SYNTHESIS OF N-OXIDES WITH A HEPATIC MICROSOMAL DRUG OXIDASE INSOLUBILIZED ON ARTIFICIAL MATRICES

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SUMMARY. Insolubilization of a hepatic microsomal mixed function flavoprotein oxidase by covalent attachment to nylon tubing, sepharose particles, or glass beads increases the thermal stability of the purified oxidase at least one-hundred fold. This oxidase catalyzes the NADPH- and oxygen-dependent oxidation of several hundred foreign compounds and of clinically useful drugs containing amine or hydrazine functional groups. Covalent attachment of the oxidase to an insoluble support does not qualitatively change its substrate specificity but some quantitative changes are observed. Reactors containing the insolubilized enzyme can be used for the production of N-oxidized drug metabolites that are difficult to prepare by direct chemical synthesis.

A mixed function flavoprotein oxidase isolated (1) from pig hepatic microsomes is known to catalyze the N-oxidation of amines (1) and of hydrazines (2). The oxidation of amines has been studied in some detail and in general the flavoprotein oxidase will catalyze the N-oxidation of any lipid soluble secondary or tertiary amine that does not have a polar group on the alpha carbon or a functional group more polar than a hydroxyl within a two carbon radius of the nitrogen. In every case, tertiary amines are oxidized to the N-oxides and secondary amines to the corresponding N-hydroxy amines.

The N-oxidation of tertiary amines has been demonstrated in man and other vertebrates both *in vivo* and *in vitro* (3-6), and the contribution of N-oxidation to the metabolism of amine drugs in different species has been reviewed by Bickel (7). While N-oxidation is considered a minor route for metabolism of many amine drugs, it is a major route for the metabolism of phenothiazine drugs (8). The N-oxidase activity of human liver tissue (9) is also several times higher than that of hepatic tissue from laboratory animals and N-oxida-

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tion may be a more significant pathway of drug metabolism in humans than in commonly used laboratory animals.

MATERIALS. The microsomal flavoprotein oxidase was isolated from pig liver microsomes as described earlier (1). Isocitrate dehydrogenase was purified from cell-free extracts of E coli by the method described for the isolation of the analogous enzyme from A vinelandi (10). $NADP^+$, NADPH and Sepharose were obtained from Sigma Chemical Company. The drug substrates were supplied by the firms indicated: chlorpromazine and prochlorperazine - Smith, Kline, and French Laboratories; brompheniramine - A. H. Robbins and Co.; guanethidine - Ciba Pharmaceutical Co.; and ethylmorphine - Merck and Co. N,N-dimethylaniline was purchased from Eastman Chemical Co. and purified by gas-liquid chromatography. The nylon tubing was obtained from Cadillac Plastics Co. of Houston, Texas; and the Corning glass beads were purchased from Pierce Chemical Co.

METHODS. Enzyme was bound to the interior surface of nylon tubing by a modification of the method of Hornby and Inman (11). Enzyme was mounted on Sepharose 6B by the method of Axen and Ernback (12). The binding procedure involved activation of the polysaccharide polymer by cyanogen bromide followed by protein coupling in slightly alkaline medium. Enzyme was bound to zirconia-clad, 1350 Å pore diameter glass beads according to directions supplied by Pierce Chemical Company (13). Glutaraldehyde was utilized as a carbonyl intermediate and the protein attached via Schiff base coupling. The bead insolubilized oxidase was stored in 0.025 M potassium phosphate buffer, pH 7.4, at 0-4° when not in use.

In operation in the configurations indicated in the Figs. the reaction medium contained 0.025 M potassium phosphate, pH 7.6, 0.2 mM $NADP^+$, 5 mM $MgCl_2$, 10 mM isocitrate and sufficient isocitric dehydrogenase to reduce no less than 1 µmole $NADP^+$/min/ml at 40°.

