absence of halide ion. They can oxidize group 2 substrates such as dimethyl sulfoxide (20), pyrogallol, guaiacol and o-dianisidine.

There are at least three reasons that haloperoxidases are very important. Firstly, halogenated intermediates are important in chemical synthesis (24). Secondly, halogenated compounds have an important role in biological activity such as hormones, steroids, pesticides and antibiotics (25,26). Thirdly, these enzymes may be used in chemical diagnostics and treatment (27). Thus they have a wide range of potential applications, e.g., as synthetic tools and diagnostic reagents. Further research on these enzymes may lead to new commercial opportunities.

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2. Myeloperoxidase

Myeloperoxidase (MPO) is considered to be one of the very important haloperoxidase enzymes. It is considered to have a basic role as part of human defensive mechanism.

2.1 Myeloperoxidase properties

MPO was first isolated from empyema fluid of tuberculous patients (2). Human and canine enzymes have been obtained in crystalline form (28,29,30). MPO was reported to be a glycoprotein with molecular weight of 146,000. It composed of two heavy (MW 57,000) and two light subunits (MW 16,000). The heavy subunits are linked by a disulfide bridge (31,32). This enzyme has two heme groups per molecule (33). The heme groups and carbohydrate chains are attached to the heavy subunits.

2.2 Functions of MPO

Neutrophils contain a large amount of MPO which has an important role in the microbicidal system of these cells in the presence of H_2O_2 and halide ion. MPO, H_2O_2 and halide ion combine to form an antimicrobial system against bacteria (38,39), fungi (40,41), viruses and mycoplasma (42).

There are two sources of H_2O_2 for the MPO-mediated antimicrobial system, leukocytic matabolism and microbial metabolism. In leukocytic metabolism, the oxygen comsumption is detected when cells are in contact with particles. The oxygen consumed is converted to H_2O_2 by NADPH oxidase and superoxide dismutase located on the plasma membrane. This has been detected by inhibition of catalase by aminotriazole (43). The efficiency of the conversion of

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Table 4 Physical and chemical properties of MPO from human leukocytes.

Properties		Reference
Molecular weight	146,000	(34)
Prosthetic group	2 hemes/enzyme	(35)
Carbohydrate content	3% w/w	(35)
Predominant sugars	mannose, glucose, N-acetylglucosamine	(36)

Table 5 Amino acid analysis of MPO (37)

Amino acid residue	Residues/molecule of enzyme
Asp	172
Thr	56
Ser	54
Glu	136
Pro	84
Gly	76
Ala	72
Cys	24
Val	48
Met	24
Ile	33
Leu	60
Tyr	24
Trp	12
Phe	48
His	12
Lys	24
Arg	96

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oxygen to H_2O_2 is not known since only a portion of H_2O_2 formed is detected. The extracellularly generated H_2O_2 diffuses into intracellular component of neutrophil while maintaining its chemical activity (44). Microorganisms can serve as the source of H_2O_2. The lactic acid bacteria such as pneumococci, streptococci and lactobacilli do not contain the cytochrome system which reduce O_2 to H_2O. Terminal oxidations are catalyzed by flavoproteins which reduce O_2 to H_2O. These microorganisms also lack catalase (45). The H_2O_2 formed may be toxic to the organisms and this toxicity is enhanced by MPO and a halide.

Chloride is present in leukocyte at a concentration which is greater than that required as a component of MPO-mediated antimicrobial system. The plasma chloride concentration in man is 1.03×10^{-1} M. The bactericidal activity of MPO and H_2O_2 is increased by chloride at a concentration of 5×10^{-6} M, with a total killing at a concentration of 5×10^{-4} M. The efficiency of transfer of chloride across the membrane of phagolysosome is not known. Chloride may enter the vacuole during phagocytosis in the layer of extracellular fluid which accompanies the ingested particle.

Bromide concentration is 1.0×10^{-4} M at physiological pH. Ritter et al (1988) (46) reported that bromide plays a significant role in halogenation even though *in vivo* levels of chloride are equal to about 1000 times greater than bromide.

Iodide is more effective than chloride in MPO-mediated antimicrobial system. A bactericidal effect was observed at an iodide concentration of 5×10^{-8} M with complete killing at a concentration of 5×10^{-6} M (39). The iodide concentration in serum is

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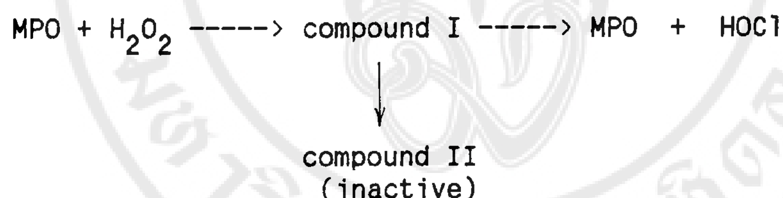
very low (less than 1 µg/100 ml). However it may be concentrated by leukocytes against an electrochemical gradient.

2.3 Reaction mechanism of MPO

The antimicrobial activity of MPO has been related to chloroperoxidatic activity of the enzyme (39). Agner (1941) (2) first observed the oxidative potential of MPO in the presence of chloride and proposed the formation of free hypochlorous acid (HOCl). Subsequently Harrison and Schultz (1976) (47) studied the chlorinating activity of MPO. They demonstrated that MPO catalyzes the peroxidation of chloride to free HOCl and the mechanism of chlorination is distinct from that of CPO. The mechanism of chlorination of chloride catalyzed by MPO is shown below (48).



In this mechanism, MPO first reacts with H_2O_2 to form compound I which contains two oxidising equivalents more than the resting enzymes and which oxidises chloride to HOCl under reformation of resting enzyme. In this step a proton is involved. Upon binding of Cl^- and a proton to MPO, the reaction of H_2O_2 with MPO is prevented. During turnover, compound I may be reduced to compound II which contains iron (IV) and is unable to oxidise Cl^- into HOCl (49).

To prove that the pathway involves hypohalous acid (HOX) formation, not an enzyme-bound intermediate (EOX), the rate of HOCl formation by the enzyme had been shown to be equal to the

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rate of chlorination of the substrate. On the other hand, if EOX is the halogenating species, then the reaction rate, substrate specificity and product selectivity of the enzyme reaction should be quite different from that of chemical halogenation processes. The substrate, H_2O_2 and Cl^- were pumped through the immobilized MPO on a millipore membrane. Under set conditions, a turnover number of 100 min^{-1} was obtained both in the absence of substrate (i.e. HOCl was measured in the effluent) and in the presence of substrate (i.e. chlorinated substrate was measured in the effluent). Thus the rate of HOCl formation of substrate halogenation was shown to be equal to the rate of substrate halogenation, and HOX mechanism was shown for MPO.

In contrast to MPO, haloperoxidatic mechanism for CPO from *Caldariomyces fumago* has not been agreed upon yet. Some scientists point out that HOCl is its halogenation intermediate by showing absence of stereoselectivity in CPO catalyzed halogenation (50, 51,52). The others believe that the EOX intermediate is formed as they observe the differences in substrate specificity occured between enzymatic and chemical chlorination (12,53). Thus it is still the question whether the favored pathway involves HOX.

The HOCl formed by MPO may affect the microorganisms in many ways (54,55,56). Firstly it is the effective SH reagent. Secondly it oxidizes iodide to iodine. When a suitable iodine acceptor is present in the reaction mixture, iodination occurs. The iodination of bacterial protein by MPO, H_2O_2 and iodide is stimulated by chloride when iodide concentration is low. Thirdly, HOCl reacts with nitrogeneous compounds to form chloroamines which are toxic to microorganisms (57,58,59). Fourthly, hypochlorite (OCl^-)

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reacts with H_2O_2 to form oxygen. The metastable intermediate, singlet oxygen, is toxic to microorganisms. It dissipates its energy by reaction with certain organic compounds (60); chemiluminescense can be observed when this occurs (61).

When iodide is the cofactor, the death of the microorganisms is associated with the iodination of bacterial protein (38). The products of iodide oxidation are also the strong oxidizing agents. They may cause the toxicity such as oxidation of sulfhydryl groups 62).

The mechanism of bromide-dependent system is similar to that of iodide system. The bromination and oxidation of essential components cause the microbicidal effect. Bromination of bacteria by the MPO-H_2O_2-Br^- system has been demonstrated using ^{82}Br (63). The formation of singlet oxygen by this system is toxic to microorganisms.

The polymorphonuclear leukocytes deficient in MPO have decreased microbicidal activity. MPO could be replaced by LPO when the iodide ion was in the system. The effectiveness of MPO and LPO was also comparable when bromide ions were employed. However. when chloride ions were used LPO was considerably less effective than was MPO (39).

Bromoperoxidases from marine algae are also known to synthesize many brominated organic compounds (4,64). Many of these halometabolites are antibacterial and antifungal. It is generally considered that these compounds act as defensive substances.

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2.4 Solution reaction of MPO

In the assay of MPO activity for biochemical study, 3,3'-dimethoxybenzidine(65),tetramethylbenzidine (66) and other classical peroxidase substrates such as guaiacol, o-dianisidine are generally used. The only substrate used for haloperoxidatic assay MPO and also for the other haloperoxidases is monochlorodimedone (48).

Monochlorodimedone has been used as standard haloperoxidase substrate since 1963. In that year Beckwith and Hager (1963) (67) found that extracts of *Caldariomyces fumago* catalyzed the peroxidative chlorination of 1,3-cyclopentanedione to yield 2-chloro-1,3-cyclopentanedione or monochlorodimedone and then the reaction proceeded further to yield 2,2-dichloro-1,3-cyclopentanedione or dichlorodimedone. This latter compound is reduced to the naturally occuring compound, caldariomycin, by growing cultures. Monochlorodimedone has absorption maximum at 290 nm but the absorbance rapidly decreases due to the formation of dichlorodimedone. This halogenation can be detected by using a spectrophotometer.

The bromination of monochlorodimedone by MPO is similar to the chlorination reaction (48).

2.5 Cytochemistry of MPO

For more than 70 years, the reaction for MPO activity has been mainly concerned with cytochemical differentiation of cells of granulocytic origin and lymphoblastic origin. This reaction remains one of the most widely used reactions in hematology laboratory.

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With the initial use of benzidine as substrate (68), the MPO cytochemical staining can be conducted with various substrates such as 3-amino-9-ethylcarbazole (69), homovanillic acid (70), pyrocathecol-8-phenylenediamine (71), 2,7-fluorenediamine (72), diaminobenzidine (73), o-tolidine (74), 4-chloro-1-napthol (75) and basic blue 93 (76). These substrates are oxidised or polymerized by peroxidase activity of MPO except for basic blue 93 whose reaction with MPO is not well defined.

2.6 Polyacrylamide gel staining of MPO and other haloperoxidases

Up to now, staining of polyacrylamide for haloperoxidase activity is not clear and specific. Pember *et al* (1982) (77) and Olsen *et al* (1986) (78) still used 3,3'-diaminobenzidine and H_2O_2 for MPO staining in non-denaturing polyacrylamide gel electrophoresis. Pickard and Hashimoto (1982) (79) stained CPO with 3,3', 5,5'-tetramethylbenzidine. Menthey and Hager (1981) (80) stained BPO from green algae by using o-dianisidine as substrate. Itoh *et al* (1985) (81) used pyrogallol for staining BPO of red algae and Wever *et al* (1985) (82) detected BPO from brown algae by using the combination of o-dianisidine and phenol red to give brown bands. They also stain peroxidase activity of the samples.

At present, monochlorodimedone (MCD) is the most popular haloperoxidase substrate. Detection of haloperoxidase activity by using this substrate requires a spectrophometer with ultraviolet capability. This may be a limiting factor. Staining of cells containing MPO, CPO, BPO and LPO activity has been based on ordinary

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peroxidatic reaction (68-76), therefore unspecific. Also, gel staining after electrophoresis of these enzymes and their extracts has also been unspecific because peroxidatic substrates are used (77-82).

AIMS OF THESIS

1. Comparison of a novel substrate, phenol red, for assay of MPO activity with that of MCD.
2. Identification of the final product from reaction-specific MPO-catalyzed phenol red reaction.
3. Applications of phenol red in cytochemistry of polymorphonuclear leukocytes.
4. Applications of phenol red in staining of chloroperoxidatic and bromoperoxidatic activity in polyacrylamide gel.

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CHAPTER II

MATERIALS AND METHODS

Name of chemicals	MW	Company
Acetic acid	60.05	Carlo Erba
Acrylamide	71.10	Sigma
Ammonium hydroxide (NH_4OH)	35.05	Ajax
Ammonium persulfate($(NH_4)_2S_2O_8$)	228.70	May & Baker
Amyl alcohol	88.15	Carlo Erba
Ampholine pH range 3-10	-	LKB
Ampholine pH range 2-5,9-11	-	Pharmacia
Bacto-agar	-	Difco
Benzene	78.00	Fisher
Bromophenol blue	670.00	Sigma
Cetyltrimethylammonium bromide (CETAB)	364.46	Fluka
Chloroform deuterated ($CDCl_3$)	120.39	Fluka
Copper sulfate	249.68	Sigma
3,3'-Diaminobenzidine tetra-hydrochloride (DAB)	306.10	Sigma
Dimethylsulfoxide d_6-deuterated	84.17	Fluka
Ethanol	46.07	Merck
Ethyl acetate	88.11	Fisher
Ethylenediamine tetraacetic acid (EDTA)	336.20	Sigma
Formaldehyde	30.03	Merck
Glycerol	92.10	Fisher

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Name of chemicals	MW	Company
Hydrogen peroxide (H_2O_2)	34.02	Chemikit
Isopropanol	60.10	Fisher
Methanol	32.00	Merck
N,N'-Methylene bisacrylamide	154.20	Sigma
Monochlorodimedone (MCD)	174.60	Sigma
Phenol red	374.60	Sigma
Phosphoric acid (H_3PO_4)	98.00	Carlo Erba
Potassium bromide (KBr)	119.01	Merck
Potassium chloride (KCl)	74.58	Merck
Potassium phosphate monobasic (KH_2PO_4)	136.09	Fisher
Potassium phosphate dibasic (K_2HPO_4)	174.20	J.T.Baker
Potassium cyanide (KCN)	65.12	Carlo Erba
Pyridine	84.14	Fisher
Safranin O	350.85	Sigma
Silica gel GF_{254}	-	Merck
Sodium acetate	82.04	Sigma
Sodium azide (NaN_3)	65.01	Fluka
Sodium bicarbonate ($NaHCO_3$)	24.01	J.T.Baker
Sodium carbonate (Na_2CO_3)	105.99	J.T.Baker
Sodium chloride (NaCl)	58.44	Carlo Erba
Sodium hydroxide (NaOH)	40.00	Merck
Sucrose	342.30	Sigma

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Name of chemicals	MW	Company
N,N,N'N'-Tetramethylethylene-diamine (TEMED)	116.20	Eastman
Trisma base (Tris)	121.10	Sigma

The other chemicals were all of analytical grade.

METHODS

1. Preparation of haloperoxidases

1.1 Bromoperoxidase

BPO was extracted from a Thai seaweed from Pattani province. Ten grams of the seaweed was ground in 0.1 M potassium phosphate buffer pH 7.0 using a motar and pestle. The homogenate was filtered through cheesecloth. Then it was centrifuged at 10,000 rpm for 10 min at $4^{o}C$ to remove debris. The supernatant was concentrated with aquacide and it was used for further study.

1.2 Myeloperoxidase

MPO was prepared from normal human polymorphonuclear leukocytes. Fresh buffy coat was obtained from human blood. Leukocytes and erythrocytes were separated by centrifugation at 10,000 rpm for 10 min. The contaminated erythrocytes were lysed in hypotonic solution (0.15 M NH_4Cl, 0.01 M $NaHCO_3$ and 0.1 mM EDTA) for 1 hr. After centrifugation at 10,000 rpm for 10 min the leukocyte pellet was resuspended in 0.1 M potassium phosphate buffer pH 7.4 containing 0.5 % CETAB and 0.1 M Na_2SO_4. Homogenate was made using a Teflon-glass homogenizer at 4° C for 5 min followed by soni-

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cation at 60 cycle/sec 50 % duty for 30 sec and centrifugation at 10,000 rpm for 10 min. The supernatant was dialysed against 0.1 M potassium phosphate buffer pH 7 to remove CETAB. The dialysate was used for assay MPO activity

MPO from normal human leukocyte was also purchased as purified lyophilized powder from Calbiochem.

1.3 Chloroperoxidase and Lactoperoxidase

They were obtained from Sigma. CPO was from *Caldariomyces fumago*. It was the purified suspension in 0.1 M sodium phosphate buffer pH 4. LPO was in lyophilyzed powder form. It was obtained from bovine milk. They were kept below 0° C.

2. Non-colorimetric assay of haloperoxidase

The haloperoxidase activity was assayed spectrophotometrically by measuring the decrease in absorbance of MCD at 290 nm at 25° C (80).

The reaction mixture of 3 ml contained 2.8 ml of 0.1 M potassium phosphate buffer, 6×10^{-5} M MCD 2×10^{-2} M KBr or KCl and 2×10^{-4} M H_2O_2. The reaction was started by the addition of 0.05 ml of the enzyme solution and mixed in a 3 ml cuvette. Blanks consisted of all reaction components except the enzyme solution.

The enzyme activity was measured by the decrease in absorbance at 290 nm (ε = 21.6 mM^{-1} cm^{-1}) at 25° C.

$$\text{unit/ml of enzyme activity} = \frac{\Delta A_{290}/\text{min} \times V}{\varepsilon \times v}$$

V = volume of the solution in cuvette (ml)

v = volume of the enzyme used (ml)

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3. Colorimetric assay for peroxidatic activity

Peroxidase activity was measured according to the method of Herzog et al (1973) (83) by using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as chromogen.

DAB solution was prepared by dissolving 0.018 g of DAB in 0.05 M potassium phosphate buffer pH 4.3. This solution is usually stable for several hours. However, DAB undergoes slight auto-oxidation in light. So it should be kept in the dark. Larger quantities can be stored for a few days at 4° C.

2.9 ml of DAB solution and 0.05 ml of peroxidase solution were mixed in a 3 ml cuvette. The reaction was started by the addition of 0.05 ml of 0.001 M H_2O_2. The change in absorbance at 465 nm at 25° C was recorded at various time in a Shimadzu UV-160 spectrophometer using DAB solution as blank. The enzyme activity was expressed as ΔA_{465}/min.

4. Investigation of phenol red as the organic substrate for the haloperoxidases.

Phenol red has been tested by using each haloperoxidase in various pH optimum conditions.

The other substances which have phenol red like structure (bromophenol blue, bromocresol green, bromocresol purple, bromothymol blue, thymol blue, cresol red, m-cresol purple and pyrocatechol violet) have also been tested. Only phenol red, chlorophenol red and bromophenol red had suitable proterties as MPO substrate and stain. Phenol red was chosen because it is the simplest and most common of the three.

