Ethanol and Lactic Acid Sensors Using Electrodes Coated with Dehydrogenase-Collagen Membranes

Shuichi SUZUKI, Fujio TAKAHASHI, Ikuo SATOH, and Nobuyuki SONOBE

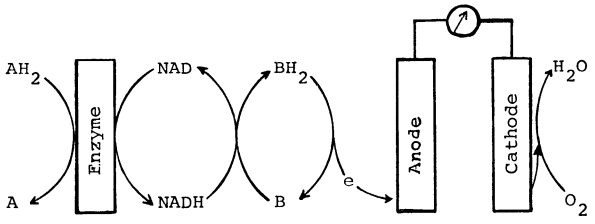
Research Laboratory of Resources Utilization, Tokyo Institute of Technology, Ookayama, Meguro-ku, Tokyo 152

(Received May 7, 1975)

Electrochemical and enzymatic reactions were studied for the purpose of making sensors of ethanol and lactic acid. The dehydrogenase-collagen membrane coated onto a platinum plate was prepared by the electroshaping method. The substrates were dehydrogenated and nicotinamide adenine dinucleotide (NAD) was reduced to form the reduced NAD (NADH) by an enzymatic reaction on the collagen membrane. Flavin mononucleotide (FMN) was chosen as an electroactive substance which could oxidize NADH to return NAD. The reduced FMN ($FMNH_2$) was easily oxidized at the anode and a current was obtained. From the above reactions, the concentrations of ethanol and lactic acid in solutions were found to be determined by a current-substrate concentration relation. The accuracy was estimated to be within 10% error. The activity and stability of enzymes immobilized in the collagen membrane were investigated.

Since 1962, the characteristics of biochemical reaction cells have been studied in this laboratory.[1] These cells have been found not to generate a large quantity of power. However, these cells have a specificity that only one substrate in the mixture selectively reacts in the enzymatic reaction to form an electroactive substance which in turn reacts at the electrode to generate electricity. One of the present authors has already reported on the application of several sensors for the detection and analysis of hydrogen peroxide and uric acid using biochemical reaction cells.[2] It has been found that NAD can be utilized as one of electroactive substances in the biochemical reaction cells.[3] It has been found that NAD is involved in more than 200 kinds of enzymatic reactions as a coenzyme.[4] Therefore, it is expected that many kinds of substrates might be detected or determined by the measurement of the anodic reaction of NADH produced by the enzymatic reaction with each substrate.

The purpose of this study is to produce a sensor which combines the electrochemical reaction and the enzymatic reaction with NAD. The necessary conditions for the sensor are (1) to obtain the relative value of the potential or current corresponding to the substrate concentration, (2) to allow the electrochemical reaction to occur with a constant concentration of NAD, and (3) to be capable of using the enzyme repeatedly. It is already known that NADH is hardly oxidized at the anode electrochemically.[3] Therefore, it was proposed that another redox system, as an electroactive substance which can easily react with NADH, be introduced in the biochemical reaction cell as follows:



AH_2: Substrate, A: product, B,BH_2: electroactive substance

This paper reports on the studies of electrochemical and enzymatic reactions for making ethanol and lactic acid sensors.

Experimental

Materials and Preparation of Collagen Membrane. Alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH) were supplied by Oriental Yeast Co. All the other chemicals were of reagent grade or the best commercially available. ADH-collagen membranes were prepared, according to the method of Karube, as shown in Fig. 1, by an electrochemical method using collagen fibril suspensoid which was obtained from hide.[5]

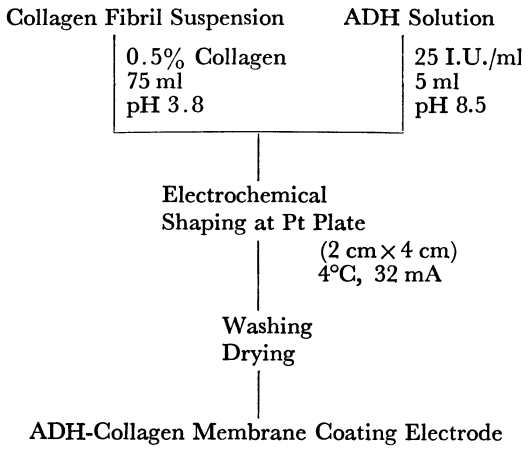


Fig. 1. Preparation of ADH-collagen membrane.

