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Analytical determination of nicotinamide using bacterial electrodes

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Summary

Two bacterial electrodes have been studied for the determination of nicotinamide (vitamin PP) with a linear range of 2.8×10^{-4} M to 2×10^{-2} M. The used strains, although taxonomicaly different and differently improved (*E. coli* mutated and *B. pumilus* induced), present the same nicotinamide deaminase activity, able to be used for analytical assays. Their long-term stability (more than 100-fold higher than the purified enzyme) is realized by the regeneration of living cells on the electrode itself. The parameters involved in this type of electrode construction are discussed. This type of bacterial electrodes presents a very good selectivity for nicotinamide in multivitamin pharmaceutical formulations.

Introduction

We have recently proposed and described bacterial electrodes suitable for applications to urea and asparagine determinations (Vincké et al., 1983a and b).

Nicotinamide deaminase (nicotinamide amidohydrolase EC 3.5.1.19), a hydrolyzing enzyme involved in nicotinamide adenine dinucleotide (NAD) metabolism was found to be present in microorganisms such as *Aspergillus niger*, *Escherichia coli* as well as in vertebrate tissues (rabbit and rat liver) (Sarma et al., 1964; Pardee et al., 1971; Bray et al., 1949, 1950; Petrack et al., 1965). The role of nicotinamide deaminase in the maintenance of the cellular NAD level has been studied by Sarma

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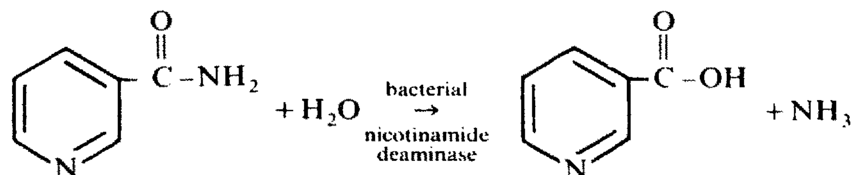
et al. (1961). According to these investigations, the enzyme deamidates nicotinamide to nicotinic acid and the nicotinic acid formed is utilized for NAD-biosynthesis via the Preiss and Handler pathway.

This enzyme is not yet commercially available, because the half-life of the enzyme extracted from a mutated strain of *E. coli* and purified 200-fold is only about 2 h at 37°C (Pardese et al., 1971). For this reason, we propose the use of bacterial modified electrodes for the quantitative determination of nicotinamide (vitamin PP). Only in recent years, the important role of this new applied biotechnology has been studied (Rechnitz et al., 1977; Kobos and Rechnitz, 1977; Wollenberger et al., 1980; Walters et al., 1980; Karube et al., 1980; Vincké et al., 1983a and b).

Guilbault (1977, 1980) has shown great interest to combine the sensitivity of the electrochemical sensors and the specificity of the enzymatic process. Enzyme electrodes give good results for their applications in clinical biochemistry (Mascini and Guilbault, 1977; Huang et al., 1977; Fischer et al., 1982; Vincké et al., 1984a).

Microbiological and enzyme electrodes, as electrochemical techniques, allow principally to work in real time of the reaction process, give an absolute value ($\delta C/\delta t$) of substrate concentrations and produce electrical signals which are able to feed a microcomputer for data processing. The subject of this work concerns the analytical applications of several bacterial strains "nicotinamide deaminase +". The microbiological electrodes are specially used for the determination of substrates for which specific enzymes are very unstable or not yet commercially available.

The interest of the enzymatic deamination, following the reaction:



is to present a greater selectivity than the chemical hydrolysis (Vincké et al., 1983c) for the determination of the vitamin PP.

The enzyme instability and the low enzyme level in microorganisms require an optimalization of the microbiological and physicochemical factors involved in the construction and the manipulation of this bacterial electrode. The influence of the physicochemical properties (type of membrane, temperature, pH, ionic strength of the buffers, cofactors), biochemical and microbiological factors (choices of the strains, mutation or induction modes, growth media, incubation times, other enzymic interferences and storage) on the electrode response will be described further.