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The colorimetric assay of this dye was the same as that of MCD. The change in maximum absorption of the substrate was recorded at various times. The enzyme activity was expressed as unit/ml.

5. Stability test of phenol red and MCD

Haloperoxidase substrates stability under UV light was done by exposed 1.6×10^{-5} M phenol red and 1.0×10^{-4} M MCD under 60 cycle/sec UV lamp at room temperature. The solutions were measured absorbance at absorption maximum every 15 min for 1 hr.

The stability of these substrates when kept at 4° C and room temperature were performed as above. But the absorbance of the solutions were measured every week.

6. Determination of $K_{m_{app}}$ values

The $K_{m_{app}}$ values for MPO substrates, H_2O_2 and halide ions were determined by using 0.05 ml of the enzyme with varying concentration of one of substrate in the presence of a fixed concentration of the second and third substrates.

Haloperoxidase activity was determined as described before. $K_{m_{app}}$ values were determined from a Cornish-Bowden plot of enzyme activity and substrate concentration (84).

7. pH optimum of MPO

The MPO activity was assayed in 0.1 M potassium phosphate buffer of various pH value. The assay condition was the same as described before. The substrate phenol red was used in comparison with MCD. The halide ions were chloride and bromide. Results were expressed as percentage of the maximum MPO activity.

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8. Preparation of phenol red brominated product by MPO

Phenol red was converted to its brominated products by normal human MPO. KBr and H_2O_2 were added until the solution turned to purplish blue. The brominated products were pooled and lyophilized. Phenol red chlorinated products were prepared by the same method but KCl was used instead of KBr.

9. Purification of phenol red brominated products by thin layer chromatography

Thin layer plates of Silica gel GF_{254} were prepared by spreading the slurry of Silica gel GF_{254} and water 50 % w/v on clean glass plates with the aid of an applicator. The plates were dried at 110° C for 1 hr and stored in a desiccator until used.

For seperation of phenol red brominated products. The TLC plates were developed with amyl alcohol-ethanol-conc.NH_4OH (50:45:5) according to Stahl (1969) (85). The seperated components were scraped off and extracted with distilled water. The extracted product was lyophilized for further identification.

10. Identification of phenol red brominated products

10.1 Thin layer chromatography (TLC)

The products were spotted on microslide coated with Silica gel GF_{254}. The solvent system was amyl alcohol-ethanol-conc.NH_4OH (50:45:5). The R_f values of the products were compared with those of standard sulfonphthalein dyes.

The R_f value of products were further confirmed with authentic substance by TLC in various solvent systems according to Stahl (1969) (85). These solvent systems were benzene-isopropanol-

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acetic acid (60:40:1), ethyl acetate-pyridine-water (60:30:10) and ethyl acetate-methanol- 5 M NH_4OH (60:30:10)

10.2 UV-VIS spectroscopy

Ultraviolet and visible spectra of the final product were obtained on a Shimadzu UV-240 spectrometer. They were compared with those of sulfonphthalein dyes.

The isobestic points, molar extinction coefficients, absorption maxima and pK_a values were determined as follows.

The isobestic points were determined by scanning the spectra of the substances at various pH. The common points of spectral intersection were the isobestic points.

The molar extinction coefficients were obtained by scanning spectra at various concentrations of products and sulfonphthalein dyes. Absorbance at λ_{max} of these substances was plot versus their concentrations. The linear slope was converted to molar extinction coefficient.

The pK_a values were determined by ploting the absorbance at λ_{max} of the substances versus pH. The inflection points were the pK_a values.

10.3 Infrared spectroscopy (IR)

The infrared spectra of the final phenol red brominated and chlorinated products and some sulfonphthalein dyes were obtained on a Jasco model A-302 and a Perkin-Elmer model 683 infrared spectrometer. The samples were treated by the Nujol mull method.

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10.4 Nuclear magnetic resonance spectroscopy (NMR)

The NMR spectra of phenol red and its brominated product and bromophenol blue were obtained on a JEOL model FM-3602 and a JEOL model FX-90Q NMR spectrometer at 60 and 90 MHz respectively. The samples were dissolved in d_6-DMSO and $CDCl_3$. Tetramethylsilane was used as an internal standard.

10.5 Mass spectrometry (MS)

Mass spectra were obtained using a JEOL model DX-30 mass spectrometer. The samples were heated up to 300° C and mass spectra were recorded.

10.6 High performance liquid chromatography (HPLC)

HPLC was performed using a reverse phase system. The column (4.6 mm x 25 cm) was packed with Spherisorb S 5 ODS 2. The mobile phase was 57.5 % methanol-0.01M potassium phosphate buffer pH 3.4. The samples were eluted at the flow rate of 1 ml/min at pressure 2400 psi and detected by UV-VIS spectrometer at 430 nm.

11. Cytochemical demonstration of MPO activity in polymorphonuclear leukocytes with phenol red, benzidine and Sudan black.

The air dried EDTA blood and bone marrow smears were performed from 40 normal persons and 48 leukemic patients. The leukemic patients were classified according to FAB (French-American-British cooperative group) classification. The patients were distributed as follows; 5 acute myelogeneous leukemias without maturation (M_1), 5 acute myelogeneous leukemias with maturation (M_2), 5 promyelocytic leukemias (M_3), 4 acute myelomonocytic leukemias (M_4), 4 acute monocytic leukemias (M_5), 2 acute lymphocytic

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leukemias and 23 chronic myelocytic leukemias. The smear was fixed for 1-2 sec in 10 % formalin-ethanol (10 ml of 37% formaldehyde and 90 ml of absolute ethanol) and air dried.

The microslide was incubated in the incubation mixture (0.2 M sodium acetate buffer pH 5 18 ml, phenol red 8 mg, 3 M KBr 0.25 ml, 0.5 % $CuSO_4$ 1 ml,3 % H_2O_2 0.05 ml and Safranin O 20 mg) for 3 min at room temperature. The solution was removed by wrist action and the slide blotted until dry.

The conventional peroxidase staining by benzidine was performed by fixing the smeared slide in 0.5 % $CuSO_4$ for 1 min and saturated benzidine solution and 1 drop of 3 % H_2O_2 was applied to the slide for 5 min. After washing with tap water the slides were countersteined with 1 % Safranin O for 2 min. The slides were washed in running tap water and air dried.

Sudan black staining was performed by fixing air dried smears in formalin vapour for 1 min and washing in tap water. The fixed air dried slides were immersed in Sudan black staining solution (0.18 g Sudan black, 0.5 g phenol and 0.1 g disodium hydrogen phosphate in 100 ml distilled water) for 1 hr and then washed in 70 % ethanol followed by rinsing in tap water. They were counter-stained with M.G.C. for 5 min.

12. Determination of isoelectric point by isoelectric focusing

Isoelectric focusing of haloperoxidases was performed in 7.5-10 % polyacrylamide gel containing 10% glycerol and 2 % ampholine at a suitable pH range of each enzyme. The gel was polymerized with 10 % ammonium persulfate and TEMED. Polymerization was allowed to proceed at room temperature for 1 hr. After gel polyme-

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rization, the water layer was removed and the top of the gel surface was rinsed three times with distilled water.

The sample was dissolved into a 1 in 15 solution of ampholine containing 15 % sucrose and loaded into well of gel. The reservoir buffers were 0.02 M sodium hydroxide and 0.02 M phosphoric acid for the cathode and anode respectively. The sample was focused at constant 300 V for 7 hr at room temperature.

After the focusing was completed, the gel was removed from the glass plate. The pH profile of the gel was obtained by cutting the gel (without any loaded sample during electrofocusing) into 0.5 cm/section. 2 ml of distilled water was added into each section and left at room temperature for 2 hr. The pH of the solution was measured by a pH meter.

Haloperoxidase activity staining was performed by soaking the gel in staining solution (5 x 10^{-5} M phenol red, 1 x 10^{-3} M H_2O_2 and 5 x 10^{-2} M KBr in 0.1 M phosphate buffer pH 5.8). The solution was changed 3 times. Blue bands developed in the gel. Photographs were immediately taken.

Classical peroxidase activity staining by using DAB was also performed (83). This solution consisted of 5 x 10^{-4} M DAB and 1 x 10^{-3} M H_2O_2 . Brown bands represented peroxidase activity.

13. CETAB-Polyacrylamide gel electrophoresis of MPO

The slab gel was prepared according to the method of Akin et al (1985) (86). A 7.5 % polyacrylamide gel was mixed with 0.6 % H_3PO_4, 0.036 M Tris pH 6.0 and TEMED. For initiation of polymerization, the gel was overlaid with water-saturated n-butanol and exposed to fluorescent light. Polymerization was completed within 2 hr.

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The sample was mixed with 0.3 % H_3PO_4, 0.018 M Tris pH 6, 0.3 % CETAB. Glycerol was added to each sample to a final concentration of 5 % v/v. Pyronin Y was used as a tracking dye.

The running buffer used was 0.3 % H_3PO_4 and 0.018 M Tris pH 6. The upper chamber contained 0.3 % CETAB and polarity was reversed so that the sample was migrated toward the cathode at the bottom of the gel. Electrophoresis was performed at a constant voltage of 150 V at room temperature for 8 hr.

The gel was stained for haloperoxidase activity as described in isoelectric focusing of the haloperoxidase.

14. Rapid detection of bromoperoxidatic activity by agarose gel

50 mg of agar was dissolved in 5 ml of 0.1 M potassium phosphate buffer pH 5.8. This agar solution contained 5×10^{-5} M phenol red and 5×10^{-2} M KBr. Then the mixture solution was heated until agar was completely dissolved. After that, the solution was left at room temperature until the temperature decreased to approximately 45° C, H_2O_2 was added to the solution at final concentration of 1×10^{-3} M. This solution was poured onto the cleaned glass microslide (5ml/slide) and left until it was in gel state. The small wells on the gel were made by a punching machine. The test extract sample was dropped into the well with the aid of a microsyringe. The test seaweeds were immersed in the gel.

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CHAPTER III

RESULTS

1. Investigation of phenol red as substrate for haloperoxidases

Phenol red, a sulfonphthalein dye, was used as substrate for MPO and other haloperoxidases. When it reacted with MPO and other haloperoxidases in the presence of H_2O_2 and bromide ion, the color changed from yellow to purplish blue. The optical spectrum showed that phenol red (ε = 19.76 $mM^{-1}cm^{-1}$ at 430 nm) was converted to a final purplished blue product with an absorption maximum at 590 nm (ε= 63.49 $mM^{-1}cm^{-1}$ at 590 nm) (Fig. 1 A). In the presence of chloride ion, a final reddish-brown product (ε = 15.98 $mM^{-1}cm^{-1}$ at 575 nm) was obtained (Fig.1 B).

After bromination reaction catalyzed by MPO, the substrate bromophenol red (λ_{max} 435 nm) and chlorophenol red (λ_{max} 575 nm) changed color to blue (λ_{max} 590 and 591 nm respectively). The other substrates, bromophenol blue (λ_{max} 590 nm), bromocresol green (λ_{max} 612 nm), bromocresol purple (λ_{max} 585 nm), bromothymol blue (λ_{max} 615 nm), thymol blue (λ_{max} 590 nm), cresol red (λ_{max} 425 nm), m-cresol purple (λ_{max} 435 nm) and pyrocathecol violet (λ_{max} 441 nm) did not change their absorption maxima after addition of MPO, H_2O_2 and KBr into the reaction mixture at pH 5.8.

The standard substrate for assay of haloperoxidases at present is MCD (ε= 20.1 $mM^{-1}cm^{-1}$ at 290 nm), which after bromination by MPO is converted to chlorobromodimedone (ε= 0.20 $mM^{-1}cm^{-1}$ at 290 nm) (Figure 2A). The rate of reaction is usually followed by measuring decrease of absorbance at 290 nm. The chlorination of MCD by MPO gave the product dichlorodimedone (ε = 0.20 $mM^{-1}cm^{-1}$ at 290 nm) (Figure 2 B).

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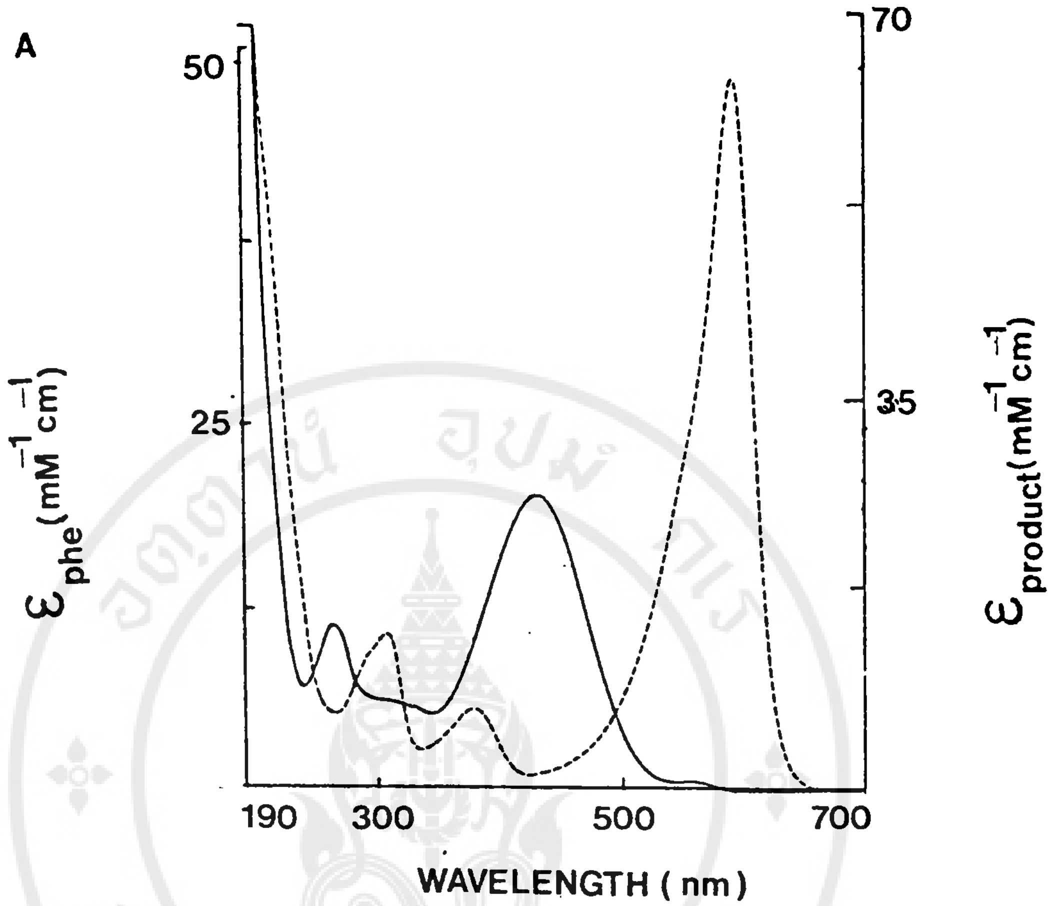

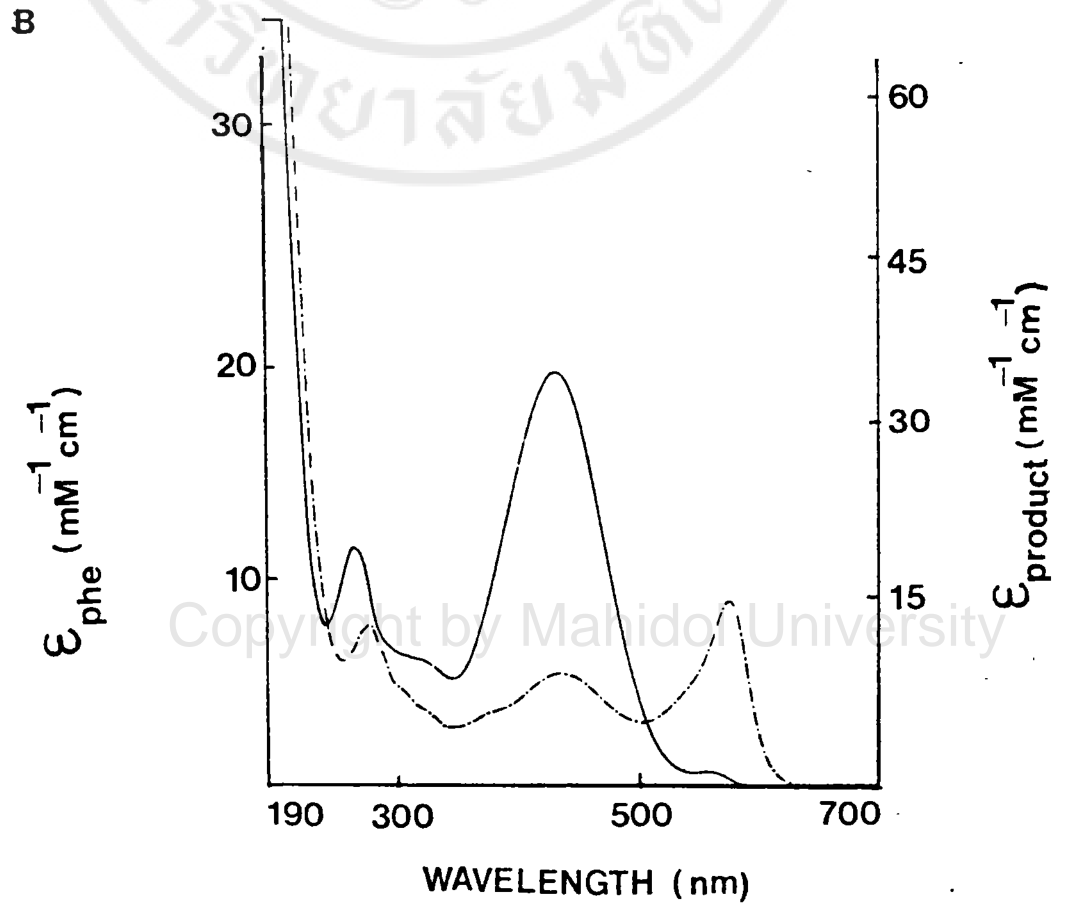

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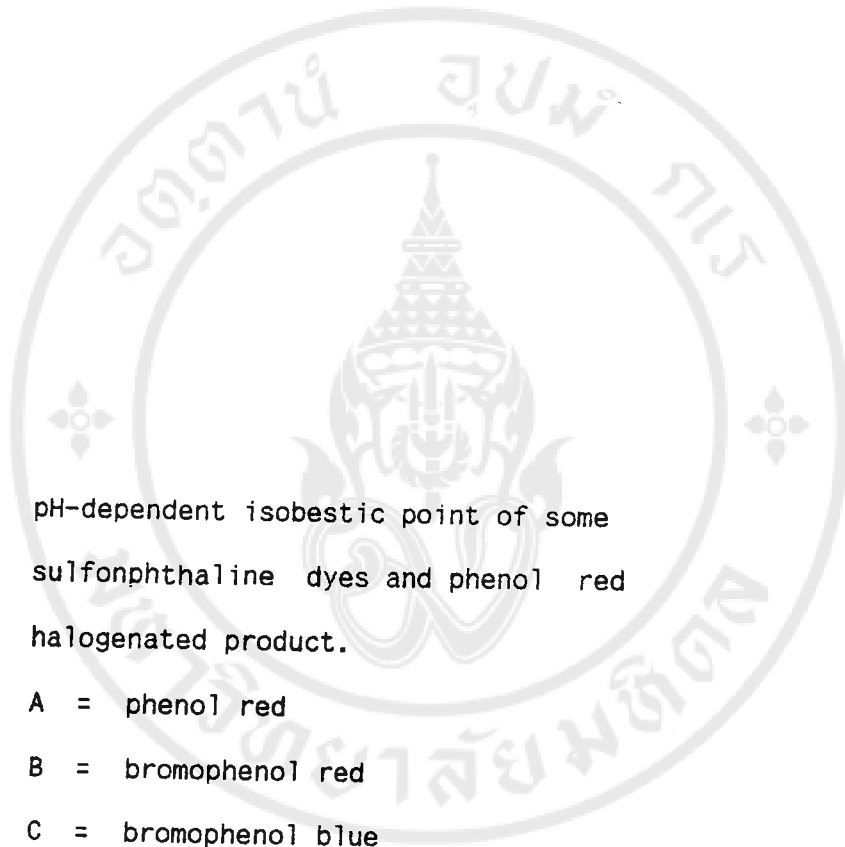

Figure 3 pH-dependent isobestic point of some sulfonphthaline dyes and phenol red halogenated product.

