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ADSORPTION PHENOMENA IN THE NAD^+/NADH SYSTEM AT GLASSY CARBON ELECTRODES

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ABSTRACT

A variety of electrochemical approaches has been used to investigate the adsorption of NAD^+, NADH and the NAD-NAD dimer from aqueous solution at glassy carbon electrodes (GCE) with supplementary studies of adsorption at pyrolytic graphite and platinum electrodes from aqueous media and at GCE from DMSO solution. The following hypotheses are advanced concerning the adsorption orientation: at carbon electrodes, on which NADH is not adsorbed, NAD^+ produced by anodic oxidation of the NADH is first rapidly adsorbed in a planar configuration relative to the electrode surface, which is probably bound to the surface through the adenine moiety; there is then a relatively slow reorientation of the adsorbed NADH molecules to a perpendicular orientation relative to the electrode surface, which adsorbate is more tightly bound to the surface than the planar oriented adsorbate and which likely involves interaction between parallel adenine and pyridinium rings. Reduction (one-electron process) of NAD^+ at the GCE produces the NAD-NAD dimer, which, at a clean electrode surface, involves a diffusion-controlled process and an adsorption-controlled process; the latter is due to formation of adsorbed dimer, which is more strongly adsorbed than NAD^+. The dimer is oxidized at the GCE only if it is adsorbed. The factors controlling and involved in the adsorption processes have been examined with particular reference to the use of anodic voltammetry for the analytical determination of NADH.

INTRODUCTION

Previous studies [1,2] on the electrochemical two-electron ($2e$) oxidation at carbon electrodes in aqueous media of dihydronicotinamide adenine dinucleotide (NADH) to yield the corresponding nicotinamide (NAD^+), indicated the analytical chemical and biochemical interest in this reaction, described the effect of experimental conditions and variables in producing a prewave or prepeak in the current-potential relations as a result of the adsorption on the electrode of NAD^+ produced in the oxidation, and set forth procedures for the voltammetric oxidation of NADH, which would avoid the overlapping waves or peaks due to the presence of both the adsorption-controlled and diffusion-controlled processes by suppressing the current due to the production of adsorbed molecules of NAD^+. These studies involved further elucidation of the factors affecting the

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oxidation and may lead to a better understanding of the mechanism for the electrochemical oxidation of NADH.

The present paper describes a more detailed consideration of the factors determining the adsorption of NAD^+ on glassy carbon electrodes from aqueous media and of the nature of the adsorbate as affected by experimental conditions. Brief complementary studies were made of the adsorption of NAD^+ on pyrolytic graphite and platinum electrodes, since these electrodes have been used in studies and analytical procedures involving the oxidation of NADH. The adsorption of NAD^+ onto glassy carbon electrodes from dimethylsulfoxide solution was also examined. A variety of electrochemical approaches was used.

Since NAD^+ is initially reduced electrochemically at the carbon electrode as well as at the mercury electrode in a 1*e* process to form a free radical which rapidly dimerizes [3], the adsorption of the dimer was also examined.

There is no evidence to support the adsorption of NADH to any appreciable extent on the electrode surfaces examined under the conditions used.

EXPERIMENTAL

Unless otherwise specified, chemicals, electrodes, apparatus and procedures were the same as previously described [1,2].

Chemicals

1,4-Dihydronicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide (NAD^+) and adenosine-5'-diphosphoribose sodium salt (ADPR) (P-L Biochemical ChromatoPure grade) were used as received on the basis of polarographic and spectrophotometric examination. Reagent grade chemicals were obtained from J.T. Baker except for 2-amino-2-methyl-1,3-propanediol (Tris) from Sigma, tetra-n-butylammonium perchlorate from G. Frederick Smith, and dimethylsulfoxide (DMSO) from Fisher.

Electrodes

The working glassy carbon electrodes (GCE) used were those previously designated as GC-1 and GC-2 [1]; their areas were 0.230 cm^2 for GC-1 and 0.254 cm^2 for GC-2. The working pyrolytic graphite electrode (PGE) (area = 0.383 cm^2) and the working platinum electrode (PE) (area = 0.0090 cm^2) were also previously described [1,2]. These were used as stationary or rotating disk electrodes. The reference electrode was an aqueous saturated calomel electrode, to which all potentials cited are referred. The counter electrode was a platinum gauze.

Apparatus

Electrochemical measurements were made with a Princeton Applied Research 170 multipurpose instrument and a three-compartment water-jacketed cell [4], whose counter and reference electrode compartments were filled with the background solution and whose temperature, unless otherwise specified, was 25°C.

For cyclic voltammetry, a Tektronix 5103N cathode ray oscilloscope with suitable modules and cameras was used. Electrodes were rotated by a Sargent synchronous motor (10 rps). CPK space-filling Ealing precision molecular models were used for behavior and orientation simulation.

Electrode preparation

A pretreated or *clean* electrode is one which has been subjected to the Blaedel and Jenkins procedure [5], i.e., the electrode was conditioned (15 sweeps from 1.50 to −1.50 V at 0.1 V s^{-1}) and pretreated (1.50 V for 2 min; −1.50 V for 2 min; repeated twice) in the background solution. Following pretreatment, background cyclic voltammograms (0.00 to 0.75 V; scan rate (v) = 2 mV s^{-1}) were recorded at the rotating disk electrode (RDE) until successive cycles yielded superposable curves; NADH was added and the RDE was held at the initial potential of 0 V for 2 min before recording each voltammetric curve. Electrode pretreatment was unnecessary between measurements on the same NADH solution. Between experiments on different NADH solutions, the electrode was rinsed with distilled water, wiped with a paper tissue, and stored while immersed in distilled water.