Materials and Methods

Apparatus

An ammonia gas sensing electrode (Tacussel pNH$_3$-1 or a Universal Sensors type 0197335821) is used for the construction of the bacterial electrodes. Potentials are

monitored with a Tacussel Minisis 6000 millivoltmeter in conjunction with a Göertz recorder, Model Servogor 120. Measurements are performed in a thermostatic cell at 30.0 ± 0.2°C.

Reagents

All solutions are prepared with tridistilled water.

All chemicals used are of analytical or pharmacopoeia pure grade (Merck and Difco).

Test organisms

Two types of strains were used. Collections strains, namely *Escherichia coli* ATCC 27 195 and *Bacillus subtilis* ATCC 6 633, and wild isolates of *Bacillus cereus* and *Bacillus pumilus*. The identity of the bacterial strains has been controlled by means of classical biochemical reactions.

Culture media:

(1) The stock cultures of bacterial strains are maintained at 4°C on Heart Infusion Agar (H.I.A Difco) slants.

(2) Medium 1. Medium for induction (0.2% substrate):

yeast extract	1 g
tryptone	1 g
NH_4Cl	2 g
KH_2PO_4	4 g
Na_2HPO_4	4 g
Fe^{2+} (< $FeSO_4$ $_7H_2O$)	10 mg
Mg^{2+} (< $MgCl_2$)	28 mg
distilled water	1 litre

The stock solution is autoclaved at 121°C for 15 min by amounts of 100 ml. To this solution 0.2 g of substrate (nicotinamide in this case) are extemporaneously added and the total solution is sterilized by filtration.

(3) Medium 2. Brain Heart Infusion (B.H.I. Difco):

calf brain (infusion of)	200 g
beef heart (infusion of)	250 g
peptone	10 g
NaCl	5 g
Na_2HPO_4	2.5 g
dextrose	2 g

37 g of this medium are dissolved in 1 litre of distilled water and autoclaved at 121°C for 15 min.

Methods

Before fixing the experimental conditions, factors of optimalization of the bacterial electrode responses were fixed.

(1) General preparation of bacterial electrodes

Two electrodes are constructed using either *E. coli* ATCC 27 195 (mutated strain) after 3 subcultures in 100 ml B.H.I for 18–24 h, or *Bacillus pumilus* with first an induction step (Medium 1) followed by two subcultures in 100 ml B.H.I. for the same time.

In each case, the third suspension is filtrated through a Millipore cellulose acetate membrane (HAWP 047 SO, porosity = 0.45 μm). The filter saturated with micro-organisms is washed with sodium chloride 0.9% solution. Then an adequate part of this saturated membrane is taken and fixed between the hydrophobic membrane of the electrode and another filtration membrane as recently described (Vincké et al., 1983a).

(2) Nicotinamide determination procedure

Before each measurement, the bacterial electrode is conditioned in order to reach its baseline at pH 7.80 in a fresh buffered solution of 0.01 M Tris (hydroxymethyl)-aminomethane-hydrochloric acid (Tris-HCl) containing 4×10^{-3} M of magnesium chloride. Quantitative determinations of nicotinamide are carried out in the same buffer at $30.0 \pm 0.2\,°C$ by potentiometry:

$$E = E_0 + S \cdot \log[\text{nicotinamide}]$$

where S = slope of the linear portion of the standard curve.

After runing the experimentals, the electrode is stored at +30 °C in a B.H.I. medium under orbital agitation (60 r.p.m.). This storage mode insures the regeneration of the bacterial enzyme activity.

Results and Discussion

(A) Choice of experimental conditions

(1) Bacterial growth

The investigated bacteria are members of two very different taxonomic groups: *E. coli* and several Bacillus species (*B. pumilus, B. subtilis* and *B. cereus*).

E. coli pncH9 (ATCC 27 195) is a mutant of *E. coli* K 12-2000α, characterized by a 50-fold higher deaminase activity and isolated by mutagenesis and selection on minimal plates containing nicotinamide as the sole nitrogen source (Pardee et al., 1971). But, the nicotinamide deaminase activity maintains a value of several orders of magnitude lower than the activity of many enzymes involved in major bacterial pathways.