In the case of LDH-collagen membrane, the collagen fibril in the suspensoid was treated with pepsin to digest and to produce an almost transparent solution before adding LDH. The LDH-collagen membrane-coated anode was dipped in a glutaraldehyde solution and then washed with distilled water. NAD, NADH, and FMN were determined by UV spectrophotometry. The ADH was analyzed by measuring the zinc content in the enzyme by atomic absorption spectrophotometry. The ADH and LDH activities were determined by measuring NADH which was formed as a product of the enzymatic reaction.

Instruments. Absorption spectra and atomic absorption spectra were obtained using a Shimadzu spectrophotometer UV-200 and a Varian atomic absorption spectrophotometer A-A1000, respectively. A Toa Electric recorder EPR-2T and a Kikusui 114 Millivolt-ammeter were used for measuring the current. The potentials were determined with reference to a saturated calomel electrode.

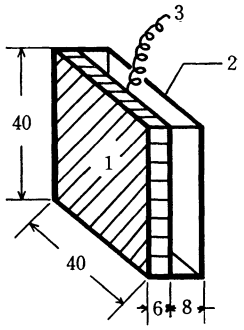


Fig. 2. The cathode.
1. Carbon, 2. plastic plate, 3. lead wire. unit: mm

Procedure and Measurement of Electrochemical Reaction. The electrochemical reaction was carried out with the following mounting: Anode: an intact or enzyme-collagen membrane-coated platinum plate (2 cm × 4 cm). Cathode: a box-shaped cathode, as shown in Fig. 2. One surface of the carbon plate (4 cm × 4 cm × 0.6 cm) was soaked in the catholyte and the other side was exposed to air. The anolyte was 0.1 mol/l of a phosphate buffer solution (pH 7.7) containing substrates (ethanol or lactic acid), NAD and an electroactive substance. The enzyme was added to the anolyte when an intact platinum plate was used as the anode. The catholyte was 0.1 mol/l of a phosphate buffer solution (pH 7.7). The anolyte and catholyte in each beaker were connected *via* an agar bridge. When the circuit of the biochemical reaction cell was open, the anode potential went to less noble because of the enzymatic reaction in the anolyte, as shown in Fig. 3. Nearly 30 min after the enzymatic reaction were required to reach equilibrium in the anolyte. Then the discharge was started and the current was measured. During this procedure, nitrogen gas was bubbled into the anolyte.

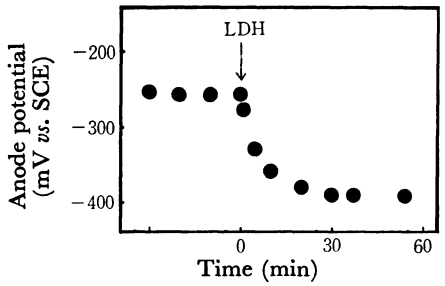


Fig. 3. Anode potential–time curve.

Results

The Electroactive Substances. FMN, thioglycolate, methyl viologen and phenazine methosulfate (PMS) were used as electroactive substances which reacted with NADH. In this biochemical reaction cell, 40 ml of an anolyte containing 1.5 mmol/l of the electroactive substance and 1 mmol/l of NADH, and an intact platinum anode were used. The values of current obtained from short-circuit discharging after the redox reaction between the NADH and the electroactive substance are summarized in Table 1. In the case when the anolyte contained only NADH, the current decreased to zero within 5 min after discharging. PMS oxidized NADH so rapidly that a high current was observed. But the PMS was unstable. It appears that the electrochemical reactions of thioglycolate and methylviologen are remarkably slow. It was found that an almost constant current was maintained after 1 minute in the case of FMN. From these results FMN was chosen as the electroactive substance for subsequent experiments.