A *covered* electrode, i.e., one coated with adsorbed NAD^+ (cf. text) was prepared by rotating the electrode, held at 0.75 V, in a 1 m*M* NADH solution for 1 h. Reproducible results with such an electrode were obtained by merely rinsing it with distilled water, storing it in the air when not in use, and not exposing it to potentials more negative than −0.5 V (cf. ref. 2 for details). When used, the only requirement is to rotate the electrode, held at the initial potential of 0 V, for 2 min before recording each voltammetric curve.

Each new electrode was first conditioned and pretreated, and then used either as a clean electrode or as one covered with adsorbed NAD^+.

BEHAVIOR OF NADH AT A GLASSY CARBON ELECTRODE

Emphasis was placed on the use of the GCE designate as GC-2 [1]; the adsorption of NAD^+ is less at this electrode than at the PGE and at the other type of GCE examined. The primary approach was through the voltammetric current-potential (i—E) curves produced at a clean, i.e., pretreated, GC-2 electrode on the electrochemical oxidation of NADH, which normally shows two anodic peaks at carbon electrodes on sweep voltammetry at a stationary electrode or derivative pulse polarography at a rotated electrode, if the NADH concentration exceeds ca. 0.15 m*M* [1]; the less positive peak is due to the oxidation of NADH to adsorbed NAD^+. The first peak is adsorption-controlled; the second peak is diffusion-controlled.

On addition of 2 m*M* NAD^+ to a 1 m*M* NADH solution (background: 0.5 *M* KCl as supporting electrolyte; 0.05 *M* phosphate or 0.05 *M* Tris as pH 7.0 buffer), the derivative pulse polarographic curves do not show any detectable peak corresponding to an adsorption-controlled process (Fig. 1). Voltammetric curves at a stationary electrode do not show an adsorption-controlled peak when the potential scan rate, v, is less than 20 V s^{-1}; such a peak becomes apparent when v exceeds 20 V s^{-1}. Identical voltammetric curves are recorded between pH 5 and 9.5.

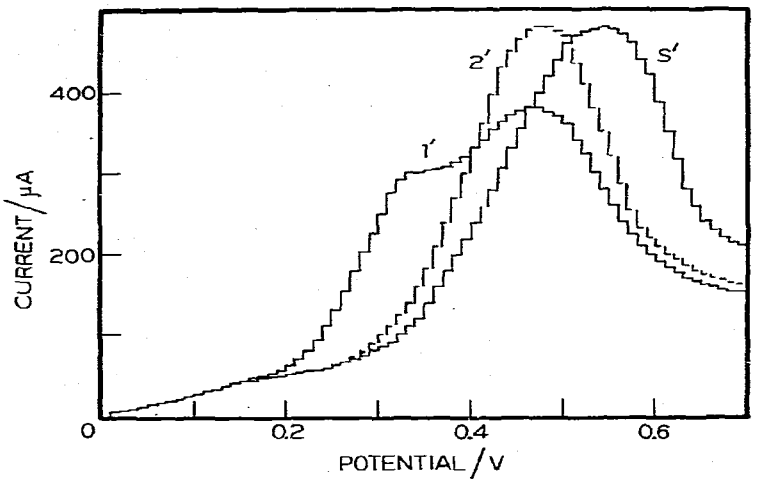


Fig. 1. Derivative pulse voltammograms at the rotated GCE (GC-2). Scan rate, v = 2 mV s^{-1}. Pulse characteristics: time interval between successive pulses = 5 s; sample duration time at the end of each pulse = 5 ms. Background: 0.5 *M* KCl; 0.05 *M* phosphate buffer; pH 7.0. (1') 1 m*M* NADH at a pretreated electrode. (2') Same as (1') but with 2 m*M* NAD^+ added. (S') 1 m*M* NADH with the RDE held at 0.75 V for 1 h in a 1 m*M* NADH solution or for 0.5 h in a 1 m*M* NADH, 2 m*M* NAD^+ solution before the experiment.

On repeated continuous cyclic potential scanning between 0.0 and 0.8 V at the RDE (v = 2 mV s^{-1} , the half-wave potential ($E_{1/2}$) of the wave slowly becomes more positive with time while the limiting current (i_Q) remains practically constant (Fig. 2); after ca. 2 h, the curve reaches a steady-state shape (curve

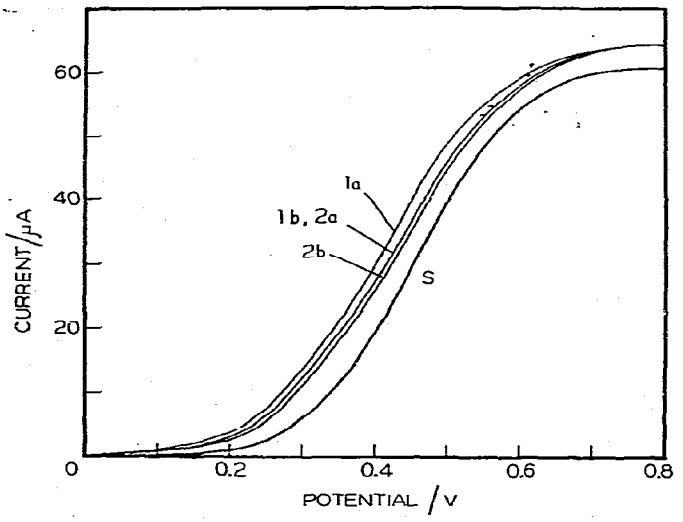


Fig. 2. Effect of time on voltammetric curves at v = 2 mV s^{-1} for NADH at the rotating GCE (10 rps), corrected for background current. Solution composition: 0.85 m*M* NADH; 2 m*M* NAD^+; 0.5 *M* KCl; 0.05 *M* Tris buffer; pH 7.1. Number indicates the chronological order of the cycle; a indicates a scan with increasingly positive potential and b with decreasing potential; S identifies the steady state pattern obtained after ca. 2 h (with forward and back scans). (Reproduced with permission from ref. 2.)

