CONTINUOUS-FLOW METHODS FOR THE DETERMINATION OF RESERPINE

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CONTINUOUS-FLOW METHODS FOR

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CHAPTER I

INTRODUCTION

There has been a growing awareness in the recent years that drug quality control by manufacturers and drug quality testing by enforcement agencies, must be increased to ensure safe and effective drug supply. One type of specification test in this regard is called the content uniformity test (1). It is applied to tablets, capsules and containers of sterile solids or suspensions in which the active ingredient is present in low quantities (50 mg or less). Variations in composition of individual units is undesirable and substantial differences in content of active ingredient(s) must be avoided. Wherever practicable it is necessary to demonstrate the homogeneity of the dosage form. Since this specification is directed at determining uniformity within a given batch or lot, rather than simply determining uniformity between batches, it has greatly increased the number of samples to be analyzed. The Food and Drug Administration requires that tests be performed on at least 10 to as many as 30 separate individual samples in triplicate. This has proved burdensome whether it occurs in a pharmaceutical industry or in a drug enforcement laboratory and involves considerable demand on personnel and

facilities.

Automation of routine analytical procedures should provide a means of saving time and manpower, which ultimately makes them cost efficient. One of the means by which this can be accomplished is by implementing continuous-flow systems. Continuous flow analysis is perceived as a unique way available to the analytical chemist for performing the task of solution mixing and transportation of the reaction products to the point of detection for the determination. They provide faster and more economical methods of doing bulk analysis. A large number of samples can be processed with a good degree of precision and accuracy and the reagent consumption is reduced. It frees the technician from spending much of the time doing routine operations and also minimizes human errors.

Continuous flow procedures can be broadly divided into segmented and unsegmented methods. In the segmented methods, air bubbles are introduced into the carrier stream between samples to control dispersion and preserve sample integrity. It was first described by Skeggs (2) in 1957 and based on it the "Auto Analyzers" were produced and marketed by Technicon Corporation. In the unsegmented continuous-flow systems the air bubbles are eliminated. Although examples of this nature are seen in the literature from earlier times (3) they have gained popularity and acceptance under the name of 'flow injection analysis' (FIA)

coined by Ruzicka and Hansen (4) in 1975. In FIA, a sample is inserted into the carrier stream. The sample plug is carried by the stream during which time physical and chemical changes may occur. The detection system continuously senses the flow stream and yields a transient signal when the sample plug passes through the flow cell. Neither physical equilibrium (homogeneity) nor chemical equilibrium (completness of reaction) need to be attained when the signal is detected. The instrumentation necessary to implement a FIA system is relatively inexpensive and can be assembled in a laboratory. The signal in FIA is a plot of the analytical response with time. The response can be absorbance, fluorescence intensity, current, etc. The peak height is directly proportional to the analyte concentration, although in some cases the area under the peak is integrated. The time from the start of the signal to the return to baseline dictates the number of injections that can be performed per hour. The increasing need for rapid methods for the determination of pharmaceuticals can be met with continuous-flow analysis. Reserpine is a pharmaceutical that is recognized for its double therapeutic value as an antihypertensive and a sedative agent. It is widely used for the treatment of moderate to mild hypertension accompanied by anxiety and emotional disturbances in which its tranquilizing action may be helpful (5). It is an alkaloid obtained from certain species of Rauwolfia, mainly <u>Rauwolfia serpentina.</u> There are

several pharmaceutical formulations in which reserpine is the single active ingredient or acts in combination with other drugs, including Rauwolfia serpentina preparations. It is important to determine the exact amount of drug present in these formulations. The literature contains a number of methods for the separation and determination reserpine. A few automated methods for the determination of reserpine are reported but they involve extractions and separations and the number of samples analyzed per hour is small. A brief review of the methods is presented in Chapter II.

The most commonly used methods involve the oxidation of reserpine to 3,4-didehydroreserpine which is then determined by spectrophotometric or fluorescent measurements. When a small concentration of a suitable catalyst is used, the rate of oxidation is increased. The amount of products formed at any particular time is greater and allows the determination of lower concentrations of the chemical species. The inherent dynamic characteristics of a continuous-flow system are well suited for catalytic methods of analysis which are kinetic in nature. The use of catalysts in continuous-flow systems is reviewed in Chapter III.

The work described in this thesis comprises of studies with two different catalysts for the oxidation of reserpine, namely:

1. The enzyme, peroxidase (from horseradish EC 1.11.1.7), was tested to catalyze the aerobic oxidation of reserpine.

Inhibition of the enzyme during the oxidation prevents its effective use as an immobilized catalyst in a continuous-flow system. Studies on the cause of inactivation and the type of inhibition of the horseradish peroxidase are described in Chapter IV.

2. The oxidation of reserpine by periodate ions is catalyzed by Mn(II) or Mn(IV). Implementation of successful continuous-flow systems based on this catalysis is discussed in Chapter V.

These studies were directed to develop a continuous-flow system of easy, practical application for the repititive determination of reserpine in pharmaceutical formulations.

CHAPTER II

PROPERTIES AND ANALYTICAL CHEMISTRY OF RESERPINE

Some properties of reserpine

Reserpine and rescinnamine are the primary active alkaloids among the Rauwolfia serpentina species. Other alkaloids present are structurally similar to reserpic acid and are devoid of any pharmacological activity. They include ajmaline, isoajmalinine, isoajmaline, serpentine, rauwolfine, and sarpagin (6).

Reserpine was first isolated from the source in 1952 (7) and is obtained from the root of the herb. Although it has been totally synthesized it is not economically feasible to produce it in that fashion (8). Reserpine has the chemical name 1,2-didehydro-2,7-dihydro-11,17α-dimethoxy-3β,20α-Yohimban-16β-carboxylic acid methyl ester, 18β-trimethoxybenzoate ester with the empirical formula $C_{33}H_{40}N_2O_9$. It is an odorless, white to pale buff colored, crystalline substance. It is almost insoluble in water and slightly soluble in ethanol and ether. It is freely soluble in acetic acid and chloroform. It is weakly basic and the solubility decreases as the pH increases. Reserpine is also

more soluble in the less polar solvents.

The major routes of degradation of reserpine are hydrolysis, epimerization and oxidation (Fig. 1). Reserpine undergoes hydrolysis of the two ester groups splitting off trimethoxybenzoic acid and methanol leaving behind reserpic acid. It has six asymmetric carbons. Although the reserpine molecule contains six asymmetric carbons, only C-3 undergoes inversion to give 3-isoreserpine which occurs mainly in highly acidic solutions. Oxidation of reserpine occurs chemically or in the presence of light. Photo-oxidation of reserpine produces a greenish yellow fluorescent substance which on further oxidation turns into a reddish brown color with a blue fluorescence. The initial oxidation product was identified as 3,4-didehydroreserpine (9). The blue fluorescent product was shown to be 3,4,5,6-tetrahydroreserpine (10). Degradation of pharmaceutical formulations occurs mainly by oxidation. Epimerization and hydrolysis are not considered as a significant source of degradation. Banes (9) reported significant levels of oxidation products in injections and elixirs but hardly any evidence in tablets. With thin-layer chromatography, Wright and Tang (10) found trace amounts of 3,4-didehydroreserpine in all commercial samples they analyzed, but found no traces of the tetrahydro compound.

Reserpine metabolism occurs via the oxidative demethylation of the 4 position of the 3,4,5-trimethoxy benzoyl group and hydrolysis of the ester linkage. The

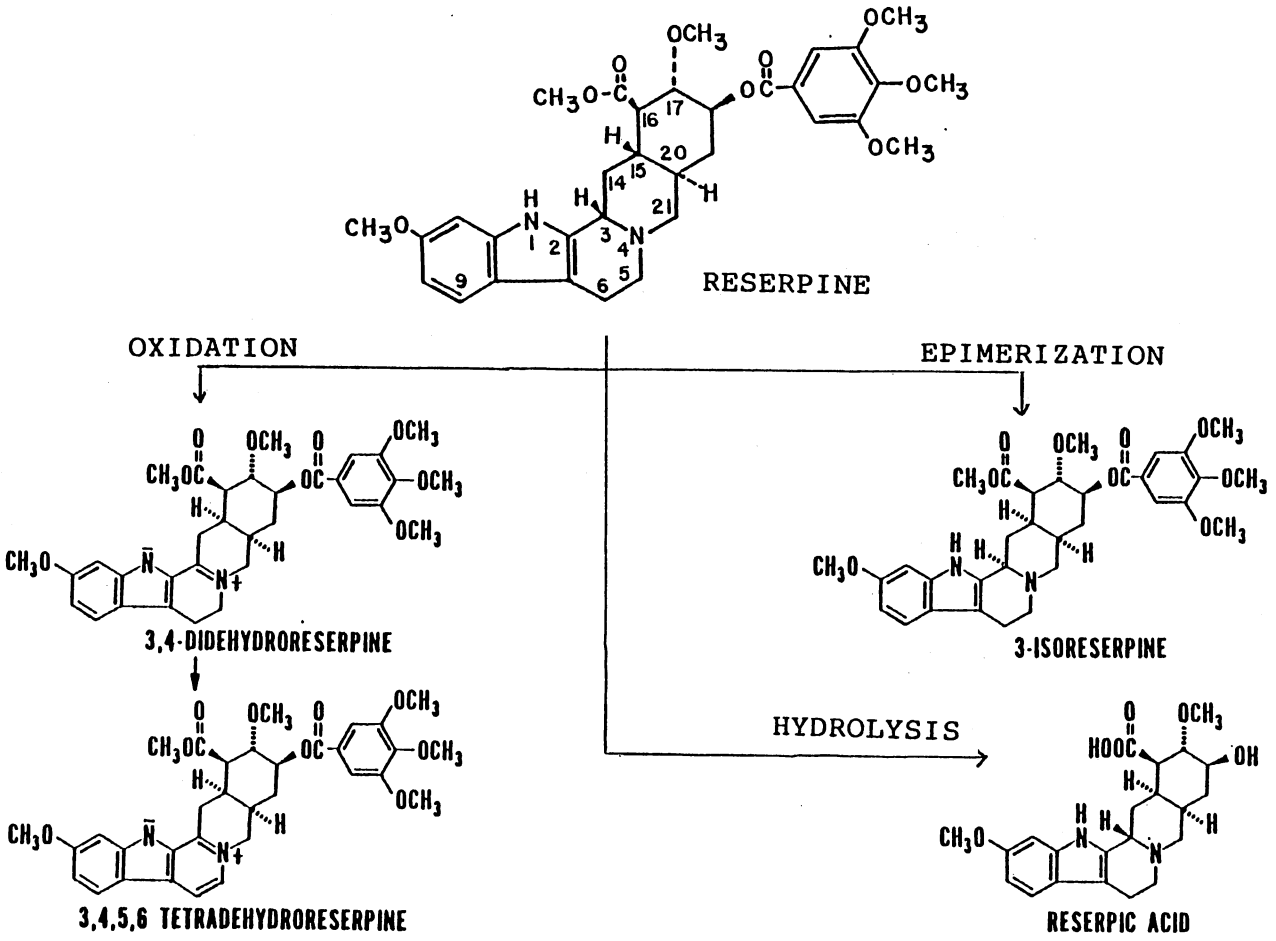


Figure 1. Major Degradation Products of Reserpine

products of metabolism include methyl reserpate, 3,4,5-trimethoxy benzoic acid. It is noted that 3-isoreserpine, didehydroreserpine and the tetrahydroreserpine are absent from the tissues analyzed after administering measurable quantities of the drug (11).

Methods of determination of reserpine

Due to its therapeutic importance and wide use since the 1950's, several methods have been developed for the determination of reserpine. The dosage of the drug varies and is adjusted according to the individual needs of the patient. Several forms of the drug containing different amounts of reserpine are available. Some of them are: tablets containing 0.1-5.0 mg; capsules containing 0.25-0.75 mg; elixirs containing 0.25-2.5 mg/ 5 ml; injections containing 2.5-5.0 mg in 2-10 ml containers. Rauwolfia serpentina is administered in the form of powdered roots or tablets with strengths ranging from 50-200 mg. Methods which give low limits of detection are necessary to determine the reserpine- rescinnamine group alkaloids in Rauwolfia serpentina preparations. Biological samples like tissue, blood plasma and urine are also tested for reserpine in clinical laboratories. Due to interferences from degradation products, related alkaloids and excipients, separation is often necessary before determination. A brief review of the different separation and detection techniques found in the literature is presented.

Chromatographic methods

Separation of reserpine on thin-layer chromatography (TLC), paper chromatography, and column chromatography has been extensively studied and reviewed by Schirmer (12). A number of thin-layer systems have been developed for the separation of structurally similar alkaloids of rauwolfia. Silica gel and alumina have been widely used as adsorbent materials. A variety of solvent systems have also been used for the separation of alkaloids. Solvent systems with low eluent power work best for the slightly basic compounds. Spots can be located by fluorescence in UV light. Some chromogenic reactions can also be used for identification. Spraying with 1% ammonium vanadate, 1% ferric chloride, or 0.5% phosphomolybdic acid in 50% nitric acid yields a yellow-green color turning greenish brown on standing. A 1% ceric sulfate solution in 10% sulfuric acid gives a greenish brown color turning brown when heated to 105^{o}C (13). Quantitative determination of reserpine has been made following TLC separation. The adsorbent is removed by scraping or by suction and shaken with a suitable solvent to extract the alkaloid. The resultant suspension is filtered and the filtrate is evaluated spectrophotometrically. A non treated zone of the same size is similarly treated and used as blank.

Paper chromatographic systems have been used to separate reserpine from its synthetic precursors and related

alkaloids. Several solvent systems have used such as mixtures of benzene, methanol and methyl ethyl ether saturated with 2% formic acid. In some cases, 50% ethanolic formamide was used as stationary phase and benzene as eluent (14). Reserpine can be visualized on paper chromatograms under mercury lamps after spraying with 0.0025% solution of fluorescein in 0.5 M ammonia or under a UV lamp after spraying with a 3% solution of sodium nitroprusside in 50% trichloroacetic acid to give a strong green fluorescent or by spraying with Dragendroff reagent (2% potassium bismuth tetraiodide in 0.01 N HCl) (15). After separation reserpine is extracted and the filtrate is quantitatively determined by UV or visible spectrophotometry or by electrochemical methods. Reserpine is also separated from rescinnamine and reserpic acid by electrophoresis on paper with 5 M acetic acid as electrolyte (12).

Column containing various trap layers was used for the separation of excipients from reserpine formulations. A layer of diatomaceous silica treated with 2% citric acid is followed by a layer containing saturated sodium bicarbonate. Chloroform is used as the eluting solvent. Basic degradation products like methyl reserpate and reserpic acid are removed along with any coloring agents like indigotin or tartrazine that may interfere with color measurements (16).

Gas Chromatography has been described as a means of separation and determination of reserpine from Rauwolfia serpentina roots. Reserpine was hydrolyzed by refluxing

with sodium hydroxide for 90 min resulting in the formation of trimethoxy benzoic acid, which was then cooled to 0°C and esterified with diazo methane (17) and the solution of the ester was injected into the gas chromatograph. A faster method involved the transesterification of the trimethoxy benzoic acid moiety with Methelute (0.2 M trimethylanilinium hydroxide in methanol). Reserpine was determined in several pharmaceutical preparations by this method. A linear response of 10-200 µg/ml was obtained. Rescinnamine does not react with Methelute and can be separated (18).

High performance liquid chromatography (HPLC) has been used for the separation and determination of reserpine and has become increasingly popular because of the speed and sensitivity of the technique and decreased possibility of interferences (19). Reserpine was determined in commercial samples and rauwolfia preparations by liquid chromatography on a normal phase column and eluting with methanol. Flurometric determination that followed gave competitive sensitivity (20,21). Liquid chromatography followed by amperometric detection at 0.65 V for reserpine and 0.72 V for rescinnamine gave low limits of detection. This technique was used to determine reserpine in complex biological matrices without elaborate pretreatment (22). Reserpine in plasma was extracted into benzene, oxidized by vanadium pentoxide in phosphoric acid and chromatographed on a octadecylsilane column by ion-pairing with heptane sulfonate ions. Detection limit of 100 pg of reserpine/ml

of plasma was obtained (23). Simultaneous determination of reserpine and hydrochlorothiazide in a two component tablet formulation was achieved. The components were extracted with tetrahydrofuran and chromatographed on a silica gel column with a mixture of diethylamine, chloroform, and propanol in hexane as mobile phase. A linear calibration curve was obtained in the range of 4-40 µg/ml (24).

Electrochemical methods

Due to the redox behavior of reserpine it can be determined by electrochemical methods. In acid media the alkaloids containing the 6-methoxy indole group undergo a one electron oxidation with the introduction of an -OH group. As previously mentioned, this has been exploited for the amperometric detection, using glassy carbon electrodes, of reserpine and rescinnamine in liquid chromatographic effluents (22). Strong accumulation of the drug on the electrode surface was used as the basis for a highly sensitive adsorptive stripping voltammetric procedure for the determination of reserpine and rescinnamine. Due to the preconcentration of the analyte, low limits of detection of 2×10^{-9} M and 3×10^{-9} M, respectively were achieved by this method (25). Studies on the polarographic determination of reserpine, using a.c. polarography in aprotic organic solvent systems, yielded detection limits in the range of 10^{-5} M and were effectively used for single tablet analysis (26).

Spectrophotometric methods

Reserpine absorbs infrared radiation in the 5.0-6.5 um region. When extracted into chloroform, the band intensity can be measured for the quantitation of the compound in pharmaceuticals (27). The ultraviolet spectra of the indole alkaloids show three bands at 216 nm, 268 nm, and 296 nm with molar absorptivities of 55,700, 15,700, and 9,660 $mole^{-1}$ cm^2. In the quantitative determination of reserpine the absorbance of the sample is commonly measured at 268 nm. Interferences from similar alkaloids and degradation products require isolation prior to measurement. Extraction of reserpine from roots and crude preparation of Rauwolfia serpentina was followed by UV determination (28). Reserpine content of formulations was determined by UV spectrophotometry after electrophoresis of 5 N acetic acid solution (29).

Colorimetric methods are the most rapid and offer low enough detection limits for the determination of reserpine in pharmaceutical formulations. The most widely used method involves the oxidation of reserpine to the greenish-yellow 3,4-didehydroreserpine by treatment of an acidified alcoholic solution of reserpine with sodium nitrite (30). The absorbance is measured at 390 nm. Similar alkaloids like rauwolscine and yohimbine, do not interfere. However, reserpic acid, methyl reserpate and rescinnamine exihibit similar absorption. The same procedure is used for the

determination of the active ingredients of Rauwolfia preparations after extraction (31). Linear concentration ranges of 5-15 μg/ml have been obtained. Several other chromogenic reagents have been used for colorimetrically determining reserpine and are listed in Table I. The reaction of tertiary amines with citric acid was utilized to develope a method for the determination of trace amounts of reserpine in biological systems. It gave the lowest limit of detection of the colorimetric methods and a linear range of 0.5-40 μg/ml (34). The hydroxamic acid method was the only method in which the ester group (necessary for the pharmacological activity of the alkaloid) was involved. The hydroxamate complex, formed in the presence of hydroxylamine hydrochloride and ferric ammonium sulfate, has a red-violet color that absorbs at 535 nm. The linear range obtained, 20-250 μg/ml, was suitable for determination of reserpine in pharmaceutical formulations (34).

The greenish-yellow oxidized reserpine is also highly fluorescent. Hence, the sensitivity of the colorimetric method can be enhanced by fluorometric determination. Several reagents have been used for the development of the greenish-yellow fluorescence and are listed in Table II. The product in all cases is 3,4-didehydroreserpine and exhibits emission maximum at about 500 nm. Nitrite oxidation followed by fluorometric determination has become the method accepted by the United States Pharmacopeia for determination of reserpine (35,44). When hydrogen peroxide

TABLE I

REAGENTS USED FOR THE COLORIMETRIC DETERMINATION OF RESERPINE

Reagent	Wavelength nm	Range or (LD)	Comments	Ref.
Sodium nitrite	390	5-15 ug/ml	No interferences from related alkaloids	30
Vanillin	532	(17 ug/ml)	-	32
Bromocresol purple	402	(2.8 ug/ml)	-	33
Citric acid	505	0.5-40 ug/ml	Low limit of detection	34
Hydroxylamine-Ferric ammonium sulfate	535	20-250 ug/ml	Complex formation with the pharmacologically active ester group	34

TABLE II

REAGENTS USED FOR THE DEVELOPMENT OF FLUORESCENCE BY OXIDATION OF RESERPINE

Reagent	Ref.
Sodium nitrite	35
Hydrogen peroxide	36
Vanadium pentoxide	37
p toluene sulfonic acid	38
Hexa-amminecobalt(III)-tricarbonatocobaltate(III)	39

was used as the oxidant heating was required to enhance the fluorescence intensity. Concentrations of reserpine as low as 0.02 µg/ml were measurable by this method (36). Vanadium pentoxide oxidation gave wide concentration range in linear calibration curves, 4 ng-2 µg/ml. The intensity of the fluorescence was increased by the saturation of the solution with phosphoric acid. The method was used to analyze Rauwolfia serpentina tablets and root powders (37). It is also the official method for single tablet determinatons (45). A recent method based on the formation of yellow-green fluorescence by oxidation of an acetic acid solution of reserpine tablets with hexa-ammine cobalt(III) tricarbonatocobaltate (III) (HCTC) gave a linear range of 0.01-0.24 µg/ml. The oxidation product exhibits maximum fluorescence at 420 nm and has a structure different from 3,4-didehydroreserpine (39).