Influence of Substrate, NAD, and FMN to the Current. The anode reaction process in process (1) includes 3 steps. For the purpose of the assignment of the rate-determining step in process (1), the current was measured in the ethanol-ADH system and in the lactic acid-LDH system in the anolyte containing various concentrations of the substrate, NAD and FMN. An intact platinum plate was used as the anode in the anolyte. The results are shown in Figs. 4, 5 and 6. The values of the current are thoughtsto be reliable to within 10%. The substrate concentration–current relation did not fall on a straight line.

Electrochemical Reaction Using an Enzyme-Collagen Mem-

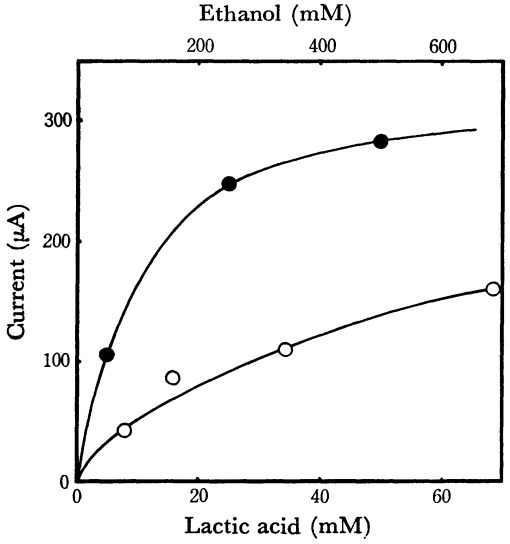


Fig. 4. Substrate–current relation.
—○—: Ethanol, —●—: lactic acid

Table 1. Electroactive substances

Compound (1.5 mmol/l)	NADH (mmol/l)	Anode potential (mV *vs.* SCE)	Current (μA) 1 min after closing the circuit
None	1	−262	10
Thioglycolic acid	1	−132	0
Methylviologen	1	−134	0
Phenazine methosulfate[a)]	1	−155	150
FMN	1	−440	207
FMN	0	−180	0

0.1 mol/l of a phosphate buffer (pH 7.7), 25 °C. a) 2 mmol/l.

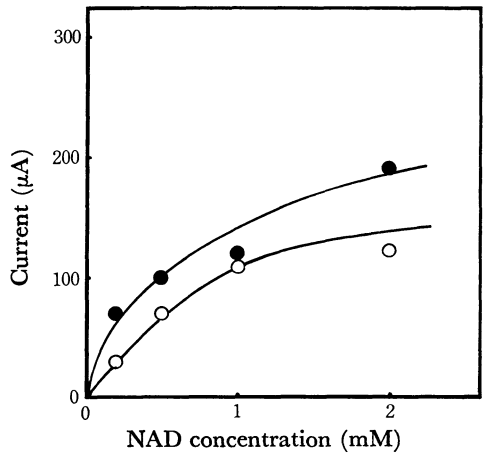


Fig. 5. NAD-current relation.
—○—: Ethanol, —●—: lactic acid.

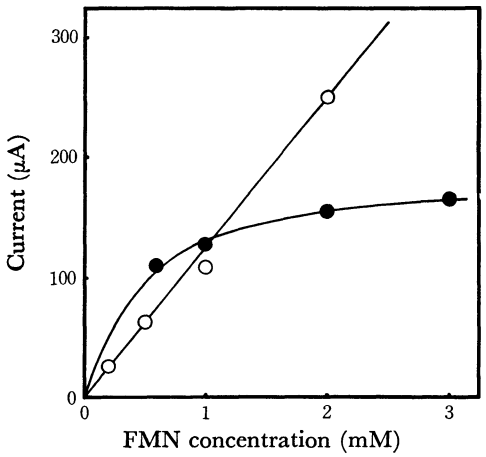


Fig. 6. FMN-current relation.
—○—: Ethanol, —●— lactic acid.

brane-Coated Anode. The enzyme was immobilized in the collagen membrane, so that the enzyme might be used repeatedly. The anode reaction was studied using an anode coated with an enzyme-collagen membrane, which was prepared by means of the electrochemical process shown in Fig. 1. The enzyme content, activities and other properties of the enzyme-collagen membrane were determined (Table 2). The substrate-current relation was measured in the same way as in Fig. 4. The results (Fig. 7) showed a tendency similar to that in Fig. 4, but the current was lower. The same anode coated with an enzyme-collagen membrane was used repeatedly and its activity was determined with the hope of maintaining the same activity for each measurement. The anolyte contained 0.34 mol/l of ethanol, 1 mmol/l of NAD and 2 mmol/l of FMN.

