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Analytical Applications of Reactors Containing Immobilized Enzymes

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Abstract

The theory for analytical packed-enzyme reactors is discussed and it is shown that a 100% conversion efficiency gives many advantages. This concept has been applied to methods for substrate determinations of urea, amino acids, and glucose. Enzyme reactors have also been used in the effluent from a chromatographic column to enhance selectivity and sensitivity for cholesterol. Enzyme reactors for the determination of inhibitors, e.g. mercury ions, should be designed differently. A low conversion efficiency gives high sensitivity and a linear response.

Index Entries: Analysis, with immobilized enzyme reactors; reactors, immobilized enzyme; chromatography, on packed-enzyme reactors.

Immobilized enzymes packed into small reactors and used as components of flow systems have a number of analytical applications that have so far only partly been exploited. This paper will discuss how experiments can be designed so that the advantages of the reactor concept can be utilized.

Theory

Conversion Efficiency

Following the Goldstein-Katchalski treatment of reactor kinetics *(1)*, it is easy to show *(2)* that the necessary size of the reactor can be calculated for systems with Michaelis-Menten Kinetics.

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$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \overset{k_2}{\rightarrow} P + E \quad (1)$$

If a solution with the substrate concentration s flows through a reactor with the area A and the length L with a rate of q mL/min and the product concentration at the outlet is assumed to be p, the quantities are related by

$$AL\ k_2E_tq^{-1} = p + K_m \ln s/(s - p) \quad (2)$$

In order to determine the amount of enzyme required to convert, say, 99% of the substrate to products, $p/s = 0.99$ is inserted into the logarithmic expression, which then becomes 4.6 (for 99.9%, it becomes 7). The quantity k_2E_t is first determined experimentally from Eq. (2) with a small reactor; a literature value of K_m for soluble enzyme seems to be adequate *(2)*. The dimensions of a reactor giving complete conversion can then be calculated when q and s are known. Only the product AL enters the equation, showing that the form of the reactor is unimportant as long as channelling can be neglected. Whenever possible a reactor giving complete conversion of substrate to products should be selected. Table 1 enumerates the advantages of this approach.

TABLE 1
Factors Affecting the Analytical Response of Enzyme Reactors

Factor (small changes)	Response: Incomplete conversion, <100%	Response: Complete conversion, 100%
1. Flow rate	Proportional	No effect
2. Temperature	Varies	No effect
3. pH	Varies	No effect
4. Ionic strength	Varies	No effect
5. Small additions of inhibitors or activators	Varies	No effect
6. Substrate dependence	Not completely linear	Linear up to a maximum concentration

Conversion Time

At first it may be intuitively felt that a system that relies on a complete conversion of the substrate will be slow because the reaction should need some time to go to completion. That this is not so is shown mathematically by Eq. (2); the time as a variable has been replaced by the flow rate. The time necessary to convert the substrate within a very small solution volume is the time between the entrance at the reactor front and the time of its appearance at the reactor outlet. The reactor is filled completely with enzyme–glass so that the dead volume becomes small. The time delay owing to the insertion of a reactor in the system has so far been unimportant in all analytical systems.

Packing of Reactors

Methods based on the attainment of a mass transfer-dependent steady-state (e.g., enzyme electrodes) will be affected by changes in the thickness of the enzyme layer. In an enzyme reactor, the corresponding factors are dead volume and channeling. The first can easily be controlled by using a rigid support *(3)* in order to prevent compression of the packing by the flow. The second is not important if the percentage conversion of the reactor is high. In the determination of inhibitors, on the other hand, a reproducible utilization of the available enzyme is important. It is impossible to pack a very small reactor sufficiently well. Since the inhibitor starts to bind at the first part of the reactor and proceeds downwards only when the first sites are filled, there is a way around the difficulties, namely to utilize only about half of the inhibitor-binding capacity of the reactor. This ensures that very little inhibitor is lost even if there is some channeling.

Applications

Splitting of a Substrate

Figure 1 shows a flow system for the determination of urea *(3)*. Urea samples are introduced through a sample loop or an injection port, mixed with a phosphate buffer, pH 7.0, and then fed into a reactor containing urease immobilized with glutaraldehyde to porous glass. The amount of ammonia is monitored with an ammonia-selective gas electrode. One advantage is immediately apparent, namely, that the arrangement permits independent pH selection for the reactor and the detector. The ammonia electrode response is proportional to the logarithm of the ammonia concentration, which is one of the factors that contributes to the large dynamic range of the method.