A = phenol red

B = bromophenol red

C = bromophenol blue

D = phenol red final brominated product

E = chlorophenol red

F = phenol red final chlorinated product

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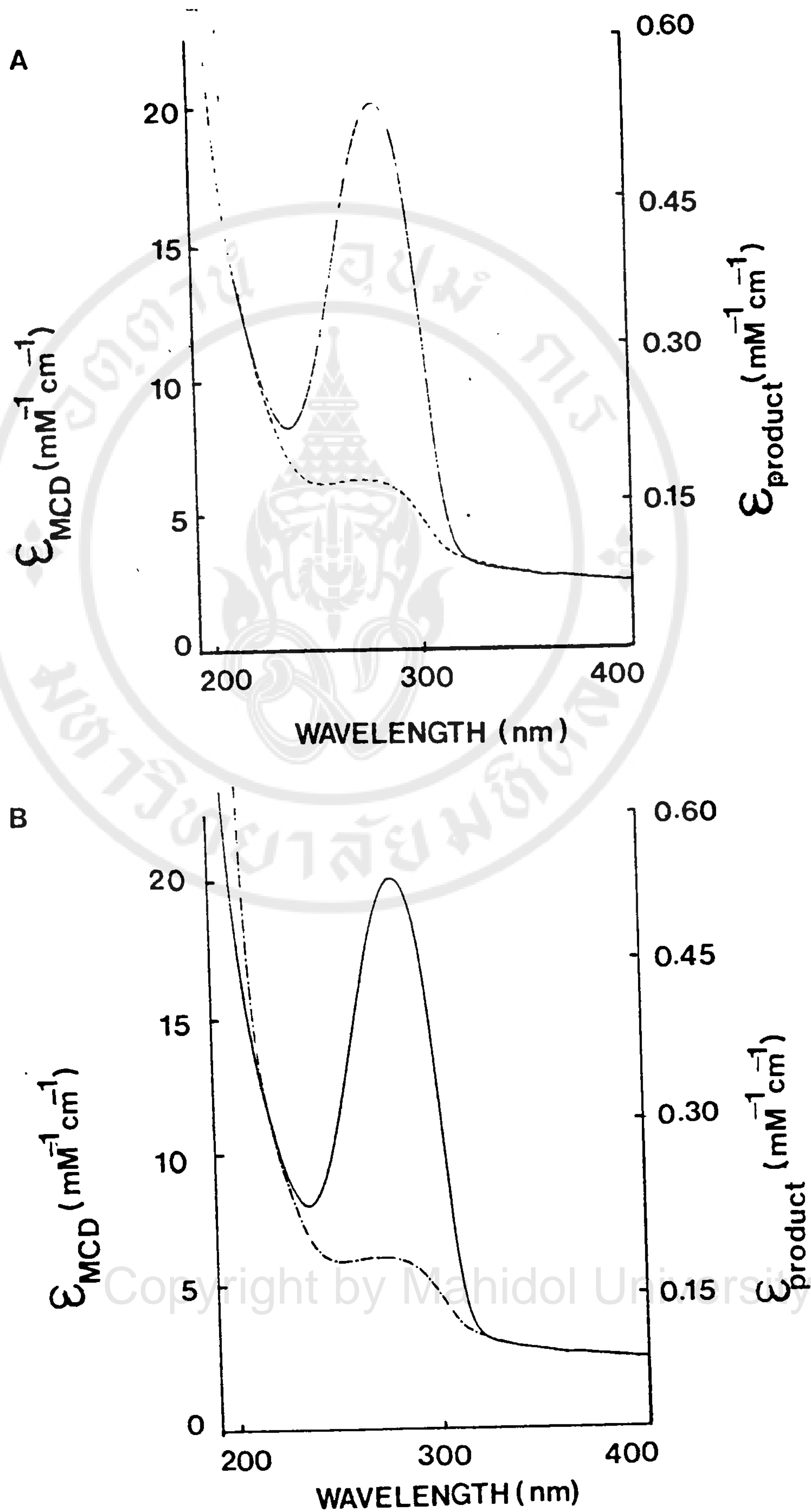

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Figure 2 Absorption spectra of MCD and its final halogenated product.

A = MCD and its final brominated product

B = MCD and its final chlorinated product

—— MCD

----- MCD brominated product

—·—·- MCD chlorinated product

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2. Identification of phenol red final halogenated products by MPO

2.1 Thin layer chromatography (TLC)

For identification purpose, the products obtained from the MPO-catalyzed halogenation reaction were subjected to TLC on Silica gel GF_{254}. The solvent system used was amyl alcohol-ethanol-acetic acid (40:50:5). R_f values of the main phenol red haloganated products were compared with those of sulfonphthalein dyes as shown in Table 6. The R_f values of MPO final brominated and chlorinated product were nearly equal to those of bromophenol blue and chlorophenol red.

The R_f values of these products were also confirmed by TLC in various solvent systems. They were benzene-isopropanol-acetic acid (60:40:1), ethyl acetate-pyridine-water (60:30:10), ethyl acetate-methanol-5 M NH_4OH (60:30:10). It was found that the final brominated and chlorinated product always moved together with bromophenol blue and chlorophenol red respectively.

These final halogenated products were scraped from TLC plates and extracted with water. Their absorption maximum, millimolar extinction coefficient, K_a and pH-dependent isobestic points were compared with sulfonphthalein dyes as shown in Table 7 and Figure 3.

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Table 6 R_f values of sulfonphthalein dyes by TLC on Silica gel GF_{254}

Dye	R_f	color of spot
phenol red	0.33	orange
bromophenol red	0.50	orange
chlorophenol red	0.52	brown
bromophenol blue	0.54	purple
phenol red final brominated product	0.54	purple
phenol red final chlorinated product	0.52	brown

$$R_f = \frac{\text{distance of spot center from start point}}{\text{distance of solvent front from start point}}$$

Solvent system was amyl alcohol-ethanol-conc. NH_4OH (50:45:5)

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Table 7 Absorption maxima (λmax), millimolar extinction coefficient (ε), pK_a and pH-dependent isobestic points of some sulfonphthalein dye and MPO halogenated products of phenol red.

Dye	λ_{max}	$\varepsilon(mM^{-1}cm^{-1})$	pK_a	isobestic point
phenol red	430	19.76	7.8	477
bromophenol red	430	10.82	6.1	487
bromophenol blue	590	63.49	4.1	494
chlorophenol red	575	15.98	6.0	485
phenol red final brominated product	590	63.52	4.1	495
phenol red final chlorinated product	575	15.84	6.0	485

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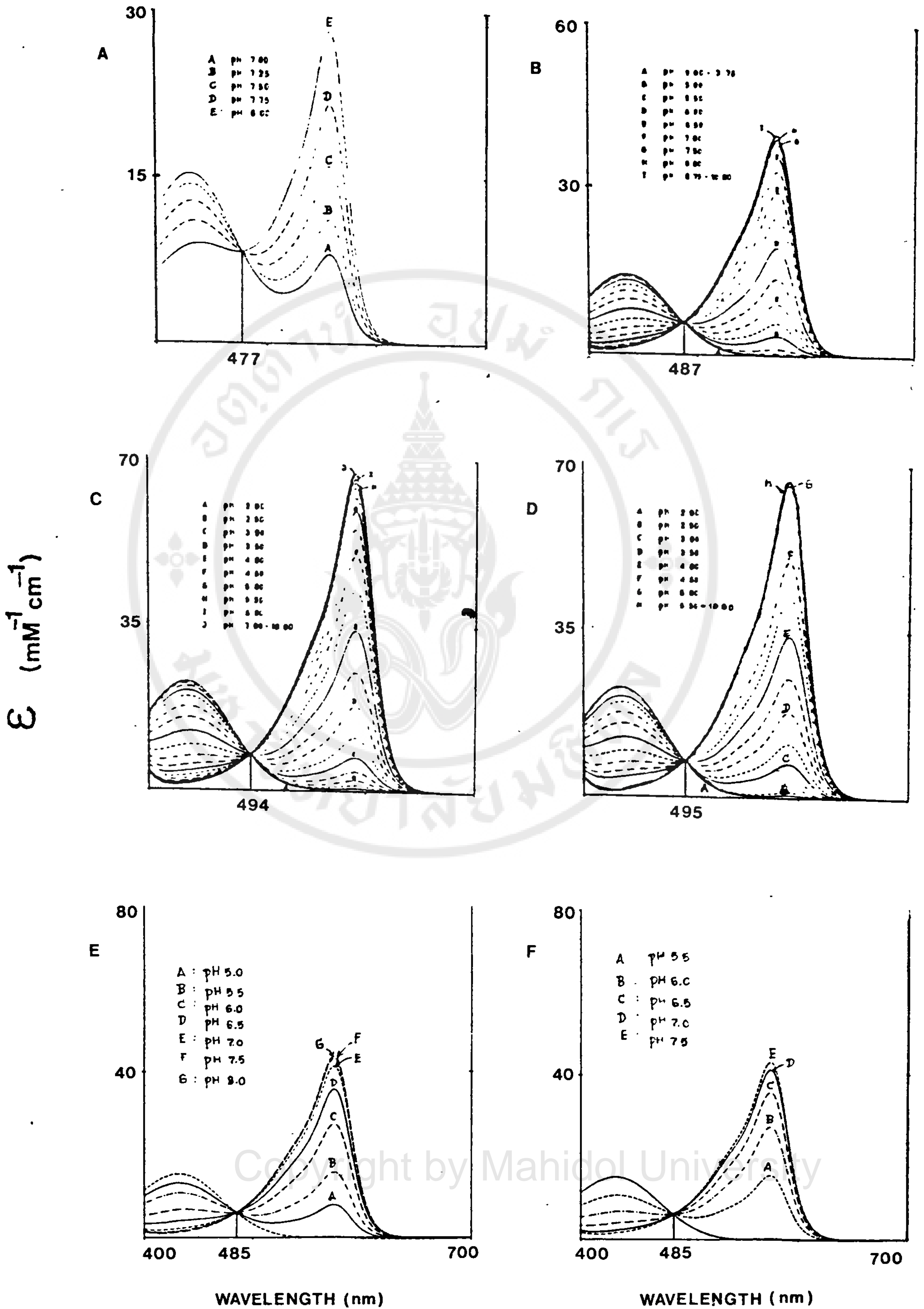

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2.2 Infrared spectroscopy (IR)

Identification of MPO final halogenated products by IR is shown in Figures 3 and 4. The samples were prepared in Nujol, which by itself showed C-H stretching band at 2800-3000 cm^{-1} and C-H bending band at 1370-1460 cm^{-1} (Figure 4 A). The IR spectra of bromophenol blue and MPO final brominated product and chlorophenol red and MPO final chlorinated product are shown in Figures 4 B, 4 C, 5 A and 5 B respectively. They all show O-H stretching band near 3300 cm^{-1}, C=O stretching band near 1600 cm^{-1}, C-O stretching band near 1000 cm^{-1}, C=C stretching band near 1600 cm^{-1} and C-S stretching band near 1200-1300 cm^{-1}. Between 900-1450 cm^{-1}, which is the fingerprint region, the final brominated and chlorinated products showed the same IR pattern as bromophenol blue and chlorophenol red. The C-Br band which should be at 500 cm^{-1} was not clear. The small C-Cl band was observed near 600 cm^{-1}.

2.3 Nuclear magnetic resonance spectroscopy (^{1}H-NMR)

NMR spectrum of phenol red showed two kinds of protons, one kind as multiplet (δ = 7.4-8.2), the other kind showed a clear AB aromatic splitting (δ = 6.75 and δ = 7.12) (Figure 6). Bromophenol blue showed one singlet (δ = 7.55) and multiplet (δ = 7.0-8.1). The multiplet overlapped with the singlet as shown by shoulder at the right side of the singlet (Figure 7 A). Integration showed 1:1:1 ratio for phenol red NMR peaks and 1:1 ratio for bromophenol blue The MPO final brominated product showed the same pattern as bromophenol blue (Figure 7 B).

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Figure 4 Infrared spectra of

A = nujol

B = bromophenol blue

C = phenol red final brominated product

The spectra were recorded by a Jasco model A-302 infrared spectrometer.

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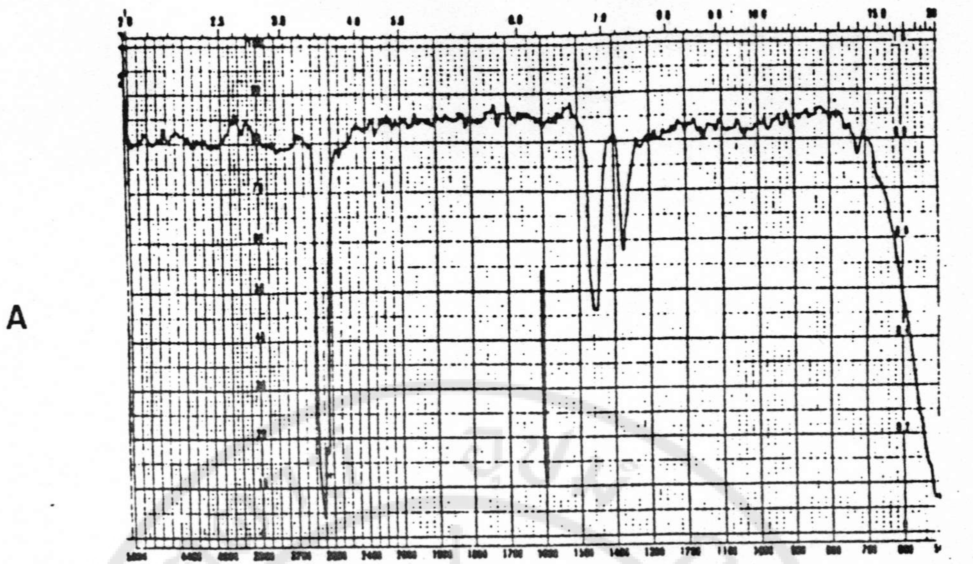
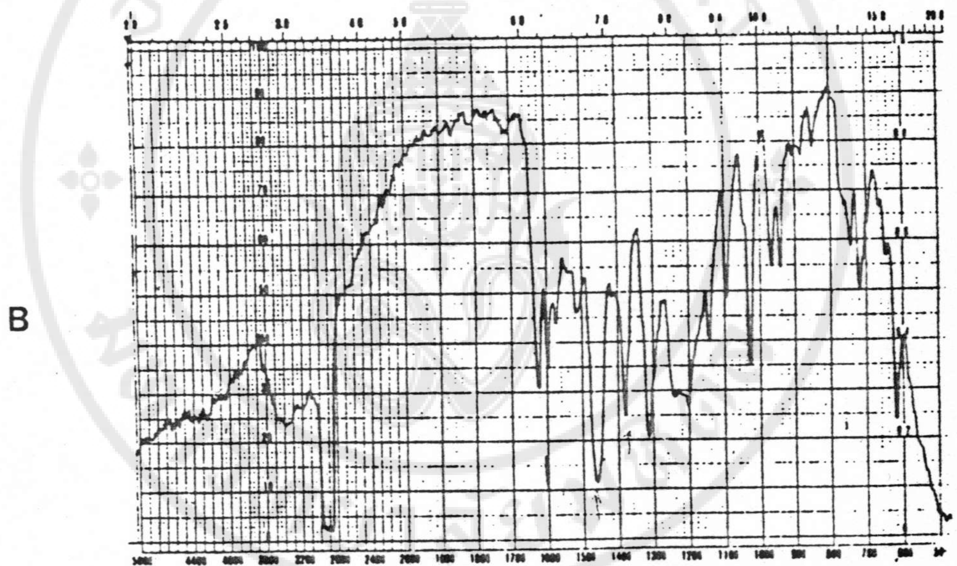
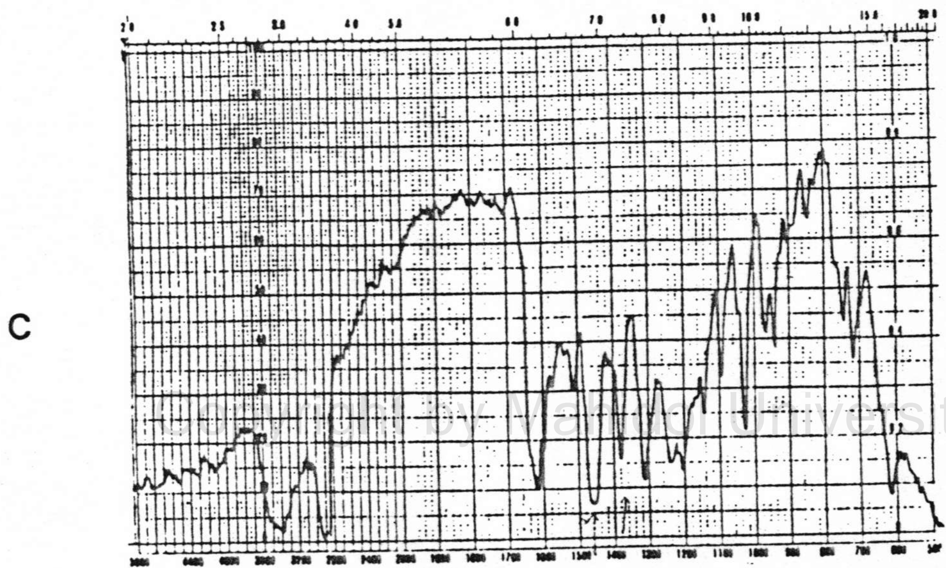
A