Automated methods

There have been a few attempts to develop automated methods for the determination of reserpine in tablets and Rauwolfia serpentina preparations. The samples are analyzed using the Technicon Auto-Analyzer. A simple manual procedure is usually employed for the extraction of reserpine using chloroform or methanol as solvent. The reaction with acidified sodium nitrite was followed by spectrophotometric (40) or fluorescence measurements (41). UV absorption was also used for the determination of

reserpine (42). The sampling rate was set at 20 samples/h. Vanadium pentoxide oxidation followed by fluorimetric determination was automated for single tablet analysis (43). The procedures involve extraction and separation steps and the sample throughput is low. The auto-analyzer is based on the principle of segmented continuous-flow. Methods using unsegmented continuous-flow systems for the determination of reserpine have not been found in the literature.

CHAPTER III

CATALYTIC METHODS OF DETERMINATION IN CONTINUOUS-FLOW SYSTEMS

Catalytic methods of determinations encompass all chemical determinations based on monitoring the rate of a catalyzed reaction. They diverge from the traditional view that substances in solution must be in equilibrium for determination. Due to the dynamic nature of the measurement it is essential to control reaction conditions strictly and monitor the concentrations of the reactants or products as a function of time, accurately. Continuous-flow methods are eminently suited for kinetic determinations because the reaction conditions can be controlled by careful selection of parameters such as pumping rate, dimensions of tubing, and temperature of the reaction coil. This makes the system less operator sensitive and more reliable.

This is not a comprehensive review on the subject. Fundamentals of analytical determinations based on catalytic reactions are reviewed to give some background information, to be able to better understand and interpret the outcome of the current work. Some examples are provided to illustrate the advantages of doing catalytic determinations using continuous-flow systems. The methods discussed here are

classified as enzymatic or non-enzymatic, based on the type of catalyst used.

Specific catalytic properties of some enzymes have opened a whole new area of analysis. Enzyme catalyzed reactions are used to determine both enzyme activities and substrate concentrations of clinical importance. Regeneration of the enzyme in the catalytic cycle is particularly relevant to continuous-flow systems because they can be reused. Immobilization of the enzyme on different inert supports and the different configurations of the reactors add to the efficiency of the enzymatic determination. Non-enzymatic methods are directed towards the determination of the catalyst itself, which are in most cases transition metal ions. Some anions are also represented. It is possible to determine trace amounts of the chemical species. Rapid rates of sampling can be obtained using continuous-flow systems.

Kinetics of Catalytic Reactions

In a non-enzymatic homogeneous reaction system the catalytic cycle for the reaction

$$R + X \rightarrow P \qquad (1)$$

where R (species monitored) and X are the reactants, P the product(s), and C the catalyst, is given by

$$\begin{aligned} R + C &\rightarrow RC \\ RC + X &\rightarrow C + P \end{aligned} \qquad (2)$$

The overall rate is given by the sum of the rates of the

catalyzed and uncatalyzed reactions.

$$-\frac{d[R]}{R} = k_u dt + k_c [C]_0 dt \qquad (3)$$

where k_u and k_c are the rate coefficients of the uncatalyzed and catalyzed reactions and subscript "0" indicates initial concentration.

Integrating equation (3) between fixed times t_1 and t_2, $[R]_1$ and $[R]_2$

$$\ln \frac{[R]_1}{[R]_2} = \Delta t \, [k_u + k_c [C]_0] \qquad (4)$$

where $\Delta t = t_2 - t_1$. If $\ln([R]_1/[R]_2) = \Delta^*[R]$,

$$\Delta^*[R] = k_u \Delta t + \Delta t \, k_c [C]_0 \qquad (5)$$

If Δt = constant (fixed time), $\Delta^*[R]$ is directly proportional to the initial concentration of the catalyst and gives the basis for the construction of calibration curves. Straight line plots of $\Delta R = [R]_2 - [R]_1$ against $[C]_0$ are obtained within limited concentration ranges.

If $\Delta^*[R]$ is a constant K^*, from equation 5, we get

$$\frac{1}{\Delta t} = \frac{k_u}{K^*} + \frac{k_c \, [C]_0}{K^*} \qquad (6)$$

This provides the basis for the variable-time procedure in which case plots of $1/\Delta t$ versus $[C]_0$ should be linear. This approach is better suited for the determination of catalysts, including enzymes (46).

The catalytic reactions involving enzymes are best described by the Michaelis-Menten mechanism:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [ES] \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P + E \qquad (7)$$

where E=enzyme, S=substrate, [ES]=addition complex and P=products. Assuming steady state conditions the following expression is obtained (1):

$$-\frac{d[S]}{dt} = \frac{k_2[S][E]_0}{K_m + [S]} \qquad (8)$$

where K_m is the Michaelis-Menten constant equal to $[(k_{-1}+k_2)/k_1]$. Again the rate of change of concentration of the reactant (substrate) is directly proportional to the enzyme activity.

When $[S] >> k_m$, the initial rate (IR) is proportional to enzyme concentration.

$$(IR) = (IR)\ max = k_2\ [E] \qquad (9)$$

When $[S] << k_m$ the initial rate is directly proportional to substrate concentration.

$$(IR) = \text{constant}\ [S] \qquad (10)$$

Sensitivity, Limits of Detection, and Selectivity

The major advantages of catalytic methods are their low limits of detection and high sensitivity. Limit of detection (LD) is defined as the lowest quantity detected with high probability and can be calculated from the expression

$$LD = \bar{b} + 3s_b \qquad (11)$$

where $\bar{b}$ is the average of the blank readings, and s_b is the standard deviation of those readings. The limit of

detection is different from the limit of determination (LD_m), which is the minimum quantity that can be actually measured. The limit of determination is calculated as follows:

$$LD_m = \bar{b} + 10s_b \qquad (12)$$

The limit of detection and the limit of determination are expressed in units of concentration.

Sensitivity and limit of detection are used synonymously in many textbooks and journal articles. The two terms, however, represent different concepts. Sensitivity of a method is a measure of the change in signal with concentration of the species determined and is represented by the slope of the calibration curve. From equation 5, it can be seen that, for the fixed-time procedure the sensitivity is given by $\Delta t k_c$. Therefore, the larger the value of k_c, the higher the sensitivity of the method. The limit of detection is dictated by the ratio k_u/k_c. In order to get low limits of detection, the rate coefficient of the catalyzed reaction, k_c, must be maximized and the uncatalyzed rate k_u, must be minimized. For the variable-time approach,it can be seen from equation 6, that the limit of detection is related to k_u/K^* and the sensitivity is given by k_c/K^*. Hence, a small value of k_u and large value of k_c will again yield lower limits of detection and higher sensitivity.

While sensitivity and limits of detection are

considered as the major advantages of catalytic determinations, selectivity limits the practical applications of these determinations. Selectivity denotes the extent of interferences by other chemical species in the determination of a given species by a given method. A reaction is said to be specific if there are no interferences. Among catalytic methods, this is common only for enzyme catalyzed reactions. Nonenzymatic methods usually offer very poor selectivity. The catalytic activity of metal ions is related to their size, charge and coordination sphere. Hence, similar species have similar catalytic activity.

Mathematical expressions have been derived from rate equations to quantitatively determine the selectivity of a catalyst. Applications of these are, however, not found in the literature. It has been found possible to improve selectivity by varying pH, temperature and other reaction conditions (47). Some examples of this kind are listed below:

(1) Zirconium and hafnium catalyze the reaction between I^- and H_2O_2. The activity of the species is related to the stability of the hydroxo complexes formed, which depends on the hydrogen ion concentrations. Zirconium can be selectively determined at pH 1.1 and Hafnium at pH 2.2.

(2) The reaction between I^- and BrO_3^- is catalyzed by Mo(VI), W(VI), and Cr(VI). The rate

coefficients for the reaction are different for the different metal ions in the temperature range 0 to 40°C. Selectivity of the catalyst can be acheived by varying the temperature.

(3) Ruthenium and osmium can be selectively determined by varying the ratio of As(III)/Ce(IV) concentration in the indicator reaction, Ce(IV) + As(III) → Ce(III) + As(V).

(4) Addition of an inhibitor or an activator can help in selectively determining Ru and Os using the system, 1-naphthylamine + NO_3^-. Addition of 1,10 phenanthroline allows the determination of Ru because Os catalysis is inhibited by complexation. On the other hand, Os catalysis is activated by 8-hydroxyquinoline and osmium can be determined, separately.

(5) The species of interest can be extracted into the organic phase and determined directly. Silver is extracted into a nitrobenzene, dioxane, and water mixture with 1,10-phenanthroline and determined by the Bromopyrogallol Red + $S_2O_8^=$ system.

Enzymatic Methods of Determination

A large number of enzymatic determinations are performed in the clinical laboratories and, consequently, there is a need for rapid analysis of large number of samples. It is therefore not surprising that the bulk of catalytic continuous-flow methods are enzymatic in nature. The enzymatic methods used in FIA are classified into two

sections: determinations using soluble enzymes and determinations using immobilized enzymes. Table III list the major enzyme catalyzed reactions that are relevant to this discussion. Tables IV and V compare the different features of the FIA system using soluble and immobilized enzymes. The linear concentration ranges and sampling rates for the methods are tabulated. Before evaluating the individual methods the advantages of using enzymes as analytical reagents and the role of enzyme reactors in FIA is briefly discussed.

The two important features that make enzymes attractive as analytical reagents are: (i) The reaction between the enzyme and substrate is highly selective. (ii) Enzymes are regenerated in the catalytic cycle permiting the economical use of expensive enzymes as reagents.

Enzymes can be used in solution or as immobilized reagents. The advantage of using one over the other depends on the specific nature of the application. In the case of the more stable and less expensive enzymes, their use in the soluble form is favored. Enzyme solutions can be reused by recirculation in closed-flow through systems (48). Less stable and more expensive enzymes are better utilized in the immobilized form. In many cases the immobilized enzymes retain the activity for longer time than in solution. For example, uricase, which is relatively expensive is unstable in solution and looses 90% of its activity in about 15 days.

TABLE III

EXAMPLES OF ENZYME CATALYZED REACTIONS
AND SUBSTRATES DETERMINED

Glucose

$$\alpha\text{-D-Glucose} \xrightarrow{\text{INVERTASE}} \beta\text{-D-Glucose}$$

$$\beta\text{-D-Glucose} \xrightarrow[NAD^+]{GOD} \beta\text{D-Glucono-lactone} + NADH + H^+ \quad (58)$$

$$NADH + NH_4^+ + \alpha\text{ keto glutarate} \xrightarrow{GDH} NAD^+ + \text{glutamate} + H_2O \quad (60)$$

$$\text{Glucose} + O_2 \xrightarrow{GOD} \text{Gluconic acid} + H_2O_2 \quad (62)$$

$$\text{LDADCF} + H_2O_2 \xrightarrow{HRP} \text{DCF} + 2H_2O$$

Ethanol

$$CH_3CH_2OH + NAD^+ \xrightarrow{ADH} CH_3CHO + NADH + H^+ \quad (66)$$

Urea

$$2NH_2CONH_2 \xrightarrow{UREASE} 2NH_3 + CO_2 + H_2O \quad (63)$$

Penicillin

$$\text{Penicillin} + H_2O \xrightarrow{PEN} \text{Penicilloic acid} \quad (54)$$

Lactate

$$\text{L-lactate} + NAD^+ \xrightarrow{LDH} \text{Pyruvate} + NADH \quad (75)$$

GDH: Glucose dehydrogenase
GOD: Glucose oxidase
HRP: Horseradish peroxidase
ADH: Alcohol Dehydrogenese
LDH: Lactate dehydrogenase
PEN: Penicillinase

TABLE IV

FEATURES OF ENZYMATIC METHODS
USING SOLUBLE ENZYMES

Species	Enzyme	Detection	Range or (LD)	S/h	Other features	Ref
Glucose	Glucose dehydrogenase	Spectro-photometric	0.5-15mM	100	stopped-flow merging-zone	58
	Glucose oxidase	Fluorescence	(10 M)	60	capillary FIA laser excitation	62
Urea	Urease	Potentiometric	4-20mM	60	merging zone	63
Chol-estrol	Cholestrol oxidase	Chemi-luminescence	(0.2mg/l)	60	microporous membrane	64
Ethanol	Alcohol dehydrogenase	Fluorescence	0.125-2.0g/l	40	stopped-flow merging zone	66

TABLE V

FEATURES OF ENZYMATIC METHODS
USING IMMOBILIZED ENZYMES

Species	Enzyme	Detection	Range or (LD)	S/h	Other features	Ref
Glucose	Glucose oxidase	Spectro-photometric	1.6×10^{-4}-1.6×10^{-2}M	-	dialysis	67
	Glucose oxidase	Electro-chemical	3μM-3mM	-	modified electrode	69
Sucrose	Invertase	Amperometric	10^{-4}-10^{-2}M	30	packed	68
Glucose	Glucose oxidase				columns	
Hydrogen peroxide	Peroxidase	Fluorescence	(0.5pM)	120	-	72
	Peroxidase	Fluorescence	-	-	membrane entrapment	74
Urea chemical	Urease	Electro-	10^{-4}-10^{-1}M	-	dialysis	67
Penicillin	Penicillin-ase	pH	(5×10^{-3}M)	150	SBSR	54
Lactate	Lactate dehydro-genase	Electro-chemical	10^{-6}-8×10^{-5}M	30	-	75

However, when uricase was immobilized on controlled-pore glass (49), about 90% of the activity was retained up to almost one year.

Enzymes can be immobilized by entrapment, physical adsorption or covalent bonding. Covalent attachment on inert matrices (eg. glass and nylon) offers the greatest advantage in continuous flow systems (50). Attachment occurs via the amino or hydroxy groups on the enzyme surface. One of the widely used methods involves the bonding of the enzyme to alkylamino glass via glutaraldehyde attachment (49).

Different types of enzyme reactors have been used in continuous flow systems (51). They are broadly classified as packed reactors, single bead string reactors, and open tubular reactors. The reactor shape and dimensions affect the dispersion of the sample plug and hence the analytical signal obtained.

The behavior of packed columns is well known from chromatography. The dispersion is related to the particle size. Smaller the diameter of the packing material smaller the dispersion. Packed reactors offer the largest surface area per unit length and allows for large enzyme loading and maximum conversion of substrate to product (52) and is therefore popular. They, however, suffer from high pressure drops due to resistance to flow.

Reign et al. (53) proposed the use of a single bead string reactor (SBSR) to reduce dispersion in homogeneous

systems. Glass beads of diameter 60 to 80% that of the tube is used. The beads increase the radial dispersion and therefore reduce dilution and longitudinal dispersion of the sample plug. These reactors are mainly used as mixing devices to facilitate solvent extraction and chemical reactions. Enzymes have been immobilized on the beads and also on the tube walls (54) for use in the determination of penicillin.

Open tubular reactors (OTR) have the largest sample throughput and very low back pressures. The coiling of the reactor produces secondary flows which reduce dispersion. These however have the lowest surface area per unit length. The area of glass capillary is enhanced by the growth of "whiskers" by etching with ammonium bifluoride (55). This increases the area for immobilization of enzymes by three orders of magnitude. A recent innovation (56) in immobilized enzyme OTRs has been reported. The surface area was increased by embedding an inert support material with inherently large surface area . Control pore glass (CPG) was embedded on Teflon and Tygon tubes. The reactors have the advantages of an open tubular reactors and the surface area offered by other kinds of reactors. The schematic in Figure 2 shows the various reactor configurations mentioned above.

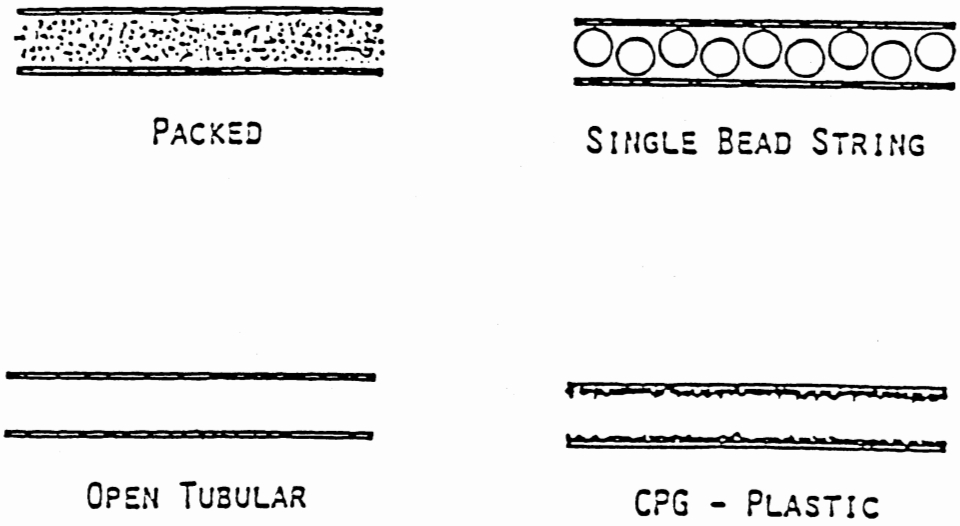


Figure 2. Schematic Representation of Major Reactor Configurations (From Ref. 51)

Some Typical Determinations Based on Soluble Enzymes

Glucose. The determination of glucose in biological samples is of great importance in the clinical diagnosis of various diseases, both in routine analysis and in emergency situations. Flow injection analysis has provided a very effective way of determining this species. Several investigations have been reported in this area. They are all geared towards the development of a procedure that has a low reagent consumption and a high sample throughput.

A photometric method based on the reaction of glucose with glucose dehydrogenase(GDH, E.C.1.1.1.47) and coenzyme NAD^+ was introduced by Ruzicka and Hansen (57) for the determination of glucose in blood serum by FIA. The NADH formed acts as the indicator and can be spectrophotometrically monitored at 340 nm. This method was further improved by the introduction of the merging zone principle in combination with a stopped-flow operation (58). The reagent consumption was reduced to less than 1 unit of GDH per determination. With the stopped-flow method determinations were made at a rate of 100 samples per hour.

The catalytic determination of enzymes in closed-loop system was shown to be possible by physical removal of the enzyme after signal detection. An example of this was the repetitive determination of glucose oxidase (E.C.1.1.2.3) by monitoring the oxygen consumption from the oxidation of

glucose by glucose oxidase and using sponge traps of phenoxyacetylcellulose (59). The deterioration of the base line seen when the enzyme was continuously recirculated after 25 injections did not occur until after 425 injections. The enzyme was stripped off and recovered with its catalytic activity retained.

Roehring et al. (60) demonstrated the use of closed-loop systems for the determination of glucose with regeneration and recycling of the coenzyme and enzymes. Using the GDH reaction in the presence of NAD^+; the NADH produced was converted back to NAD^+ by glutamate dehydrogenase (GLDH. E.C.1.4.1.3). The recirculation is done by (a) aspiration and (b) gravity. The process allows up to 300 determinations of glucose in serum with 40 ml of solution continuously circulating.

A microporous membrane has been used by Nieman et al. (61) to separate a pressurized reservoir containing glucose oxidase from an analyte stream containing glucose, luminol, and peroxidase. The glucose oxidase is forced at the rate of a few microliters per minute into the stream containing the analyte. The hydrogen peroxide produced was determined by luminol chemiluminescence in the presence of peroxidase (E.C.1.11.1.7). The carrier stream flows at a faster rate of few milliliters per minute. The flow rate ratio minimizes enzyme consumption and reduces sample contamination.

Determination of glucose or any oxidase dependent

reaction was possible by following the reaction between H_2O_2 and leucodiacetyldichlorofluroescein (LDADCF) to give fluorescent dichlorofluorescein (DCF), in the presence of glucose oxidase (GOD) and horseradish peroxidase (HRP). Kelly and Christian (62) used this reaction to determine GOD. Using an Argon ion laser excitation at 488 nm, the fluorescence was monitored at 525 nm. They found it could be applied to a broad range of oxidase systems. The sensitivity of the system was highly increased. The procedure was used together with capillary FIA as a means of reducing reagent consumption and sample size. A glass capillary of 0.2 i.d. and a 10 ul sample loop were used. This allowed for reduced dispersion and longer reaction times to enhance sensitivity. A limit of detection of 0.2 ng/ml was found possible. For most determinations of glucose such a low limit of detection is not necessary. The sophistication of the system is seen as reduntant.

The glucose oxidase catalyzed oxidation of D glucose was used to illustrate the substrate determination in a continuously circulating reagent mixture (46). The depletion of oxygen was monitored amperometrically using a three electrode system. The time between injection and the signal maximum was 2 seconds. The working range covered the normal blood serum glucose levels, 50-100 mg/100ml. The time for return to baseline was 5 sec from the time of injection and 2 sec from the time of the transient signal. Exploiting the delay time, the injection rate was extended

to 1700 determinations/h. The enzyme solution was diluted continually and the contamination from the serum sample only occured after about 10,000 injections. This method has the highest sampling rate in FIA.

Urea. Ruzicka et al. (63) developed an enzymatic determination of urea in aqueous and serum samples by FIA, with pH measurement. The main drawback of using pH measurements for biological samples was the different concentrations of buffers encountered. Buffering capacity was kept constant. In the range of 4 to 15 mM, the change in pH was linear with urea concentration. A sampling rate of 60/h is obtained. The enzyme (urease, E.C.3.5.1.5) consumption was 25 units per determination which is rather high. This could be further reduced by using merging zones.

Cholesterol. Nieman et al. (64) used a microporous membrane chemiluminecence cell containing cholesterol oxidase (E.C.1.1.3.6) to determine cholesterol in blood serum samples. The hydrogen peroxide reacts with luminol in the presence of HRP to produce chemiluminescence. A limit of detection of 0.2 mg/l was obtained with a sample throughput of 60/h with only 0.01 unit of enzyme used per sample. The major advantages of the method are the low enzyme consumption and rapidity of the determination.