Unless the solution is made strongly alkaline, a fraction of the product of the en-

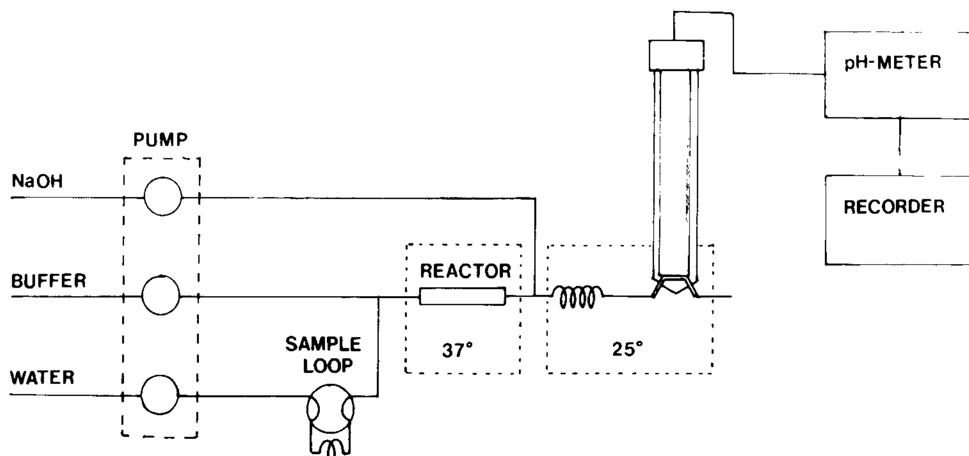

Fig. 1. Experimental arrangement for urea determination (reproduced from *Anal. Chim. Acta* with permission.)

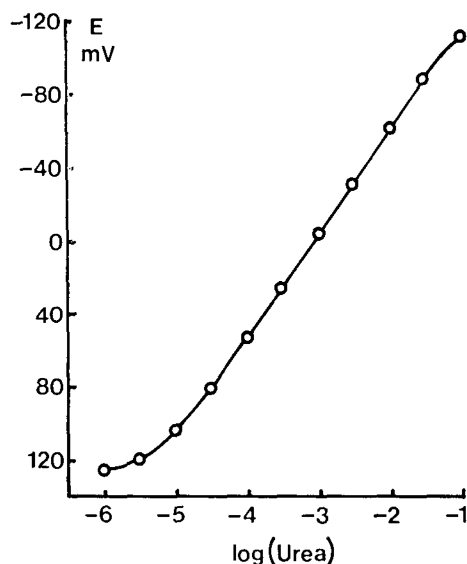

Fig. 2. Calibration curve for urea determination (reproduced from *Anal. Chim. Acta* with permission).

zymatic reaction will be present as NH_4^+ ions. The ratio NH_3/NH_4^+ will depend on pH and is thus sensitive to, e.g., the pH or the buffer capacity of the sample. Addition of strong alkali, as shown in Fig. 1, causes a complete conversion of the product to ammonia and makes the system immune to pH-affecting factors.

The reactor was 45 mm long and the id was 3.4 mL, so that the total volume became 400 μL. This proved to be sufficient to give a quantitative conversion of up to 3×10^{-2} *M* urea to products at a flow rate of 40 mL/h (see the calibration curve in Fig. 2).

Reactors for Systems Requiring a Co-substrate

Figure 3 shows an analysis of L-leucine using L-amino acid oxidase (*4*) immobilized on porous glass. If oxygen for the reaction is provided only from that dissolved in the buffer and sample solution, the upper limit of measurements are limited to about 5×10^{-4} *M* amino acid. Therefore catalase (1/3) was immobilized to porous glass and mixed with the immobilized amino acid oxidase (2/3). Hydrogen peroxide was then added to the buffer so that oxygen could be produced within the reactor where it is needed. The upper limit is set by oxygen bubble formation in the absence of substrate.

Gorton and Bhatti *(4)* showed that glucose determination can be made with a reactor containing immobilized glucose oxidase. An in-line microdialyzer was used to remove proteins from the samples. The normal co-substrate, oxygen, was replaced by benzoquinone. The quinone/hydroquinone ratio was monitored with gold electrodes and the original concentration of glucose in the sample was evaluated via a derived expression. The reactor was designed to give complete conversion of β-D-glucose to gluconic acid, but nevertheless the system lost some of the advantages mentioned in Table 1. The reason is that the dialyzed fraction is dependent on the flow rate and the temperature of the dialyzer.