B



C



WAVE NUMBER (cm^{-1})

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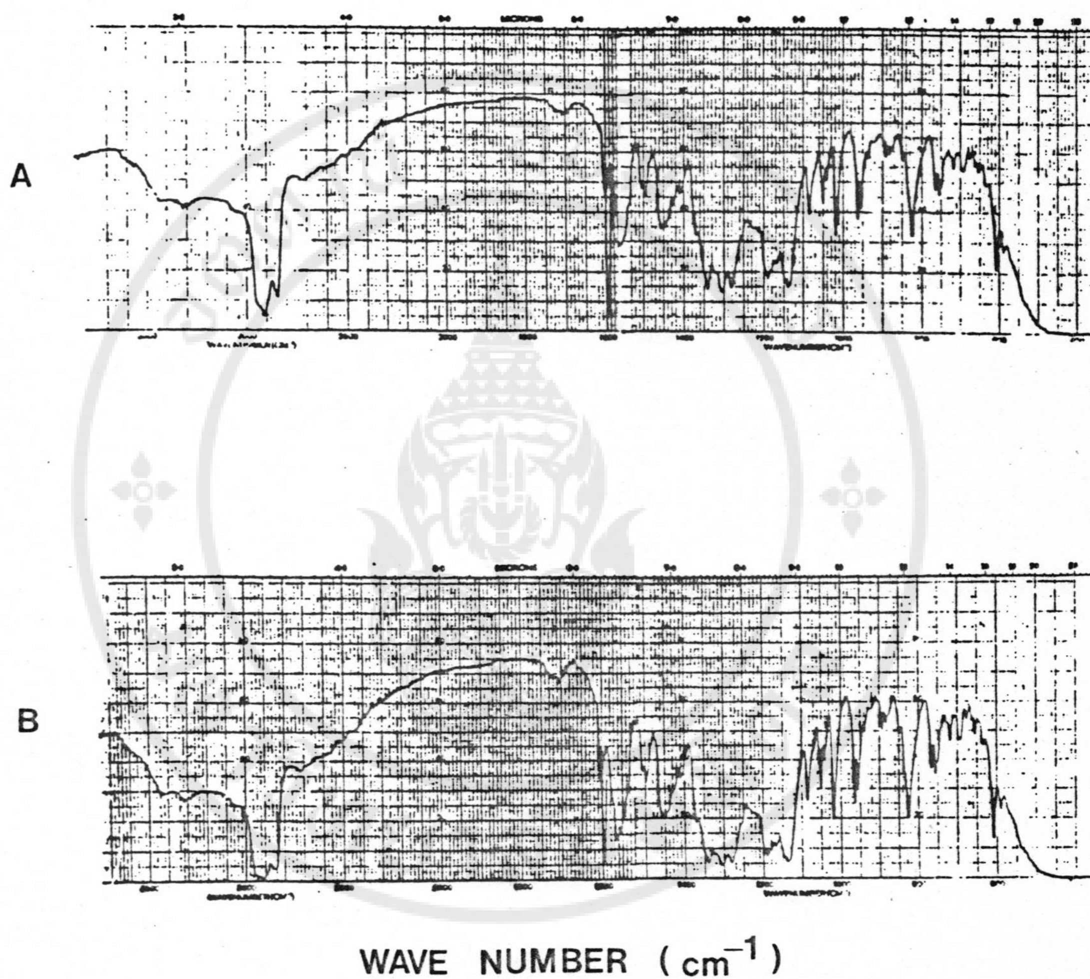

Figure 5. Infrared spectra of

A = chlorophenol red

B = phenol red final chlorinated product

The spectra were recorded by a Perkin-Elmer model 683 infrared spectrometer.

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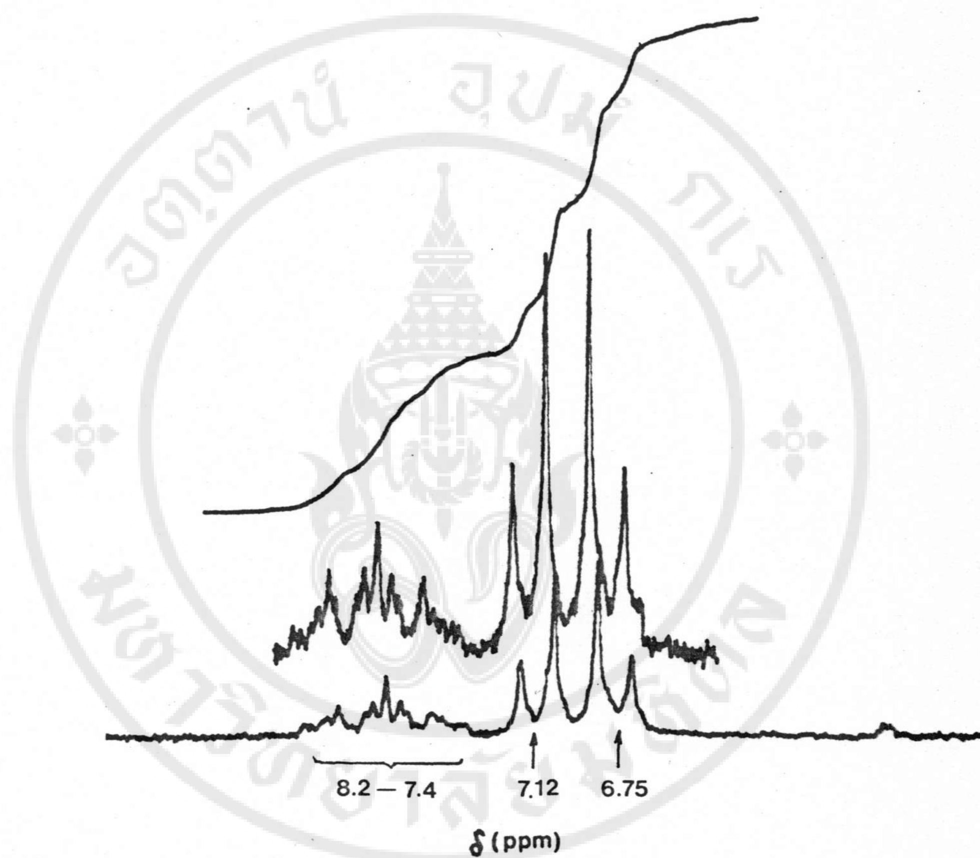

Figure 6 NMR spectrum of phenol red. The spectrum was recorded by a JEOL model FM-3602 NMR spectrometer at 60 MHz. Solvent peaks (δ = 2.3,4.5) and peak of tetramethylsilane (δ = 0) have been omitted.

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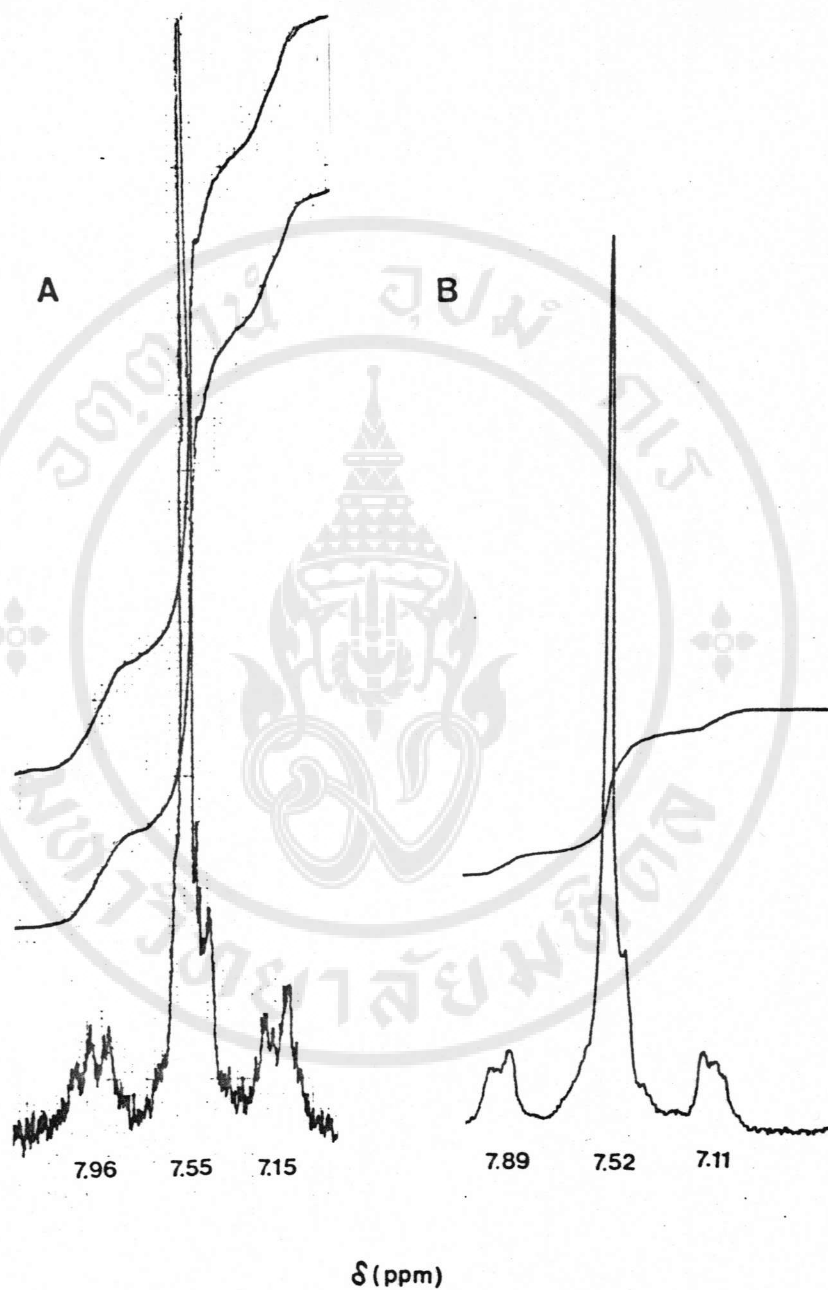

Figure 7 NMR spectra of

A = bromophenol blue

B = phenol red final brominated product

Solvent peaks (δ = 2.3, 4.5, 6.7, 8.2) and peak of tetramethylsilane (δ = 0) have been omitted.

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2.4 Mass spectrometry (MS)

Mass spectrometry of bromophenol blue and MPO brominated product show the presence of molecular ion peaks at m/e 666, 668, 670, 672 and 674 in a ratio of 1:4:6:4:1 (Figure 8). The fragmentation gave rise to the peaks at m/e 587, 508, 429 and 350.

2.5 HPLC and reaction intermediates

The MPO catalyzed bromination reaction was stopped at various time and injected into HPLC reverse phase system. At 0 min of reaction peak I appeared at retention time 3.16 min. At time passed, peak II, III, IV and V were detected at retention time 3.69, 4.95, 5.95 and 7.28 respectively (Figure 9). The peak area of peak I decreased until totally disappeared at 40 min of reaction. Peak II which could be detected at 1 min of the reaction also showed decreasing peak area as time passed. Peak III showed its highest peak area at 5 min of reaction and then decreased. Peak IV always increased. Peak V increased at first and disappeared at 40 min. The plot between peak area and time of reaction was shown in Figure 10. The plot between peak area and amount of the 5 peaks injected into the column showed linear relationship upto 2×10^{-5} M.

Bromophenol red which has retention time 4.95 min like peak III was tried to use as substrate of MPO. At first of the reaction peak 4.95 min decreasd and peak 7.28 which has the same retention time as peak IV appeared. This peak then decreased whereas peak at 6.12 min appeared.

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Peaks II and V were collected from the column. Reaction of peak V fraction with MPO gave only one peak at 6.02 min.

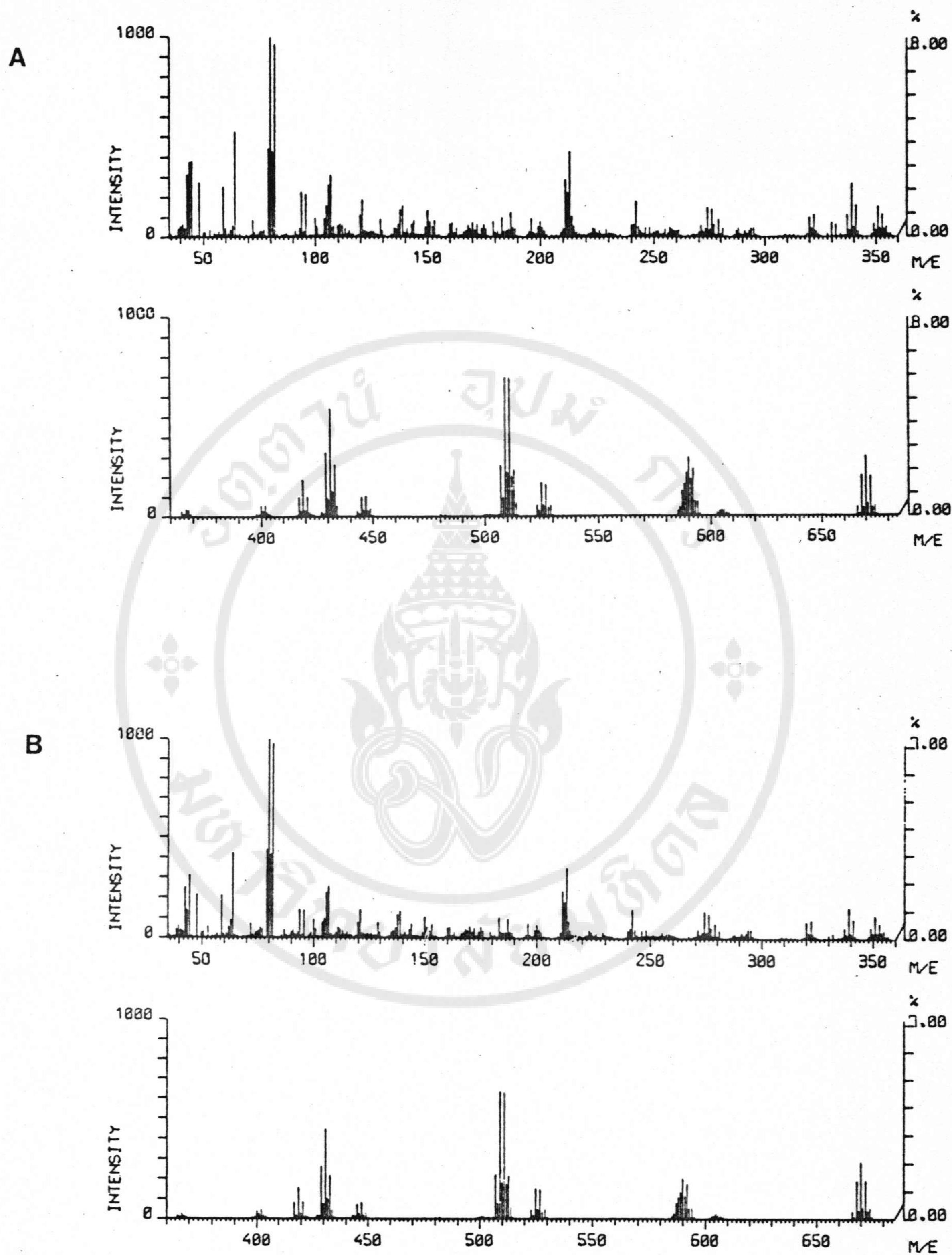

Figure 8 Mass spectra of

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A = bropmophenol blue

B = phenol red final brominated product

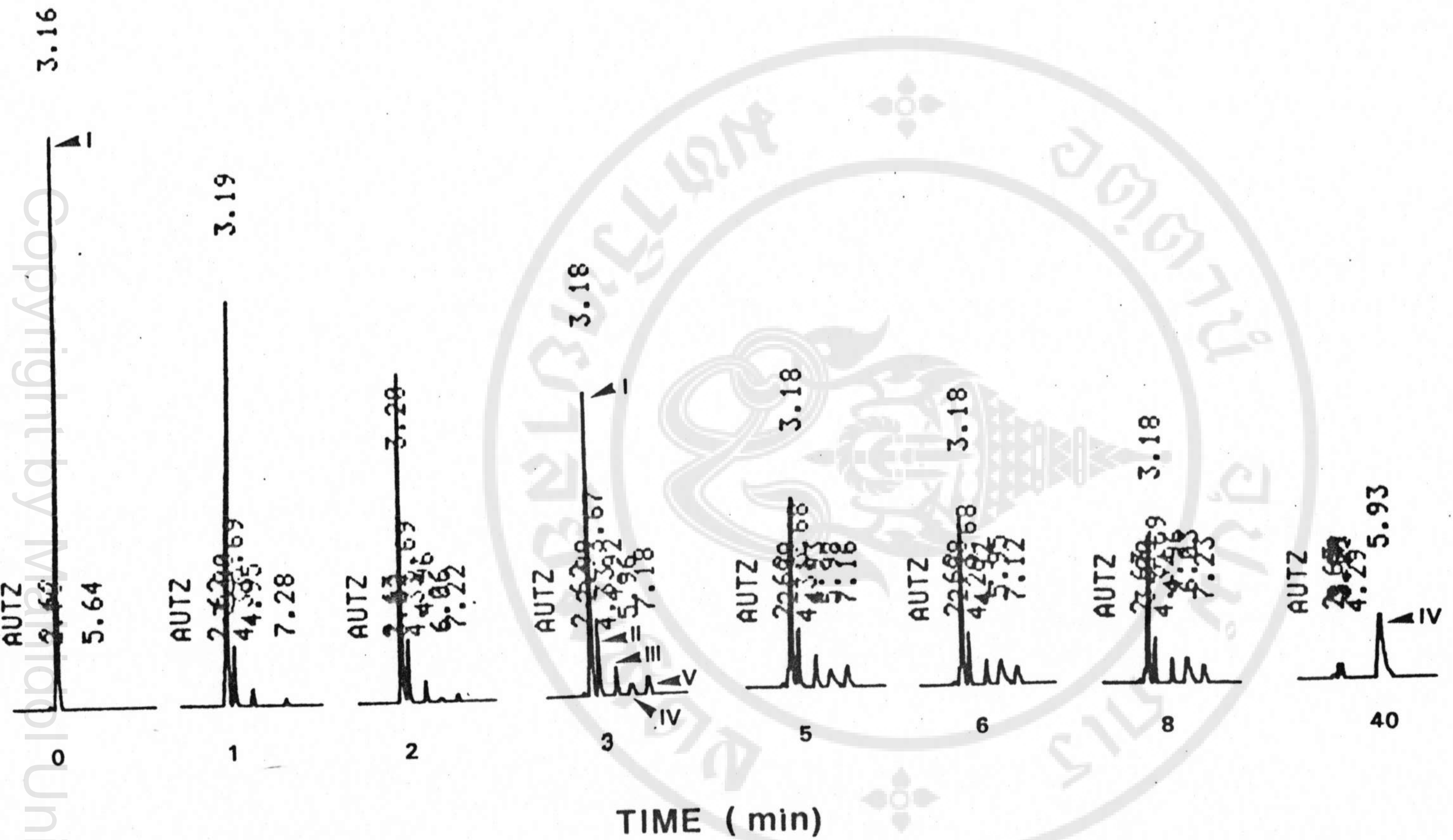

Figure 9 HPLC peaks of phenol red and its brominated products formed at various times. The reaction was catalyzed by MPO.