Ethanol. Determination of ethanol is another frequently performed operation in clinical and forensic laboratories. Catalytic oxidation by alcohol dehydrogenase

(E.C.1.1.1.1) in the presence of the coenzyme NAD^+ is employed. The NADH produced is spectrophotometrically monitored at 340 nm. Worsfold et al. (65) proposed a stopped-flow FIA procedure for the determination of blood alcohol. The sample was diluted and introduced into the system directly. A kinetic determination was performed in which the rate of increase of NADH being formed was monitored. The blood alcohol was analyzed at a rate of 70-80 samples/h. Alcohol in several beverages was also determined by the same method at a rate of 120 samples/ h.

In a recent technical note Fernandez Gomez et al. (66) claim to have developed a method for the determination of blood alcohol by the above reaction that was more rapid and sensitive. The samples did not require pretreatment. The use of merging zones (small reagent and sample size) together with stopped-flow (high sensitivity) and flurometric detection made this a highly competitive procedure. A sampling frequency of 40/h was obtained. The concentration range determined was 0.125-2.0 g/l within which lies the legal level of alcohol permitted. The method uses small volumes of sample and has high precision and sampling rate and uses a rather inexpensive apparatus.

Some Typical Determinations Using Immobilized Enzymes

Glucose. A spectrophotometric determination of glucose using an immobilized enzyme reactor for a flow system was

developed by Gorton and Ogren (67), using glucose oxidase. The hydrogen peroxide formed was converted to a colored complex with 4-amino phenazone and N,N-dimethyl aniline. The reaction was catalyzed by immobilized peroxidase and the product monitored through a flow cell. Calibration curves for glucose were in the range of 1.6×10^{-4} to 1.6×10^{-2} M. In determining glucose, in serum samples, a dialysis membrane was used in the FIA system for the separation of the proteinic materials.

Townshend and Masoom (68) determined sucrose and glucose in mixtures with immobilized invertase (E.C.3.2.1.26)/ mutarotase (E.C.5.1.3.3) and glucose oxidase on controlled-pore glass in tandem. For the sequential determination of sucrose and glucose a bypass around the invertase column controlled the flow to pass through either both columns or glucose oxidase column alone. Thus, two sample injections, with or without the bypass, allowed both components to be determined. This was further refined by splitting the sample, one portion passing through both columns and the other only through the glucose oxidase column. A delay coil allows the two signals to be obtained in succession. The first from sucrose + glucose and the second from glucose only. A flow through amperometric detector was used for determining the H_2O_2 produced. The two sugars were determined in standard mixtures within 25 s. This was also applied to determine the sugars in soft drinks.

Jonsson and Gorton (69) constructed a glucose electrode by adsorbing glucose oxidase on a modified electrode consisting of Pd/Au sputtered on graphite. 0.8 units /cm^2 of GOD was adsorbed. This electrode was directly used in a FIA manifold for the determination of H_2O_2 formed by glucose oxidation. The decrease in over voltage of the H_2O_2 is related to the formal potential of the $Pd/Pd(OH)_2$ redox couple. The properties of this glucose sensor gave a linear calibration curve between 3 uM-3mM when an oxygen saturated carrier was used. The stability depended on the pH, sampling frequency and applied potential. The reoxidation of the catalytic surface was crucial in the recycling mechanism.

D-Glucose concentration in serum samples of 10 ul have been determined by following the chemiluminescence from the reaction of luminol with H_2O_2 (70). The sample was first passed through a column of immobilized glucose oxidase and through the flow cell containing immobilized peroxidase. A sampling rate of 20/h and a limit of detection of $5x10^{-6}$ M for H_2O_2 was obtained.

Thompson and Crouch (71) developed a stopped-flow instrument in which a nylon tube containing the immobilized enzyme was fitted into the observation cell. The enzyme reaction occurs under static conditions. The kinetics is controlled by molecular diffusion and the inherent enzyme reaction rate. Glucose was determined in a fixed-time mode over a linear concentration of 0-10 mM.

Hydrogen peroxide. In the presence of peroxidase, 3(p-hydroxyphenyl) propionic acid gave a fluorescence when reacted with H_2O_2 and the mixture was passed through a periodate treated enzyme on aminopropyl glass beads (72). The product stream was monitored with a conventional fluorescence instrument. The samples of hydrogen peroxide was determined at the rate of 120 samples/h. The limit of detection was as low as 0.5 pM using a 20 µl injection volume. The immobilized enzyme could be reused for a year without loss of activity. This is a highly sensitive and rapid method for hydrogen peroxide determination in systems generating H_2O_2 and is of clinical importance.

Dasgupta et al. (73,74) have focussed on the determination of trace atmospheric gases and hydrogen peroxide has been of particular interest in acid precipitations. The fluorometric determination based on the oxidation of p-hydroxyphenyl acetic acid to the fluorescent dimer by peroxidase mediation has been the method of choice. The immobilization of peroxidase on macroporous single bead string reactors gave good enzymic activity per unit reactor volume. An interesting alternative is one in which the enzyme is entrapped in a membrane. Microporous hollow fibers were taken as a bundle, folded to form a U. One end was closed, and the exterior of the bundle coated with epoxy. The reactor was then filled with peroxidase and ready to use. The reactor has much larger enzyme activity per unit reactor volume than any other immobilization

procedure and also provides the characteristics of enzyme in solution. However, the stability gained by some enzymes after immobilization is not realized. The system can not be used for the determination of other organic peroxides like methyl and hydroxymethyl peroxide because of severe loss of enzyme activity.

Urea. Gorton and Ogren (67) have also determined urea in biological samples, using immobilized urease reactors for converting urea to ammonia. The ammonia was detected using an ammonia sensing electrode. Samples were analyzed over the range of concentrations of 10^{-4} to 10^{-1} M. Normal urea concentrations in blood and urine samples lie in the linear portion of this curve. The samples required an on-line dialysis. The ammonia electrode shows a memory effect that prevents the determination of low concentration samples immediately after a sample of high concentration.

Penicillin. Pharmaceutical industries and clinical laboratories are required to determine the content of penicillins in samples on a routine basis. The enzymatic determination of penicillin is based on its hydrolysis to penicillinoic acid by penicillinase. Gnanasekaran and Mottola (54) immobilized penicillinase (E.C.3.5.2.6) on glass beads via glutaraldehyde coupling and packed them in a single bead string reactor to convert penicillin to the acid. The pH changes were monitored with a combination glass electrode. Penicillin was determined selectively in

the range, 0.05-0.50 mM. The activity of the enzyme was retained up to 97% after ten months of daily use. Penicillin content of pharmaceutical samples (tablets, injectibles and fermentation broth) were determined and the activity was found comparable to the manufacturers' specifications.

L-Lactate. Immobilized lactate dehydrogenase (E.C.1.1.1.2.7)was used for the determination of L-Lactate in a continuous flow system by Yao et al. (75). In the presence of the enzyme, lactate was oxidized to pyruvate, while the redox cofactor NAD^+ was reduced to NADH. The NADH formed was amperometrically monitored. The peak current is directly proportional to the L-lactate concentrations in the range 1 to 80 x 10^{-6} M. The immobilized enzyme retained its activity up to 90 days, with repetitive use. Sample injections at the rate of 30/h were possible.

Schmidt et al. (76) constructed an enzyme reactor with Eupergit (epoxyacryl resin beads)-bound dehydrogenases. It was coupled with graphite electrodes used as NADH sensors for the determination of different dehydrogenase substrates and applied to food analysis and process control. Some examples are D- and L-Lactate in butter, L-glutamate in beef, and ethanol in beer. The method is highly sensitive and the reactor is very stable for the use in flow system. Thompson and Crouch also used their stopped-flow instrument (71) for the determination of lactate by immobilizing

lactate dehydrogenase inside an open nylon tube. Linear concentration ranges of 0-45 μM were obtained with a sample throughput of about 90 samples/h.

Non-Enzymatic Methods of Determination

Oxidation-reduction catalysis by transition metal ions are the most common non-enzymatic catalytic reactions utilized in analytical determinations. The various indicator reactions of interest are listed in Table VI. A few examples in which catalytic reactions are used to determine the substrate are also included. Classification is based on the catalytic species. Different detection methods are considered and evaluated on the basis of the sensitivity of the method, limits of detection, linear concentration range, sampling rate and interferences from other metal ions. Special instrumental and flow manifold characteristics are highlighted. Some of these features are included in Table VII.

Cobalt

Various indicator reactions have been used for the determination of Co(II) in batch systems (77). However, the adaptation to flow systems was not always simple. The residence time was usually small to reduce sample dispersion, and hence the sensitivity was low. The stopped-flow technique was employed by Yamane (78) for greater sensitivity in a method based on the Co(II) catalysis of the

TABLE VI

INDICATOR REACTIONS USED FOR THE DETERMINATION OF SOME CATALYTIC SPECIES

Co(II)	
SPADNS + H_2O_2 ⟶	(78)
Protocatechuic acid + H_2O_2 ⟶	(79)
Galic acid + H_2O_2 ⟶	(81)
Cu(II)	
2,2'-dipyridyl ketone hydrazone + O_2 ⟶	(87-90)
Luminol + H_2O_2 ⟶	(92)
Mn(II)	
Hydroxynaphthol Blue + H_2O_2 ⟶	(96)
Mn(II) and Fe(III)	
Salicylaldehyde thiocarbazone + H_2O_2 ⟶	(98)
V(IV)	
Chromotropic acid + BrO_3^- ⟶	(103)
Sulfide	
Iodine + Azide ⟶	(104)
Iodide	
As(III) + Ce(IV) ⟶	(105)

TABLE VII

FEATURES OF NON-ENZYMATIC CONTINUOUS-FLOW DETERMINATIONS

Species	Detection	Interferences	Range or (LD)	S/h	Ref
Co	Spectrophotometric	Zn, Fe, Ni, Cr	5-200ppb	60	78
Co	Spectrophotometric	Ni, Mn, Fe	(0.2ppb)	50	79
Co	Chemiluminescence	Ca, Mg, Fe, Mn	(4ppb)	20	81
Co	Chemiluminescence	-	(0.01pg/µl)	-	82
Cu	Spectrophotometric	-	(0.25µg/ml)	325	84
Cu	Chemiluminescence	Fe, Cr	(3pg/µl)	60	85
Cu	Fluorescence	Al, Cr, Sn	8-300ng/ml	100	89
Cu	Fluorescence	Al, Cr, Sn	0.2-300ng/ml	70	90
Mn	Spectrophotometric	-	2-5ng/ml	-	97
Mn,Fe	Spectrophotometric	-	48-200ng/ml 40-600ng/ml	30	98
Mn	Spectrophotometric	Mg, Ni, Cd, Pb	0.2-1300ng/ml	45	99

TABLE VII (Continued)

Species	Detection	Interferences	Range or (LD)	S/h	Ref
Mo	Spectrophotometric	W, Cr	(0.7µg/l)	90	100
Mo,W	Spectrophotometric	-	0.3-8µg/l 0.4-13µg/ml	12	101
Zn,Cd	Chemiluminescence	Ni, Co	10-100ng/ml 20-200ng/ml	20	102
V	Spectrophotometric	-	10-160ppb	60	103
S	Amperometry	-	(0.1mg/l)	-	104
I	Thermochemical	-	(0.15ng)	-	105
I,SCN	Spectrophtometric	Mn, Fe, Hg, Ag	0.1-2ppm 0.2-20ppm	20	106

oxidation of 2(4-sulfophenylazo)-1,8-dihydroxynaphthalene-3,6-disulfonic acid (SPADNS) by H_2O_2, in alkaline media. Absorbance measurements were made at 520 nm. With adequate reaction time, as little as, 5 ppb of cobalt were determined at a rate of 60 samples per hour. The method was very straightforward and used a simple flow injection manifold. Interferences from Zn and Fe were observed when present above 1 ppm and Ni and Cr above 2 ppm. The same author (79) introduced later a new method for the determination of Co(II) in flow systems based on the catalytic oxidation of protocatechuic acid by H_2O_2 and spectrophotometric determination. The rate of the uncatalyzed reaction was small in comparison with the catalyzed reaction rate and therefore provided high sensitivity. This method also had improved selectivity. Nickel, manganese, and iron showed positive interferences only when present in 100 fold excess of cobalt. The detection limit, 0.2 ppb, was lower than that obtained with SPADNS oxidation. Determination of Co(II) by the catalysis of H_2O_2 oxidation of organic dyes, Stilbazo, and Pyrocatechol Violet gave comparable sensitivity and limits of detection to the previously mentioned methods. The flow injection manifold is more complicated by the presence of coiled tubes to dampen the pump pulses and sample injection shock (80).

Chemiluminescence methods provide lower limits of detection. Nakahara et al (81), for instance, used galic

acid-H_2O_2- NaOH system with a little methanol to obtain a limit of detection of 4 ppb. The linear range was of three orders of magnitude. The poor selectivity observed in many chemiluminesence (CL) studies was not observed here. The galic acid CL system is very selective for cobalt. Townshend and co-workers (82) studied the Co catalyzed oxidation of luminol by H_2O_2 in a FIA system. Instead of a mixing coil a coiled flow cell was placed in front of the detector. The response was fast, precise, and the method was sensitive. The blank emission was low because the CL stimulated by other trace metals decay before entering the cell. A limit of detection of 0.1 pg/µl was obtained. In a novel method, CL of luminol is ultrasonically induced in the presence of Co(II) in alkaline medium containing dissolved oxygen (83). This proved to be a highly sensitive and selective method. Limits of detection of 0.07 pg/µl sample is the lowest obtained by any method. The selectivity is comparable to the galic acid method.

Copper.

Analytical methods for the determination of copper based on its catalytic activity are numerous. A few of these procedures offer good sensitivity and low limits of detection and can be applied to biological samples. Ramasamy and Mottola (84) reported the determination of copper in human blood serum based on the catalytic effect of

Cu(II) ions on the oxidation of thiosulfate by Fe (III) in acidic medium. The catalyst was removed electrochemically permitting the use of a closed loop system. At the same time, reoxidation of Fe(II) to Fe(III) occured maintaining the concentration of the monitored species constant. The formation of iron-thiocyanate complex was monitored spectrophotometrically. A limit of detection for Cu(II) in human blood samples of 0.25 µg/mL was obtained and 325 determinations/ hour were performed.

Yamada and Suzuki (85,86) performed various studies on the chemiluminescent determination of Cu(II) by Flow Injection. A flavin mono nucleotide-hydrogen peroxide-phosphate buffer system was used. The optimum conditions for determination of Cu(II) gave a linear calibration curve of three orders of magnitude and a detection limit of 3 pg/µl at a sampling rate of 60 per hour. They also used the catalytic decomposition of hydrogen peroxide in the presence of 1,10-phenanthroline and a cationic surfactant micelle to increase the sensitivity. This also gave more selectivity than any other CL system and a limit of detection of 0.3 pg/µL injection. Metals like Cr(III), Mn(II), Ni(II), and Co(II) gave no emission.

Valcarcel and co-workers (87-90) have studied the fluoresence of 2,2'-dipyridyl ketone hydrazone (DPKH). When oxidized by atmospheric oxygen in the presence of Cu(II) it gave an intense blue fluoresence (λex= 350nm; λem= 429nm). The oxidation requires a basic medium but the fluoresence

takes place in a highly acidic medium. A series of developments were made to arrive at a method that gave rapid determinations and low detection limits. In the conventional equilibrium method (87) two steps were necessary for developing maximum fluoresence; the oxidation product was generated slowly (90 min) in neutral solution. The linear concentration range was 0.4-1.0 ng/ml. When a kinetic measurement was made using an intermediate pH of 6.3, it was neither rapid (4 min/sample) nor did it give low limits of detection (0.1 μg/ml). In the Flow Injection method (89) the oxidation was performed in alkaline solution at optimum pH and then an acidic stream was introduced to allow maximum fluorescence. The method gave a linear calibration curve of (8-300 ng/ml) and fast determinations (100 samples per hour). It lacked selectivity due to interference by Al(III), Cr(III), EDTA, and Sn(II) at concentration levels similar to that of Cu(II) and the method is not very feasible for determinations in real samples. When a stopped-flow technique (90) was used the interfering ions did not affect the signal even in the ratio of 100:1 to copper. The limit of detection was also improved to 0.3 ng/ml. This method was used to determine Cu(II) in food and blood serum. The results obtained were comparable to those obtained by atomic absorption after extraction. The reaction was extended to the simultaneous determination of Cu(II) and Hg(II) (91).

The catalytic effect of Cu(II) on the

luminol-H_2O_2 reaction luminescence has been studied extensively and used for the determination of proteins. Rule and Seitz (92), for instance, evaluated the system studying the effect of flow rate, sample volume and reaction kinetics on the magnitude, precision, and stability of the CL response for the determination of H_2O_2. A sample throughput of 360/hour was found possible.

Protein determinations by FIA were carried out by Hara et al. (93,94). The catalytic activity of Cu(II) on the luminol-H_2O_2 was found to decrease in the presence of proteins. A new method was developed based on this phenomenon. Small amounts of proteins are economically determined over a wide concentration range, 10^{-4}-10^{-1} g/L, with a sampling rate of 30 samples per hour and a limit of detection of 0.2 μg/ml. Proteins in human and bovine serum albumin have been determined using the same reaction.

The FIA determination of o-amino acids with CL monitoring by Hara and co workers (95) was also based on the reduction of the catalytic activity of Cu(II) on the luminol-H_2O_2 reaction in the presence of an o-amino acid. L-Aspartic acid can be determined in a concentration range of $1x10^{-6}$-$1x10^{-4}$ M with a LD of 2.7 ng/ml. The method does not have competitive sensitivity but determination can be carried out without derivatization of the amino acid.

Manganese and Iron

The catalytic oxidation of hydroxynaphthol blue by hydrogen peroxide has been used for the determination of manganese in flow systems (96,97). The limit of detection is 2-5 ng/ml. Lazaro et al. (98) introduced two methods for the determination of Mn(II) and Fe(III) in mixtures, based on their catalytic action on the oxidation of salicylaldehyde thiocarbazone (SAT) by hydrogen peroxide. Different products are obtained depending on the oxidant concentration. From an analytical view point, the most interesting product is N-nitroaminomethane sulfonic acid which fluoresces in ammoniacal solution (λex=357nm; λem=433nm). The simultaneous determination is acheived in two ways: sequential and differential. In (a) the system is optimized for each catalyst separately by optimizing the variables in the indicator reaction.

When Fe(III) was injected into the Mn(II) stream no signal was produced and the response was due to Mn(II) only. When Mn(II) was injected into the iron stream the linear calibration range was small. The amount of Fe(III) was determined by subtracting the peak height due to Mn(II).

In method (b) two portions of the sample were divided and reached the detector at different times. Two peaks were obtained for each injection which have relative proportions. It is advantageous that these methods use a single manifold and detection system. Calibration ranges of 48-200 and

40-600 ng/ml are obtained, respectively. The sampling rate is 30/h.

Ultra trace amounts of Mn(II) were determined by Blanco et al.(99). Manganese catalyzes the oxdation of succinimide dioxime in a basic medium. The reaction product can be spectrophotometrically monitored at 695 nm. Linear graphs of catalysts concentration vs peak height obtained at different temperatures. The linear concentration ranges obtained at 25 ^{o}C, 35 ^{o}C, and 45 ^{o}C were 50-1300 ng/ml, 1-30 ng/ml, and 0.2-10 ng/ml, respectively. Hence, by controling the temperature Mn can be determined in a wide concentration range. A sampling frequency of 45/h was obtained. Manganese determination in food products like rice and coffee is illustrated by the authors.

Molybdenum and Tungsten

An FIA method for the determination of molybdenum based on its catalytic activity on the oxidation of iodide by hydrogen peroxide in acidic medium has been reported (100). The triiodide formed can be spectrophotometrically monitored at 350 nm. Molybdenum was determined in natural water samples without need for preconcentration at a rate of 90 samples/h with a limit of detection of 0.7 µg/l. The calibration curves were linear over three orders of magnitude in concentration.

A new type of ion selective electrode was used as a detector in an FIA system for the catalytic determination of

Mo(VI) and W(VI) (102). The electrode was based on a AgS/AgI membrane and the change in iodide concentration was monitored with the aid of a flow-through electrode.

When molybdenum and tungsten are present together the latter may be masked out by citrate; thus, a simultaneous determination is possible. The concentration range and sampling rate, however, were small. Molybdenum and tungsten were determined in the range 0.3-8 μg/ml and 0.4-13 μg/ml, respectively, at a sampling rate of 12 samples/h.

Zinc and Cadmium

Zinc and cadmium can be successfully eluted from an ion exchange column and subsequently determined by their inhibition of the Co(II)-catalyzed chemiluminescence of luminol. Townshend and Burguera (102) found that when Zn(II) or Cd(II) are added to the system the emission intensity decreases. Thus sequential release of the ions from the column causes negative peaks. Inhibitor concentrations of 10-100 ng Zn/ml and 20-200 ng Cd/ml showed linear dependency. The limits of detection were 5 ng/ml for Zn(II) and 3 ng/ml for Cd(II). Sampling rates were in the neighborhood of 20 samples/h. The method was both sensitive and rapid and could be extended to other combinations of metal ions.

Vanadium

Yamane and Fukasawa (103) developed a catalytic

photometric method for the determination of V(IV) in which catalysis of the chromotropic acid- bromate reaction is followed spectrophotometrically. The adaptation to a flow system was simple and a sensitive and rapid determination possible. Vanadium in the range of 10-160 ppb gave a linear response and an injection rate of 60 /h was found possible. There is no mention of possible interferences from other metal ions.