S in Fig. 2). The difference in i_Q between curves 1 and S corresponds to the NADH electrolyzed during the 2 h. Curve S can be more rapidly obtained by holding the RDE in the solution at 0.75 V for 30 min. Such a $E_{1/2}$ shift occurs at the same slow rate between pH 5 and 9.5. If NAD^+ is not originally added to the solution, a longer time (1 h) is needed to reach the steady state.

A similar shift is seen at a stationary electrode at considerably higher v (0.5 V s^{-1}) until the voltammetric curve reaches a steady state shape. Holding the RDE at 0.75 V also affects the derivative pulse polarographic curves and a steady state curve S' as shown on Fig. 1 may be obtained after 0.5 h in a 2 m*M* NAD^+, 1 m*M* NADH solution or 1 h in a 1 m*M* NADH solution.

NATURE OF THE ELECTRODE SURFACE

The steady state curves obtained, e.g., as the result of holding a glassy carbon RDE at 0.75 V in an NADH solution of 1 h (or longer) or of repeated cycling between 0.0 and 0.8 V, most probably result from an effective saturation coverage of the electrode surface with adsorbed NAD^+ molecules.

Presence of rather strongly adsorbed NAD^+ on the electrode surface was confirmed as follows. After rotation at 0.75 V for 1 h in 1 m*M* NADH solution (0.5 *M* KCl; 0.05 *M* phosphate buffer; pH 9.0), the electrode was removed, rinsed with distilled water, and immersed in a deoxygenated background solution; the voltammogram was then recorded at the stationary electrode between 0.0 and

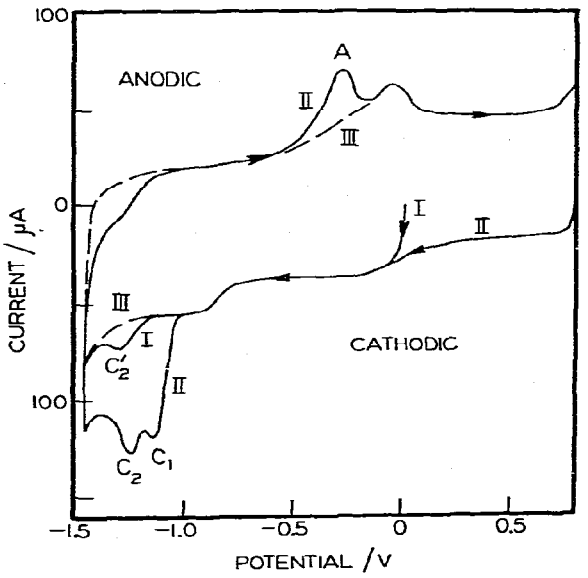


Fig. 3. Voltammograms at the stationary GCE (GC-2) at v = 0.5 V s^{-1}. Background: 0.5 *M* KCl; 0.05 *M* phosphate buffer; pH 7.0. (I) Electrode covered with adsorbed NAD^+ (span of 0.0 to −1.5 V). (II) Pretreated electrode in the presence of 1 m*M* NAD^+ (span from 0.7 to −1.5 V and return; triangular sweep). (III) Same as (II) with background solution only (dashed line, which is omitted where it coincides with curves I or II). Preparation and previous treatment of the electrodes are described in the text. Arrowheads indicate the direction of the scan.

—1.5 V (Fig. 3, curve I). In a cyclic experiment, a cathodic peak C_2' (E_p = —1.28 V) appears on the first scan while only the background current is recorded on the second scan. Peak C_2' can be identified as corresponding to the reduction of NAD^+ on the basis of past studies [6], and on the basis of the subsequently described and discussed results on the behavior of NAD^+ and of the NAD-NAD dimer formed on reduction of NAD^+ at the GCE. Consequently, the area of peak C_2', corrected for the background current, corresponds to the electrical charge consumed by reduction of the amount of NAD^+ adsorbed on the electrode. Assuming that adsorbed NAD^+ forms a monolayer, the area occupied by one molecule of adsorbed NAD^+ is 90 ± 10 Å^2 (mean and standard deviation for 3 experiments at 3 different pH values), since the reduction of NAD^+ leading to the dimer is a 1*e* process [3].

ORIENTATION OF ADSORBED NAD^+: FAST AND SLOW ADSORPTION PROCESSES

The adenine moitey is assumed to be the site involved in the adsorption of NAD^+ at the mercury electrode [7]. This may be assumed to be still true at the GCE, at least in a first step in which adsorption and desorption processes may occur rather rapidly as generally admitted.