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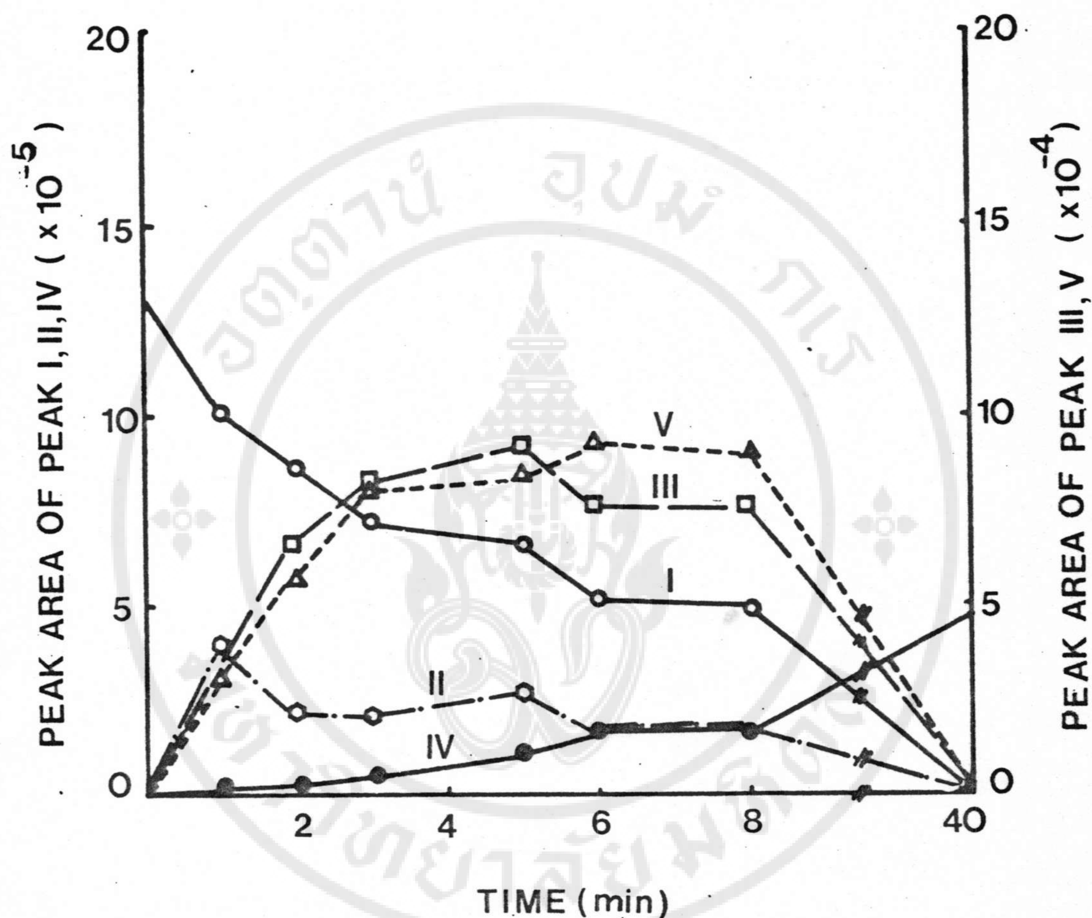

Figure 10 HPLC peak area of phenol red and its brominated products formed at various time. The reaction was catalyzed by MPO.

○——○ peak I ⬡—·—⬡ peak II

□—·—□ peak III ●——● peak IV

△----△ peak V

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Peak II reacted with MPO to give 3 peaks at retention time 4.90, 5.98 and 7.22 min.

The chlorination products of phenol red by MPO gave 3 peaks at retention time 3.16, 3.62 and 4.43 min respectively. There are 2 minor peaks at 6.12 and 7.55 min. The overnight reaction gave a major peak at 4.43.

Standard sulfonphthalein dyes were also injected into the HPLC at the same condition. Phenol red, bromophenol red, bromophenol blue and chlorophenol red gave retention time at 3.14, 4.95, 5.97 and 4.40 respectively. In addition, spiking the reaction solutions with phenol red, bromophenol red, bromophenol blue and chlorophenol red was also carried out. In the bromination of phenol red, it was found that phenol red co-chromatographed with peak I, bromophenol red with peak III and bromophenol blue with peak IV.

3. Kinetic parameters of phenol red as substrate for MPO

Phenol red and MCD solutions followed Beer's law up to 1 x 10^{-4} M. They were stable under UV light (60 cycle/sec) for at least 1 hr. They were also stable when kept at room temperature and at 4° C for at least 35 days. The initial rate velocity of MPO in bromination reaction of phenol red at 2 x 10^{-5} M is shown in Figure 11. The absorbance at 430 nm decreased to 0 in 30 sec. When this initial rate velocity was followed at 590 nm lag time was observed for about 1 to 10 sec of the reaction if the rate was slow.

$K_{m_{app}}$ values for bromination and chlorination of MCD, phenol red and chlorophenol red were determined by measurements of the initial rate velocity at various concentrations of substrates. The

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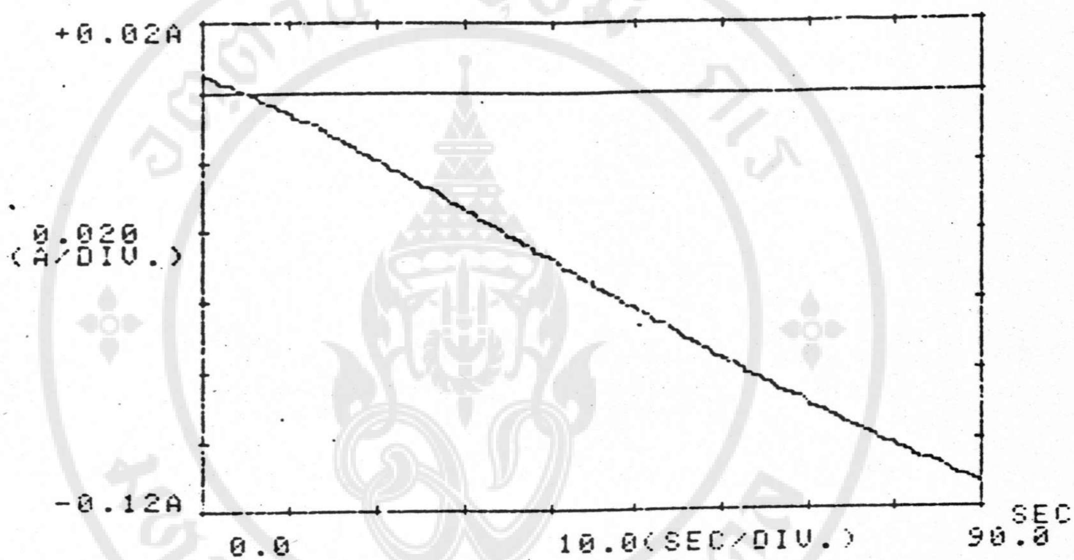

Figure 11 Initial rate velocity of MPO in bromination reaction of phenol red (λ_{max} 430 nm)

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$K_{m_{app}}$ values were calculated from Cornish-Bowden plots. They are summarized in Table 8. Both Cornish-Bowden and Lineweaver-Burk plots of the substrates are shown in Figure 12.

Table 8 $K_{m_{app}}$ values of myeloperoxidase

Substrate	K_m value			
	organic substrate (M)	H_2O_2 (M)	KBr (M)	KCl (M)
MCD	2.6×10^{-5}	1.0×10^{-4}	3.0×10^{-2}	3.0×10^{-1}
phenol red	8.0×10^{-6}	3.5×10^{-4}	4.5×10^{-3}	5.6×10^{-1}
chlorophenol red	2.3×10^{-5}	3.5×10^{-5}	2.7×10^{-3}	-

The pH optimum of MPO for these substrates is shown in Figure 13. MPO exhibited optimum pH at 5 and 5.8 for chlorination and bromination of MCD and phenol red.

4. <u>Cytochemical demonstration of MPO activity in granules of polymorphonuclear leukocytes by phenol red staining</u>

The phenol red staining was shown to have particular staining pattern that could differentiate between lymphoid and non-lymphoid series and also early monocytic and myelocytic series.

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Figure 12 $K_{m_{app}}$ values of phenol red, H_2O_2, KBr and KCl. These substrates were catalyzed by MPO.

A = plot between velocity (ΔA_{430}/min) and substrate concentration (M).

B = Lineweaver-Burk plot between 1/v and 1/S

C = Cornish-Bowden plot between v and S

The substrates used for $K_{m_{app}}$ determination

1 phenol red

2 H_2O_2

3 KBr

4 KCl

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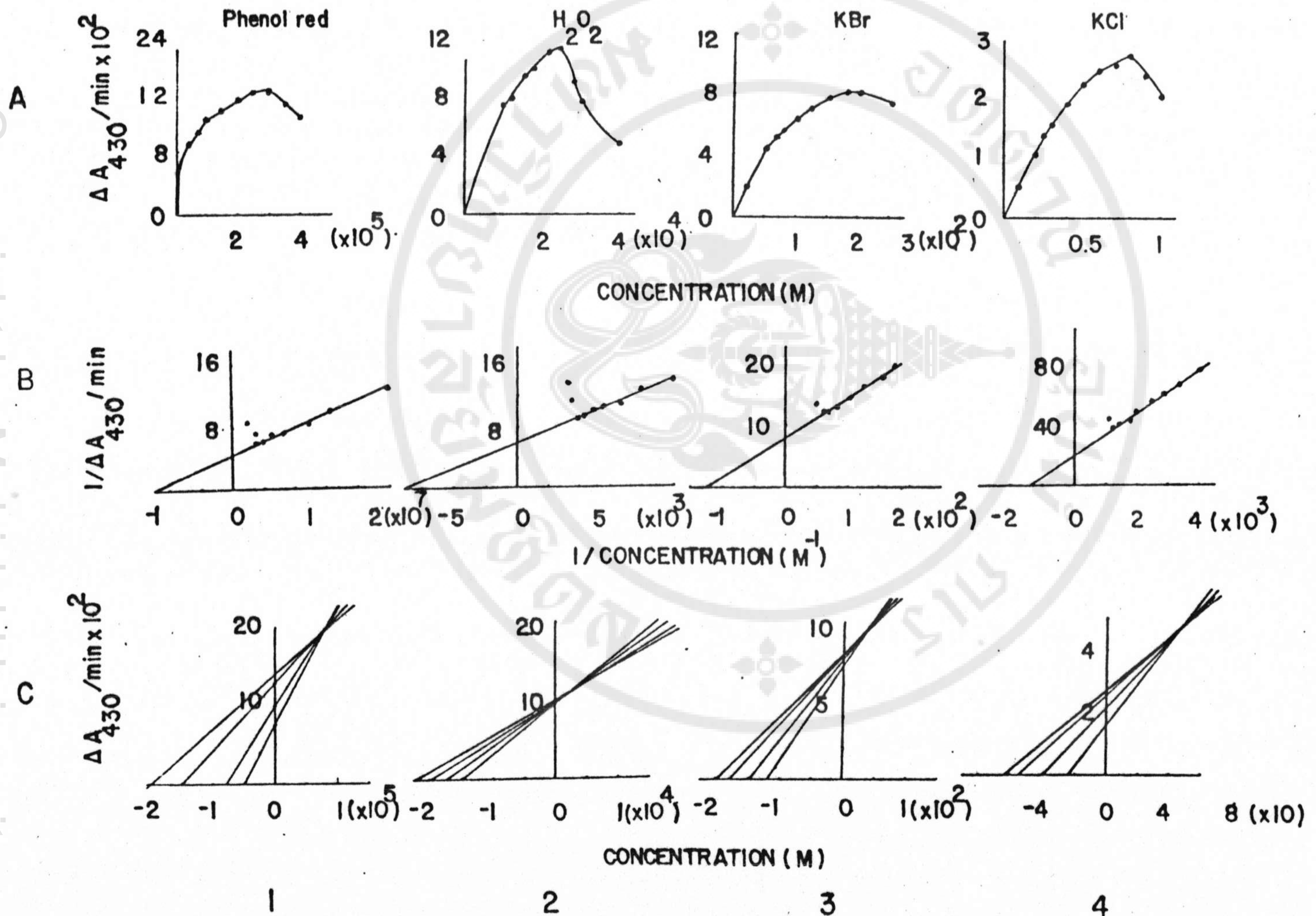
A
B
C
Phenol red
H_2O_2
KBr
KCl
ΔA_{430}/min x 10^2
$1/\Delta A_{430}$/min
CONCENTRATION (M)
1/CONCENTRATION (M^{-1})
CONCENTRATION (M)
1
2
3
4

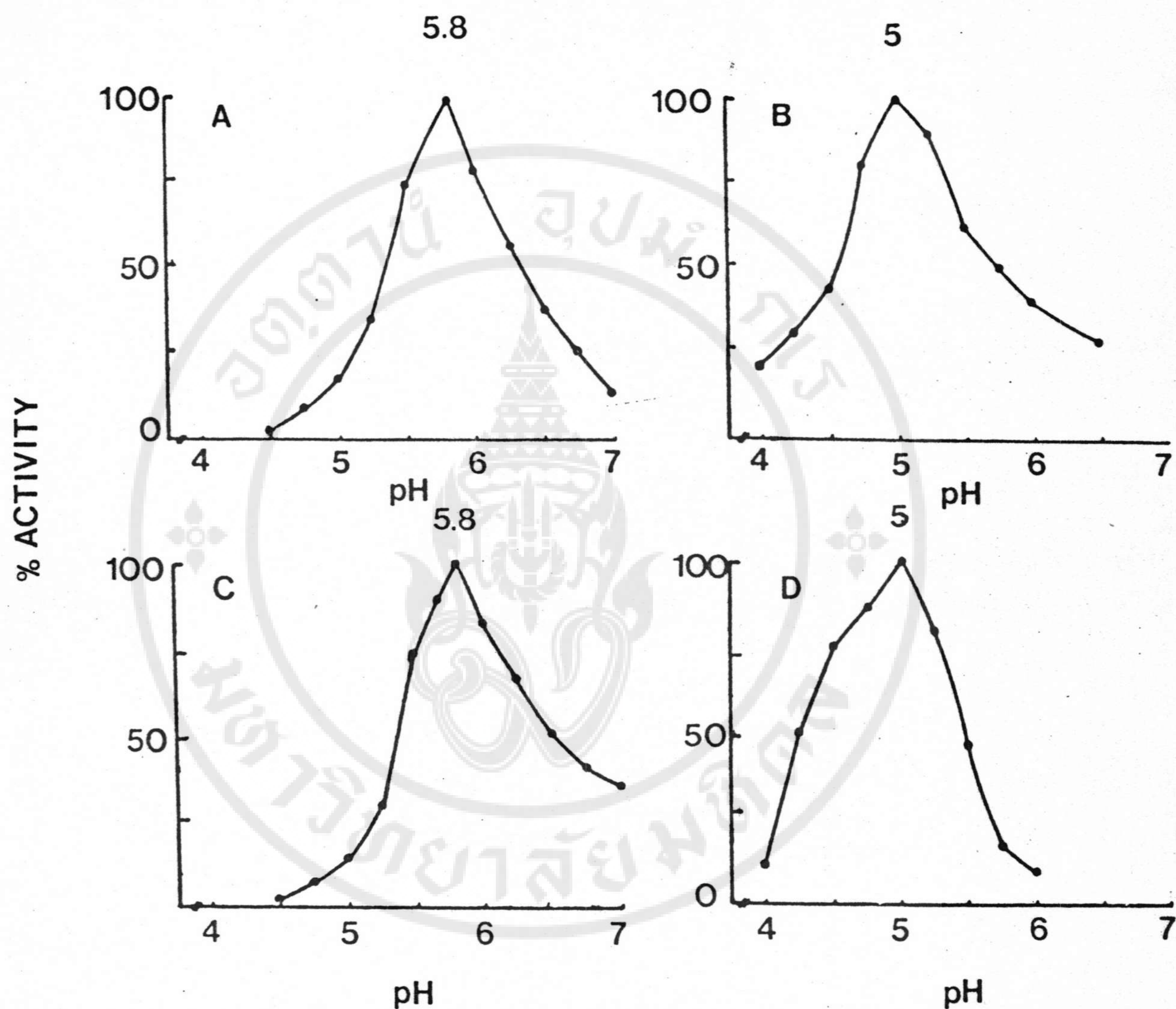

Figure 13 pH-rate profile of MPO with various substrates and halide ions

A = MCD and KBr

B = MCD and KCl

C = phenol red and KBr

D = phenol red and KCl

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4.1 Cell series in peripheral blood from normal subjects

4.1.1 Granulocytic series

After staining with phenol red, KBr and H_2O_2 the intense purplish blue granules were found in cells of granulocytic series with increasing positivity with progressive maturity as shown in Figure 14. Myeloblasts (Figure 14 A) possesed a few small purplish blue granules localized near the nucleus. The form of positivity became coarse and more extensive in promyelocytes (Figure 14 B), while myelocytes (Figure 14 C) and metamyelocytes (Figure 14 E) and band forms of the neutrophil series had increasing numerous stained granules. Polymorphonuclear leukocytes showed very strong positivity, the granules filling the cytoplasm and often obscuring part of the nucleus (Figure 14 F). Eosinophil cells of all stages of maturity showed a strongly positive reaction than neutrophil series (Figure 14 G). The granules were not seen in basophils.

4.1.2 Monocytic series

A few fine and coarse purplish blue granules scattered discretely over the cells was very characteristic of cells of the monocytes and their precusor which could be easily distinguished from granulocytic series (Figure 14 H).

4.1.3 Lymphocytic series and platelet

As shown in Figure 15 A and B, lymphocyte and platelet show negative staining by phenol red. Red blood cells also exhibited no purplish blue color.