Sulfur(II)

The iodine/azide reaction induced by S(II) has been used to determine several S(II) compounds (104). Iodine consumption in the reaction was detected by biamperometry with Pt electrodes. Different sulfur compounds have different induction times. Some of the compounds examined were sodium sulfide, thiosulfate, cystine, glutathione and thiourea. Detection limits of 0.1 mg/l to 0.2 mg/l were observed. The linear calibration ranges depended on the concentration of iodine in the mixture.

Iodide

A commonly used catalytic method for the determination of iodide is based on the redox reaction between Ce(IV) and As(III). Low catalyst concentrations cause large increase in reaction rates and a proportional increase in heat produced. Coupling of a catalytic method with thermochemical detection can give low limits detection.

Carr et al (105), for example, demonstrated it for the determination of I^- in a flow system. Cerium and arsenic streams were mixed into which a sample of I^- was injected. A heat pulse resulted and was detected with a differential temperature measurement system. A minimum detectable quantity (MDQ) of 0.15 ng/ml was obtained; the best reported value for a thermochemical method.

The simultaneous spectrophotometric determination of iodide and thiocyanate by FIA has been proposed by Tanaka et al. (106). The redox exchange between Ce(IV) and As(III) was used as indicator reaction. The catalytic activity of thiocyanate was destroyed by reacting with Ce(IV) and was restored by a large excess of As(III). Selective inactivation of the reactivity of the thiocyanate in the presence of iodide was possible. In the FIA system, a double injection technique was used. One of the samples was introduced into the stream of Ce(IV), followed by an excess of As(III). In the other case the sample was introduced into a mixture containing both Ce(IV) and As(III). The first peaks were due to iodide and thiocyanate, while the second was due to the presence of iodide alone. The disappearance of the yellow color of Ce(IV) was monitored at 254 nm and calibration curves showed good linearity. The range of concentrations of this method for thiocyanate was 0.2 ppm to 20 ppm and for iodide was 0.1 ppm to 2 ppm. A sampling rate of 20/h was obtained.

In these studies, both enzymatic and non-enzymatic

catalysis have been tried for the oxidation of reserpine. The enzyme catalysis centered on the aerobic oxidation of reserpine catalyzed by peroxidase is the subject of discussion in the next chapter. As indicated in Chapter I, the enzyme was rendered inactive, making it less attractive as an analytical reagent. The search for a alternate catalyst resulted in the choice of Mn(II) as the catalyst for the oxidation of reserpine by periodate ions. Develpoment of an analytical method based on this reaction is the focus of Chapter V.

CHAPTER IV

STUDIES ON THE PEROXIDASE PROMOTION OF THE OXIDATION OF RESERPINE AND ITS INHIBITION BY 3,4-DIDEHYDRORESERPINE

The usefulness of enzymes as analytical reagents center around their selectivity towards certain substrates and their ability to catalyze reactions at low concentrations (107). Their regeneration in the catalytic cycle suggests that repeated use of the same enzyme preparation is possible. When enzymes are immobilized on inert supports they offer some further advantages and they can be easily separated and recovered from the reaction mixtures and they can be used as reactors in continuous-flow systems as discussed in Chapter III. It is however critical, that the enzyme retains its activity and is not inhibited during the course of the reaction or its use in immobilized form becomes an exercise in futility.

Peroxidase [E.C. 1.11.1.7], obtained most commonly from horseradish, consists of a colorless protein (apo-enzyme) combined with an iron-porphyrin. The molecular weight of peroxidase is about 40,000. It is present in almost all living things, although its exact biological functions are not totally known and continue to be a point of intrigue.

One of its main functions is that of a hydrogen peroxide scavenger. Apart from its biological functions, it is widely used as an indicator or marker in controlling the deterioration of processed food quality in the food industry (108). It is also one of the first enzymes that was used as an analytical reagent (catalyzes the hydrogen peroxide oxidation of substrates) (109). Inactivation of peroxidase is, therefore, of interest to the food industry and becomes of relevance to analytical chemistry.

Peroxidase is known to be inhibited reversibly and irreversibly by a variety of chemical species (110). Peroxidase inactivation by an excess of hydrogen peroxide, which can attach to the sixth coordination site of the iron atom in the prosthetic group, is reported and shown to cause problems when used as an immobilized reagent (111). The inhibition of peroxidase described in this study was observed while attempting to develop an analytical method for the determination of certain pharmaceuticals. Peroxidase was found to accelarate the oxidation of reserpine by dissolved air (Figure 3). The enzyme was immobilized on controlled-pore glass following the procedure of Olsson (112). Amino groups were bonded to the controlled-pore glass support by refluxing with 3-aminopropyltrimethoxysilane and the enzyme was bound to the support by glutaraldehyde coupling. The immobilized peroxidase was used in a packed enzyme reactor for the determination of reserpine in a continuous-flow system by

monitoring the product of oxidation, 3,4-didehydroreserpine which has a greenish yellow color and absorbs at 385 nm. The absorbance, however, decreased with repetitive injections indicating a loss of peroxidase activity (113). Experimental studies conducted to elucidate the type of inhibition of peroxidase during the aerobic oxidation of reserpine are described and the findings reported here. Since the inhibition occurs after the initial accelerating effect on the aerobic oxidation of reserpine, the overall process can be classified as promotion (114).

Experimental

Reagents and solutions

All the reagents used were of analytical grade unless otherwise indicated. The solutions were prepared with deionized water further purified by distillation in an all-borosilicate glass still with a quartz immersion heater (Wheaton Instruments, Millville, NJ).

Reserpine was obtained from Sigma Chemical Co., St. Louis, MO and dissolved in 5.0 N acetic acid and diluted with phosphate buffer (Clarks and Lubs buffer(115)), at pH = 7.00. Freshly prepared solutions were used to minimize the degradation of the solution due to oxidation. The oxidation product of reserpine was obtained by exposing a solution of reserpine to sunlight until a constant absorbance was obtained at 385 nm (molar absorptivity: 10,778 M^{-1} cm^{-1}).

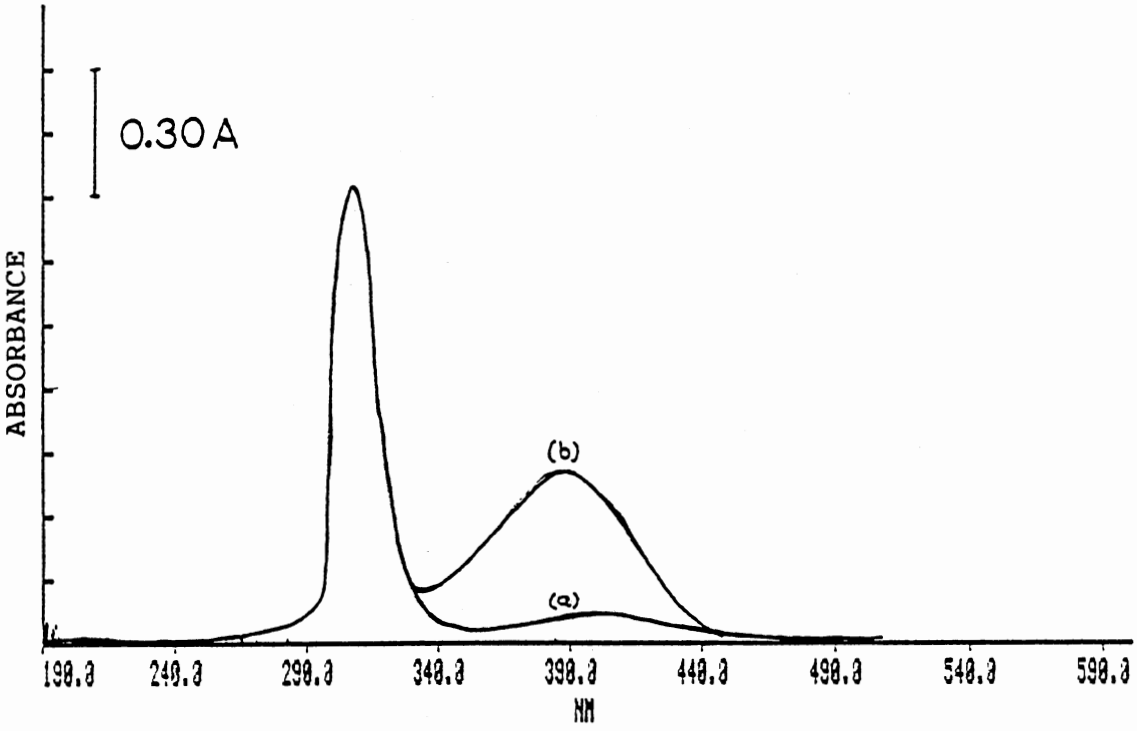


Figure 3. Spectra of (a) Reserpine and (b) 3,4-didehydroreserpine in the presence of Peroxidase

The horseradish peroxidase, Type I and VI, were obtained from Sigma. A third type, which consisted of an ultrapure preparation containing only one isoenzyme C, was obtained from BM Biochemica (Indianapolis, IN) and was used for crossreference. Enzyme solutions were prepared daily with phosphate buffer. The 4-aminoantipyrine used for enzyme activity determination (116) and rate studies was also obtained from Sigma.

The measurements of reaction rates were performed at 20 ± 1°C.

Apparatus

A Perkin-Elmer Lambda 3840 UV/VIS linear diode array spectrophotometer, operated by a Perkin-Elmer 7300 professional computer (Perkin-Elmer Inc., Norwalk, CT), was used for the collection of spectral information and for the monitoring of reaction rates. An Orion Model 601 (Orion Research Inc., Cambridge, MA) digital pH meter and an epoxy-body combination electrode (Sensorex, Westminster, CA) were used for pH measurements.

Procedure

The activity of peroxidase was determined by a method in which 4-aminoantipyrine is used as the oxidizable substrate (116). 1.4 mL of phenol (0.17 M)/ 4-aminoantipyrine (0.0025 M) solution and 1.5 mL of hydrogen peroxide (0.0017 M) were taken in a cuvette and allowed to

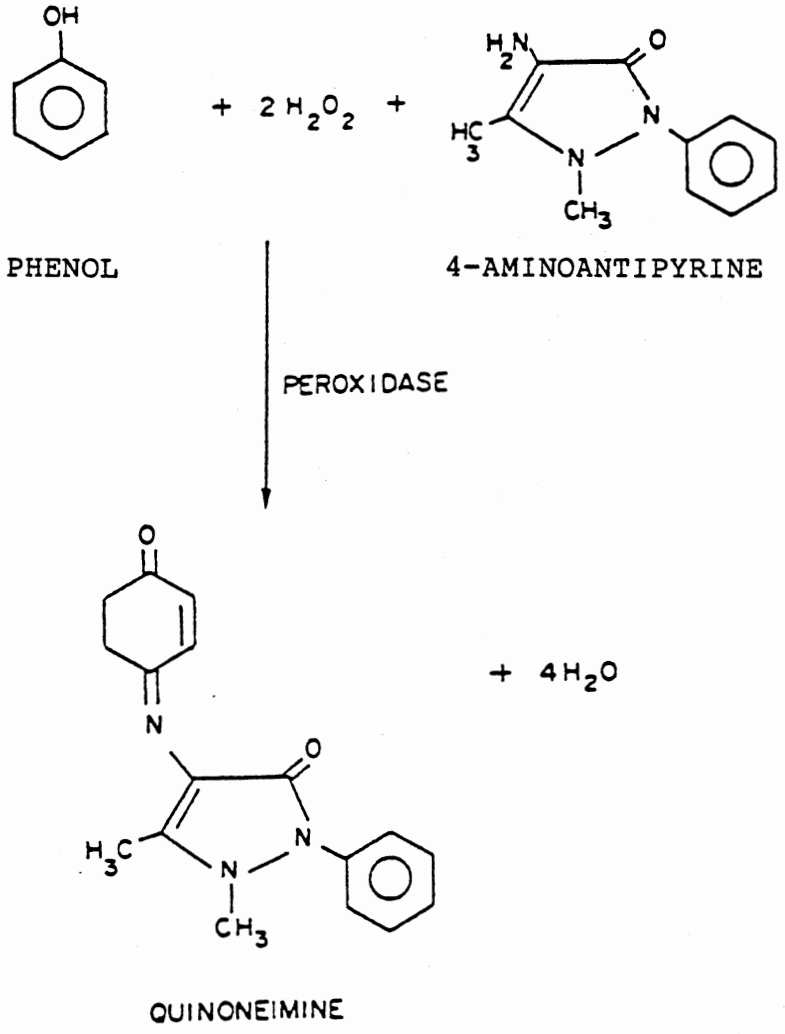


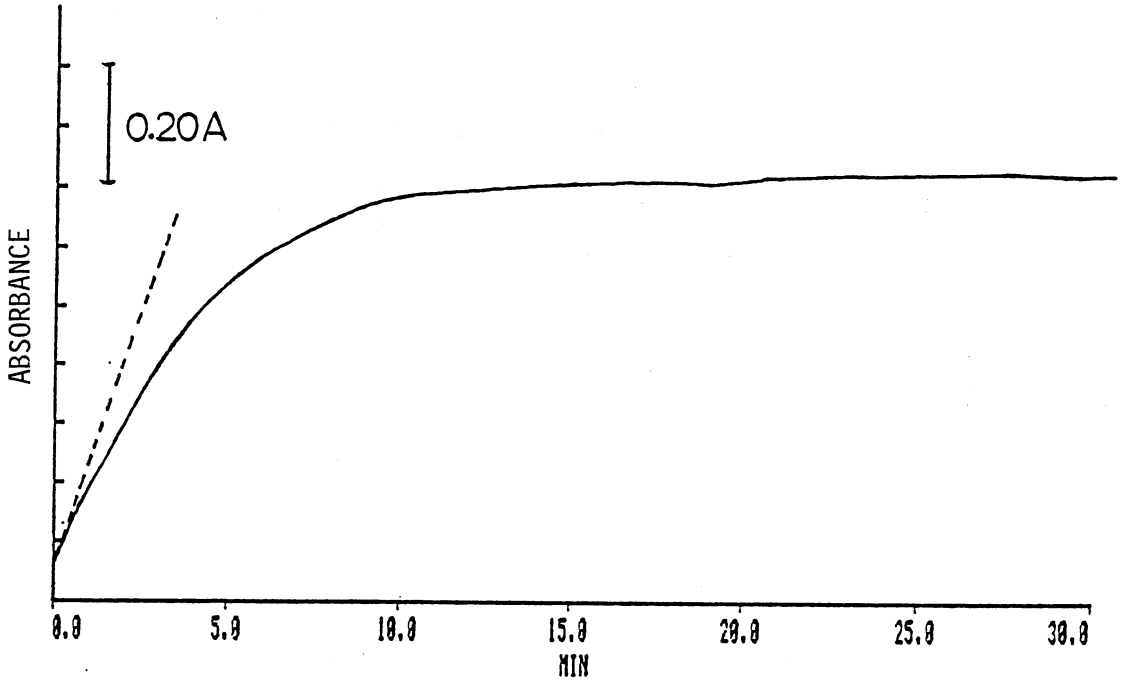
Figure 4. Mechanism for the Peroxidase Catalyzed Formation of the Quinoneimine Dye

reach equilibrium. 0.1 mL of enzyme (0.05-0.25 units mL^{-1}) was then added to it. The enzyme catalyzes the reaction between 4-aminoantipyrine and phenol, in the presence of hydrogen peroxide. The product of the enzymatic reaction is a quinoneimine dye (Figure 4) with an absorption peak at 510 nm. The reaction rate was determined by measuring the increase in absorbance resulting from the decomposition of hydrogen peroxide. By definition, one unit (U) of peroxidase is the amount of enzyme which will decompose 1 umole of peroxide in one minute at 25 °C and pH = 7.00. The activity can be calculated as follows:

$$\text{Units/mg} = \frac{A_{510}/\text{min}}{6.58 \times \text{mg enzyme/ml reaction mixture}} \quad (13)$$

To establish the need for the presence of oxygen in the oxidation of reserpine, nitrogen gas was first bubbled through the solutions of reserpine, peroxidase, and buffer. An aqueous solution containing reserpine (4.8×10^{-4} M) and peroxidase (5.0×10^{-6} M) was taken in a cuvette and tightly covered to prevent air from entering. The oxidation was monitored by observing the change in absorbance at 385 nm. The cover was then removed and oxygen was bubbled through the solution. When the absorbance reached a constant value more reserpine was added to see if the enzyme was still active. The activity of the enzyme present in the cuvette after reaction was also independently determined by the 4-aminoantipyrine method mentioned above.

A peroxidase solution (5×10^{-6} M) of initial



--- Slope Used to Calculate Initial Rate

Figure 5. A Typical Absorbance vs. Time Curve for the Oxidation of Reserpine by Dissolved Oxygen in the Presence of Peroxidase

activity of 180.4 U. mg^{-1} M was separately incubated with reserpine (4.8×10^{-4} M), oxidized reserpine (1×10^{-5} M), and buffer (0.1 M), in individual vials and in an atmosphere of nitrogen to determine which of these chemical species was responsible for the inactivation of peroxidase. Aliquots of these solutions containing peroxidase were withdrawn after 5 min, 30 min, and 1 h incubation time and the activity was then determined by the 4-aminoantipyrine-phenol method.

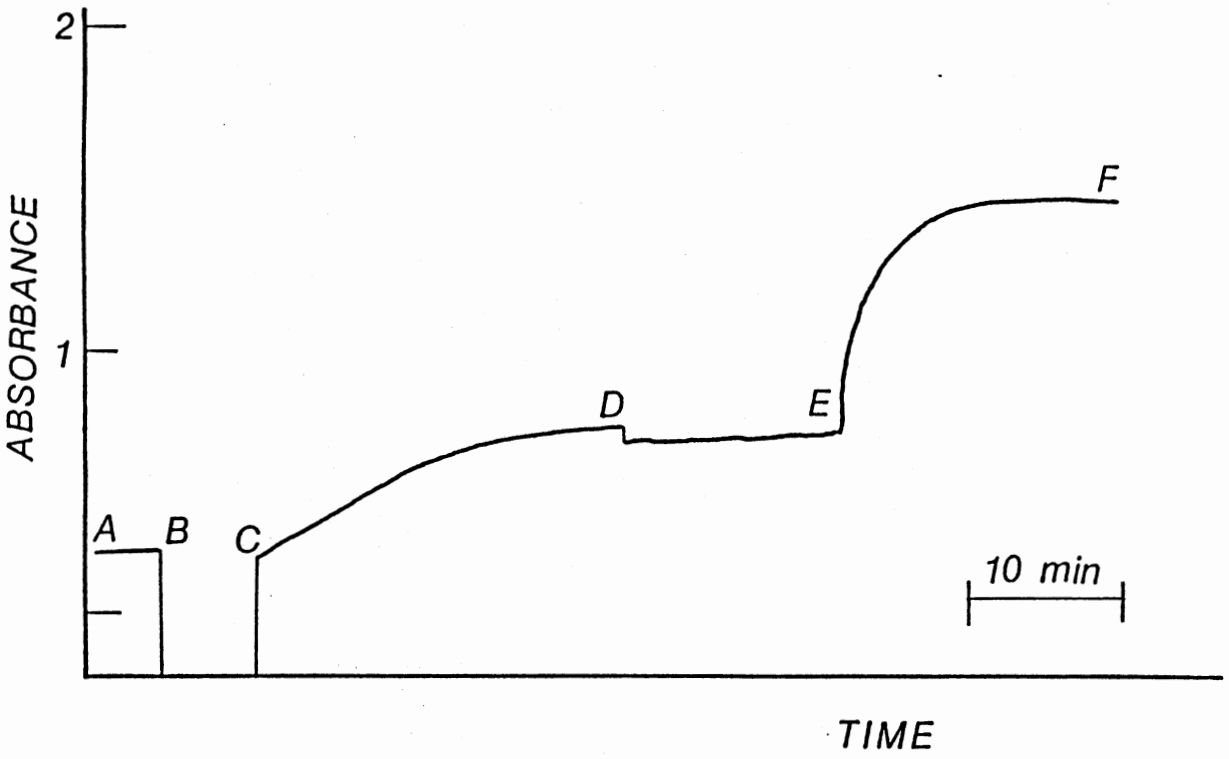
Initial rate studies were conducted by monitoring the change in absorbance with time and all the measurements were made within 2% reaction completion. The absorbance measurements were made every 3 seconds, until maximum absorbance was reached. A typical absorbance versus time curve for the oxidation of reserpine is shown in Figure 5. A tabular output of all the data points, listing the time of measurement and the absorbance measured was also obtained. The initial rate was calculated using this data. The slope was obtained by a linear least squares fit of the data points corresponding to less than 2% conversion. From the slope and the molar absorptivity of 3,4-didehydroreserpine, the change in concentration with time was determined.

Initial rates were determined for Sigma Type I, VI and BM Biochemica preparations of horseradish peroxidase. A buffered solution of reserpine (4.8×10^{-4} M) was taken in a cuvette and the enzyme (with different activities) was added to initiate the reaction. The change in absorbance

with time, which determined the initial rates was plotted against the activity of the enzyme. The effect of different inhibitor concentrations (ϵ = 10,778 M^{-1} cm^{-1}; 0.8 x 10^{-5} M-1.0 x 10^{-4} M) on the initial reaction rates were measured at different substrate concentrations. The aerobic oxidation of reserpine and the hydrogen peroxide oxidation of 4-aminoantipyrine were used as models to determine the type of inhibition. Reserpine concentrations ranged from 6.67 x 10^{-6} to 1.33 x 10^{-4} M and 4-aminoantipyrine concentrations ranged from 8.3 x 10^{-6} to 8.3 x 10^{-4} M. Due to the difference in reaction rates, enzyme concentration was 25 U. ml^{-1} for reserpine as substrate and 1 U. ml^{-1} for 4-aminoantipyrine as substrate.