Planar adenine, based on its crystal structure, has an area of 42 Å^2 [8] but, considering the unsymmetrical shape of the molecule and the resultant difficulty in packing on the surface, the actual area occupied by an adenine molecule was estimated to be 50 to 60 Å^2 and was found to be 55 ± 4 Å^2 as a result of analysis of the capacitance curves for adenine at a mercury electrode, at least for the first adsorption layer [9]. The same result was found with deoxyadenosine and the authors concluded [9] that the deoxyribose group is tilted away from the electrode surface. However, these results do not seem to apply to the area occupied by a flat orientation of the adenine side of NAD^+ on the electrode since, if NAD^+ is assumed to have the conformation of a folded molecule with the adenine and pyridinium rings being parallel [10], the minimum projected area corresponding to a molecular model of NAD^+ in such an orientation is at least 125 Å^2, while the minimum projected area, when the planes of the adenine and pyridinium rings are assumed to be perpendicular to the electrode surface, is about 85 Å^2. Moreover, in the latter orientation, purine-pyrimidine type association [11] can occur in the adsorbed state between the parallel adenine and pyridinium rings of stacked adsorbed NAD^+ molecules as assumed in similar cases [12—14].

Rapid adsorption: planar conformation

We may assume then that, in a first step, a rather fast adsorption-desorption process occurs involving planar adsorption of NAD^+. This adsorption, due to the adenine site, may account for the adsorption-controlled process observed on normal and pulse polarography at the pretreated RDE and on linear sweep voltammetry at a stationary pretreated electrode [1]. As this process is rapid, there is an equilibrium between the activity of NAD^+ in the bulk of the solution and that of adsorbed NAD^+; as previously noted, a bulk concentration of 2 m*M* NAD^+ is sufficient to cause the adsorption-controlled process to disappear on

normal and pulse polarography and on linear sweep voltammetry when v is less than 20 V s^{-1}. However, as the current due to the adsorption-controlled process is proportional to v [1], a prepeak corresponding to this process appears on linear sweep voltammetry when v exceeds 20 V s^{-1}, showing that the concentration of adsorbed NAD^+ in equilibrium with a 2 mM bulk concentration is not sufficient to block all of the sites of the electrode surface for planar adsorption of NAD^+.

Slow reorientation: perpendicular conformation

In a second step, a reorientation may slowly occur, leading to the association of perpendicularly oriented adsorbed molecules as shown schematically in Fig. 4. In such a case, there are two possible orientations for adsorbed NAD^+ molecules: the phosphate groups are close to the electrode surface or the opposite. The negatively charged phosphate groups may be attracted by the positively charged electrode surface but, on the other hand, the sugar-phosphate groups are hydrophilic while the adenine and pyridine rings tends to be hydrophobic and exhibit an aromatic azabenzene character which may favor binding to the carbon ring structure of the electrode.

According to the assumption just outlined, the steady-state voltammetric curves S and S′ (Figs. 2 and 1) are obtained when the electrode surface is totally covered with associated, perpendicularly oriented adsorbed molecules. At a potential more negative than −1.3 V, the adsorbed NAD^+ is reduced and, consequently, desorbed since curves similar to curves 1a and 1′ in Figs. 2 and 1 are recorded for an initial potential more negative than −1.3 V with a NADH solution.

Interaction between the adenine and pyridinium rings of adsorbed NAD^+ molecules seems necessary in order to produce a coverage of the electrode

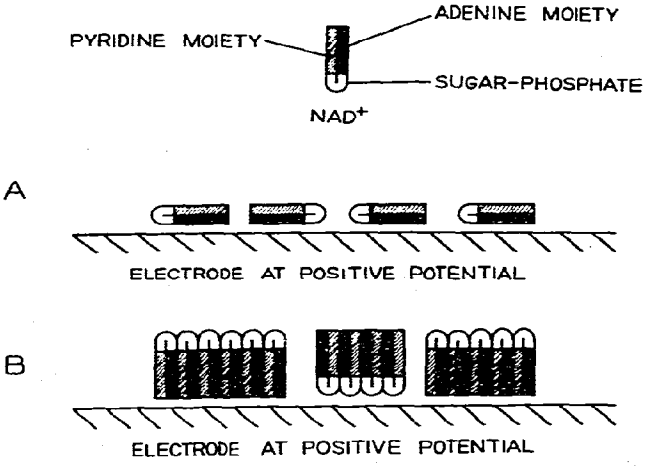


Fig. 4. Schematic representations of possible orientations of NAD^+ at a mercury electrode/aqueous solution interface. (A) Planar adsorption. (B) Adsorption and association of perpendicularly oriented molecules.

involving perpendicularly oriented adsorbed molecules since a pretreated RDE held for 1 h at 0.75 V in a 1 m*M* ADPR solution behaves differently from the same electrode held for 1 h at 0.75 V in 1 m*M* NAD^+. In the former case, $E_{1/2}$ for NADH oxidation shifts positively when NADH is added and successive voltammetric curves are recorded, until it reaches the value corresponding to the steady-state curve S. In the second case, the first scan after NADH addition gives curve S. With ADPR, interaction between adenine and pyridinium rings is not possible since the ADPR molecule is identical to the NAD^+ molecule without its pyridinium ring. However, as ADPR contains the adenine moiety, its presence in the solution makes the adsorption-controlled process, due to the production of adsorbed NAD^+ in the flat orientation, disappear from the voltammetric curves corresponding to the electrochemical oxidation of NADH at a pretreated electrode, just as NADH would behave in the presence of the same bulk concentration of NAD^+.

Slow adsorption-desorption

Slow desorption of the adsorbed NAD^+ from the surface of a covered electrode also occurs. For example, voltammetric curves analogous to curves 1a and 1′ of Figs. 2 and 1 are again recorded if a covered RDE, which originally gave curves S and S′, has been held at 0.0 V for more than a hour. However, the dependence of the desorption rate upon the electrode potential has not been systematically examined.