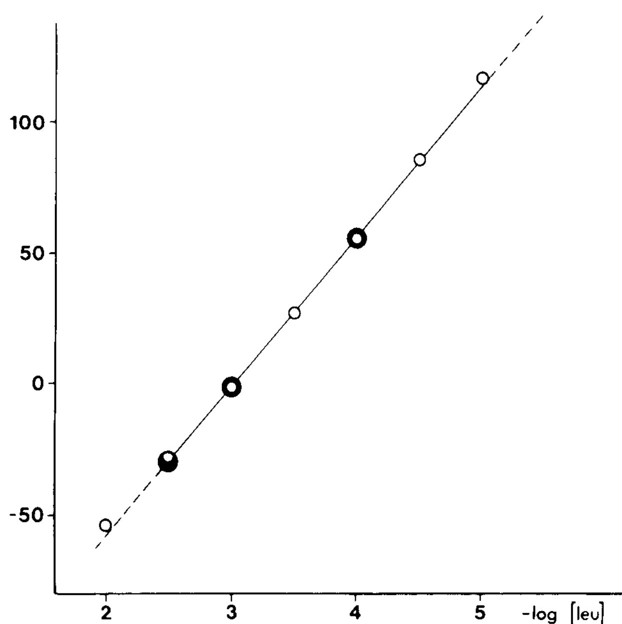

Fig. 3. Calibration curve for the determination of L-leucine using L-amino acid oxidase. Filled large circles denote calibrations with NH_4Cl (reproduced from *Anal. Chim. Acta* with permission).

Enzyme Reactors in Chromatography

Oxidized cholesterols separated by HPLC can be monitored with a UV-detector only with difficulty. Insertion of a reactor containing immobilized cholesterol oxidase into the column outlet improves the detection since the conjugated systems of the products have a high molar absorptivity and absorb at more convenient wavelengths *(6)*. The application is interesting for the topic of this paper because it demonstrates the behavior of a system in which the conversion was substantially less than 100%. The activity of the enzyme decreased when the percentage of organic solvent was increased. A high percentage of solvent was necessary to obtain separation on the column, however. Furthermore the concentration in the effluent was much lower than K_m. Further purification of the enzyme increased the specific activity of the enzyme glass as well as its stability. Nevertheless the final compromise resulted in a system in which the reactor converted 72% of the cholesterol to products at the selected flow rate. Figure 4 shows, as expected, that the calibration curve deviates from linearity.

There is another aspect, which is illustrated by Table 2. The enzyme has a broad activity for a number of sterols with a 3β-hydroxy group present. The relative activity between these differs much less in the enzyme reactor, than for a corresponding comparison with soluble enzyme. This is a consequence of the difference in form of the kinetic expression for a reactor and for a soluble enzyme. Although Eq. (2) does not hold for the cholesterol oxidase, it can be seen that the p/s ratio decreases slower with k_2 and K_m than it would do in the normal Michaelis-Menten equation. This consequence is advantageous for a chromatographic detection sys-

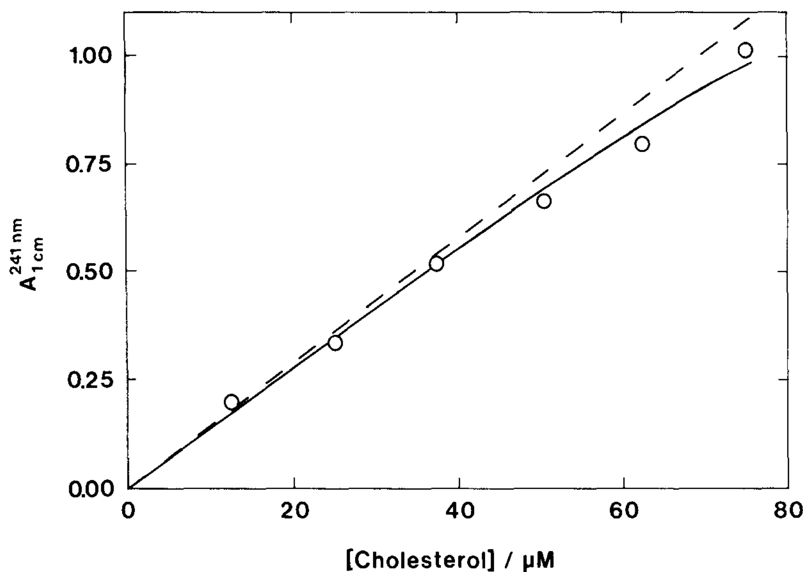

Fig. 4. Calibration of a reactor containing cholesteroloxidase with cholesterol at 1 mL/min. The dotted line indicates the expected response for 100% conversion efficiency (reproduced from *Anal. Chim. Acta* with permission.)

tem in which a group selectivity is desired. In an analytical system for determination of the normal substrate of the enzyme on the other hand the loss of selectivity may be disadvantageous.