Results and Discussion

The oxidation of reserpine to 3,4-didehydroreserpine by atmospheric oxidation at room temperature occurs slowly. In the presence of horseradish peroxidase the reaction rate is considerably increased. Dissolved oxygen is essential for the oxidation to take place, probably as a co-substrate. Figure 6 shows the change in absorbance in the presence and absence of oxygen. There is no visible oxidation in the presence of nitrogen but when oxygen is bubbled through, the absorbance continues to increase until a plateau is reached. If the enzyme remains active at the end of the catalytic cycle, addition of more reserpine should result in further



AB: Constant absorbance observed when nitrogen was bubbled through the solution.
BC: The bubbling of nitrogen was discontinued and air bubbled through the solution.
CD: Increase in absorbance due to the reserpine oxidation indicating the need for O_2 (g) for the reaction to occur.
At D: Fresh reserpine is added; no noticeable oxidation due to inactivation of enzyme.
At E: Fresh enzyme added; The reserpine added at D gets oxidized.
At F: Fresh reserpine is added but absorbance remains the same.

Figure 6. Variation of Absorbance of a Solution of Reserpine Containing Peroxidase Measured at 385 nm

reaction. This was not found to be so, which indicates that the enzyme has undergone inhibition during the reaction.

Chemical Species Responsible for the Inhibition

Inhibition of enzymes can occur irreversibly due to temperature and mechanical effects. In the absence of such effects the inactivation is produced by a chemical reaction. The chemical species present in the system under study that could be responsible for the inactivation are oxygen, reserpine or 3,4-didehydro reserpine. These chemical species were incubated with the enzyme in the absence of the other and the enzyme activity determined periodically, the results of which are tabulated in Table VIII. The activity of the enzyme did not change in the presence of oxygen or of reserpine in the first one hour. However, a sharp decrease in activity (only 5% of the initial activity remained after 30 min) was observed when the enzyme was incubated with the 3,4-didehydroreserpine. This indicated that this species is responsible for the inactivation of horseradish peroxidase in this study. Bubbling more oxygen into the system after initial inactivation did not regenerate enzyme activity.

Spectral Evidence for the Absence of Heme Degradation

Irreversible inhibition of peroxidase by chemical species such as hydroxymethylhydroperoxide (HMP) and

TABLE VIII

DEPENDENCE OF HORSERADISH PEROXIDASE ACTIVITY UPON INCUBATION WITH RESERPINE AND WITH 3,4-DIDEHYDRORESERPINE

Time, min	Activity, $U.mg^{-1}$	
	Incubation with Reserpine	Incubation with 3,4-didehydroreserpine
5	177.8	122.7
30	168.7	10.0
60	151.3	1.3

Initial activity: 180.4 $U.mg^{-1}$. Incubation in the absence of dissolved oxygen.

Activity determination by the 4-aminoantipyrine/phenol method.

cyclopropanone hydrate have been reported (117, 118). When HMP was added to a peroxidase solution a green compound was formed with an absorption at 670 nm (ϵ = 30,000 M^{-1} cm^{-1}). The decrease in peroxidase activity is proportional to the increase in absorption at 670 nm. When this compound is treated with pyridine and sodium hydroxide it shows an absorption spectrum similar to a product of heme degradation. Cyclopropanone inhibition also results in a modified heme. When peroxidase (1.6×10^{-6} M) was inhibited during reserpine oxidation ([Reserpine] = 4.8×10^{-4} M), no spectral changes were observed around 670 nm. This suggests that the inhibition by 3,4-didehydroreserpine does not cause any modification of the heme.

Reaction Rate Studies to Determine the Type of Inhibition

Inhibition of an enzyme is characterized by an equilibrium between the enzyme and the inhibitor which is governed by the affinity of the enzyme for the inhibitor. The type of inhibition is related to the behavior of the inhibitor towards the active site of the enzyme. The inhibition is termed competitive if the inhibitor and substrate compete for the active site. When the inhibitor binds to the enzyme-substrate complex and not to the free enzyme it is called uncompetitive. Noncompetitive inhibition is one in which the enzyme binds itself to a site

other than the active site of the enzyme, but causes it to inactivate (119). A schematic representation of the types of inhibition using the lock and key analogy is shown in Figure 7.

A simplified mechanism for the enzyme-catalyzed reactions and the derivation leading to the Michaelis-Menten equation is described in Chapter III. The equation can be transformed into a linear form to graphically analyze data and to detect the deviations from ideal behavior. One of the best known methods is the double reciprocal (or Lineweaver and Burk) plot. By inverting both sides of the equation 8, substituting equation 9 and rearranging, the following expression can be obtained:

$$\frac{1}{IR} = \frac{1}{IR_{max}} + \frac{K_M}{IR_{max}\,[S]} \qquad (14)$$

The plot of 1/IR versus 1/[S] is linear with a y-intercept of $1/IR_{max}$ and x-intercept of $-1/K_M$ from which the values of IR_{max} and K_M can be obtained.

In the presence of an inhibitor, an additional equilibrium must be taken into account for the Michaelis-Menten mechanism. In the case of competitive inhibition it is given by

$$\begin{array}{ccccc} E & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & ES & \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} & E + P \\ I \Big\Updownarrow K_I & & & & \\ EI & & & & \end{array} \qquad (15)$$

where I = inhibitor, EI = enzyme-inhibitor complex and K_I is the equilibrium constant for the inhibition

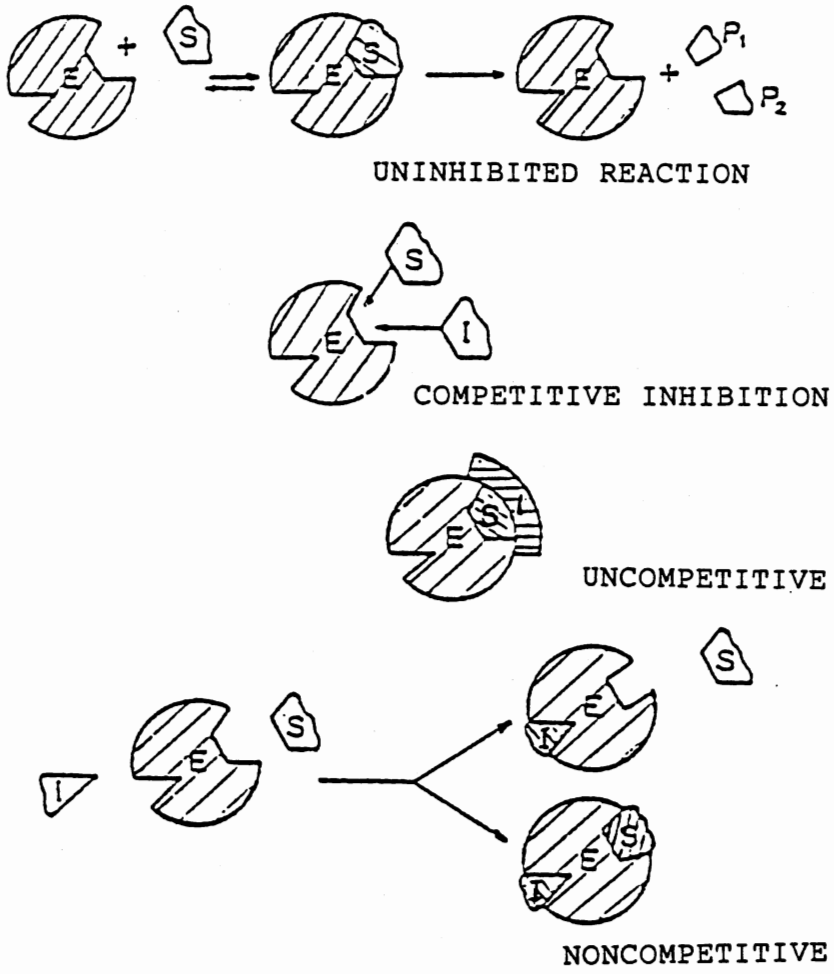


Figure 7. Schematic for the Representation of the Main Types of Inhibition Using the Lock-and -Key Analogy
E-enzyme, S-substrate, I-inhibitor, P_1 and P_2-products

reaction. Considering $[E_o] = [ES] + [EI] + [E]$, the rate equation can be written as

$$IR = \frac{IR_{max}\,[S]}{[S] + K_M\,(1 + ([I]/K_I))} \tag{16}$$

Competitive inhibition affects K_M but not IR_{max}. The Michaelis-Menten constant is increased by a factor of $(1 + ([I]/K_I))$. When I and S bind simultaneously to the enzyme instead of competing for the active site, the mechanism can be illustrated by:

$$\begin{array}{ccccc} E & + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & ES & \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P & + E \\ \Big\Uparrow\Big\Downarrow K_I & & I'\Big\Uparrow\Big\Downarrow K_I' & & \\ EI & \longrightarrow & [EIS] & & \end{array} \tag{17}$$

When a non-competitive inhibition is in effect, the dissociation of [S] from EIS is the same as the dissociation from ES, then:

$$IR = \frac{IR_{max}\,[S]}{(1 + ([I]/K_I))\,(K_M + [S])} \tag{18}$$

K_M remains unaffected by the inhibition while IR_{max} is reduced by $(1 + [I]/K_I))$. When the inhibitor binds to the enzyme-substrate complex, resulting in uncompetitve inhibition, the Michaelis-Menten reaction leads to :

$$IR = \frac{IR_{max}\,[S]}{(1 + [I]/K_I))} \times \frac{[S]}{[S] + K_M/(1 + [I]/K_I))} \tag{19}$$

where both K_M and IR_{max} are lowered by the presence of the inhibitor by a quantity of $(1 + [I]/K_I))$. Lineweaver and Burk plots (1/IR vs. 1/[S]) for the different type of inhibitions is depicted in Figure 8.

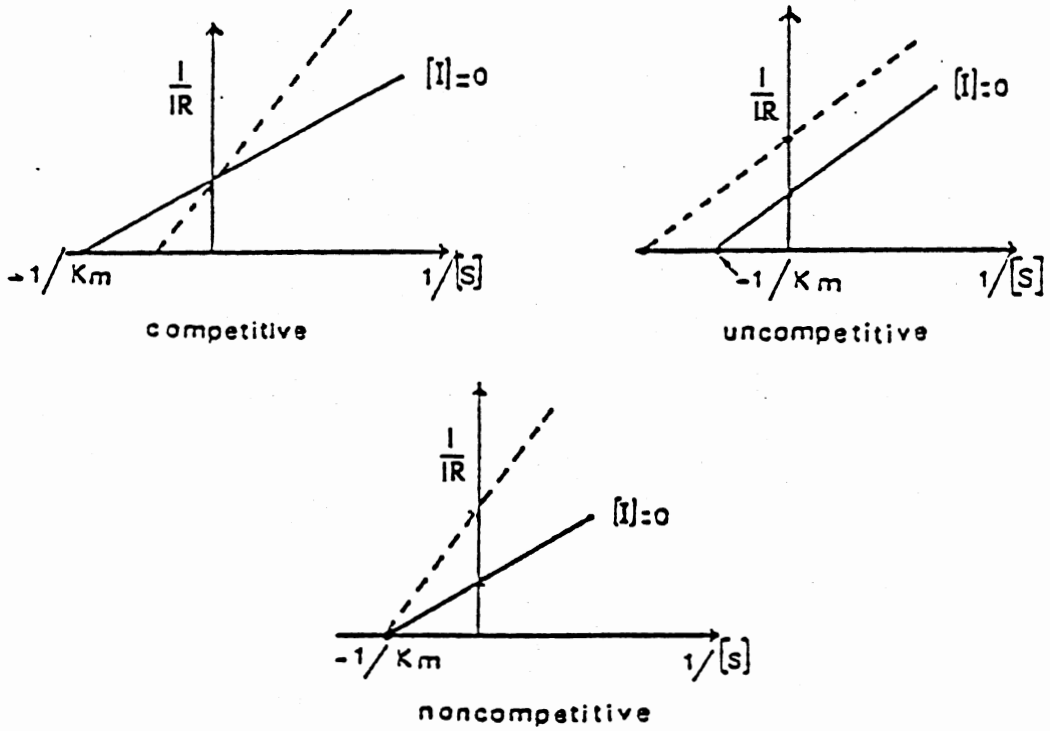


FIGURE 8. Typical Lineweaver-Burk Plots for the Different Types of Inhibition
IR-initial rate, [I]-inhibitor conc., [S]-substrate conc., K_m-Michaelis-Menten constant

The initial rates were found to be linearly dependent on the enzyme activity for the three different preparations of peroxidase. The Sigma Type VI and the BM Biochemica gave essentially the same slopes. The Sigma Type I gave slightly lower slopes than the other two types of enzymes. Further studies were conducted using the Sigma Type VI horseradish peroxidase. Initial rate studies of both the dissolved oxygen oxidation of reserpine and the hydrogen peroxide oxidation of 4-aminoantipyrine in the presence of phenol yielded the plots shown in Figures 9 and 10. In all cases the rate decreased with increasing concentration of the inhibitor, 3,4-didehydroreserpine. At lower reserpine concentrations, a lag time was observed before the product formation was noticeable. Although this lag time was seen to increase with increasing the initial concentration of the inhibitor, no quantitative correlation was possible with the available data. The plot of 1/IR versus 1/[S] for different inhibitor concentrations yielded straight lines with different slopes, which showed a tendency to converge on the x-axis. The slopes and the intercepts obtained from the Lineweaver-Burk plots are summarised in Tables IX and X. The Michaelis-Menten constants calculated from the x-intercept shows random variations. It has been already pointed out that if the inhibiton was competitive or uncompetitive in nature, the values of K_M obtained from these plots would be consistently lower or higher than the value of K_M in the absence of any inhibitor. It has to

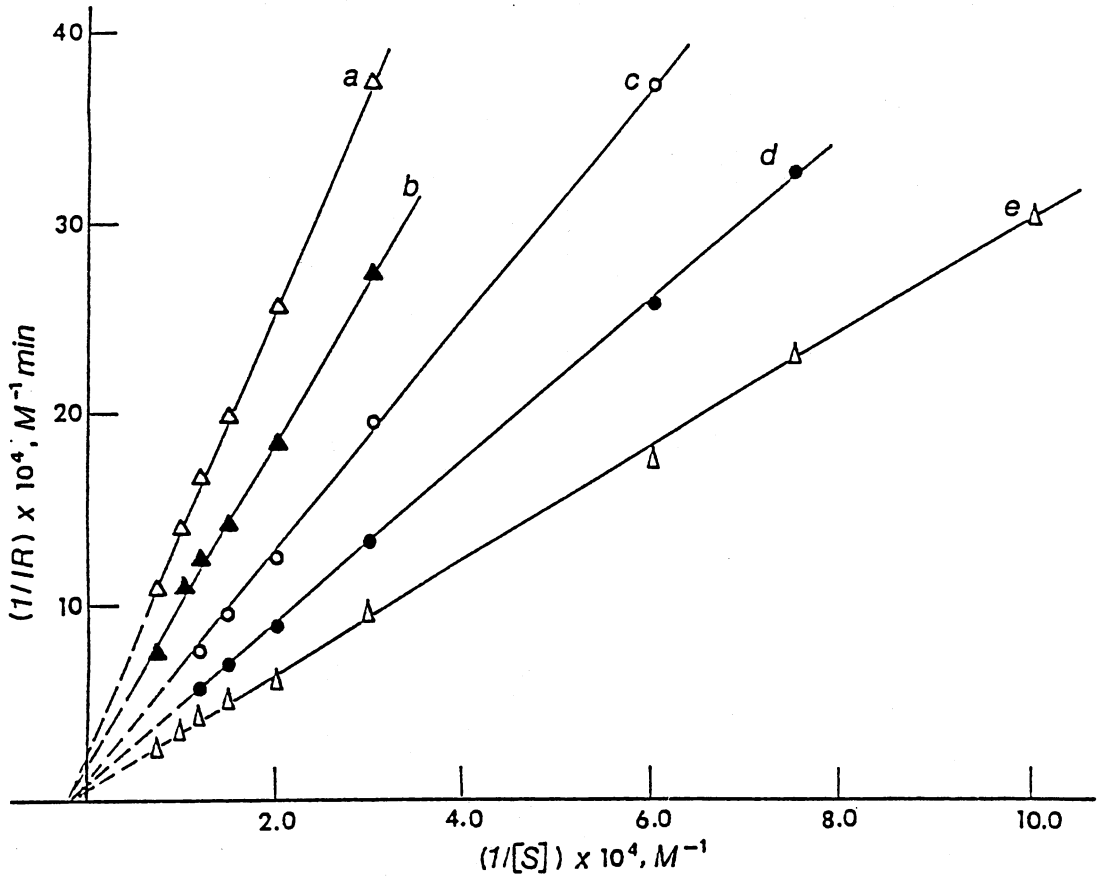


Figure 9. Lineweaver-Burk Plot for the Inhibition of Peroxidase Promotion of Reserpine Oxidation by Dissolved O_2(g) [3,4-didehydroreserpine] x 10^{-5}, M: (a) 2.5, (b) 1.67, (c) 1.33, (d) 0.83, and (e) no inhibitor added

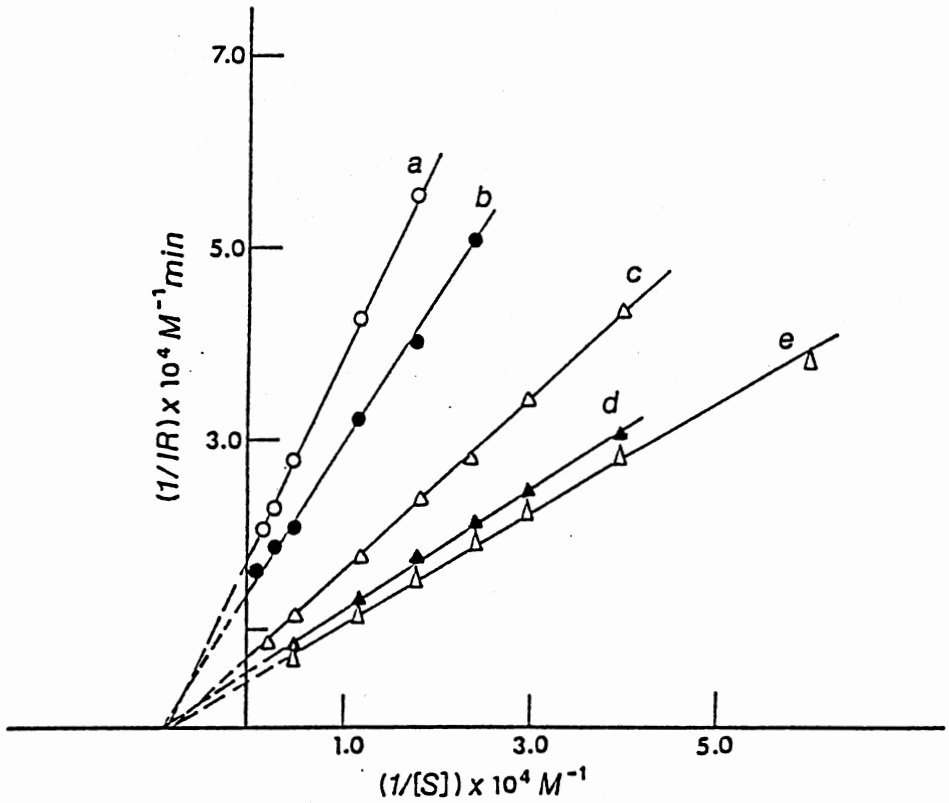


Figure 10. Lineweaver-Burk Plot for the Inhibition of Peroxidase Catalysis of 4-aminoantipyrine Oxidation by H_2O_2 [3,4-didehydroreserpine] x 10^{-5}, M: (a) 10.0, (b) 5.0, (c) 2.5, (d) 1.0, and (e) no inhibitor added

TABLE IX

EFFECT OF 3,4-DIDEHYDRORESERPINE ON THE INITIAL RATES OF OXIDATION OF RESERPINE

Initial concentration of 3,4-didehydroreserpine, M	Slope	Correlation coefficient	K_m, x 10^4 M
0	2.98	0.9994	6.85
0.83×10^{-5}	4.12	0.9999	8.17
1.33×10^{-5}	5.99	0.9993	8.33
1.67×10^{-5}	8.47	0.9972	4.85
2.50×10^{-5}	11.7	0.9998	5.15
		Average	6.67 ± 1.6

Oxidation of reserpine by dissolved oxygen. pH = 7.00, 20 ± 1 ^{o}C.

TABLE X

EFFECT OF 3,4-DIDEHYDRORESERPINE ON THE INITIAL RATES OF OXIDATION OF 4-AMINOANTIPYRINE

Initial concentration of 3,4-didehydroreserpine, M	Slope	Correlation coefficient	K'_m, x 10^4
0	0.578	0.9995	1.22
1.0×10^{-5}	0.626	0.9984	1.11
2.5×10^{-5}	0.906	0.9993	1.36
5.0×10^{-5}	1.48	0.9979	1.04
1.0×10^{-4}	2.08	0.9998	1.18
		Average	1.18 ± 0.12

Oxidation of 4-aminoantipyrine by hydrogen peroxide in the presence of phenol. pH = 7.00, 20 ± 1 ^{o}C

be concluded, therefore, that the inhibitory effect of 3,4-didehydroreserpine on the systems studied is noncompetitve in nature. There is no apparent trend of change in the Michaelis-Menten constant. The inhibtion cannot be reversed by altering the substrate concentration.