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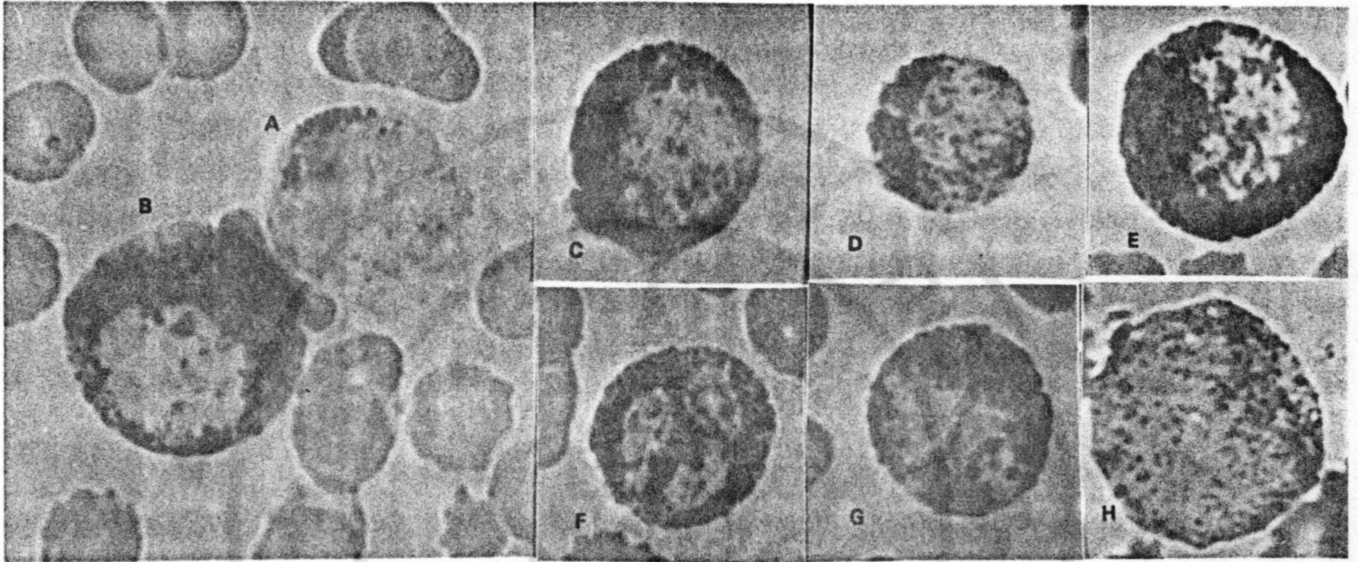

Figure 14 Myeloperoxidase staining using phenol red, H_2O_2 and KBr.

The peripheral blood was obtained from normal human subjects. The photographs were taken under microscope at magnification 1000x with blue filter.

A = myeloblast B = promyelocyte

C = myelocyte D = promyelocyte

E = metamyelocyte F = neutrophil

G = eosinophil H = monocyte

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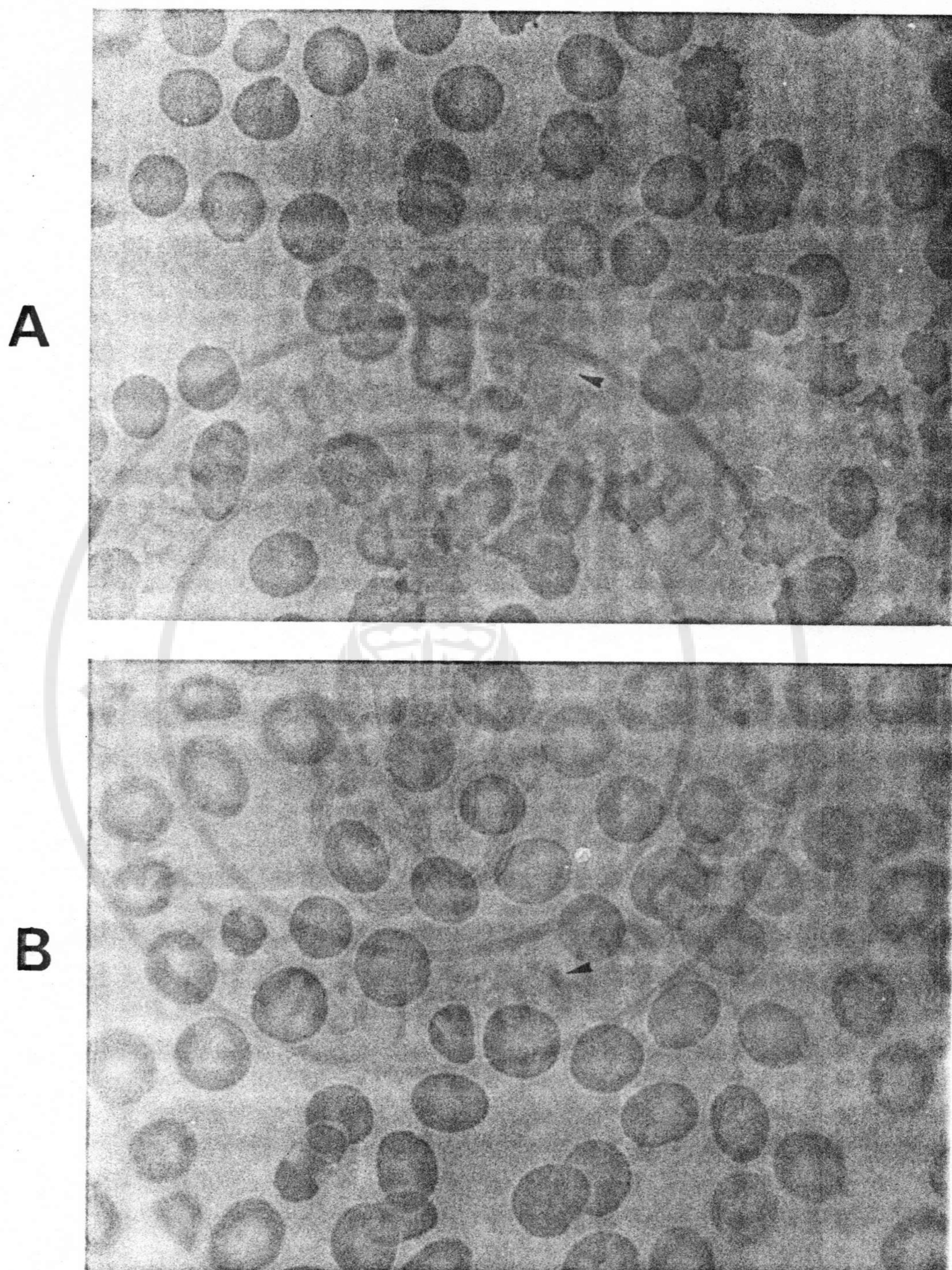

Figure 15 Phenol red staining of white blood cells of normal human subjects. The magnification was 1000x.

A = lymphocyte

B = platelet

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4.2 Peripheral blood and bone marrow from leukemic patients

The staining pattern of granulocytic and monocytic series in leukemic patients was studied by phenol red staining. Both series had positive reaction as purplish blue color in their granules. However, myeloblasts from M_1,M_2 and M_4 patients were intensely stained showing heavy localized granules in the cytoplasm (Figure 16 A,B,C,F and G) whereas monoblasts from M_4 and M_5 (Figure 16 D,E,H and I) patients had a few fine and coarse purplish blue granules scattered discretely over the cells.

4.3 Animal blood cells

The blood samples from rat, mouse, rabbit, hamster and bovine also revealed the purplish blue granules in granulocytic and monocytic series by phenol red as mentioned above.

4.4 Aging of smears before and after staining with phenol red

The blood and bone marrow smears could be kept at room temperature for 14 months before staining with phenol red. The stained smears could be stored at room temperature either in dark or exposed to light without color fading for 14 months. The comparison was made with freshly prepared blood smears.

4.5 The comparison of phenol red staining, Sudan black staining, and benzidine staining method

Phenol red staining showed purplish blue color distributed overall the granules of the granulocytic cell series. Benzidine staining gave blue product with the same staining pattern as that of phenol red (Figure 17 A) except no blue color was seen in

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Figure 16 Myeloperoxidase staining using phenol red, H_2O_2 and KBr. The blood was obtained from patients with myeloid leukemia. The photographs were taken under microscope at magnification 1000x with blue filter.

A = micromyeloblast (M_1)

B = myeloblast of myeloblastic leukemia without maturation (M_1)

C = metamyelocyte of myeloblastic leukemia with maturation (M_2)

D = monoblast of myelomonocytic leukemia (M_4)

E = myeloblast of myelomonocytic leukemia (M_4)

F = promyeloblast of myeloblastic leukemia

G = promyeloblast of hypergranular promyelocytic leukemia (M_3)

H = monoblast of monocytic leukemia (M_{5a})

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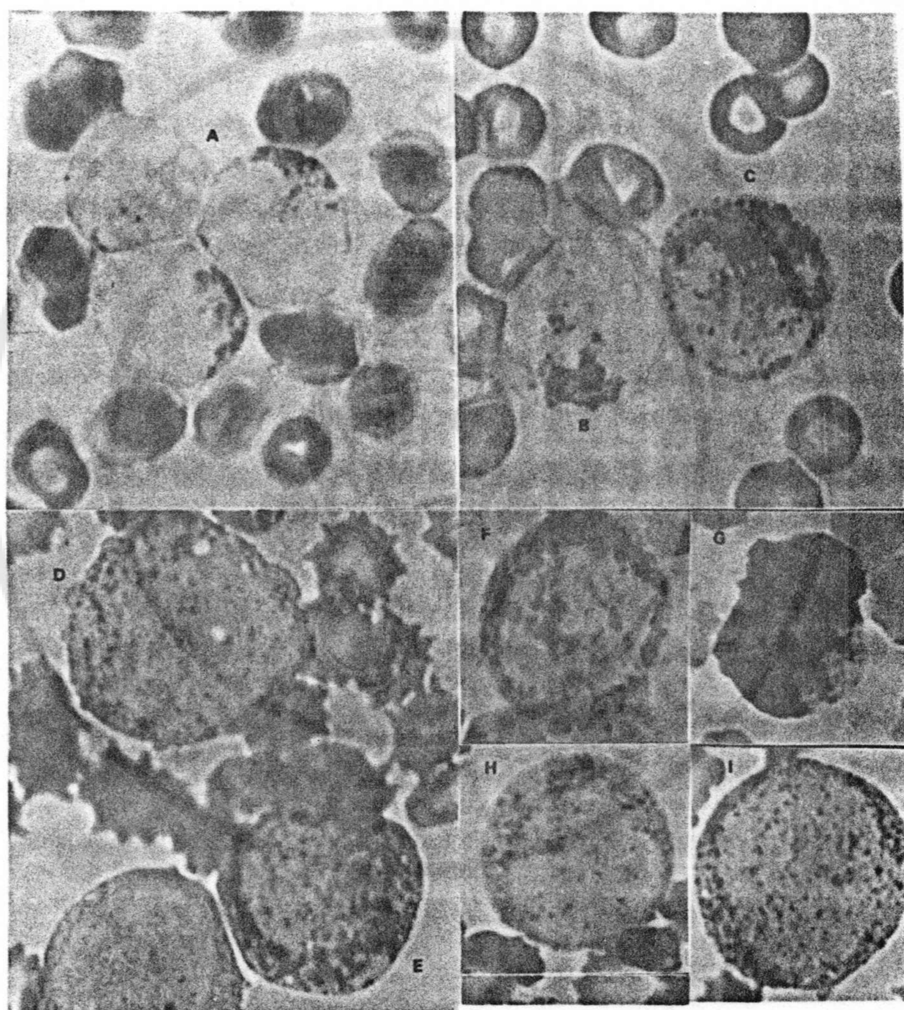
I = monoblast of monocytic leukemia (M_{5b})



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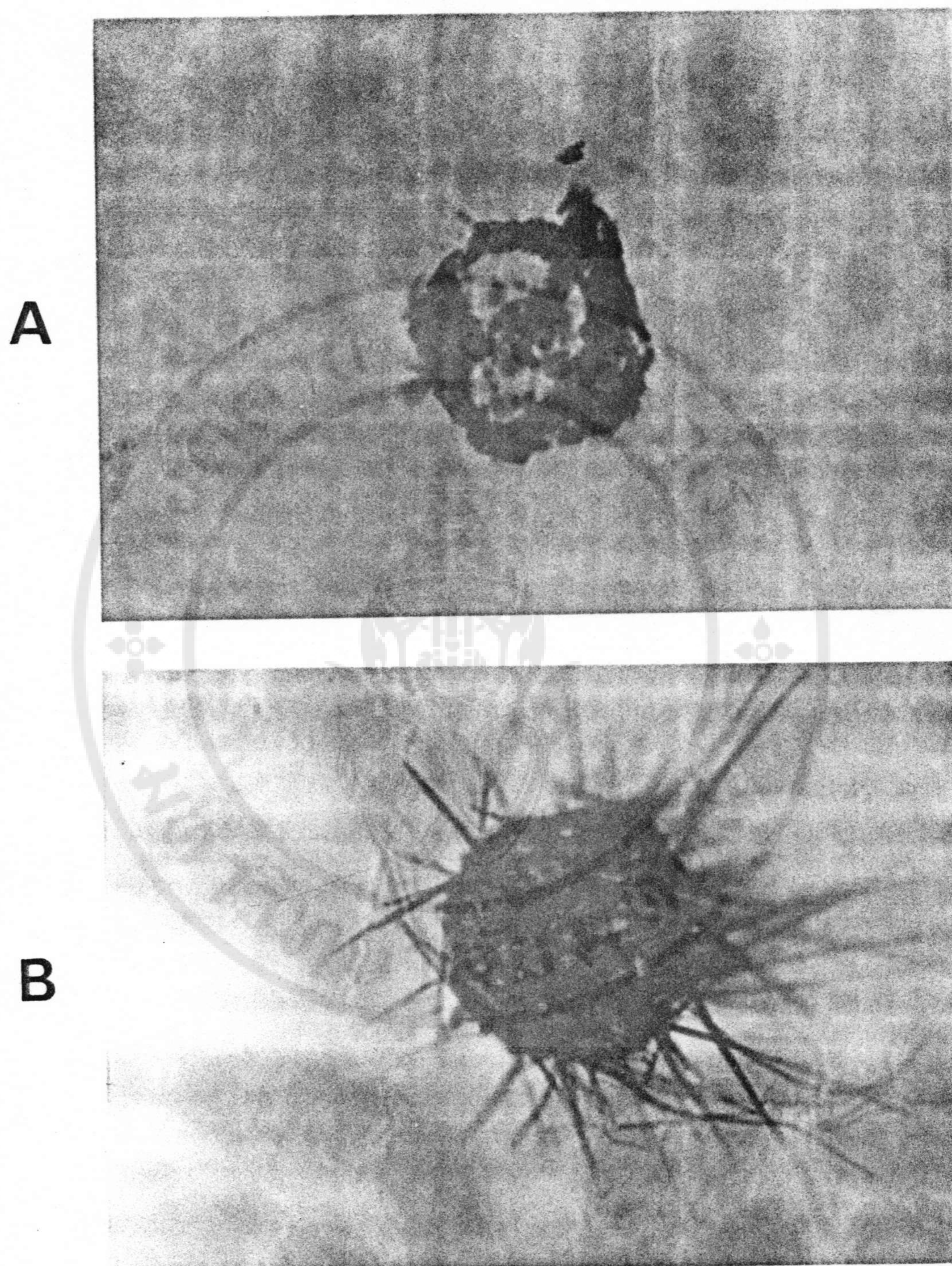

Figure 17 Benzidine staining of neutrophil from normal human subject as magnification 1000x.

A = benzidine staining

B = benzidine staining with crystal formation

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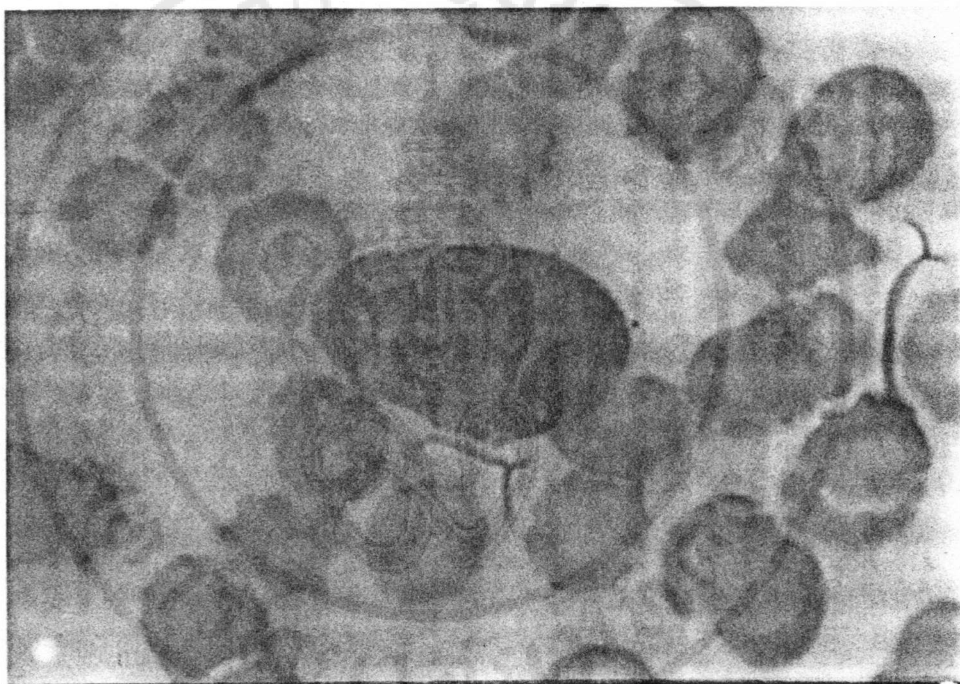

Figure 18 Sudan black staining of monocyte from monocyte from monocytic leukemic patients (M_5). The magnification was 1000x.

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early myeloblasts and monoblasts. The crystal formation was often seen in benzidine staining (Figure 17 B) Sudan black staining pattern was seen as black bodies in neutrophil granules or as shell of eosinophil granules. The early monoblast and myeloblast granules were also seen as black bodies (Figure 18). The time used for staining by phenol red, benzidine and Sudan black was 3, 7 and 70 min respectively.

In phenol red staining, the purplish blue could not be observed in peripheral blood and bone marrow smear when bromide was omitted from the staining solution. When chloride was added instead of bromide, no purplish blue color was observed. Chlorophenol red and bromophenol red could substitute phenol red.

When H_2O_2 was not added into the phenol red staining solution, pale purplish blue granules were observed. Type of buffers (phosphate buffer, citrate buffer, acetate buffer) used in preparing incubation mixture of phenol red has no effect to the staining pattern of the granules. But pH has an important role. Higher of lower pH than 5 caused no color change of phenol red as observed in slides. Sodium azide and potassium cyanide at a concentration of 10^{-3} M can also inhibit this color change.

5. Applications of phenol red in staining MPO and the other haloperoxidases in polyacrylamide gel and agarose gel

5.1 Isoelectricfocusing of MPO and other three haloperoxidases

The isoelectric points of haloperoxidases were determined by isoelectric focusing on polyacrylamide gel. A pH gradient

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were generated by ampholine in the pH range 2.5-5 for CPO, 3.5-10 for BPO and 9-11 for MPO and LPO.