The assumption of slow coverage of the electrode surface with adsorbed NAD^+ is in accord with the evolution of the voltammograms reported on Fig. 2. The difference in current values between curves 1a and S is greater in the 0.2—0.4 V interval than in the 0.5—0.7 V interval; therefore, the slope of curve S is steeper than that of curve 1a. This is probably due to the fact that, in the case of curve 1a, the current in the 0.2—0.4 V region includes a large proportion of current provided by the adsorption-controlled process while this process does not occur in the case of curve S. It is also evident that curves 1b (current vs. decreasing potential) and 2a (current vs. increasing potential) are almost superposable, whereas curves 1a and 1b of the first cycle and curves 2a and 2b of the second cycle differ, respectively, to an appreciable extent.

A similar situation holds for each couple of two successive cycles and can be explained by the fact that, on recording the i—E curve as E increases, e.g., curve 1a, the current increases, and the end of this curve and the beginning of the following one, e.g., curve 1b, correspond to a large current density (limiting current plateau) and, therefore, to an appreciable production of NAD^+ at the electrode surface, which is slowly but strongly adsorbed. Then, while recording curve 1b, the capability of the electrode surface for the adsorption of NAD^+ is slightly decreased compared to curve 1a and, at each potential value below the region corresponding to the limiting current plateau, curve 1b exhibits a smaller current than curve 1a since the amount of current due to the adsorption-controlled process is consequently smaller. The end of curve 1b and the beginning of curve 2a correspond to a low current density; in this region, the amount of NAD^+ adsorbed on the electrode surface does not change very much. Thus, while recording curve 2a, the capability of the electrode surface for the adsorp-

tion of NAD^+ is not appreciably different as compared to curve 1b; as a result, curves 2a and 1b are almost superposable.

Conclusions concerning adsorption

In summary, two adsorption processes are involved in the electrochemical oxidation of NADH, both of which are related to the adsorption of the oxidation product, NAD^+. The first process, which is rapid and which may be assumed to be due to the adsorption of NAD^+ in a planar conformation, is responsible for the adsorption-controlled process which appears on the voltammetric curves obtained with a pretreated electrode and which may be hindered by the coverage of the electrode surface with adsorbed NAD^+ as the result of an electrolytic process or of the presence of a relatively high concentration of NAD^+ in the test solution. The second process is slow and may be assumed to be due to the adsorption of NAD^+ in a perpendicular configuration; an electrode covered with NAD^+ so adsorbed provides the optimum conditions for the reproducibility of the voltammetric curves (cf. data in refs. 1 and 2).

The assumption of two different kinds of adsorption layers, as structurally defined, is justified but not conclusively established by the electrochemical data. However, the hypotheses advanced concerning the adsorption orientation provide the more economical explanation of the observed behavior (occurrence of a fast process; occurrence of a slow process producing tightly adsorbed NAD^+; area occupied by one molecule of tightly adsorbed NAD^+; difference between the adsorption behavior of ADPR and NAD^+), and are in harmony with the explanations of other authors concerning the variation in behavior of adsorbed species [9—14].

In a previous paper [15], related to the analysis of the sweep voltammetric curves obtained for the oxidation of NADH at a stationary GCE, the passivation of the electrode surface (shift of the peak potential, E_p, toward positive potential; attenuation of the peak height, i_p) and the slope of 0.58 obtained for the plot of $\ln i_p$ vs. $\ln v$ (instead of the 0.50 expected for a diffusion-controlled process) were attributed to the adsorption of NADH since the adsorption-controlled prepeak was not detected at the pretreated electrode. The non-appearance of this prepeak was probably due to the use of relatively concentrated NADH solutions (2.6 *mM*) [15], under which conditions the peaks due to the adsorption-controlled and diffusion-controlled processes overlap [1]. The present results show that, at the least, NAD^+ is more strongly adsorbed than NADH, and that the adsorption of NAD^+ is mostly responsible for the complexity of the voltammetric behavior of NADH at a pretreated GCE. No evidence has been found to support the adsorption of NADH to any appreciable extent on the electrode surface.

BEHAVIOR OF NAD^+ AND NAD-NAD DIMER AT A GCE

In order to identify peak C_2' in Fig. 3 as due to the reduction of a coated electrode, as previously described, the electrochemical reduction of NAD^+ and the oxidation of its reduction product, which is the NAD-NAD dimer, at the GCE, were examined.