The concentrations of N-methyl and N,N-dimethyl tertiary amine oxides were determined by colorimetric methods described earlier (14,15). Guanethidine oxide was separated from the parent amine by ascending chromatography on

Whatman No. 1 paper with 75/25 (v/v) methanol/water. Guanethidine and its N-oxide were located on the chromatographs by the method described by Horne and Dollard (16).

The activity of the bead insolubilized oxidase was monitored by measuring the substrate-dependent increase in oxygen consumption with a Clark type oxygen electrode in conjunction with a Heathkit (Model EN-2058) strip chart recorder. The electrode was mounted in a temperature controlled all glass cell. The reaction medium contained 0.025 M potassium phosphate buffer, pH 7.6, the isocitrate NADPH generating system and a weighed amount of the glass beads containing the bound oxidase. The reaction was started by the addition of substrate. In consecutive measurements with different substrates the beads and chamber were rinsed several times with water removed by aspiration after each addition. Since a single determination requires only 3 to 5 minutes, a large number of measurements are easily carried out with the same glass bead mounted enzyme over a two hour period.

RESULTS AND DISCUSSION. The physical configuration of the reactors is shown in Figures 1 and 2. In both configurations, the reactor temperature is controlled by circulating water, the reaction medium flow rate is controlled by pump, and either air or O_2 is bubbled through the fluid to supply oxidant for multiple-pass runs.

As shown in Table I the flavoprotein oxidase retains its activity upon insolubilization by covalent binding to the three different supporting matrices tested. The amount of enzyme bound on each of the different supporting media appears to be a function of the available surface area. Quantitative differences in activity could not be detected among the three different matrices containing the oxidase.

Covalent binding increases the thermal stability of the flavoprotein oxidase markedly. The soluble oxidase at 40° has a half-life of less than 3 minutes; whereas the half-life of the insolubilized oxidase at 40° is no less than 5 hours. The half-life of the insolubilized oxidase may be con-

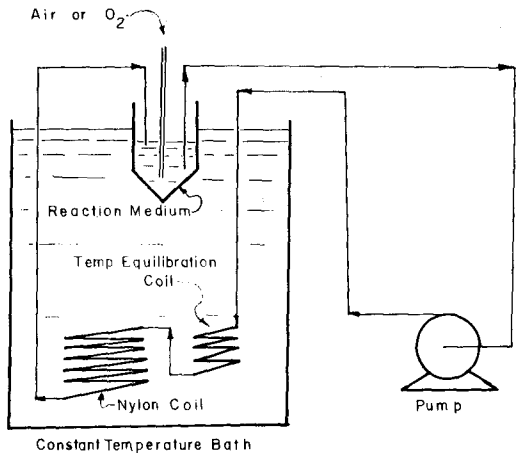

Figure 1. Configuration of reactor with oxidase mounted in nylon tubing. The arrows give the direction of fluid flow.

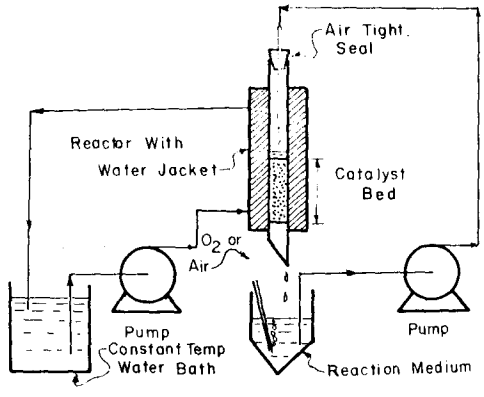

Figure 2. Configuration of reactor using bead insolubilized oxidase. The oxidase mounted on either glass beads or Sepharose is contained in the water jacketed column as indicated.

siderably greater than 5 hours since this value is based on the accumulated time of operation at 40° with intermittently used reactors over as much as a two month period. Inactivation of enzyme during storage at 4° or during the time required to flush the reaction with buffer at 40° before and after each run was not included in calculating the minimum half-time of the reactor.