5.1.1 MPO

MPO from normal human leukocytes having isoenzymes at pI of 10, 9.8, 9.5, 8.9, 8.5 and 8.4 as shown in Figure 19 A. Phenol red and DAB staining for the same gel divided into half gave the same results. Phenol red gave blue stains, whereas benzidine gave brown stains. The blue bands diffused much further than the brown bands. However, they appeared after only 1 min and 10 min for phenol red and DAB staining respectively. MPO from rat, mouse and rabbit had pI around 9-10 by phenol red staining.

5.1.2 CPO

CPO showed 2 bands of pI 3.8 and 3.6. The bromoperoxidatic activity bands appeared in 1 min. The peroxidatic bands appeared in 5-10 min. Phenol red staining for CPO activity rapidly diffused so that photographs were taken immediately after staining. DAB staining gave the stable brown bands as shown in Figure 19 B.

5.1.3 LPO

LPO from bovine milk had only 1 isoelectric point at 9.5 (Figure 19 C). This also corresponsed with peroxidatic activity staining.

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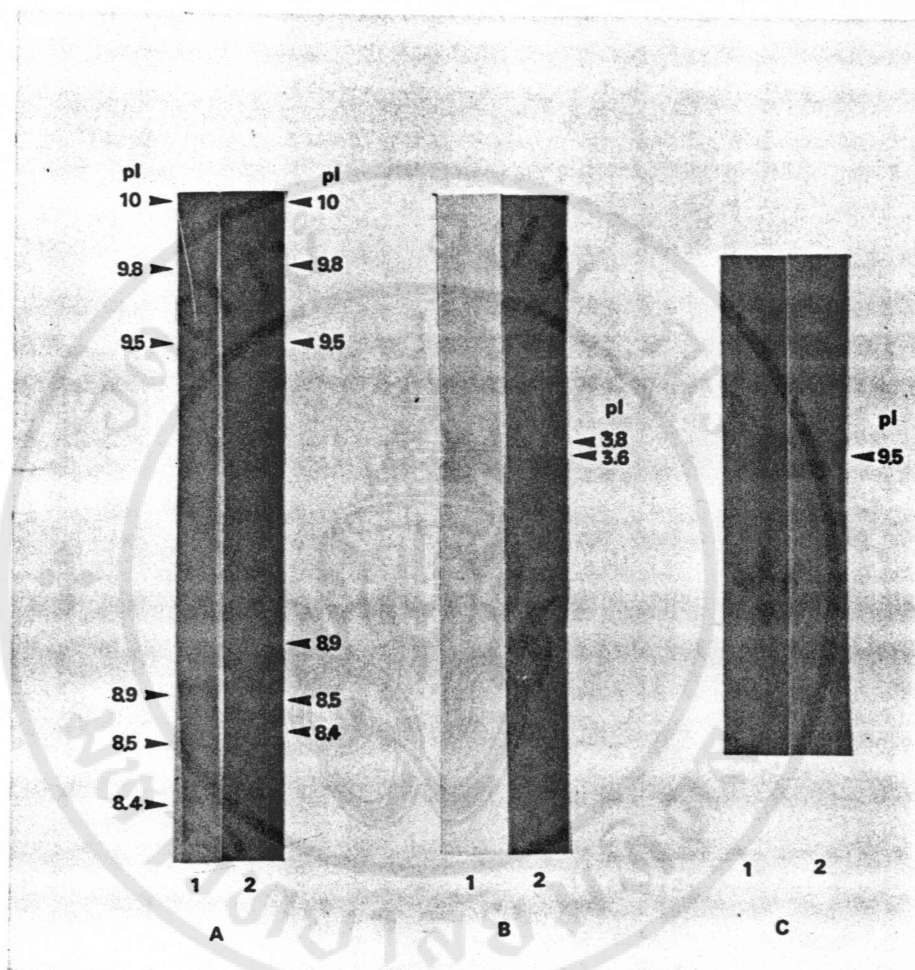

Figure 19 Isoelectric focusing gels of

A = myeloperoxidase (MPO)

B = chloroperoxidase (CPO)

C = lactoperoxidase (LPO)

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The gels were stained by

1 = phenol red, 2 = DAB

The pI values are indicated.

5.1.4 BPO

For bromoperoxidase activity staining using phenol red, H_2O_2 and KBr as substrate, the 5 blue bands appeared in the gel within 5 min. They had pI of 9, 8.8, 6.6, 6.5 and 6.2 (Figure 20 A). At least three peroxidatic isoenzymes stained by DAB (pI 7.1, 6.8 and 3.2) did not stain with phenol red. Two bromoperoxidatic isoenzymes (pI 8.8 and 6.6) did not stain with DAB. When KBr was omitted from the staining solution the blue bands could not be seen. No band appeared when H_2O_2 was omitted from both type of staining solutions. A buffer extraction of peroxidase-positive but bromoperoxidase-negative bands did not show any halogenation of MCD in the presence of H_2O_2 and chloride or bromide ion, whereas the bromoperoxidase-positive band extracts could brominate MCD. The peroxidase activity of a Thai seaweed was stained with DAB and H_2O_2. Brown bands appeared in 20 min indicating that the seaweed had 6 isoenzymes with pI 9, 7.1, 6.8, 6.4, 6.2 and 3.2 (Figure 20 B). The same result was obtained when KBr was added into the staining solution.

Both phenol red and DAB staining in polyacrylamide gel were inhibited from addition of 1×10^{-3} M of sodium azide and potassium cyanide into the staining solution. The bands did not appear when pH of the staining solution was less than 4 or higher than 6. Phenol red can be substituted by bromophenol red and chlorophenol red which gave blue bands with the same pattern as phenol red. Phosphate, Tris, acetate, barbiturate, citrate, chloride and ampholine had no effects on stainability. Iodide gave rise to a yellow color background which did not interfere with our purple

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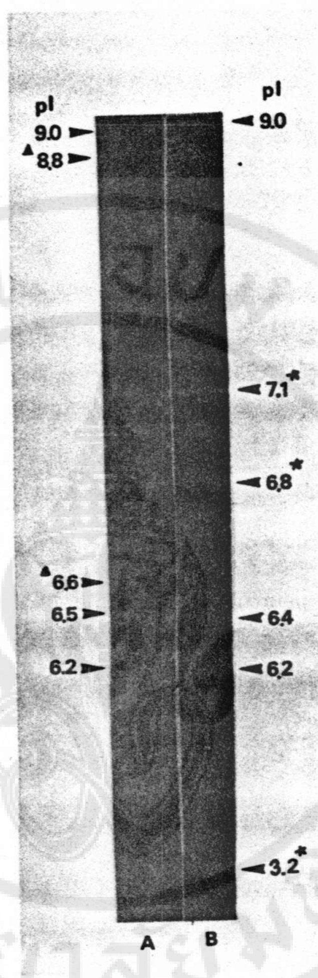

Figure 20 Isoelectric focusing gel of a seaweed bromoperoxidase stained with
A = phenol red, B = DAB
The asterisks and triangles at the top-right of pI values indicate peroxidase activity bands and bromoperoxidase activity bands which do not correspond in the two staining method. The pI values are indicated.

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band appearance.

5.2 CETAB-polyacrylamide gel electrophoresis of MPO

Crude MPO extract prepared from normal human leukocytes was compared with commercially purified human polymorphonuclear leukocyte MPO by phenol red staining of CETAB gel as shown in Figure 21. Blue band of crude MPO extract appeared at the same position as the commercial MPO.

5.3 Rapid detection of bromoperoxidatic activity by agarose gel

In field study, the assay of BPO by spectrophotometry would not be convenient. The rapid detection of BPO was developed by using agarose gel. Agar sources from commercial agar, Thai seaweed agar, Bacto agar and analytical grade agarose could be used.

The color of agarose changed from yellow to blue in the presence of BPO from Thai seaweed extracts (Figure 22 A). A relatively intact piece of seaweed itself could be checked for BPO activity by immersion in agarose gel. The blue color appeared near the tips of the seaweeds as shown in Figure 23 A. Some seaweeds did not change color of phenol red in agarose gel (Figure 23 B). The crushed seaweeds gave the same result. The extract of these seaweeds was observed to change color of phenol red solution containing H_2O_2 and KBr as well as seen in agarose gel. The seaweeds from the south and southeast of Thailand about 20 kinds have already been tested. Naturally, control agarose gel slides had no H_2O_2 or bromide ions (Figure 24).

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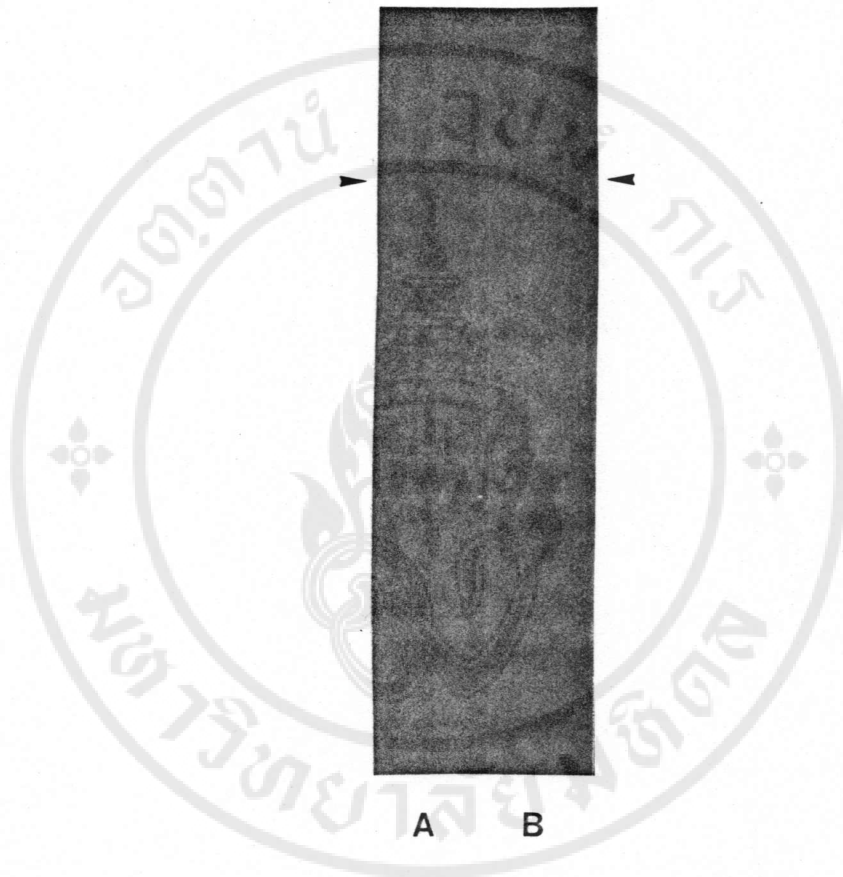

Figure 21 CETAB-PAGE pattern of MPO from normal leukocytes.

A = purified MPO

B = crude MPO extract

The gel was stained by using phenol red, H_2O_2 and KBr as substrate.

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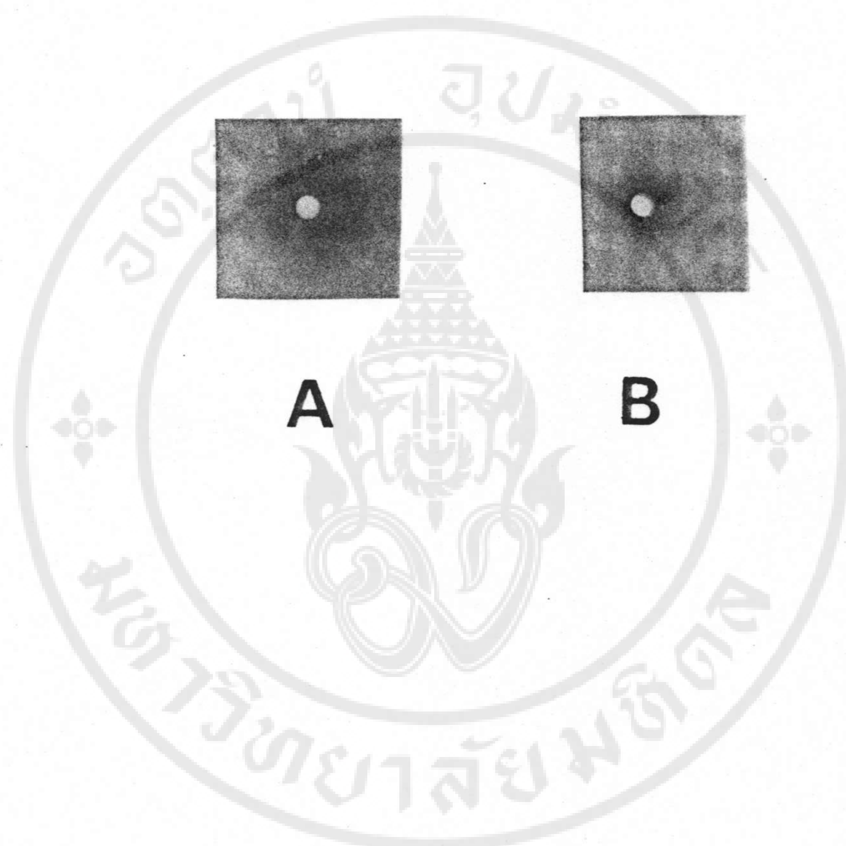

Figure 22 Rapid detection of bromoperoxidatic activity of Thai seaweed extracts by agarose gel impregnated with phenol red, H_2O_2 and KBr.

A = the seaweed extract which had bromoperoxidatic activity

B = the seaweed extract which had no bromoperoxidatic activity

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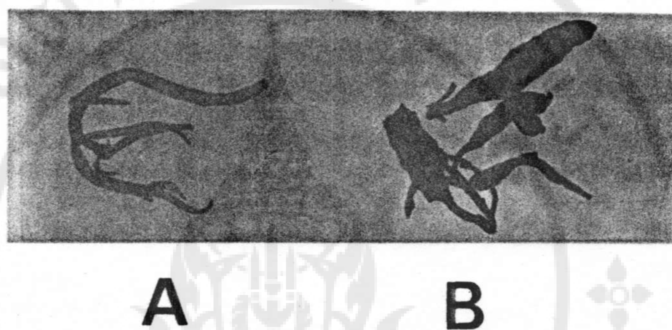

Figure 23 Rapid detection of bromoperoxidatic activity of Thai seaweeds by agarose gel impregnated with penol red, H_2O_2 and KBr.

A = the seaweed which had bromoperoxidatic activity immersed in the gel

B = the seaweed which had no bromoperoxidatic activity immersed in the gel

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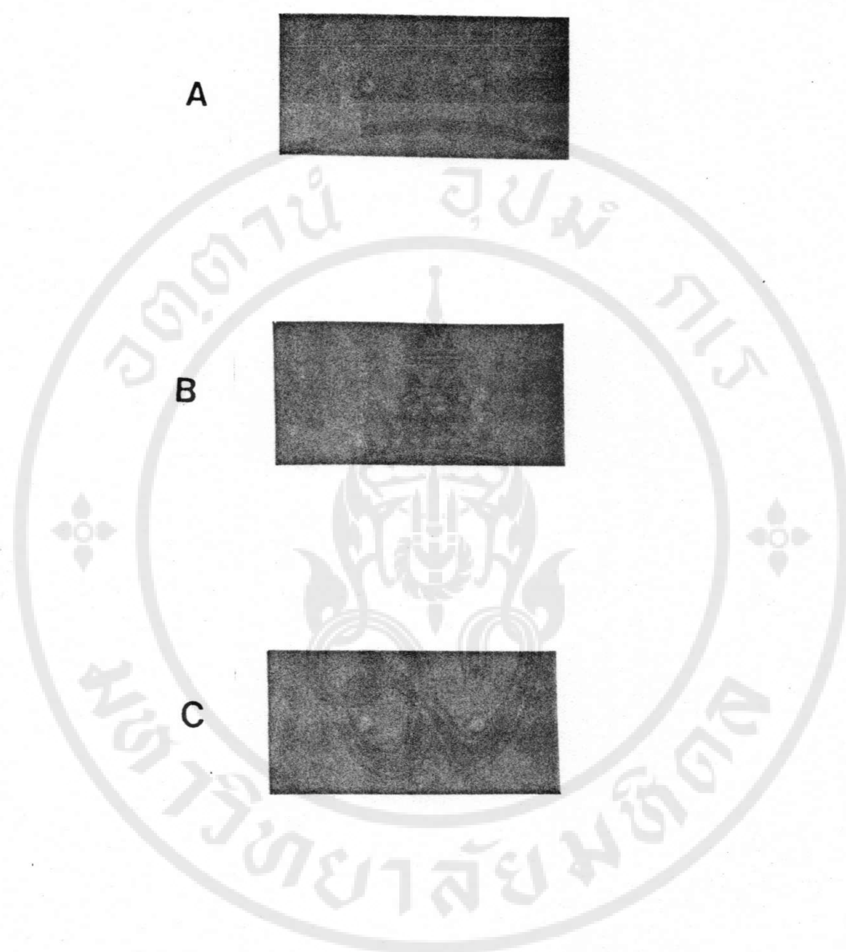

Figure 24 Control agarose gel impregnated with phenol red, H_2O_2 and KBr for detection of bromoperoxidatic activity of haloperoxidases.

A = NO KBr

B = No H_2O_2

C = No enzyme

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CHAPTER IV

DISCUSSION

1. Investigation of phenol red as substrate for haloperoxidases

The color change in sulfonphthalein dye upon enzymatic halogenation would be a useful and simple assay system for haloperoxidases because the reaction would be observed visually and it would have an advantage for screening haloperoxidase enzymes. Our thinking was that phenol red which is one of the sulfonphthalein dye should be a good haloperoxidase substrate because its halogenation product is blue and it should not undergo peroxidation or at least no color change should occur as a result of peroxidation. The results in Figure 1 show that after bromination the purplish blue product has millimolar extinction coefficient at 590 nm about 3 times more than that of phenol red at 430 nm. Phenol red is not good substrate for chlorination reaction by haloperoxidase because the chlorinated product has less millimolar extinction coefficient at 575 nm than that of phenol red at 430 nm. Besides, the reaction rate was much slower.

As our criteria are substrates which change colors after brominated by haloperoxidase to the color that can be observed visually and rapidly. Thus, the other sulfonphthalein dyes with three aromatic rings substituted by nonpolar group, for example, CH_3-, $-CH_2(CH_3)$, are considered to be bad substrates for our purpose because they did not change their absorption maxima and λ_{max} in the presence of haloperoxidases, halide ions and H_2O_2. Bromophenol red and chlorophenol red can be substituted for phenol red because they still have vacant site for bromination by haloperoxidases.

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Bromophenol blue did not change color after addition of enzyme in the reaction.

MCD, the traditional haloperoxidase substrate (6) does not have color and the halogenated products also have no color (Figure 2). Thus phenol red being yellow and yield more intensely colored product would have advantage over MCD for kinetic studies, gel and cell staining because of the specific haloperoxidatic reaction would give spectral change in the visible range as well as in the ultraviolet.