Pretreated electrode

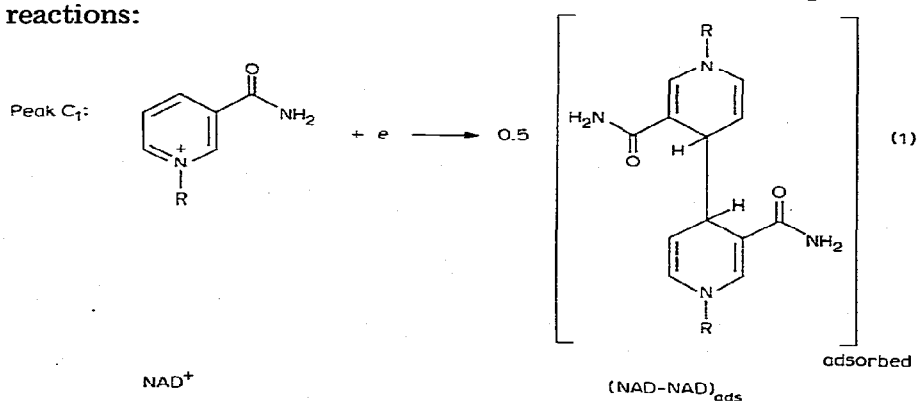
A triangular sweep voltammogram obtained with 1 m*M* NAD^+ at a stationary pretreated GCE (Fig. 3: curve II) exhibits two cathodic peaks C_1 and C_2 (E_p = —1.14 and —1.24 V), respectively, and one anodic peak A (E_p = —0.27 V). These peak potentials are pH-independent in the pH 7 to 10 range. Both peak C_1 and peak A heights increase linearly with v, showing that they correspond to adsorption controlled processes [16]. Peak C_2 height increases linearly with $v^{1/2}$ (diffusion-controlled process). If the NAD^+ solution concentration decreases, the heigts of peaks C_1 and A remain practically constant while the height of peak C_2 decreases. The heights of peaks C_1 and A only begin to decrease when the NAD^+ concentration is so low that peak C_2 does not appear. Occurrences of peaks C_1 and A are related since, if the forward scan is limited to —1.20 V (before peak C_2 appears), the peak A is practically unchanged on the backward scan.

An exhaustive electrolysis of a 1 m*M* NAD^+ solution at a pretreated rotated GCE, potential-controlled at —1.20 V, produces a solution which can be identified as approximately 0.5 m*M* in NAD-NAD dimer from its polarographic behavior (presence of anodic wave at $E_{1/2}$ = —0.28 V at the dropping mercury electrode, which produces NAD^+ [17]). The quantity of electricity consumed during the electrolysis at the rotated GCE cathode at E = —1.20 V, when corrected for the background current, corresponds to a one-electron transfer per NAD^+ molecules processed with the same precision as obtained at a mercury pool cathode [17].

Triangular sweep voltammetry on a dimer solution between —1.5 and +0.5 V at a stationary pretreated GCE shows on the forward scan an anodic peak with the same characteristics as peak A (Fig. 3), and, on the backward scan, a cathodic peak of approximately the same area (corrected for background current) as the anodic peak and having the same characteristics as peak C_1 (Fig. 3); no peak is detectable at the potential of peak C_2.

The second cathodic peak observed on reduction of NAD^+ at a stationary pretreated GCE (peak C_2 on Fig. 3) does not correspond to the production of NADH since a current due to the oxidation of NADH never appears in the region of 0 to 0.5 V of the cyclic voltammogram, not even when the potential range is extended till 0.8 V. A potential-controlled electrolysis at E = —1.40 V at a rotated pretreated GCE produces the NAD-NAD dimer (identified from its polarographic behavior).

Thus, it is safe to assume that peaks C_1, C_2 and A correspond to the following reactions:



where R represents adenosine diphosphoribose (ADPR) and most of the dimer produced is assumed to have a 4,4′ structure as at the mercury cathode [3,18].

Peak C_2: $NAD^+ + e \rightarrow 0.5$ NAD-NAD (2)

Peak A: $0.5\ (NAD\text{-}NAD)_{ads} \rightarrow NAD^+ + e$ (3)

As the product of reaction (1) is adsorbed, peak C_1 occurs at a less negative potential than peak C_2 [19].

The occurrence of two processes for the reduction of NAD^+ at a pretreated electrode, the first one being adsorption-controlled and the second one being diffusion-controlled, is confirmed by the normal and derivative pulse polarographic curves obtained at the rotated electrode with a 1 m*M* NAD^+ solution (Fig. 5). If the NAD^+ solution is 0.4 m*M* (or less), only one normal pulse polarographic wave and one derivative pulse polarographic peak are recorded at the same potentials as wave K_1 and peak K_1' of Fig. 5, respectively.

Factors influencing the adsorption of the dimer

In connection with reactions (1) and (3), the eventuality was not mentioned of the adsorption of NAD^+, which is known to occur at the mercury electrode in the same potential region [3,18]. As previously described (cf. also ref. 1), NAD^+, adsorbed at a potential greater than 0.3 V, desorbs at 0.0 V.

NAD^+ adsorbed on a coated electrode (postulated to be adsorbed in the per-

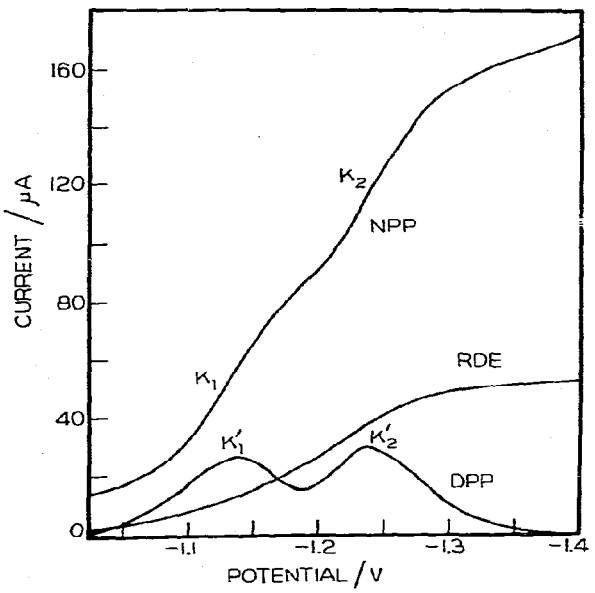


Fig. 5. Voltammetry (curve RDE), normal pulse polarography (curve NPP), and derivative pulse polarography (curve DPP) at the rotated and pretreated GCE (GC-2) for 1 m*M* NAD^+ in 0.5 *M* KCl and 0.05 *M* phosphate buffer of pH 7.0 at $v = 2$ mV s^{-1}. Pulse characteristics: time interval between successive pulses = 0.5 s; sample duration time at the end of each pulse = 5 ms.