Figure 3 illustrates that the reaction rate for the nylon tubing reactor

TABLE I. Activity of the Flavoprotein Oxidase Insolubilized on Different Supporting Media

Oxidase insolubilized on	Ethylmorphine N-oxidized[a]
Nylon tubing (Fig. I)	32 nmoles/min/meter tubing
Sepharose (Fig. II)	30 nmoles/min/ml packed gel
Glass beads (Fig. II)	60 nmoles/min/gm dry beads

[a] Flow rate 2 ml/min at 40°. Reaction rate calculated from total amount N-oxide produced in 10 minutes in 20 ml fluid reaction medium. Initial concentration of substrate - 3 mM.

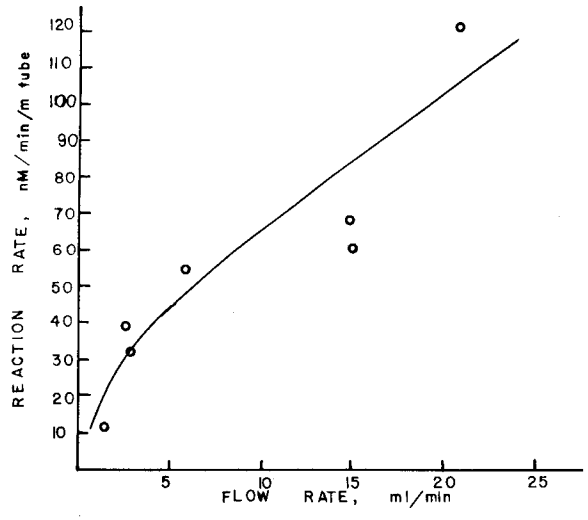

Figure 3. Rate of ethylmorphine N-oxidation as a function of liquid flow through a 3-meter nylon tube reactor. Composition of reaction medium was as given in Table I and Methods.

is a function of fluid velocity and indicates that the reaction is limited by mass transfer. This is probably also true for the reactor (Fig. 2) using bead mounted enzyme but reaction rate as a function of fluid velocity was

not measured with this system. Studies on the effects of high fluid velocities on catalyst life are also not complete.

While reaction rate appears to be limited by fluid velocity, almost complete conversion of ethylmorphine, prochlorperazine, and guanethidine to their corresponding N-oxides has been obtained by multiple passes through the reactors. In one experiment 45 micromoles of ethylmorphine N-oxide were isolated from 20 ml of the fluid reaction medium and crystallized. The low water solubility of prochlorperazine limits the amount of substrate that can be added initially but mg quantities of its oxide have been isolated from fluid reaction medium repeatedly saturated with the substrate between multiple passes through the reactor.

The potential maximum reaction rate obtainable with the glass bead mounted oxidase estimated by measuring the substrate dependent increase in oxygen consumption is shown in Table II. The rates listed were calculated from the initial rates of oxygen uptake at an oxygen concentration of 200 μM. The relative rates of oxidation of the substrates tested do not coincide

TABLE II. N-Oxidation of Amines Catalyzed by Glass Bead Mounted Oxidase[a]

Substrate	Conc. mM	μmoles substrate oxidized/ min/gm glass beads[c]
Chlorpromazine	0.5[b]	6.4
Prochlorperazine	0.1[b]	5.9
Brompheniramine	1.0	3.3
Ethylmorphine	1.0	2.8
N,N-dimethylaniline	1.0	4.3

[a] The glass beads contained approximately 5 mg covalently bound flavoprotein-oxidase/gm dry glass beads.

[b] These concentrations at or near the upper limits of solubility.

[c] Rates based on the substrate dependent increase in oxygen uptake measured polarigraphically as described under Methods.

exactly with that observed with the soluble enzyme. For example, with the soluble oxidase the first three substrates listed are all oxidized at the same rate and the last two at about one-half the rate of chlorpromazine.

The oxidation rates measured polarographically indicate that it should be possible to design a reactor capable of producing mg quantities/minute of a specific drug metabolite with as little as 1 gm of glass beads containing the bound oxidase. While the reactors described in this report have not been fully optimized their potential for synthesis of specific N-oxides of amine drugs has been demonstrated.

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