2. Identification of phenol red halogenated products catalyzed by MPO

2.1 Thin layer chromatography (TLC)

The phenol red final brominated and chlorinated products were purified and preliminarily characterized by TLC. Their R_f values were the same as bromophenol blue and chlorophenol red respectively (Table 6). Their purity was tested by pH-dependent isobestic points, absorption maxima, millimolar extinction coefficients and pK_a of phenol red final brominated and chlorinated products are the same as bromophenol blue and chlorophenol red indicating that the products were probably bromophenol blue and chlorophenol red.

2.2 Infrared spectroscopy (IR)

The IR spectra of phenol red, bromophenol blue, chlorophenol red and phenol red final halogenated products were compared especially at the fingerprint region. The brominated and chlorinated products showed the same absorption pattern as bromophenol

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blue and chlorophenol red at this region. The spectra implied that they had O-H group (3300 cm^{-1}), C=O (1620 cm^{-1}) and aromatic ring (1600 cm^{-1}) in their structures (Figure 4,5). The C-Cl stretching band (600 cm^{-1}) was found in chlorophenol red and phenol red final chlorinated product (Figure 5). The C-Br band (500 cm^{-1}) is not very useful because it lie outside the range of instrument.

Despite the near identity of the IR spectra, the fingerprint regions are not as characteristic of various halogenated products as they should be, the conclusive proofs had to come from NMR and MS studies.

2.3 Nuclear magnetic resonance spectroscopy(^{1}H-NMR)

Phenol red NMR spectrum showed 12H in its structure. H_1, H_4, H_5 and H_8 are in the same environment and in ortho position with OH group, thus their NMR peaks appeared at high field (δ = 6.75) and splitted into doublet due to the near proton. H_2, H_3, H_6 and H_7 are also in the same environment and in meta position with OH group, so they show doublet peaks at lower field (δ = 7.12). H_9, H_{10}, H_{11} and H_{12} are in different environment, so the multiplet peaks were obtained (δ = 7.4-8.2). H_{12} and H_{10} are in ortho and para position with sulfonate group whereas H_9 and H_{11} are in meta position, so H_{12} peak is at the lowest field whereas H_{11} is at the highest field of this multiplet. The integration line shows that there are 4 protons in each ring (Figure 6).

The structure of phenol red final brominated product was compared with bromophenol blue by NMR. They both show the same NMR pattern (Figure 7). The singlet peak (δ = 7.55) is caused by H_2,

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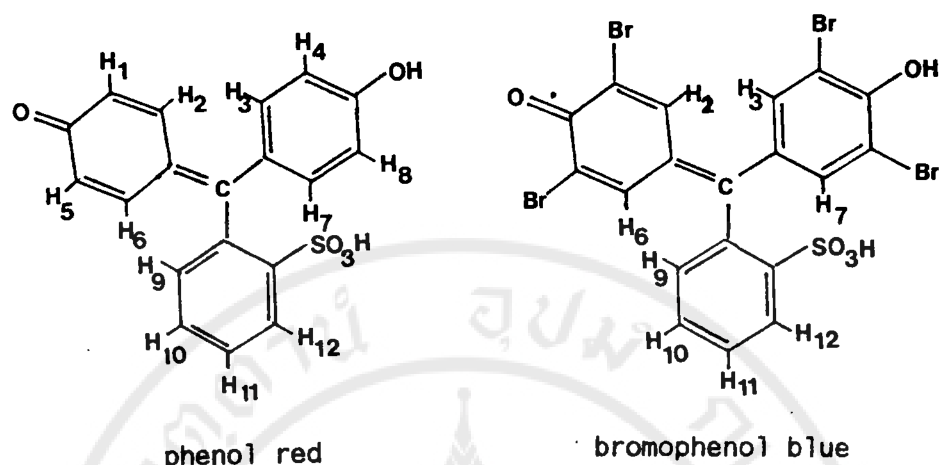

phenol red bromophenol blue

H_3, H_6 and H_7 which are in different environment and are not close to the other protons. The multiplet shifts from low field in phenol red structure to higher field (δ = 7.0-8.1) are due to the substituted bromine atoms in the structure. Thus, the multiplet peak overlaps with the singlet peak as seen to be the shoulder of the singlet peak. The integration line shows 4 protons in the singlet peak and the other four in the multiplet.

These data imply that after bromination reaction 4 protons disappear from the phenol red structure.

2.4 Mass spectrometry (MS)

Mass spectra of bromophenol blue and phenol red final brominated product appear to be very similar (Figure 8). The data imply that the product contained 4 Br atoms in the structure because the fragments 587, 508, 429 and 350 are found. The isotopic clusters produced in the spectrum were due to ^{79}Br and ^{81}Br (89).

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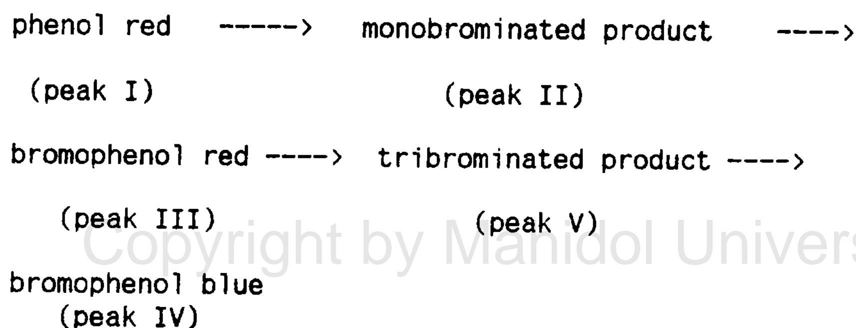
2.5 HPLC and reaction intermediates

Phenol red bromination intermediates catalyzed by MPO was analyzed by HPLC. Five peaks appeared. At 0 min of reaction peak I had the same retention time as phenol red (3.16 min) and rapidly decreased to zero at 40 min whereas at 40 min peak IV appeared and had retention time at 5.9 min the same as bromophenol blue indicating that the complete product of phenol red bromination reaction was bromophenol blue (Figure 9). During the reaction peak II, III and V were observed. Figure 10 shows a plot of peak area of these peaks versus time. Peak II rapidly decreased as time passed whereas peak III and V increased their peak area to maximum at 5-6 min and then they all disappeared at 40 min. Peak III had retention time at 4.9 min like bromophenol red. These data implied that peak II, III and V are intermediates in the reaction. Peak III was di-brominated product. Peak II should be monobrominated and peak V was expected to be tribrominated product of phenol red. This was supported by the evidence that peak III when catalyzed by MPO gave peak IV and V product. From the millimolar extinction coefficients, it is concluded that almost all of peak I was converted to peak IV.

Therefore, the sequence of the bromination reaction should be:-



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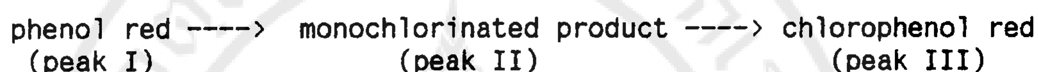
For chlorination product of phenol red catalyzed by MPO, the reaction occured slowly. There are 3 major peaks which can be detected by HPLC. They are phenol red, monochlorinated and dichlorinated product or chlorophenol red which are eluted at retention time 3.16, 3.62 and 4.43. The two minor peak at 6.12 and 7.55 min are probably be the tetra- and trichlorinated products. The sequence of chlorination reaction should be:-



Because many intermediates involved in the reaction, MPO activity was assayed by measuring the decrease in absorbance at 430 nm in order to reduce interference from the intermediates. However, the HPLC study of the reaction intermediates in MPO bromination reaction of phenol red provides the possible new substrate for MPO. This is the tribrominated intermediate. It has a single site for bromination or chlorination as MCD has. Thus, the reaction has no intermediate and lag time should not be observed because the tribrominaed product will be catalyzed to tetrabrominated product in one step. The spectrum of tribrominated phenol red showed the absorbance at 590 nm about 3 times less than that of tetrabrominated product.

Although, generally, a good substrate should undergo one step reaction to give a single product. This is not generally true for "substrate" of peroxidases and haloperoxidases used so far ,e.g., tyrosine for iodoperoxidation (90) , benzidine for peroxidation (68).

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The production of color compounds by reaction of MPO and phenol red has never been reported yet. CPO was mentioned in the undisclosed Patent by Hunter et al (1985) (87) in connection with the production of chlorophenol red and bromophenol blue. Boer et al (1987) (88) have characterized phenol red brominated product catalyzed by a vanadium containing bromoperoxidase to be bromophenol blue. We were not aware of this latter work until the middle of 1988 However, nobody has exploited MPO-catalyzed bromination reaction of phenol red and its reaction intermediates as described by us here.

3. Kinetic parameters of phenol red as substrate for MPO

$K_{m_{app}}$ values of substrates of MPO are determined by both Cornish-Bowden and Lineweaver-Burk plot (Table 8, Figure 12). The $K_{m_{app}}$ obtained by these two methods are not different too much. They are calculation of median and mean of data of $K_{m_{app}}$ values. Thus the median should give $K_{m_{app}}$ values which are closer to the real $K_{m_{app}}$ values. MPO has 10 times the apparent "affinity" of phenol red than MCD.

Whatever, the reaction mechanism of MPO catalyzed halogenation of phenol red, the $K_{m_{app}}$ values for various substrates show that for maximum initial velocity of reaction, the concentration of substrate used are in the similar order to of slightly lower than those in the halogenation of MCD.

The problem of apparent substrate inhibition cannot be explained simply. The formation of compound II from compound I may explain the H_2O_2 inhibition. The reaction of high concentration of

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hypohalous acid with H_2O_2 and enzyme may explain inhibition by high halide concentration. Perhaps high concentration of halide ion may also help block entry of H_2O_2 at the active site (49) also. The concentration of phenol red in formation of mainly mono- and dibrominated products may lead to slower decrease of absorbance at 430 nm because these intermediates absorb at 430 nm more than the tri- and tetrabrominated products. Therefore, there is an apparently slower decrease of absorbance at 430 nm.

The pH optimum for MPO activity has been found to be in the range of 5-6 (Figure 13). Thus for the enzyme activity assays, the reaction mixtures were adjusted to the pH optimum. The pH-rate profiles did not appear to be symmetrical. The presence of more than one isoenzymic forms may be one reason for this asymmetry. Substrate and product forms, e.g., hypohalous intermediates and ionizable sulfonphthalein substrate and intermediate may react differently at different pH.

4. Cytochemical demonstration of MPO in granules of polymorphonuclear leukocytes by phenol red staining

As shown in Figure 15, white blood cells in the granulocytic series showed positivity with phenol red staining of MPO in their granules. Myeloblasts (Figure 15 A) had a few small purplish blue granules localized near the nucleus. The granules became coarser and distributed overall the cells and the intensity of purplish blue color increased as myeloblasts developed to be promyelocytes, metamyelocytes and polymorphonuclear leukocytes (Figure 14 C-F). This is due to increasing MPO activity with the progressive maturity of the granulocytes. Eosinophils showed very intense purplish coarse

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granules (Figure 14 C).

For monocytes (Figure 14 H), a few fine and coarse purplish blue granules scattered discretely overall the cells showing a characteristic pattern which could be easily distinguished from granulocytic series. This property can be used to distinguish different stages of leukemias from M_1 (myeloblast, Figure 15 A) to M_5 (monoblast, Figure 15 H,I), i.e., equally well as Sudan black stain.

Sudan black staining pattern is seen as black bodies in neutrophil granules of as a shell of eosinophil granules. But phenol red staining showed purplish blue color distributed overall the granules. This is because Sudan black stain lipid component of the granules (91, 92) whereas phenol red stains MPO that localized inside the granules.

The classical MPO staining using benzidine (68) and 4-chloro-1-naphthol (75) showed the same staining pattern as phenol red except at the early blast stage which exhibited very pale or no color product because MPO activity is very low. So, they cannot be used to distinguish myeloblasts and monoblasts. They only help in differentiating granulocytic from lymphocytic cell series.

Thus, instead of having to use the conventional procedure, i.e., both benzidine type staining followed by Sudan black staining, the use of phenol red staining would at least equal the combination of the other two staining methods to achieve cell differentiation in leukemias.

In this cytochemical staining of MPO in white blood cells we found that phenol red changed color from yellow to purplish blue color of bromophenol blue as identified in the first part of this

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thesis. When bromide ion was omitted from the reaction, the purplish blue color was not observed indicating that no bromination reaction occured. When H_2O_2 was omitted from the reaction, the pale purplish blue granules were observed, implying that there is some H_2O_2 in the granules. How this arises in the granules is not known at present. But when chloride was used instead of bromide, only yellow color was observed due to the color of phenol red. Bromophenol red and chlorophenol red can be used instead of phenol red because their bromination product showed blue color of bromophenol blue of dibromodichlorophenol red. These bromination reaction indicated the bromoperoxidatic activity of MPO. The color seen from the other classical peroxidase substrate such as benzidine (68), 3-amino-9-ethylcarbazole (69), homovanillic acid (70), pyrocathecol-8-phenylenediamine (71), 2,7-fluorenediamine (72), diaminobenzidine (73), o-toluidine (74) and 4-chloro-1-napthol (75) showed very pale color product in granules of early blast stage because they stained partial peroxidase activity of MPO which is less sensitive than phenol red.

The properties which make phenol red a very good chromogen for MPO cytochemistry can be summarized as follows. Firstly, its staining pattern in normal and leukemic condition is the same as that of Sudan black which can help in distinguishing myeloid from monocytic series and at the same time differentiating granulocytic from lymphocytic cell series as effectively as benzidines. Secondly, the staining procedure is simple and less time consuming than any other methods. Thirdly the stained smears can be kept at room temperature without color fading for more than 14 months. This is

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because bromophenol blue is stable in air. And the 14 month-air dried smears gave pale purplish blue color after staining because of the sensitivity of the staining. Fourthly, it is not known to be carcinogenic, unlike some of the benzidine derivatives. Fifthly, it has no side reaction and no cryltal formation. The only disadvantage of this method is that bromophenol blue is water soluble and slides fade after a few washings with water. Thus the stained smears should be protected from water. However, water washing is not used after the staining is completed.

In conclusion, this is the first observation of MPO activity *in situ* in white blood cells. Whatever granules that contained sufficient MPO activity can be stained by phenol red. The previous study of MPO activity was from MPO extract, not *in situ* staining as our work.

5. <u>Application of phenol red in MPO staining and other haloperoxidases in polyacrylamide gel and agarose gel</u>

MPO from normal human leukocytes having isoenzymes as pI of 10, 9.8, 8.9, 8.5 and 8.4 (Figure 19 A). Phenol red and DAB staining for the same gel divided into half gave the same result. This corresponded with the work of Akin et al (1985) (85) and Miyasaki et al (1986) (31) that MPO is a basic protein. They stained MPO activity by using 3,3'-tetramethylbenzidine. The green band obtained rapidly diffused. Phenol red staining showed blue bands in 1 min whereas DAB staining of peroxidatic activity took about 10 min, because the bromoperoxidatic reaction of MPO was specific and more sensitive.

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In CETAB gel stained with phenol red (Figure 21) crude MPO extract showed blue band at the same position as the commercial MPO

indicating that they had comparable bromoperoxidatic activity bands. CETAB also prevente aggregation of MPO.

CPO (Figure 19 B) showed 2 bands of pI 3.8 and 3.6 by both method of staining. This result corresponded with the previous work of Sea and Cunningham (1979) (93) and Pickard and Hashimoto (1982) (79) who used 3,3',5,5'-tetramethylbenzidine as substrate for peroxidase activity staining. But the blue bands from phenol red staining appeared more rapidly than brown bands from DAB staining. This also indicates the specificity of CPO bromination reaction.

LPO (Figure 19 C) exhibited only 1 pI at 9.5 as same as Carlstrom's work (94) and Tenovuo's work (95). Bromination reaction is still more prominent than peroxidation reaction.

For these isoelectric focusing gels, both rod and slab were stained identically for their bromoperoxidatic and peroxidatic activity because peroxidatic and bromoperoxidatic bands happened to coincide. Otherwise, by using only DAB staining method alone (as in common practice up to now) it would not be possible to determine whether the activity stain was due to bromoperoxidatic or peroxidatic activity or both.

The advantage of using phenol red parallel with DAB staining is the ability to distinguish the chloroperoxidatic and bromoperoxidatic activity from peroxidatic activity. The crude BPO extract from Thai seaweed is a clear example. Some bands that stained for peroxidatic activity (Figure 20 B) did not stain for bromoperoxidatic activity (Figure 20 A). Also the relative intensity of peroxidase-stained bands did not correlated with that of BPO-stained bands. A previous attempt has been made to stain BPO with phenol

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red and restaining with o-dianisidine (82). At this condition the rather unsatisfactory brown bands were obtained by them instead of blue bands as ours. Subsequently the authors did not pursue this staining method.

The purplish blue and blue bands appeared in polyacrylamide gel staining of MPO, CPO, LPO and BPO as shown in Figures 19 A, B, C and 15 A are due to a mixture of brominated products of phenol red because the result from HPLC pointed out that there are 4 intermediates in bromination reaction of phenol red. If bromophenol blue, the final brominated product of phenol red, was produced solely, it would give a purplish blue and not just blue color at 5.8. When H_2O_2 of bromide ion was omitted from the staining solution, no such bands were observed. When chloride ion was used instead of bromide ion, MPO and CPO slowly gave light purplish brown bands which were hardly distinguishable from the background. Although these bands became more diffuse in 30 min, they appeared more rapidly upon staining than those bands due to DAB.

However, drying of gel in cellophane (96) can preserve the gel for more than a year. Phenol red is also stable in light and does not precipitate.

The agarose gel impregnated with phenol red, H_2O_2 and bromide ion has successfully been used by us for proving that some cells (broken white blood cells), tissues (crushed seaweed) (Figure 22,23), and organisms (*Caldariomyces fumago* in petridish) have BPO activity. This is convenient for field study because showing BPO activity does not require complex equipment such as spectrometers and centrifuges.

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CHAPTER V

SUMMARY

1. Phenol red is converted to bromophenol blue and chlorophenol red by myeloperoxidase (MPO) -catalyzed bromination and chlorination reaction respectively.
2. The intermediate in MPO-catalyzed bromination reaction of phenol red are probably mono-, di-, and tri- brominated phenol red.
3. The yellow-colored phenol red is a good substrate for kinetic assays of bromoperoxidatic enzymes by spectrophotometry. However, the tribrominated, dibrominated and dichlorinated phenol red derivatives may be better substrates in terms of simpler kinetic results and interpretation.
4. MPO-catalyzed bromination of phenol red can be used to stain granules in myelocytic and monocytic cell series of white blood cells in healthy and sick subjects. Early cells of the granulocytes can be easily distinguished.
5. This reaction specific staining can also be successfully performed on chloroperoxidatic and bromoperoxidatic enzymes in acrylamide gels.

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