Presence of nicotinamide deaminase, present as micro-constitutive enzyme, in several bacilli has been demonstrated. The immobilization of similar cells' weight gives the following enzymatic activity sequence: *B. pumilus* > *B. subtilis* > *B. cereus*.

Besides its great activity, *B. pumilus* presents two other advantages: a better growth rate and a lower sporulation velocity.

Thus, *E. coli* and *B. pumilus* will be used with their best growth rates and enzymatic activities obtained only after 3 subcultures. The third subculture shows the best slope, stability and reproducibility for all constructed electrodes. This fact is in agreement with our previous observations for other unstable and low enzymatic processes of bacteria, like the glutaminase of *E. coli* and the aspartate ammonia-lyase of *Hafnia alvei* (Vincké et al., 1984b).

In several enzymatic systems, the value is pointed out of a preliminary induction of cells in a special medium in order to increase the enzyme synthesis (Vincké et al., 1984b). The study of the effects of the induction on the two electrode responses was investigated. The enzymatic process of the mutated *E. coli* is not increased by a first treatment in the induction medium (Medium 1), followed by two subcultures in B.H.I. (Medium 2). Various substrate concentrations in the induction medium or in B.H.I. give the same results. But, as shown in Fig. 1, under the same conditions it is possible to increase the enzyme production of *B. pumilus*. After this treatment, the *B. pumilus* electrode gives the same responses (same slope and linearity) as those obtained with *E. coli* pncH9 without induction.

(2) Kinetic factors and electrode design

Great care has been taken for the electrode design: for example, studies of the

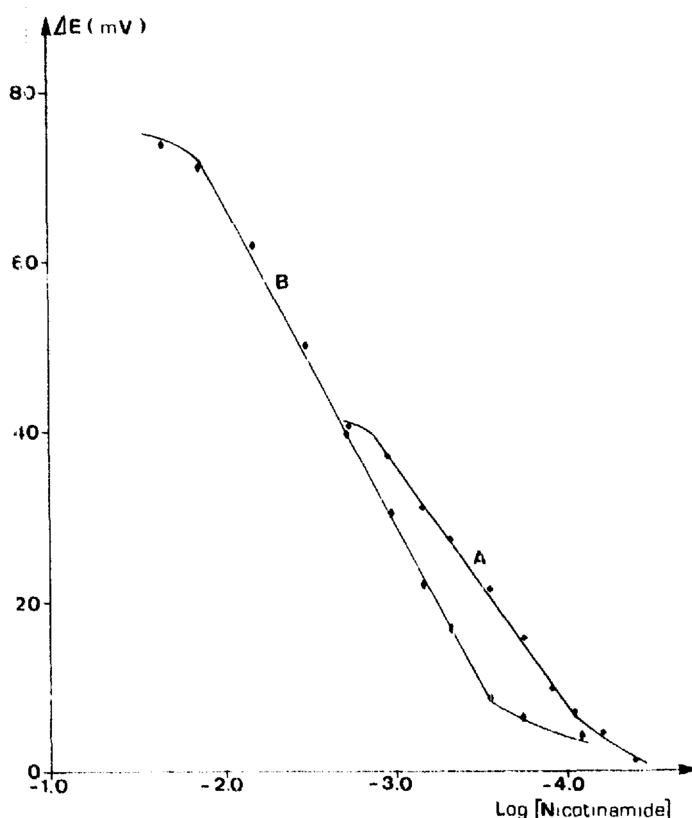


Fig. 1. Influence of the induction of *B. pumilus* on the electrode response. Buffer: Tris-HCl 0.01 M pH 7.80 + 4×10^{-3} M $MgCl_2$, 30.0 ± 0.2 °C. A: *B. pumilus* before induction. B: *B. pumilus* after induction.

parameters involved in the construction and manipulation of an enzyme electrode (gel thickness, gel activity, temperature and pH) to increase the kinetics of this electrode (Eliard et al., 1982). If, in the case of an enzyme electrode, the gel activity and the gel thickness can be separated into 2 factors in the construction of the electrode, for the bacterial electrode the activity of the cellular layer is generally intimately connected to the layer thickness.