It can also be concluded that the oxidized reserpine binds itself at a site other than the active site but distorts the site sufficiently to block the formation of the enzyme-cosubstrate-substrate complex. The size of the molecule of oxidized reserpine that leads to the inhibition points out that the attachment to the enzyme occurs via the nitrogens in the 1 and/ or 4 positions (see Figure 1, Chapter II)and it seems reasonable to assume that the attachment is not at, but close to, the active site of the enzyme. The bulky inhibitor restricts the access of the substrate to the active site by producing intramolecular distortion.

These studies on the inhibition of peroxidase suggested that it was not worthwhile to pursue the idea of using immobilized peroxidase as a catalyst for the oxidation of reserpine in continuous-flow systems. Peroxidase in solution can be used in a continuous-flow by using the merging zone technique (120). Here the amount of enzyme used is kept to a minimum by merging a plug of the enzyme with the substrate. However, the enzyme is consumed in the process and the cost of the enzyme does not justify its use in a homogeneous system. The search for a different

catalyst resulted in the use of Mn(II), sacrificing some selectivity, to catalyse the oxidation with periodate ions and is the subject of the study described in Chapter V.

CHAPTER V

CONTINUOUS-FLOW METHODS FOR THE DETERMINATION OF RESERPINE

The need for simple, reliable and rapid methods for the determination of pharmaceuticals was highlighted in Chapter I. A survey of the literature on the available analytical methods for the determination of reserpine shows the dearth of such methods (Chapter II). The most commonly used methods for the determination of dissolved reserpine involve oxidation to 3,4-didehydroreserpine and the quantitation of the product by spectrophotometric or fluorometric methods. These procedures are usually complicated, involving the use of several reagents, many extraction steps and sometimes heating. A simple procedure requiring fewer reagents and a lesser number of associated steps is presented in this chapter.

Manganese ions have been known to catalyze periodate oxidations and this catalytic action was used for the oxidation of reserpine. The spectrum obtained on the addition of Mn(II) to a solution containing reserpine and periodate is shown in Figure 11 and the 3,4-didehydroreserpine formation can be conveniently monitored at 385 nm. The overall chemical reaction when

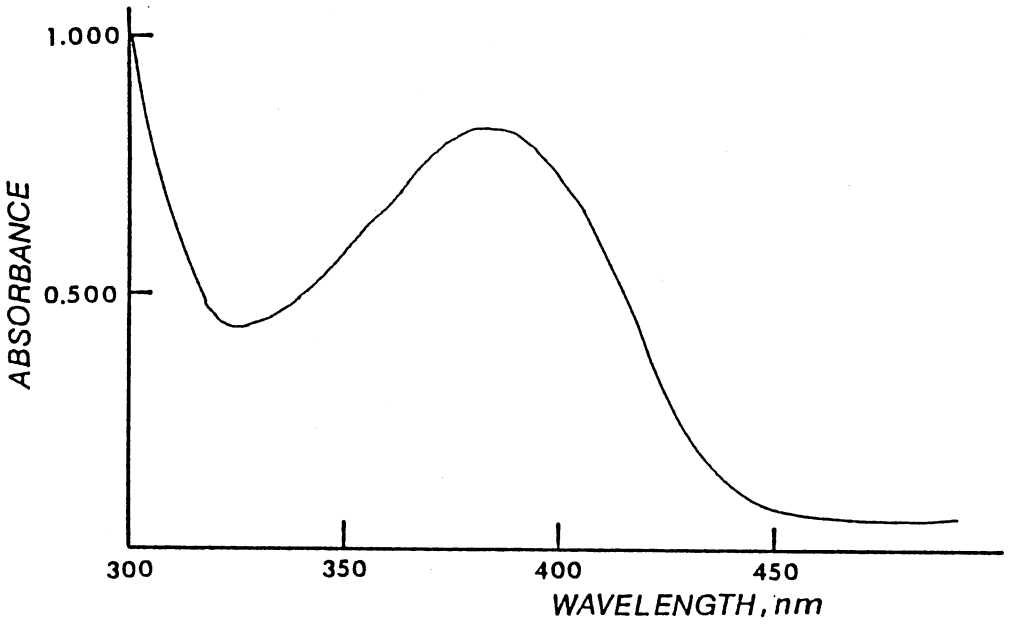


Figure 11. Spectrum of the Product Formed by the Oxidation of Reserpine by Periodate in the Presence of Mn(II)

periodate is used as an oxidant and manganese ion(s) as catalysts involve a 1:1 stoichiometry with periodate converted to iodate. The catalytic cycle is probably sustained in solution by the Mn(II)/Mn(III) couple and by the Mn(IV)/Mn(V) couple when solid MnO_2 is the catalyst.

Experimental

Reagents, Solutions, and Materials

All the reagents used were of analytical grade except when otherwise noted and the solutions were prepared with deionized water further purified by distillation.

Reserpine was dissolved in 20% acetic acid (J. T. Baker Chemical Co., Phillipsburg, NJ) which gave solutions with a pH of 2.40. The solutions were prepared daily to minimize photolytic degradation. Solutions of potassium periodate (J. T. Baker) and manganese(II) sulfate (GFS Chemicals, Columbus, OH) were also prepared similarly in acetic acid, to prevent the precipitation of reserpine. A crystalline powder of manganese dioxide [Mn(IV)] obtained from Matheson, Coleman, and Bell, Norwood, OH, was used to prepare the reactor containing manganese dioxide immobilized on the walls of Tygon tubing.

Reserpine was determined in pharmaceutical tablets "Serpasil" (0.10 mg) from Ciba and "Raudixin" (50 mg) from Squibb, purchased from a local drug store with special prescriptions from Dr. A. F. Gambill of the Oklahoma State University Health Center. The Rauwolfia Serpentina standard

powder was from United States Pharmacopeial Convention, Inc., Rockville, MD.

Teflon (0.80 mm i.d.) and Tygon (1.02 mm i.d.) tubing used as reactors were obtained from Cole-Palmer, Chicago, IL. Glass beads (Fischer Scientific, Fairlawn, NJ) of 0.5 mm diameter were used in the preparation of the single-bead-string reactor. Pump tubing (1.14 mm i.d.) was obtained from Gilson Medical Electronics, Inc., Middleton, WI.

All measurements were performed at room temperature, 20 ± 1 ^{o}C.

Apparatus

A FiAtron SHS-200 (FiAtron Systems, Inc., Milwaukee, WI) was used in conjunction with photometric detection to obtain the continuous-flow signals from the oxidation of reserpine. This commercial system used for continuous-flow analysis consists of a four channel peristaltic pump and multiinjection valve system for dispensing sample and reagent, which is controlled by a microprocessor. The pump speed can be programmed on the front panel of the instrument. The injection assembly is an all Teflon device consisting of a dual channel sample injector and a set of electrically driven four-way solenoid valves. A schematic of the valve assembly is shown in Figure 12. The valves 1 and 2 are connected by a sample loop (25 µl) , valves 3 and 4 are similarly connected. Valves 2 and 4 have exits to

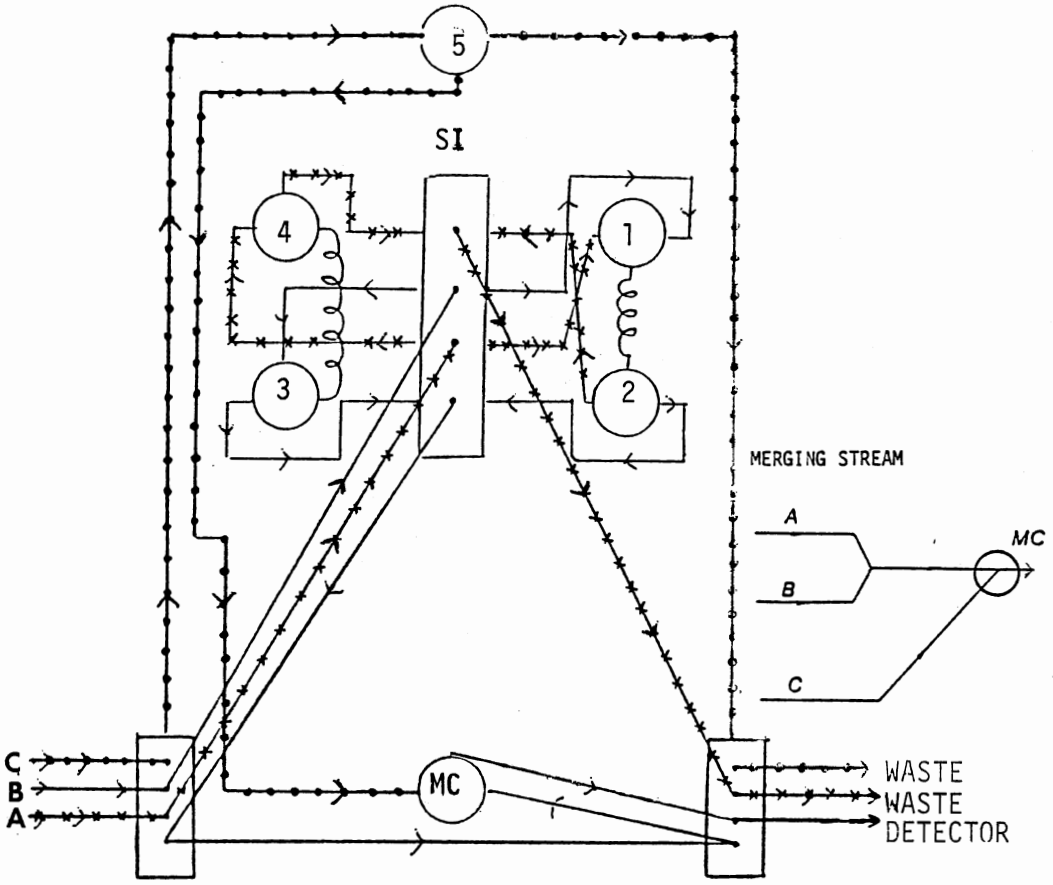


Figure 12. Schematic of the Multivalve Injection System of FiAtron SHS-200 - Filling Position (for Mode 2)
SI: Dual Channel Sample Injector
MC: Mixing Chamber
1, 2, 3, 4, and 5: Solenoid Valves
A, B, and C: Channels Through Which the Solutions A (-•-), B (——), and C (-x-) are Pumped

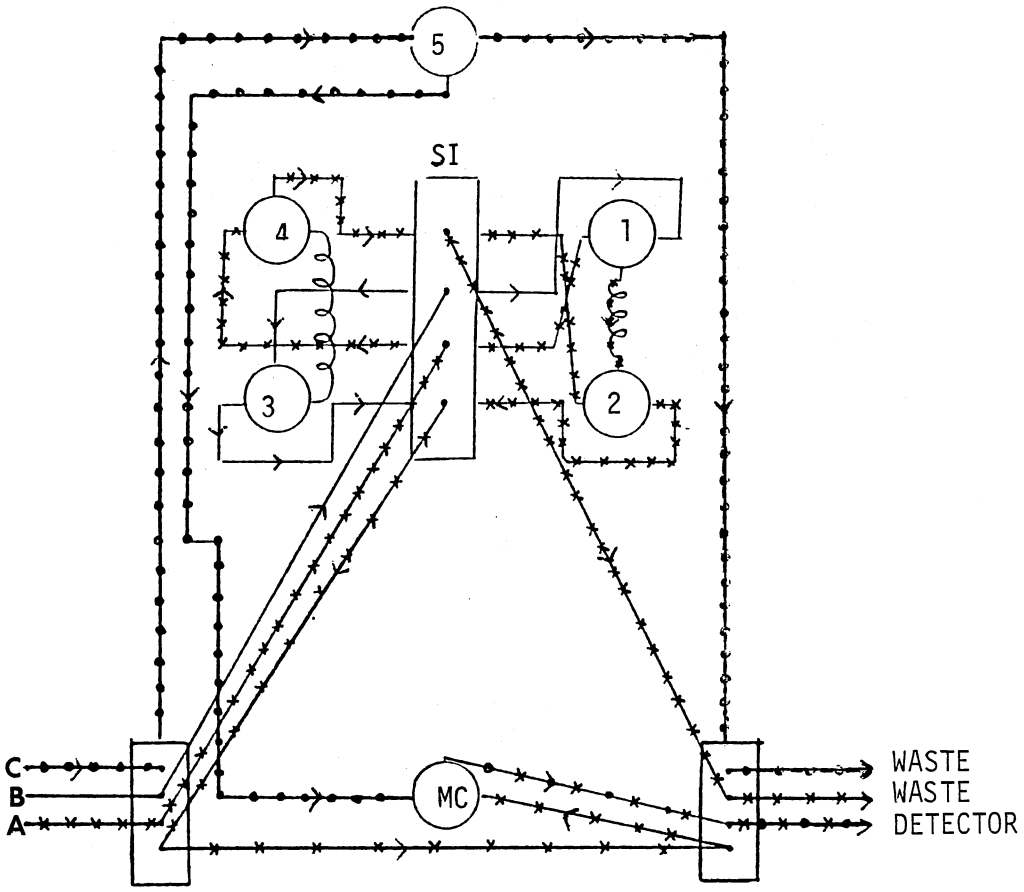


Figure 13. Schematic of the Multivalve Injection System of FiAtron SHS-200 - Injection Position
(other details, same as in Figure 12)

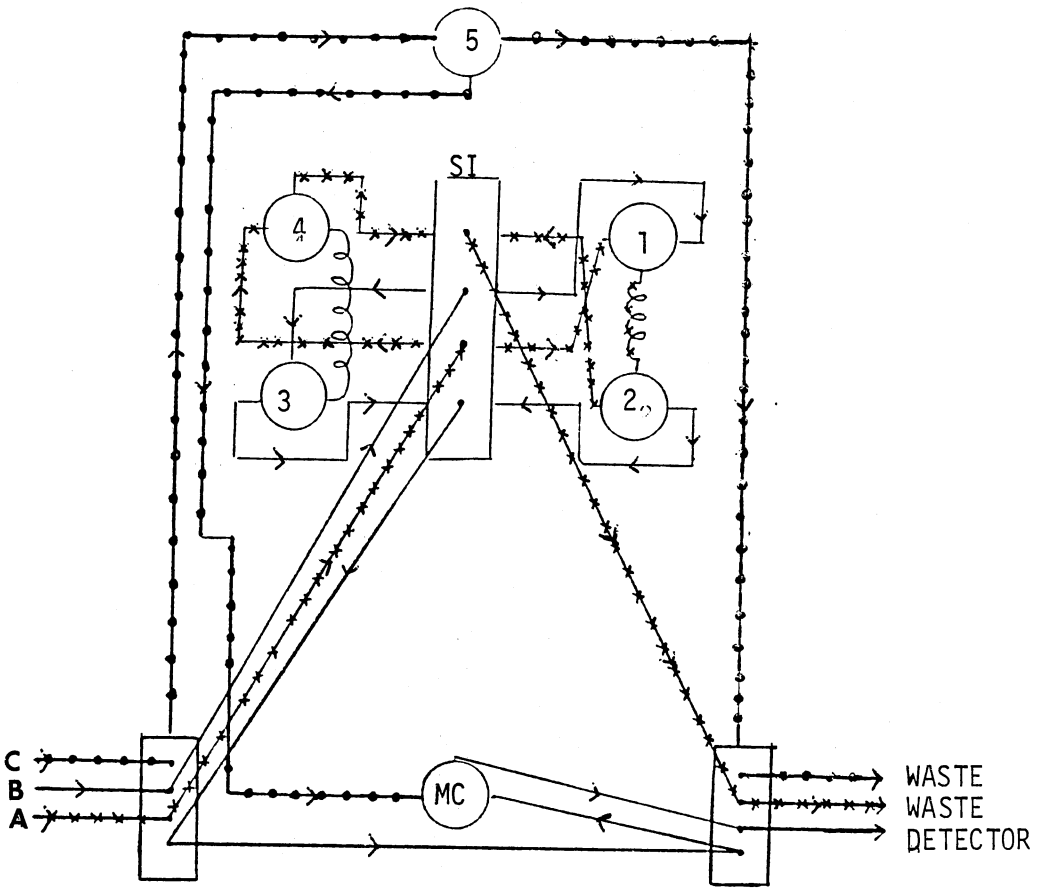


Figure 14. Schematic of the Mutivalve Injection System of FiAtron SHS-200 - Flushing Position
(other details, same as in Figure 12)

waste. A single peristaltic pump generates three streams A, B, and C. The stream A splits into two, one of which follows the path valve 1-sample loop-valve 2 and the other leads to valve 4. The stream B is also split into two, one leading to valve 3 and the other following the path valve 1-sample loop-valve 2, entering the valve through a different inlet than stream A. A fifth valve, which operates independent of the other four valves in the system, introduces a third stream, C, into a mixing chamber of 4 ul in volume to merge with the main flow stream. The valves are connected to each other by Teflon tubing (0.50 mm i.d). The different positions of the valve characterizes different operational modes. In the modes of operation applicable to this study, the dual channel sample injector simultaneously injects solutions A and B, in the form of merging streams. The time during which valves remain open for the solutions to merge corresponds to the injected volume and can be defined as T_3. The time required for pumping the solution up to the point of injection is defined by T_1. T_2 allows for the time between injections, so that there is no cross contamination of the samples. Figures 12, 13, and 14 illustrate the paths taken by the three streams during one injecton. The sum $T_1 + T_2$ dictates the number of determinations that can be performed per hour. A third solution C can be introduced into the stream through valve 5. When valve 5 remains open at all times, the mode of operation is called Mode 1. Valve 5 can also be

programmed to be open for a given time T_4, while the solution is recirculated during the rest of the cycle. This mode of operation is called Mode 2. When valve 5 remains closed at all times Mode 3 is in operation. The values of T_1, T_2, T_3, and T_4 and Modes 1, 2, and 3 can be programmed through the front panel. When the flow rate is computed and stored in memory, volumes V_1-V_4 can be used instead of the time intervals. The FiAtron can be set up to perform successive injections, automatically. A localized highly turbulent flow exists in the mixing chamber which results in mixing of the solutions. The stream that exits this chamber passes through the flow cell of the detector.

A Beckman DB-GT spectrophotometer was used as the detector with a flow cell (Helma Cells Inc., Jamaica, NY) of 10 mm pathlength and 80 µl chamber volume. A Houston Instruments 4910 Superscribe^{TM} stripchart recorder was used to obtain the readouts of analytical signals.

The preparation of MnO_2-immobilized reactors entitled the heating of the Tygon tubing in a Lab-Line Imperial IV laboratory oven (Lab-Line Instruments, Inc., Melrose Park, IL).

Procedures

Studies on the kinetics of periodate oxidation of reserpine. Kinetics of the periodate oxidation of reserpine in the presence of Mn(II) was first studied in a batch

manner. Solutions containing 1.0 to 0.4 x 10^{-4} M reserpine and 3.75 x 10^{-3} M periodate were taken in a quartz cuvette and placed in the cell compartment of a Perkin-Elmer Lambda Array Spectrophotometer. The reaction was triggered by the addition of Mn(II) (1.3 x 10^{-5} M). The solution was stirred and the change in absorbance at 385 nm with time was measured. Absorbance vs. time plots and tables containing the data were obtained. Initially, the reaction occurred slowly. A typical absorbance vs. time plot illustrating this and indicating the region where rate measurents are made is shown in Figure 15. The rates were determined as described in Chapter IV.

Determination of Reserpine With Mn(II) in Solution.