pendicular conformation) prevents the adsorption of the dimer, since no anodic current due to oxidation of the dimer is recorded for a dimer solution between —0.6 and +0.5 at a coated electrode and since only cathodic peak C_2 of Fig. 3 is recorded for a NAD^+ solution at a coated electrode. Curve I (Fig. 3) does not show a prepeak similar to C_1 of curve II and, as expected, E_p for C_2' (—1.30 V) is more negative than that of C_2 since peak C_2' corresponds to the reduction of strongly adsorbed NAD^+ (assumed perpendicular conformation), which is, consequently, more difficult to reduce than either non-adsorbed NAD^+ or less strongly adsorbed NAD^+ (assumed planar conformation) [19]. Moreover, experiments at $v = 0.5$ and 1 V s^{-1} showed that the height of peak C_2' increases linearly with v as expected for the reduction of an adsorbed reactant [16]. Consequently, it may be concluded that, when reactions (1) and (3) are observed, the NAD^+ involved in these reactions is either non-adsorbed or, if adsorbed, only adsorbed in the planar conformation with the dimer being more strongly adsorbed as at the mercury electrode [3].

At the mercury electrode, it is possible to prevent the adsorption of both NAD^+ and the dimer by adding more strongly adsorbed tetraethylammonium ions [3,18] to the solution. Such an addition does not show any appreciable effect on the voltammetric behavior of NAD^+ and of the dimer at the GCE.

However, the adsorption of the dimer at the GCE is very sensitive to the nature of the electrode pretreatment. Generally, when a GCE, whatever its previous history, is held for 2 min or more at a potential more negative than —1.4 V in a background solution and its potential is then maintained negative to 0 V, peaks such as C_1 and A of Fig. 3 due to the dimer adsorption are not obtained; however, such an electrode shows the usual behavior of a normally pretreated electrode (as described in the Experimental section) with NADH. On the other hand, if the electrode is first cleaned at —1.5 V of any adsorbed NAD^+ and is then held for 2 min or more at a potential more positive than 0.5 V (or much longer in the 0 to 0.5 V potential region), peaks such as C_1 and A are again obtainable. Such a result explains why peaks C_1, C_2 and A (Fig. 3) were recorded with an electrode pretreated as described in the Experimental section since the pretreatment ends with holding the electrode at 1.5 V for 2 min.

When a stationary electrode, coated with NAD^+, is cleaned by immersing it in a fresh background solution and running a voltammogram between 0.0 and —1.5 V (Fig. 3: curve I), a cathodic peak appears at ca. —1.3 V due to reduction of the adsorbed NAD^+, presumably to the soluble dimer since no anodic peak appears on the return scan; addition, then, of NAD^+ to the solution produces only one cathodic peak at the potential of peak C_2 of Fig. 3 on triangular sweep voltammetry (0.0 to —1.5 V), which would — in the case of a pretreated electrode — have indicated saturation coverage of the electrode with dimer. Furthermore, addition of dimer to a background solution containing an electrode treated as just described does not produce an anodic current as would result from its oxidation.

It may, accordingly, be concluded that the dimer is oxidizable at the GCE only if adsorbed and that its adsorption is hindered when the adsorption sites are blocked either by tightly adsorbed NAD^+ or by holding the electrode at a potential more negative than —1.4 V before using it (in the negative potential region).

BEHAVIOR AT GRAPHITE AND PLATINUM ELECTRODES

Pyrolytic graphite electrode.

The results on electrochemical oxidation of NADH at the PGE are generally similar to those observed for the GCE.

In order to obtain an electrode surface sufficiently covered with adsorbed NAD^+ to reach a steady state for the wave at the RDE with no adsorption-controlled process appearing at a stationary electrode on either derivative pulse polarography or sweep voltammetry (v up to 50 V s^{-1}), the rotating PGE had to be held at 0.75 V for 4 h in a 1 m*M* NADH solution compared to about 1 h for the GCE. Based on the assumption concerning adsorption orientation of NAD^+, the latter behavior may be related to the fact that the pyrolytic graphite surface consists of more or less flat oriented hexagonal carbon rings, on which the adsorption of NAD^+ in the flat orientation is probably easier than at the GCE and the process of building a coverage of the electrode surface with perpendicularly oriented adsorbed NAD^+ molecules is slower. At the covered PGE, the area occupied by an adsorbed NAD^+ molecule was estimated to be 110 ± 10 $Å^2$ compared to 90 ± 10 $Å^2$ at the GCE.

Platinum electrode

The only pretreatment necessary in obtaining reproducible i—E curves for the NADH oxidation at PE was to rotate the electrode before each scan for 2 min, while held at the initial 0.0 V potential [2]. An adsorption-controlled process did not appear on normal or derivative pulse polarography at the RDE or on sweep voltammetry at the stationary electrode.