The study of the slope (mV/dec.) of the standard curve for bacterial electrodes as a function of the enzymatic activity of the cellular layer shows that beyond a certain activity, the slope attains a maximum and constant value. Thus, we must always immobilize an adequate quantity of bacteria to reach this maximum slope value at particular pH, buffer and temperature conditions. With this maximum slope value, we obtain a long-time reproducibility of the slope and the linearity of the modified electrodes. Because of the presence of the enzymes at low concentrations in the bacteria, the bacterial membrane is generally thicker (over 200 μm) than the enzyme membrane of the corresponding enzyme electrode and so subjected to slow diffusion processes. The result is a poor response time often observed and described also by Guilbault (1983). But the enzyme kinetics are also of great importance. We have made three observations: (1) the response time of both *E. coli* and *B. pumilus* electrodes to nicotinamide is identical: 10–20 min in stationary mode; (2) this time is very similar to that obtained with the purified enzyme (Pardee et al., 1971); and (3) the kinetics of the two electrodes to asparagine with a thickness between 200 μm and 400 μm is the same (4–9 min) as that of the *Serratia marcescens* electrode (layer thickness $\pm$ 100 μm) (Vincké et al., 1983b).

So the response time of such electrodes cannot only be explained by a thickness factor.

Another factor able to increase the response time of bacterial electrodes with enzymatic activity is the use of a dialysis membrane separating the immobilized cells from the solution to be analyzed. Indeed, this cellophane membrane does not allow any excess of solution around the cells to be eliminated during the immobilization. Moreover, from the second day on, we note a swelling of the cellular layer by inclusion of the regenerating liquid. The corresponding modifications in the bacterial electrode response are a reduction of its kinetics, an increase of the baseline potential value and of the time to reach this baseline (2 or 3 h). This observation can be explained by an important retention of ammonia excreted due to the bacterial metabolism during this regeneration period. In fact, electrodes with dialysis membranes are nearly always limited in sensitivity by ammonia that cannot be removed in spite of extensive dialysis. This fact has been also previously demonstrated (Walters et al., 1980; Vincké et al., 1984b). This can be eliminated by the use of a cellulose acetate membrane (Millipore HAWP 047 SO, porosity 0.45 μm). With the porous membrane, the pressure obtained when assembling the electrode squeezes any excess solution present around the bacteria out through the filter. But above all, the porosity favours the exchange of liquids and prevents the retention of the metabolites. In consequence it maintains a low ammonia background level, which is shown by the little fluctuations of the baseline potential, its fast stabilization ($\pm$ 1 h) and the minor modifications of the response times during the life of the electrode.

(3) Buffer choice

The best slope and linearity were obtained in a Tris-HCL buffer, pH 7.80, at 30.0 ± 0.2°C. Since the low enzymatic nicotinamide deaminase activity is shown in bacteria, the use of this buffer at 0.01 M is proposed to increase the response sensitivity. Table 1 shows the influence of ionic strength of the buffer supplemented with 4×10^{-3} M $MgCl_2$ on the *E. coli* electrode response at 30.0 ± 0.2°C.

This low buffer strength is not affected by high nicotinamide concentration values (10^{-1} M). In fact, the enzyme needs a metallic cofactor: magnesium ions. But cells

TABLE 1

INFLUENCE OF THE BUFFER IONIC STRENGTH

Buffer ionic strength (Tris-HCl) + $MgCl_2$ 4×10^{-3} M	Slope (mV/dec.)	Linearity (M)
0.1 M	24	4×10^{-4}–8×10^{-3}
0.05 M	28	4×10^{-4}–1.4×10^{-2}
0.01 M	38	2.8×10^{-4}–2×10^{-2}