Figure 16 shows the schematic of the continuous-flow set-up used for the continuous-flow utilized. Periodate is pumped through channel A, Mn(II) through channel B, and reserpine through channel C. The two schemes differ only in the manner in which reserpine is added into the stream containing the Mn(II) and the periodate. The volume of periodate (V_3) merging with Mn(II) can be programmed and this dictates the volume of the plug in which the reaction occurs. In Mode 1 reserpine merges continuously with Mn(II). In Mode 2, the valve 5 opens to introduce a progammed volume (V_4) of the reserpine into the stream. During the rest of the operation the valve is closed and the reserpine solution can be recirculated. The time of periodate and reserpine introduction into the stream are

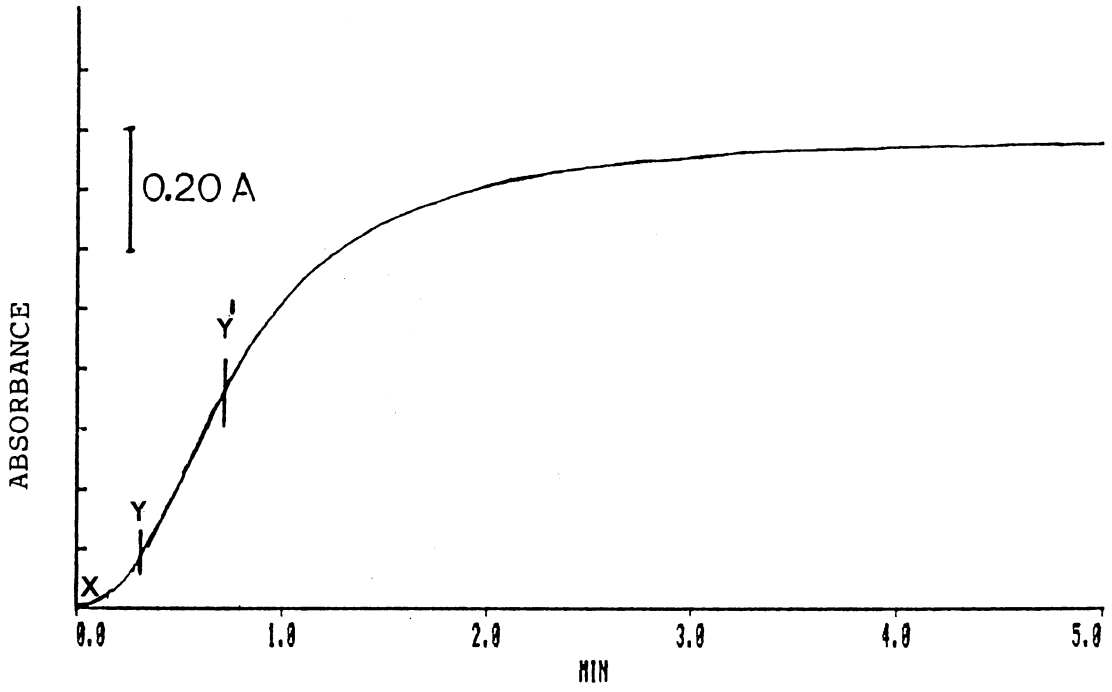


Figure 15. A Typical Absorbance vs. Time Curve for the Oxidation of Reserpine with Periodate Catalyzed by Mn(II) Indicating the Region of Initial Slow Reaction (x-y) and the Region Used to Estimate the Rate Information (y-y')

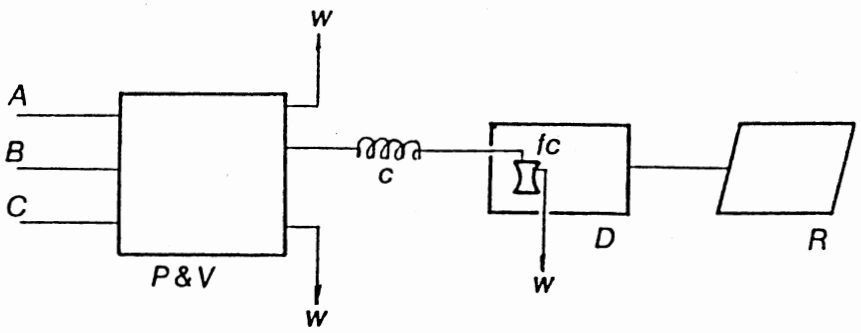


Figure 16. Experimental Set-up for the Continuous-Flow Determination of Reserpine
A: Periodate
B: Mn(II) or Reserpine
C: Reserpine
P & V: Pump and Valve of FiAtron
c: Single-Bead-String Reactor or MnO_2 reactor
fc: Flow Cell
D: Spectrophotometeric Detector
R: Recorder W: Waste

synhcronized. When the reaction plug passes through a single-bead-string mixing coil into the flow cell of a detector set at 385 nm, a transient signal is produced which is recorded. The peaks obtained are narrow and hence, the peak height is proportional to the concentration of the oxidized species. Studies were conducted to determine the optimum concentrations of periodate and Mn(II) necessary for oxidation. Flow rates and injected volumes of periodate were also optimized. Concentrations of reserpine ranging from 5×10^{-6} to 1×10^{-4} M were determined and calibration curves were obtained. A typical recording of the continuous-flow signals obtained for reserpine standards is shown in Figure 17.

Preparation of a Manganese Dioxide Reactor. The MnO_2 was immobilized on the walls of a Tygon tubing by a procedure similar to the one described in reference 56 for the immobilization of controlled-pore glass on Tygon and Teflon tubings. The Tygon used was microbore tubing of formulation S-54-HL, a modification of Tygon surgical formulation S-50-HL. A 50-cm section of the tubing was sealed at one end by heating in a flame and applying pressure with a pair of pliers. The tube was tightly packed with crystalline manganese dioxide and the open end was then sealed as before. The packed tubing was wound tightly around a glass rod of 7 mm in diameter and secured with a metal wire to maintain the shape during heating. The assembly was heated in the oven to temperatures slightly

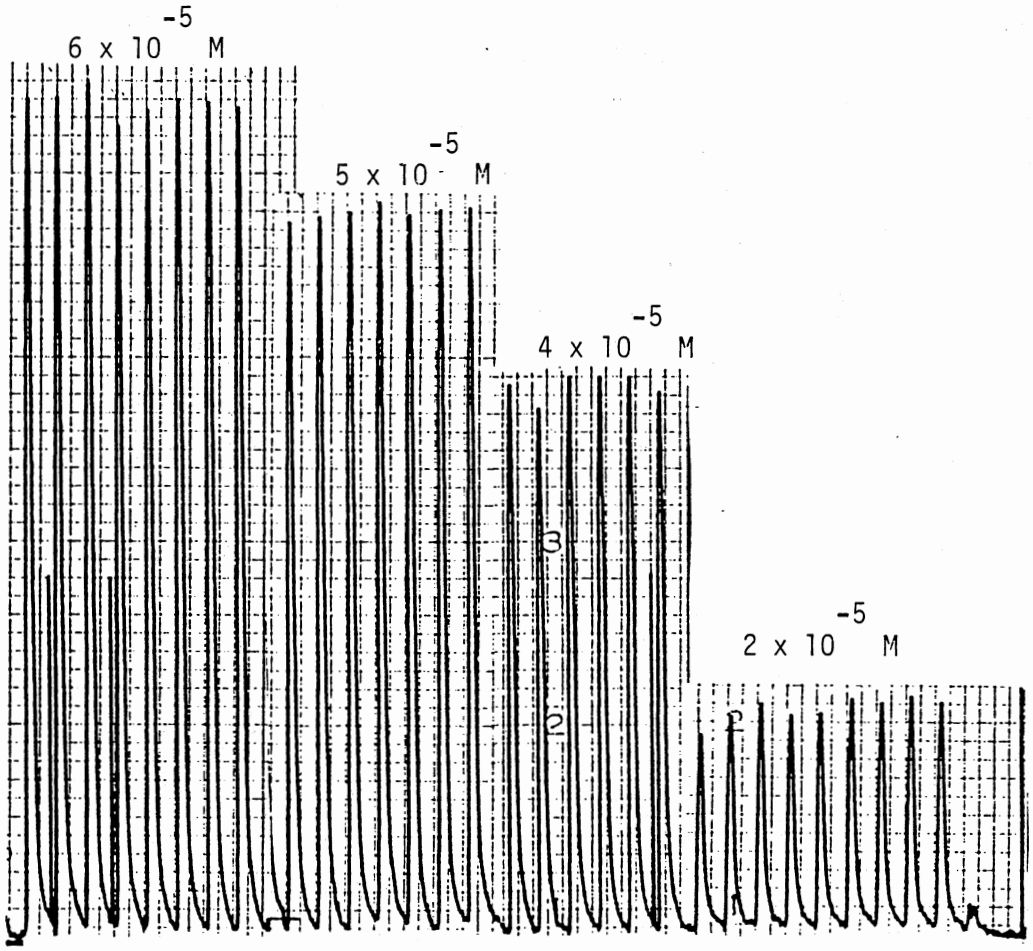


Figure 17. Typical Continuous-Flow Signals For Reserpine Flow Rate: 2.3 ml/min Chart Speed: 0.33 cm/min

above the melting point of Tygon, which was reported to be around 150 ^{o}C. It was then removed, cooled to room temperature, and the unattached particles were then shaken loose. The reactor was repeatedly washed with water to remove all unattached particles of manganese dioxide. The optimum temperature and heating time were ascertained by heating the packed tubing to temperatures of 170 ^{o}C to 200 ^{o}C and varying the time of exposure from 5 to 20 minutes. The Tygon tubing was covered effectively with MnO_2 at a temperature of 190 ^{o}C after heating for 15 min. Exposures over 20 min and higher temperatures caused the Tygon to decolorize and loose its plasticizer. At lower temperatures fewer particles of MnO_2 were attached to the surface.

Determination of Reserpine Using a Manganese Dioxide Reactor. In this scheme, the single-bead-string reactor used in the previous schemes was replaced by the MnO_2 reactor described above. The periodate solution was pumped through A, and reserpine through B (Figure 16). The solutions were then merged in the sample injector. Valve 5 remained closed at all times, since Mode 3 was in operation. The volume of reserpine merging with periodate, V_3, was programmed into the instrument. The optimum concentration of periodate from the previous experiment was used. Flow rate and sample volumes were optimized under the new conditions. A calibration curve was obtained with reserpine standards ranging in concentrations from 5×10^{-6} to 1×10^{-4}.

Determination of Reserpine in Some Pharmaceutical Preparations. The tablets were prepared for determination by grinding them individually with a mortar and pestle into a fine powder. The powder was quantitatively transfered into a dark colored bottle and stirred with 2 ml of glacial acetic acid for 5 min. The resulting extract was diluted to volume with 20% acetic acid and the insoluble material was filtered-out through a sintered glass funnel. Up to 5 repetitive injections were performed with each tablet. A blank reading was obtained by injecting the sample into the stream in the absence of periodate. At least three different samples were analysed. The amount of reserpine present was then determined by the most appropriate method, among the methods described above. The results obtained were compared with the amounts indicated on the labels.

Results and Discussion

Kinetics of periodate oxidation of Reserpine Catalyzed by Mn(II)

The reaction between periodate and reserrpine (uncatalyzed reaction) is a very slow process and no noticeable production of 3,4-didehydroreserpine is observed. The rate of oxidation of reserpine, in the presence of periodate and Mn(II) can be expressed by the equation:

$$\text{Rate} = \frac{d\ [3,4\ \text{DDR}]}{dt} = k\ [IO_4]^a\ [\text{Mn(II)}]^b\ [\text{RES}]^c \qquad (20)$$

where [3,4 DDR] = concentration of the oxidized reserpine,

[RES] = reserpine, a, b, and c are the corresponding reaction orders, and k = rate coefficient for the reaction.

Periodate is used in a 100 fold excess and can be considered to remain unchanged. The [Mn(II)] enters in a catalytic cycle and can be considered to remain unchanged, also. The orders with respect to periodate and Mn(II) in catalytic reactions are known to be 1. Equation 20 can then be rewritten with a modified rate coefficient into which the constant terms are incorporated:

$$\text{Rate} = k' [\text{RES}]^c \qquad (21)$$

where $k' = k [IO_4]^a [\text{Mn(II)}]^b$

Assuming initial rate conditions,

$$\text{Rate} = k' [\text{RES}]^c_{t=0} \qquad (22)$$

$$\log \text{Rate} = \log k' + c \log [\text{RES}]_{t=0} \qquad (23)$$

The rates measured at different concentrations of reserpine are tabulated in Table XI . log Rate was plotted against $\log [\text{Res}]_{t=0}$ (Figure 18). From equation 23, it can be seen that it should be linear. The slope of the line gives the order of the reaction with respect to reserpine and was found to be 0.91. The order of the reaction with respect to reserpine can be approximated to 1. Using the value of c = 1, the log k' can be calculated, from which a value of k' equal to 0.99 ± 0.07 min^{-1} is obtained. Using the initial concentrations and assuming first order dependence with respect to $[IO_4^-]$ and [Mn(II)], the value of k was calculated to be $1.93 \pm 0.22 \times 10^6$ M^{-2} min^{-1}.

TABLE XI

RATE OF RESERPINE OXIDATION
BY PERIODATE AND MN (II)
STUDIED IN BATCH MANNER

Initial Concentration of Reserpine, M	Slope[a] min^{-1}	Rate[b], M
1.00×10^{-4}	0.967	8.97×10^{-5}
0.80×10^{-4}	0.848	7.87×10^{-5}
0.60×10^{-4}	0.641	5.94×10^{-5}
0.40×10^{-4}	0.427	3.96×10^{-5}

[a]Slope of absorbance vs. time curve.

[b]Rate = Slope/ϵ (ϵ= 10,778 M^{-1} cm^{-1})

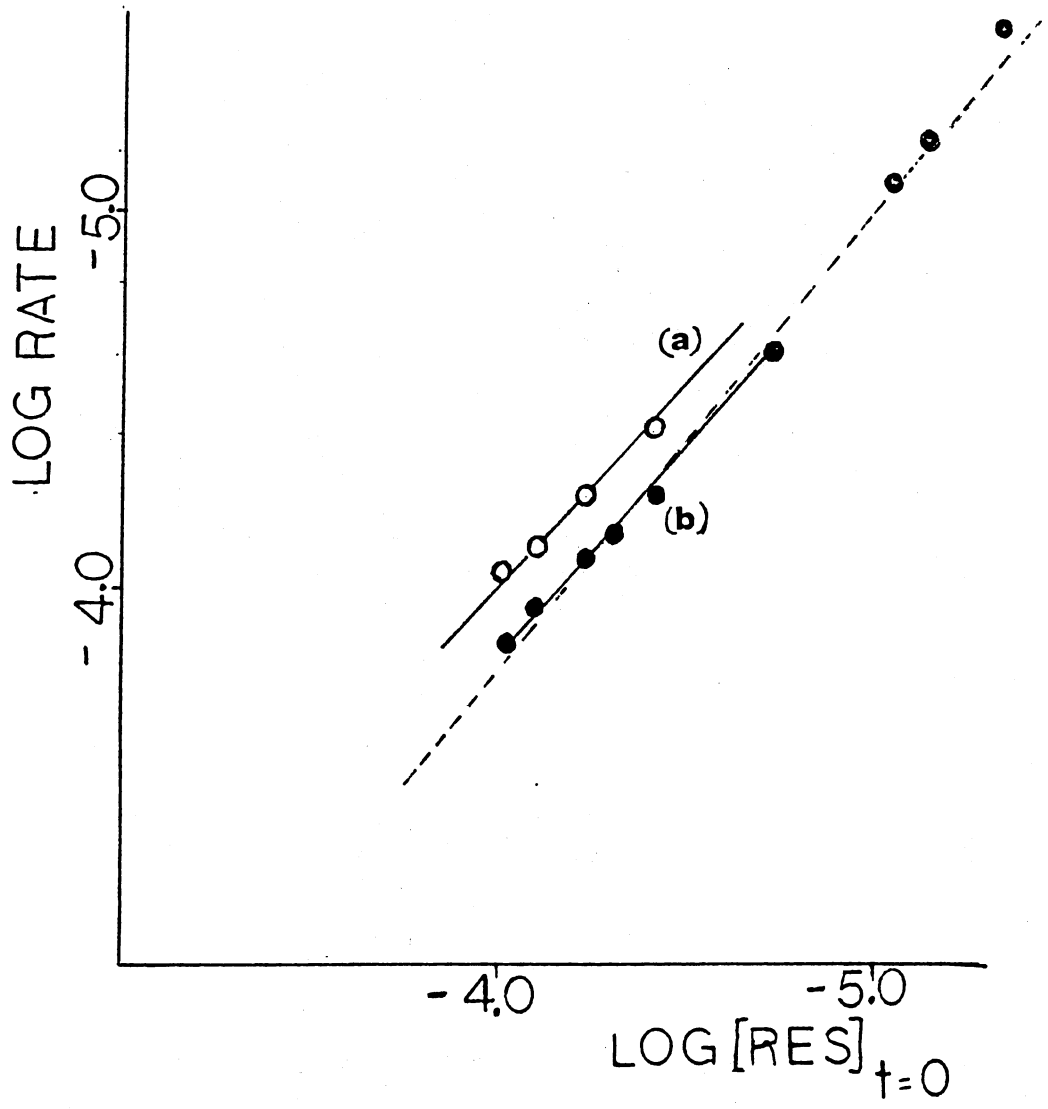


Figure 18. Plot of Log Rate vs Log [RES]
(a) in Batch System
(b) in a Continuous-Flow System

Determination of Reserpine With Mn(II) in Solution

Without Recirculation of Reserpine. Concentrations of the oxidizing agent and catalyst were optimized to provide the maximum reaction. The peak height increased sharply with periodate concentration up to a concentration of 1.0×10^{-2} M, and then started levelling off (Figure 19). The solubility of periodate in acetic acid limited the maximum available concentration to 2.00×10^{-2} M. A concentration of 1.5×10^{-2} M periodate was used in all experiments. The effect of the catalyst concentration on the peak height can be seen in Figure 20. At high concentrations of Mn(II), formation of permanganate was observed followed by a reduction in the signal height. To avoid the possibility of permanganate formation, 2×10^{-4} M Mn(II) was used. A single-bead-string reactor, 1-m in length provided increased mixing and longer reaction time. The signals were condiderably smaller with shorter mixing coils, but did not show any increase when a coil of 2-m in length was used.

When reserpine sample solutions were continuously merged with a flow stream, only a small portion actually undergoes reaction. The reaction takes place when reserpine merges with the portion of the stream which contains the periodate merged with the catalyst, [Mn(II)]. At all other times, reserpine continuously mixes with Mn(II) and flows through to the detector. Any 3,4-didehydroreserpine formed

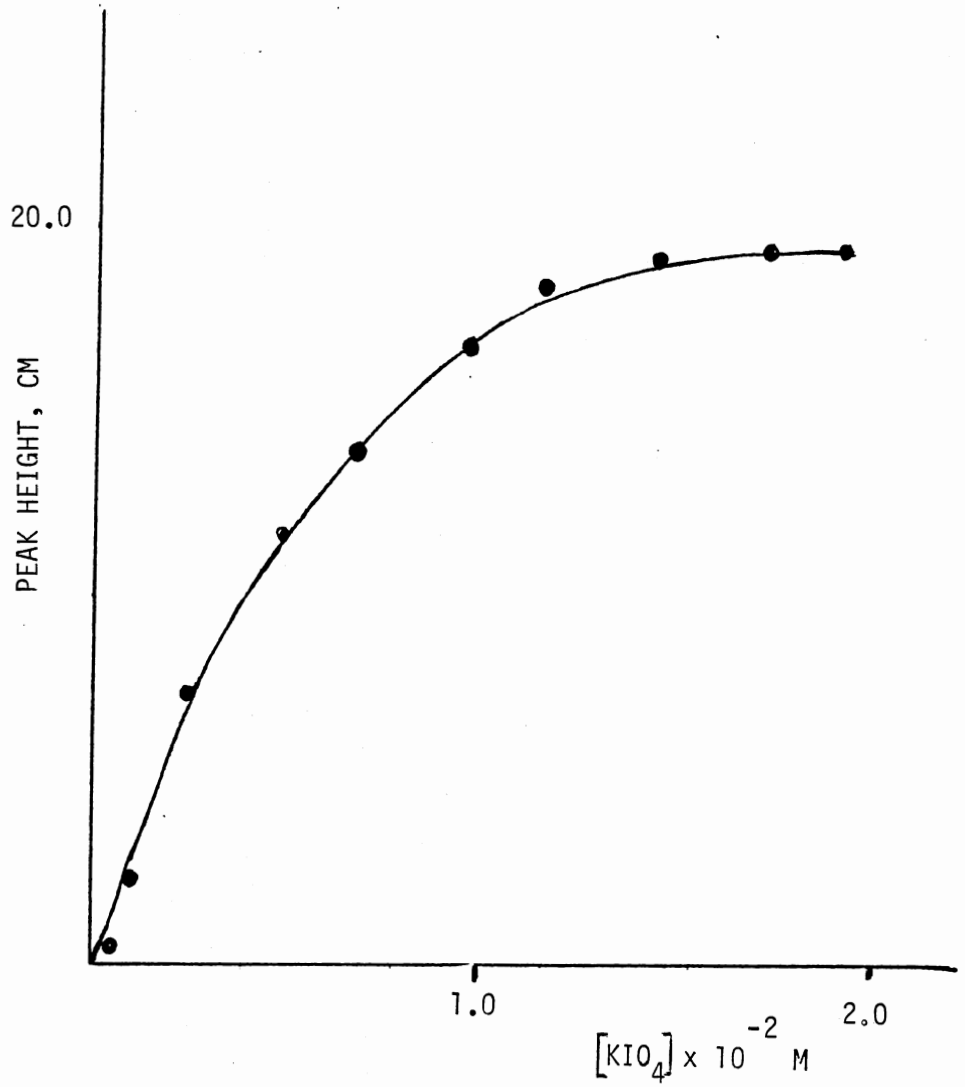


Figure 19. Effect of Periodate Concentration on Peak Height

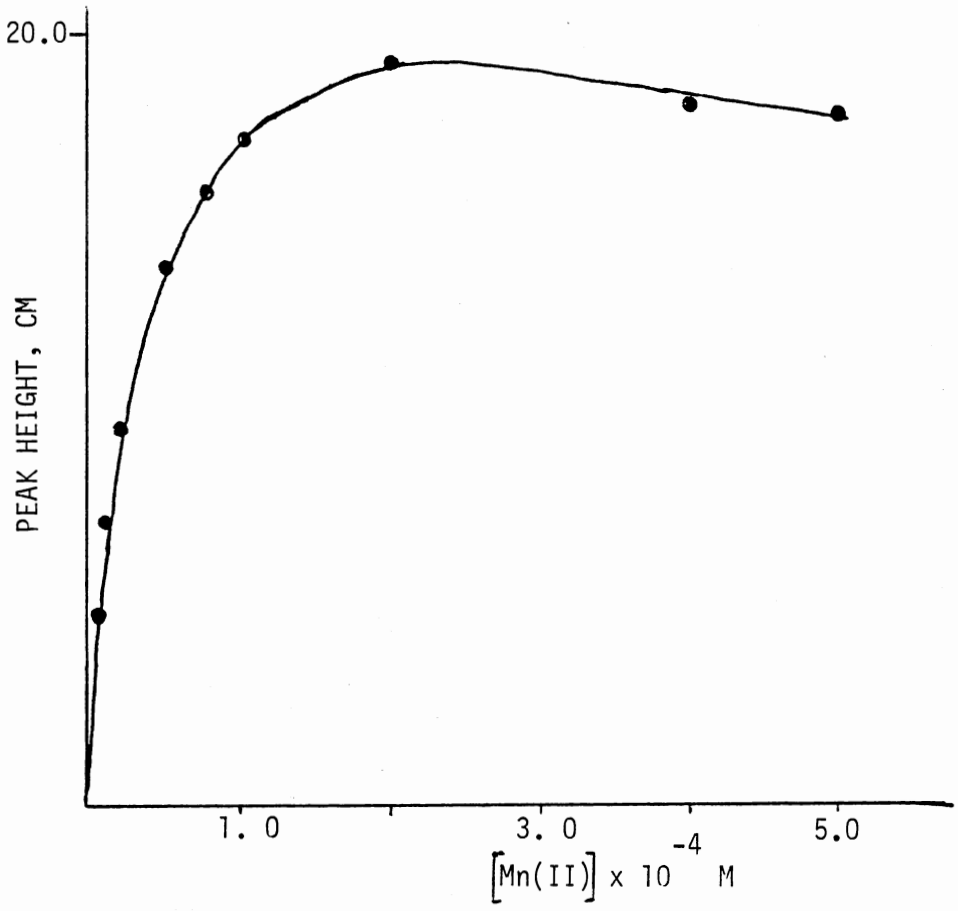


Figure 20. Effect of Catalyst [Mn(II] on Peak Height

due to the degradation of reserpine will increase the absorbance of the baseline and will not affect the signal from the oxidation of reserpine by periodate. In this mode of operation the flow is altered when reserpine merges with the flow stream and becomes twice the initial flow rate. At faster flow rates there is less dispersion of the reactants and less time allowed for the reaction, as should be expected. Figure 21 shows a plot of peak height against flow rate. The flow rate yielding maximum signals in this mode was found to be 1.75 ml/min, before merging. The injected volume of periodate also affects the reserpine signals. The best value was found to be 125 µl. Under these optimum conditions, a set of standard solutions of reserpine were determined. The plot of peak height vs. concentration was linear in the range 5×10^{-6} to 1×10^{-4} M (Figure 22). All the peak heights reported were an average of 5-10 injections with a relative standard deviation (RSD) of less than 2%. The number of injections that can be performed per hour depends on the time taken for the signal to come back to the baseline. During this time the solutions are flushed from the injection system and the tubes are filled with fresh solution. The valves are programmed to remain open for 25 s for filling, and 25 s to flush the system after injection. The total time taken for an injection was about 54s, taking into account the time taken to introduce the injected volume into the flow flow stream. Therefore, about 60 injections can be carried out