Although adsorption of NAD^+ at the PE was not seen, adsorption of other species may account for the unexpected shift of $E_{1/2}$ with pH. Compared to their values at pH 7, $E_{1/2}$ at the platinum RDE is 90 mV more positive and $dE/d\{\log[i/(i_Q - i)]\}$ is increased by 35 mV at pH 10 in both KCl and Na_2SO_4 backgrounds. Although this increase in $E_{1/2}$ agrees with the previously reported $\Delta E_{1/2}/\Delta pH$ ratio of about +30 mV/pH [20,21], it is still rather surprising, since the $\Delta E_{1/2}/\Delta pH$ ratio would be expected to be zero or negative for the grossly irreversible process [22]:

$$NADH \rightarrow NAD^+ + H^+ + 2\,e \qquad (4)$$

A reaction such as the deprotonation of NADH, which may occur near the electrode surface, would be expected to be very sensitive to changes in the electrode surface characteristics and in the basicity of the medium near the electrode.

It has been established that the potential region at the PE corresponding to the adsorption and discharge of hydroxyl anions to produce adsorbed hydroxyl groups is considerably broadened toward less positive potentials in basic media [23], and even overlaps the potential region corresponding to the oxidation of adsorbed hydrogen. Such an adsorption and discharge of hydroxyl anions above pH 8, which occurs at the same potential as the oxidation of NADH, implies a change in the electrode surface characteristics and a decrease in the basicity of

the medium in the vicinity of the electrode surface, which may account for the decrease in slope of the anodic NADH wave and for its positive $E_{1/2}$ shift.

OXIDATION OF NADH IN DMSO

Further information on the possible mechanism for the adsorption of NAD^+ and its role in the oxidation of NADH was sought by examining the oxidation of NADH (1 m*M*) in DMSO, which was 0.1 *M* in tetra-n-butylammonium perchlorate; the electrode was the GC-2.

With a pretreated electrode, no peak due to the adsorption of NAD^+ appears on derivative pulse polarography at the RDE or sweep voltammetry at the stationary electrode if v is less than 10 V s^{-1}; such a peak does appear at higher scan rates. These results are quite similar to those obtained in aqueous media when 2 m*M* NAD^+ is initially added to the solution.

It may, accordingly, be assumed that tetra-n-butylammonium (Bu_4N^+) ions adsorb at the electrode surface, and as a result, a certain fraction of the adsorption sites on this surface is occupied. NAD^+ formed by electrochemical oxidation of NADH may adsorb on the adsorption sites on the surface which are still unoccupied, but such adsorption of NAD^+ in the flat orientation would occur to a lesser extent than at an electrode surface free of adsorbed Bu_4N^+ ions. This may be the reason why the sweep voltammetric peak at the stationary electrode resulting from the process controlled by the flat-adsorption of NAD^+, appears only at v exceeding 10 V s^{-1}, since the peak height in aqueous media is proportional to v [1]. However, as in aqueous media, $E_{1/2}$ for the NADH anodic wave at the RDE from DMSO solution slowly shifted positively when the RDE had been held for a time at a potential corresponding to the oxidation of NADH. It takes 2 h at 0.8 V to reach a steady-state curve, which is longer than in aqueous media. During this time, we may assume that, as in aqueous media, the electrode becomes covered with NAD^+ adsorbed in the perpendicular orientation, which corresponds to strong adsorption coating all of the electrode surface and which may at least partially displace the Bu_4N^+ ions formerly adsorbed on this surface. The need for such a displacement may also explain why it takes a longer time to cover all the electrode surface than in aqueous media where Bu_4N^+ ions are not present. (Experiments involving Bu_4N^+ could not be done in aqueous media due to its insolubility.)

$E_{1/2}$ in DMSO at the RDE (covered with adsorbed NAD^+ by holding the electrode at 0.8 V for 2 h) is 610 ± 10 mV compared to 450 ± 10 mV in aqueous media, but the slope of the wave and the βn_a value are almost the same as in aqueous media of pH 7 or greater at the GC-2 electrode, i.e., $\beta n_a = 0.36 \pm 0.01$ in DMSO (slope^{-1} = 160 ± 5 mV).

It appears that the use of an organic solvent such as DMSO (pyridine and acetonitrile cannot be used due to the insolubility of both NAD^+ and NADH in these solvents) does not change appreciably the adsorption behavior of NAD^+ at the GCE; the only noticeable changes are related to the presence of Bu_4N^+ ions as supporting electrolyte in DMSO since they may at least partially hinder the adsorption of NAD^+ in the planar conformation.

CONCLUSIONS

In summary, in order to explain the observed behavior, we postulate that two steps or processes may occur during the adsorption of NAD^+ produced by the electrochemical oxidation of NADH at a carbon electrode:

	Step 1 (fast)		Step 2 (slow)	
NAD^+	$\rightleftharpoons$	NAD^+	$\rightleftharpoons$	NAD^+
produced at the electrode surface by NADH oxidation	sorption	adsorbed in a flat orientation (planar adenine moiety bound to the electrode surface)	reorientation	adsorbed in a perpendicular orientation (interaction between parallel adenine and pyridinium rings)

(5)

Adsorption of NAD^+ adsorbed in the perpendicular orientation provides the optimum conditions for the reproducibility of the voltammetric curves (cf. data in refs. 1 and 2).

The reduction of NAD^+ at the carbon electrode produces the NAD-NAD dimer. At a pretreated electrode, the reduction of NAD^+ shows two processes, the first being adsorption- controlled and due to the production of adsorbed dimer, and the second being diffusion controlled.

The adsorption of the NAD-NAD dimer is hindered either by that of NAD^+ (at least when tightly adsorbed in the assumed perpendicular conformation) or by holding the electrode at a potential more negative than −1.4 V for 2 min or longer before the addition of NAD^+ or dimer. The dimer is oxidizable at the carbon electrode only if adsorbed.

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