Determination of Inhibitors

It has been shown *(7)* that the design considerations for an enzyme reactor becomes completely different when an inhibitor is to be determined. A linear response of percent inhibition plotted versus the inhibitor concentration is obtained only if p/s

TABLE 2
Conversion Efficiency in an Enzyme Reactor Compared with the Relative Activity of the Soluble Enzyme, Cholesterol Oxidase *(6)*

Substrate, 50 μm	Conversion %, 1 mL/min	Relative activity in solution, %
Cholesterol	84	100
20α-Hydroxycholesterol	94	142
25-Hydroxycholesterol	91	99
7α-Hydroxycholesterol	61	18
7β-Hydroxycholesterol	52	9
7-Ketocholesterol	24	3

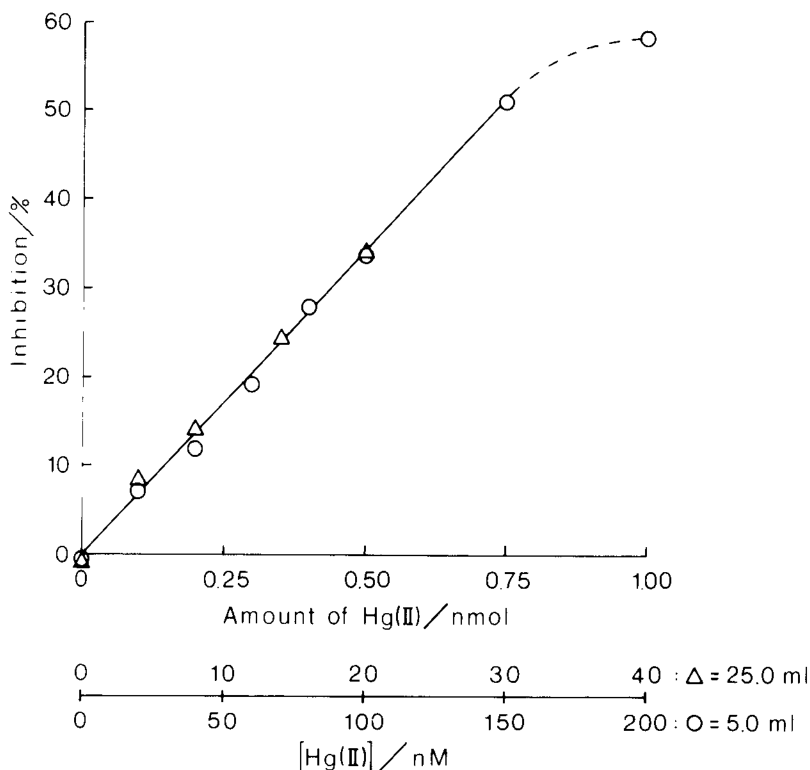

Fig. 5. Percentage inhibition as a function of the amount of mercury ions. A second scale giving the corresponding concentration in two sample sizes is also included (reproduced from *Anal. Chim. Acta* with permission).

is small. The sensitivity and also the linearity increases when the ratio K_m/s decreases.

Mercury (II) was determined via its inhibition of immobilized urease *(7)*. A buffer containing urea was pumped through a reactor containing 14 μL enzyme–glass. About 3% of the urea was converted to ammonia, which was monitored with an ammonia electrode. Since s is known, a measurement of the ammonia concentration, i.e., p, is equivalent to a determination of the enzyme activity of the urease in the reactor. A valve is switched so that a sample (5 mL aqueous solution in a syringe) can be pressed through the reactor. Any Hg^{2+} in the sample binds strongly to urease and causes inhibition. The valve is switched back and a new measurement of p is made. The percentage inhibition is calculated. A calibration plot is shown in Fig. 5. A high urea concentration, 15 m*M*, and a small reactor volume was selected in order to get a linear response with a high sensitivity.

Acknowledgment

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