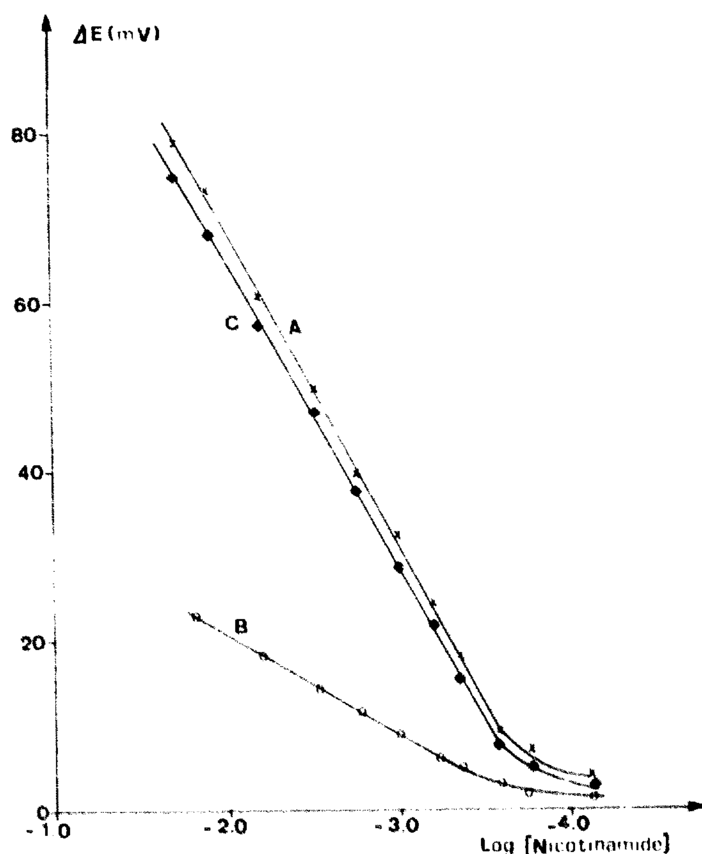


Fig. 2. Influence of magnesium ions as cofactor of nicotinamide deaminase. Buffer: Tris-HCl 0.01 M + 4×10^{-3} M $MgCl_2$, at 30.0 ± 0.2°C. A: buffer only. B: buffer + 10^{-2} M EDTA. C: buffer + 10^{-2} M EDTA + 10^{-1} M $MgCl_2$.

do not sufficiently concentrate the magnesium of the B.H.I. medium (12.18 $\mu g/ml$) for the determination of high concentrations of nicotinamide. So, we used the Tris-HCl 0.01 M pH 7.80 buffer supplemented with 4×10^{-3} M of magnesium chloride for vitamin PP determinations.

Since the magnesium ion appears as an obligatory cofactor, thus the addition of a complexing agent, such as 10^{-2} M of EDTA strongly decreases the response (Fig. 2); the enzymatic activity is restored at $\pm 97\%$ in the presence of 10^{-1} M $MgCl_2$. But the inhibition of EDTA is not restored by other cofactors such as Co^{2+}, Mn^{2+}, Fe^{2+} and Zn^{2+}. Moreover, the use of immobilized cells does not require the addition of ATP which is necessary with the purified enzyme of Pardee et al. (1971). The intracellular ATP source coming from the bacterial glycolytic pathway is sufficient to cover the requirements. These experimental conditions (Tris-HCl, 0.01 M pH $7.80 + 4 \times 10^{-3}$ M $MgCl_2$) are favourable for both *B. pumilus* and *E. coli* electrodes.

(4) Stability of the electrodes

It is well known that the stability of the purified enzyme at 30 °C is only about 20 min. The way we work strongly increases the analytical life time by regenerating the electrode in the growth medium. Table 2 compares the stability of two different types of electrodes and their corresponding analytical characteristics in Tris-HCl 0.01 M pH 7.80 buffer, with 4×10^{-3} M $MgCl_2$ at 30.0 ± 0.2 °C.

The decrease of stability after 3 days in the case of *B. pumilus* is caused by the appearance of a progressive sporulation observed by microscopy in spite of the regeneration. This fact appears also in the study of 3 other enzymatic deamination pathways of *B. pumilus*: such as asparaginase, glutaminase and histidine ammonia-lyase, where a reduction of the slope and the linearity at higher substrate concentration values is also reached after the third day. On the contrary, in *E. coli* the activity is maintained during more than 10 days.

(B) Applications

(1) Determination of nicotinamide in aqueous solutions

Quantitative determinations of nicotinamide have been realized on the two proposed electrodes (*E. coli* and *B. pumilus*). The results, as shown in Table 3, demonstrate a perfect correlation between the two electrodes.