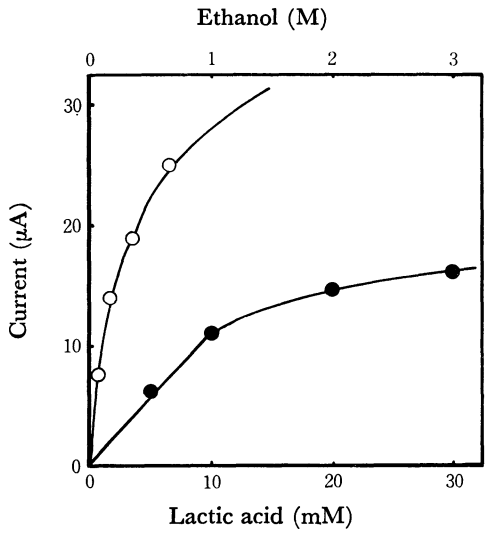


Fig. 7. Substrate-current relation (Enzyme-collagen membrane coating electrode).
—○—: Ethanol, —●— : lactic acid.

After each measurement of the current, the anolyte used was replaced by a new one and the same process was repeated. The enzyme activity was not found to decrease. The average value of current over 5 measurements was 33 ± 4 μA.

In the case of the LDH-collagen membrane, the enzyme activities were determined in a solution containing 12.5 mmol/l of lactic acid and 1 mmol/l of NAD and it was found that the activity remained constant over 8 measurements.

Discussion

It is clear that the reactions in process (1) can be utilized as a sensor. It was concluded, using the anode coated with ADH and LDH-collagen membranes, that (1) the value of the current increased non-linearly with the substrate concentration, (2) ethanol and lactic acid can be determined to within an accuracy of 10% error, (3) the enzyme activity remained constant over more than 5 measurements and (4) the necessary conditions for temperature, pH and concentrations of ethanol and lactic acid were lower than 30 °C, 6~8, 1 mol/l and 25 mmol/l, respectively.

Process (1) can be rearranged into 3 reactions as follows:

$$AH_2 + NAD^+ \longrightarrow A + NADH + H^+ \quad (2)$$

$$NADH + FMN + H^+ \longrightarrow NAD^+ + FMNH_2 \quad (3)$$

$$FMNH_2 \longrightarrow FMN + 2H^+ + 2e \quad (4)$$

If reaction (2) is the rate-determining step, the current

Table 2. Properties of enzymes in collagen membrane

Properties	Alcohol dehydrogenase	Lactate dehydrogenase
Enzyme content or activity	2% (from Zn content)	0.4 unit[a)]
pH Stability	5—10	5—10
Thermal stability	less than 35 °C	less than 35 °C
Reusability	5 times	8 times

a) In collagen membrane on platinum anode (2 cm×4 cm).

should be proportional to the substrate or total NAD concentration for a constant concentration of the NAD or substrate, respectively. However, Figs. 4 and 5 indicate that there is no linearity. If reaction (3) is the rate-determining step, the NADH concentration has to be constant during the reaction, and the concentration of $FMNH_2$ formed in the reaction could be negligible because of the rapid oxidation at the anode. The reaction velocity should be proportional to the product of the NADH concentration and the total FMN concentration. In turn, this velocity would be proportional to the current. If reaction (4) is the rate-determining step, the $FMNH_2$ concentration has to be constant in a steady state. Accordingly, the current remains constant during the electrochemical reaction, thus,

$$i \propto [FMNH_2] \quad (5)$$

From the result in Fig. 6, it can be said that reaction (4) should be the rate-determining step in lactic acid case, although it cannot be determined which step is the rate-determining one, reaction (3) or (4), in the ethanol case.

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