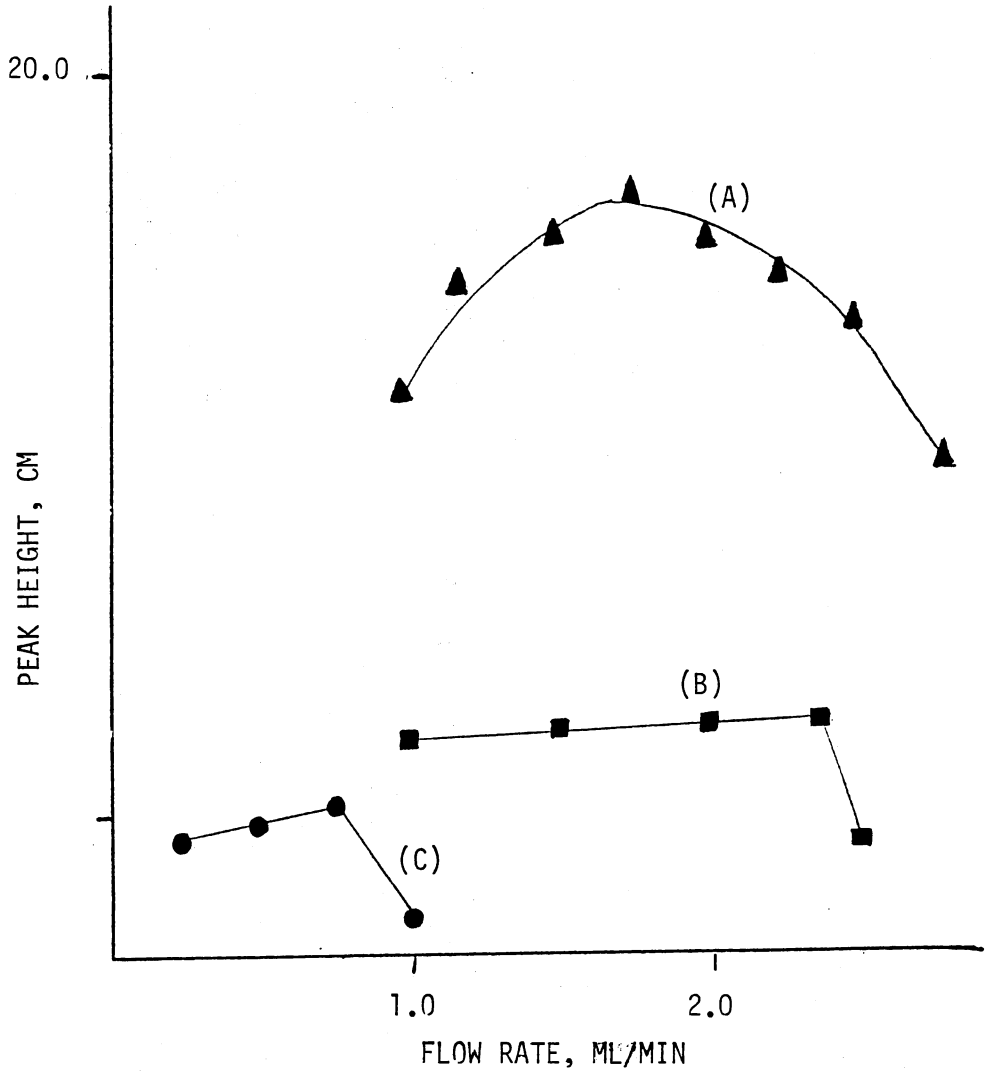


Figure 21. Effect of Flow Rate on Peak Height for the Three Modes of Operation
A: Mode 1
B: Mode 2
C: Mode 3

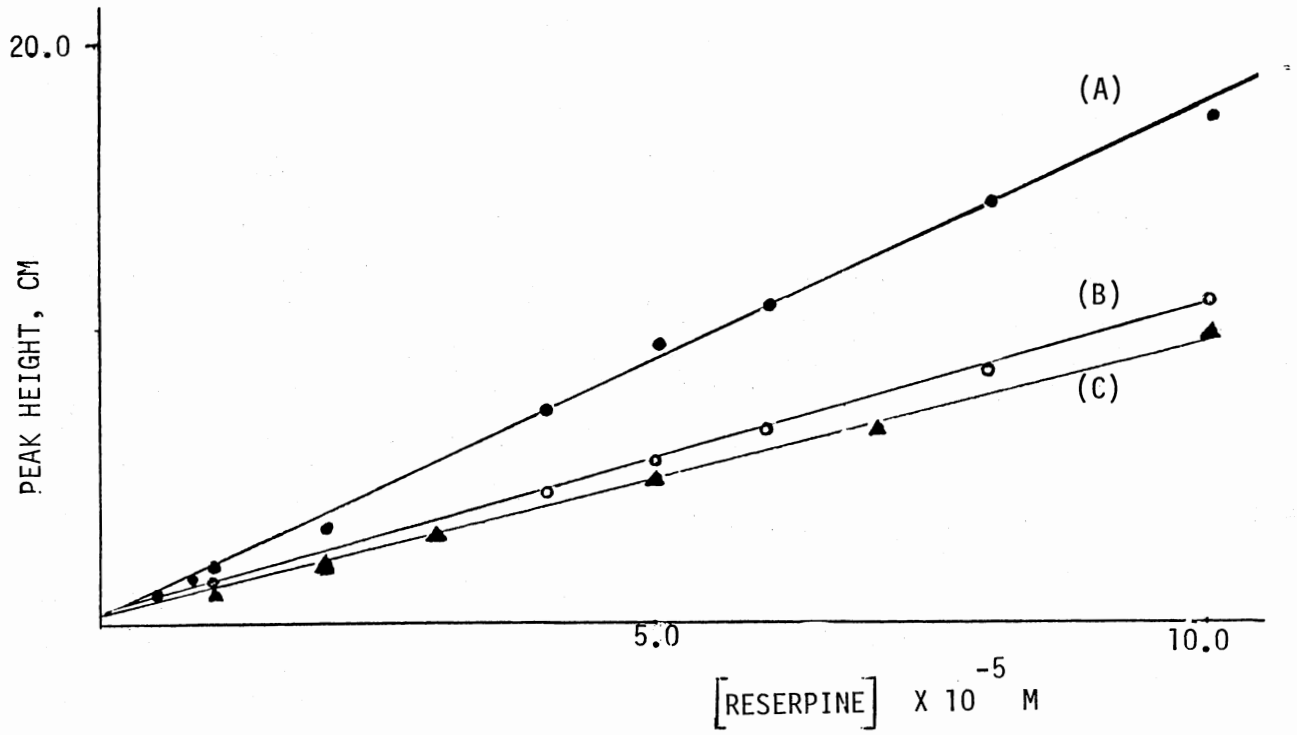


Figure 22. Calibration Curves for the Different Modes of Determination of Reserpine
A: Mn(II) in Solution - Without Recirculation
B: Mn(II) in Solution - With Recirculation
C: Solid Mn(IV) Reactor

per hour.

With Recirculation of Reserpine. In this mode , a fixed volume of reserpine is injected into the stream. Volume of reserpine injected is equal to the volume of periodate. Otherwise, reserpine is pumped back to the reservoir and recirculated. Recirculation becomes critical when working with small sample volumes. Although the injection volume itself is negligible, the FiAtron uses a considerable amount of solution to "prime" the tubings. The baseline in this circumstance is dictated by the absorbance of the Mn(II) solution. When reserpine and periodate are injected into the system in the absence of the other, no signal was observed under the reaction conditions. The concentrations of periodate (1.5×10^{-2} M) and Mn(II) (2.0×10^{-4} M) were identical to the concentrations used in the previous experiment. The flow rate remained unchanged through the system. The best flow rate for the system was found to be 2.3 ml/min (Figure 21). The effect of volume of periodate (and reserpine) was more marked in this case. When the volume was less than 400 µl, the peak heights were considerably small and above 400 µl there was little effect. It has to be inferred that all the injected periodate does not merge with reserpine and so there is correspondingly, less product formed. When a larger volume is injected the fraction of the injected volume undergoing reaction is increased. An injection volume of 450 µl was used for the rest of the experiments. A series of standard

reserpine solutions were injected under the optimum conditions and a linear working range 1 x 10^{-5} M to 1 x 10^{-4} M was obtained, as shown in Figure 22. The peak heights were smaller compared to the continuous-flow method without recirculation (35% less sensitive). The peak heights obtained were an average of 5-10 injections with less than 2% RSD. The time taken for cycle of filling, injection, and flushing added up to 1 min, allowing 60 injections per hour.

A kinetic analysis of the continuous-flow signals performed. The residence time employed in these studies was twice the lower rate and the time was independent of the reserpine concentration. Considering that the peak measurements were made when the system was still evolving toward the equilibrium composition, the rate of the reaction was assumed to be $[3,4\ DDR]_{\text{at peak}}/\Delta t_{\text{at peak}}$, where the [3,4DDR] is the concentration of 3,4-didehydroreserpine at the peak and Δt is the time between the injection and the peak maximum (corresponding to the residence time of the plug). All other assumptions were the same as in the batch calculation. The peak concentration can be calculated from the peak height and absorbance (1 cm = 0.04 A; molar absorptivity = 10,778 $M^{-1}\ cm^{-1}$). The Rate was calculated at different inital concentrations of reserpine (Table XII). A plot of log Rate vs. log $[Res]_{t=0}$ was linear (Figure 18). From equation 23, the slope gives the order with respect to

TABLE XII

RATE OF RESERPINE OXIDATION
BY PERIODATE AND MN (II)
STUDIED IN A FLOW SYSTEM

Initial Concentration of Reserpine, M	Peak Height[a] cm	Δt[b] min	Rate[c] M min^{-1}
1.00×10^{-4}	18.1	0.50	1.34×10^{-4}
0.80×10^{-4}	15.1	0.50	1.12×10^{-4}
0.60×10^{-4}	11.4	0.51	0.83×10^{-4}
0.50×10^{-4}	9.8	0.52	0.70×10^{-4}
0.40×10^{-4}	7.6	0.52	0.54×10^{-4}
0.20×10^{-4}	3.2	0.52	0.23×10^{-4}
0.10×10^{-4}	1.2	0.54	0.08×10^{-4}
0.08×10^{-4}	0.4	0.54	0.06×10^{-4}
0.05×10^{-4}	0.2	0.57	0.03×10^{-4}

[a] Absorbance at peak maximum = peak height/cm x 0.04 A/cm.

[b] Time at peak maximum

[c] Rate = $(\text{Absorbance}/\Delta t)$ at peak $\times \epsilon^{-1}$ (ϵ = 10,778 M^{-1} cm^{-1}).

reserpine and the intercept gives the value of k'. A slope of 1.2 was obtained when all the points were considered. When the slope was calculated using six out of the nine points, c was equal to 1, which gave an order of 1 with respect to reserpine. The points deviating from slope of 1 correspond to relatively low concentrations of reserpine with larger inherent uncertainty in the measurements. The value of k' was calculated using c = 1, and was found to be equal to 1.33 ± 0.09 min^{-1}. Assuming first order with respect to $[IO_4^-]$ amd [Mn(II)] whose initial concentrations are 1.5 x 10^{-2} and 2 x 10^{-4} M, respectively, the value of k was found to be 4.4 ± 0.30 x 10^6 M^{-2} min^{-1}. The magnitude of the rate coefficent from the batch was 1.93 x 10^6 M^{-2} min^{-1}, as already mentioned. The two values are comparable and the larger value of k obtained from continuous-flow peaks can be attributed to the efficiency of the turbulent mixing in the mixing chamber of the FiAtron unit.

Determination of Reserpine Using a Manganese Dioxide Reactor

The continuous-flow manifold was simplified in this scheme by the elimination of one of the reagent streams. Reserpine is merged with the periodate and the reaction plug passes through the reactor, when it comes in contact with the MnO_2, which acts as a catalyst for the oxidation.

The MnO_2 was attached firmly to the sides of the tubing and was not easily detached by the flow. Deterioration of the reactor was not observed after 100 injections, over a period of one week. The reactor was stored between use after washing thoroughly with water and drying it by pumping air through. There was no permanaganate formation observed during the reaction, either. The concentration of periodate used remained the same and was 1.5×10^{-2} M. A very slow flow rate was necessary to allow enough time for reaction. The optimum flow rate and injection volume were 0.75 ml/min and 250 ul, respectively. The calibration curve obtained here was comparable in sensitivity with the continuous-flow method with recirculation . A working range of 1×10^{-5}-1×10^{-4}Mwas obtained (Figure 22). The number of injections was also limited to 45 per hour.

The important features of the three schemes for the continuous-flow determination of reserpine are compared in Table XIII. Each of the method has its advantages and disadvantages. The sensitivity (from the slope of the calibration curve) was higher when Mn (II) in solution is used but large amounts of the sample were wasted. When the volume of sample available was limted or the sample was dissolved in small quantities of solvent to increase its concentration, replicate determinations on the same sample can be done by recirculating the solution without cross contamination. The use of manganese dioxide reactor reduces

TABLE XIII

SOME FEATURES OF THE CONTINUOUS-FLOW METHODS FOR THE DETERMINATION OF RESERPINE

	Mode 1	Mode 2	Mode 3
Flow Rate, ml/min	1.75	2.30	0.75
Injected Volume, µl	125	450	250
Slope of Calibration curve, cm/M x 10^5	1.82	1.18	1.07
LD, 10^{-6} M	5.0	7.5	8.2
Correlation Coefficient	0.9998	0.9993	0.9996
Number of Injections/h	60	60	45

LD: Limit of Detection (based on three times the standard deviation of the intercept)

reagent consumption and is more economical. Less time is required for reagent preparation, because the reactor can be reused (Mn(II) in solution has to prepared fresh every week. The sensitivity of the methods described here is less than the official methods (30, 37), it is a feasible alternative because of its simplicity and lack of rigorous reaction conditions. The analytical throughput is greater than any knowm automated method for the determination of reserpine (40, 42).

Determination of Reserpine in Pharmaceutical Preparations

Tablets of "Serpasil" labeled to contain 0.1 mg and "Raudixin" labeled to contain 50 mg of Rauwolfia serpentina, as well as USP standard Rauwolfia serpentina powder (100 mg), were analyzed using the different strategies already described. Rauwolfia serpentina contains reserpine and rescinnamine which are both pharmacologically active and yield similar products on oxidation and are therefore determined together as reserpine. Reserpine constitutes between 0.15%-0.20% w/w of the preparation. Table XIV lists the sample volume after sample preparation and the number of injections amenable with each of the modes described. Due to the large absorbance of the coating material, the "Raudixin" tablets were washed with water to remove the layer, dried and powdered, before dissolving in

TABLE XIV

SAMPLE VOLUME AFTER SAMPLE PREPARATION AND THE NUMBER OF POSSIBLE INJECTIONS WITH EACH OF THE METHODS

Sample	Operational mode	Sample Volume ml	Number of injections/ sample[a]
Serpasil	Mode 1	10.0	5
	Mode 3	8.0	4
Raudixin	Mode 1	5.0	2
	Mode 2	5.0	4
USP std. Rauwolfia Serpentina	Mode 1	10.0	4
	Mode 3	8.0	4

[a] This corresponds to the number of injections amenable by the different modes per tablet or 100 mg of Rauwolfia serpentina

TABLE XV

DETERMINATION OF RESERPINE IN PHARMACEUTICAL FORMULATIONS

Sample	Serpasil	Raudixin[e]	USP std. Rauwolfia Serpentina[a,e]
Peak height, cm	2.93[b] 1.81[d]	5.01[b] 3.51[c]	4.90[b] 3.54[d]
% RSD	2.3 3.4	7.2 5.9	2.0 2.6
Concentration of Reserpine, x 10^{-5} M	1.62 1.69	2.77 2.98	2.69 3.31
Reserpine, mg/tablet	0.099 0.104	0.084 0.091	0.164 0.161
% of label	98.7 103.7	96.0 104.0	93.7 92.0

[a] Reserpine was determined in 100 mg of powder

[b] Mode 1; [c] Mode 2; [d] Mode 3

[e] Reserpine content of Rauwolfia serpentina is between 0.15 and 0.20%. The percentages reported here are based on 0.175% content.

[f] Reserpine Tablets contain not less than 90.0% and not more than 110% of the labeled amount of reserpine, according to USP.

acetic acid. When "Serpasil" extract was injected into a stream of acetic acid, no peaks were observed, showing the absence of any oxidative degradation of the sample. The Rauwolfia serpentina preparations gave positive blank readings, which were subtracted from the peak heights to obtain the calibration curve. This may be due to the oxidative derivatives which occur naturally in the roots and are extracted together with the active ingredients (31). The concentration of reserpine in the sample was obtained from the corresponding calibration curve. The amount of reserpine (molecular weight = 608.7) present was calculated in these samples and compared with the labeled amounts on each of these samples. The correlation was found to be good. The concentration of the reserpine extract falls within the working curve of the proposed methods. A summary of comparative results and other pertinent information for the analysis is given in Table XV. Although the sensitivity and limits of detection are lower with MnO_2 reactor, it gives satisfactory results for the practical application. The use of a solid catalyst reactor has the advantage of simplicity and convenience. When lower limits of detection are necessary, the continuous-flow sytem described here can be used with fluorometric detection.

CHAPTER VI

CONCLUSIONS

The work described demonstrates the use of catalysts (both enzyme and metal ion) to promote/catalyze the oxidation of reserpine to 3,4-didehydroreserpine and its subsequent determination in a continuous-flow system by spectrophotometric monitoring of the oxidation product [oxidation with periodate in the presence of Mn(II or IV)].

The enzyme, peroxidase, used to promote the oxidation of reserpine by dissolved air was rendered inactive by the product of oxidation. This is one of the hazards of working with enzymes, especially when they are used as immobilized reagents. Using solutions of peroxidase in a homogeneous system was not considered to be a viable alternative because of the cost involved. Kinetic studies shed some light on the type of inhibition and how it occured. The calculation of the Michaelis-Menten constant, from the Lineweaver-Burk plots showed that it remained relatively unchanged, indicating that a non-competitive form of inhibition was taking place.

The alternate choice of using manganese ions to catalyse the periodate oxidation of reserpine was found viable. When Mn(II) in solution was used, continuously

mixing with periodate, the sensitivity of the method was better, than when a small volume of the catalyst was merged with the sample and the oxidizing agent. However, in this mode of operation, waste of reagent in large quantities, occurred. The wasting of reagent was avoided when the catalyst, MnO_2, was immobilized on the walls of Tygon tubing and used as an open-tubular reactor. The sensitivity of this method was lower but met the requirements for the determination of reserpine in single tablets. The continuous-flow implementations illustrated here can be used with flurometric detection when better sensitivity and limit of detection is needed.

Using a commercial apparatus like FiAtron for solution handling had its advantages and disadvantages. One of the main disadvantages of the system was the need for a larger sample volume (when compared with using a sliding injection valve and sample loop). This is due to the fact that although the actual volume of sample undergoing reaction is small, it is being continuously pumped to waste. The user is also limited by the lack of flexibility with respect to the length and diameter of tubing used in the valve system, affecting the flow rate and residence time. However, the microprocessor controlled solenoid valves allow repetitive injections of precise quantities of reagent and sample into the main flow stream with very good reproducibility. Merging two or three solution streams can be implemented with relative ease. The instrument when used in conjunction

with a sampler can be set up to do a large number of injections with minimum human involvement.

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