TABLE 2

STABILITY AND ANALYTICAL CHARACTERISTICS OF BOTH ELECTRODES

Type of electrode	Slope (mV/dec.)	Linearity (M)	Stability (days)	Numbers of tested electrodes
E. coli ATCC 27195	38 ± 1	$2.8 \times 10^{-4}-2 \times 10^{-2}$	5	10
B. pumilus after induction	38 ± 2	$2.8 \times 10^{-4}-1.4 \times 10^{-2}$	3	5

(2) Application in galenic forms

The quantitative determinations of vitamin PP in the galenic formulations have been possible on pulverized tablets without filtration and in multivitamin ampoules at lower concentrations of 1×10^{-3} M of nicotinamide.

We recorded that the two strains have the same specificity for nicotinamide and are not influenced by the presence of other vitamins of the B group.

TABLE 3

DETERMINATION OF NICOTINAMIDE IN Tris-HCl 0.01 M pH 7.80 BUFFER + $MgCl_2$ 4×10^{-4} M AT 30.0 ± 0.2°C

Nicotinamide added (mg/l)	Nicotinamide found [a] (mg/l)		Standard deviation (mg/l)		Coefficient of Variation (%)	
	E. coli	*B. pumilus*	*E. coli*	*B. pumilus*	*E. coli*	*B. pumilus*
34.1	34.5	34.0	1.1	1.3	3.2	3.8
58.3	57.9	58.5	1.8	1.6	3.1	2.7
82.5	82.7	83.1	2.0	2.1	2.4	2.5
130.5	130.5	129.9	3.1	2.9	2.4	2.2
225.3	224.5	226.1	4.9	4.7	2.2	2.1
410.6	411.1	409.3	10.0	10.8	2.4	2.6
849.8	845.1	853.2	13.9	15.2	1.6	1.8

[a] Average of 5 measurements.

TABLE 4

TABLETS

Formulation: nicotinamide 100 mg–Amyl. Mayd.–Calc. Stear.–Polyvidon.–Talc: Q.S. ad Tablett. compress. un.

Treatment of average sample	Added (mg/l)	Found [a] (mg/l)	Standard deviation (mg/l)	CV (%)
Without filtration	97.2	97.1	2.2	2.3
With filtration	97.2	96.7	2.9	3.0

[a] Average of 5 measurements with *B. pumilus* electrode.

TABLE 5

AMPOULES: VITAMINS OF B-GROUP

Formulation: Thiamin. chlorid. 10 mg–Riboflavin. (sub. forma Natr. Riboflavin. phosphoric.) 4 mg–Pyridoxin. chlorid. 4 mg–Nicotinamide. 40 mg–Dexpanthenol 6 mg–cyanocobalamin. 0,008 mg–Biotin. 0, 50 mg–Benzylic. Alcohol–Aqua ad 2 ml.

Theoretical (mg/l)	Found [a] (mg/l)		Standard deviation (mg/l)		CV (%)	
	B. pumilus	*E. coli*	*B. pumilus*	*E. coli*	*B. pumilus*	*E. coli*
97.0	96.1	96.6	1.8	2.3	1.9	2.4

[a] Average of 3 measurements.

Conclusions

The use of bacterial electrodes finds all its significance in the analytical application of enzymatic systems of low activities, showing a strong instability incompatible with the commercialization of the enzyme. Various enzymes have no specificity from the taxonomic point of view, such as nicotinamide deaminase found in *E. coli*, several other bacilli and also in *Aspergillus niger*, several lactobacilli and yeasts. According to the type of medium to be analyzed (pharmaceutical formulations, biological media, nutriments, etc.), the choice of the strain will be imposed by the interfering compounds, which are specific to the strain. Thus in the case of vitamin PP determinations, both studied electrodes are selective in commercial multivitaminic forms, but they will not always be selective in other mixtures. We have also shown the importance of the induction of micro-constitutive enzymes, which will in certain cases be of interest due to their simplicity and take the place of the mutagenetic techniques. We have indicated the influence of the microbiological factors in the improvement of the strains intended for the analytical determination of substrates and the interest of electrochemical methods in the biochemical study of the microorganisms.

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