POLAROGRAPHIC BEHAVIOR OF NUCLEOSIDES AND NUCLEOTIDES OF PURINES, PYRIMIDINES, PYRIDINES, AND FLAVINS

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I. POLAROGRAPHIC INVESTIGATION OF ORGANIC COMPOUNDS

The value of electrical measurements in elucidating and utilizing the oxidation–reduction behavior of organic compounds, including those of biological importance, has long been recognized. However, because of the fact that few organic redox systems behave reversibly, valid potentiometric data have been obtained for only a limited number of compounds. Where such data have been systematically gathered and analyzed, the results have been fruitful, *e.g.*, the work of Michaelis, Clark, and their collaborators, which was largely centered in the 1920's and early 1930's. Since then, the bulk of the studies of the redox behavior of organic compounds has been based on polarography, generally at the dropping mercury electrode (dme). Frequently, half-wave potential data on organic compounds so determined are the only energetic data readily obtainable by electrochemical measurement. For this and other reasons, such data, including their variation with

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experimental conditions, have become of considerable interest in connection with various studies of the nature and properties of organic compounds.

While much of the considerable literature, which has accumulated on the polarographic behavior of compounds of biological interest, is excellent, a considerable portion is fragmentary in its coverage. The lack of a systematic approach is indicated by the fact that, while several papers on the polarographic reduction of purines had appeared prior to 1962, none had examined purine itself, had performed macroscale electrolyses of a series of compounds in order to isolate reaction products and to determine the number of electrons actually transferred per molecule electrolyzed, or had correlated the mechanistic features of the electrochemical reduction of the purines with their chemical behavior and extent of substitution.

The present review involves a comprehensive examination of the electrochemical behavior in aqueous solution of biologically important nucleosides and nucleotides and related model compounds with emphasis on the elucidation of the pathways involved in their oxidation and reduction at solution–electrode interfaces, as revealed by polarography and derived techniques, *e.g.*, conventional direct-current constant-potential polarography at the dme, cyclic voltammetry, controlled electrode potential electrolysis, chronopotentiometry, alternating current polarography, and oscillographic polarography. Attention will be focused on electrochemical reduction, since, until relatively recently, systematic study of the electrochemistry of organic compounds was largely confined to their reduction at mercury electrodes, except for the rather limited number which were sufficiently reversible to be studied polarographically or potentiometrically at platinum electrodes. Study of electrochemical oxidation was limited by lack of an electrode, which would not itself be oxidized at the relatively positive potentials necessary for investigating organic compounds, whose oxidation generally involves a high activation overpotential.

Where possible, account has been taken of the effect of variation in experimental conditions, *e.g.*, pH, ionic strength, specific solution components, and temperature, and an attempt has been made to elucidate the redox reaction mechanisms for the individual compounds and related series of compounds, which optimally include, *inter alia*, specification of reaction sites in the molecule, evaluation of the role of adsorption in relation to electron-transfer processes and determination of adsorption sites in the molecule, and the explication of the sequence of charge transfer, protonation, and intervening non-electron-transfer chemical reactions in the over-all redox reaction. In many instances, interpretive comments have been supplied on the data and explanations given in the literature; at times, the fragmentary nature of the latter have caused the present comments to be set as questions.

The inclusion of the electrochemical behavior of the nucleosides and nucleotides of the pyrimidines, purines, pyridines, and flavins in a single review is based on the general relationship involved; *i.e.*, all of these compounds consist of a heterocyclic nitrogen base to which a sugar or sugar-phosphate moiety is attached. These nucleosides and nucleotides can serve either as a monomeric unit in polymeric naturally occurring ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) and in synthetic polynucleotides, or as coenzymes for a class of group- and hydrogen-transferring enzymes.

A. POLAROGRAPHY

It is assumed that the reader is acquainted with the basic principles of polarography at the dme. Reviews of varying degree of comprehensiveness of the theoretical and practical fundamentals are readily available, *e.g.*, ref 3–7. Electrolysis at controlled electrode potential, which is very useful for the preparation of sufficient product for isolation, identification, characterization, and determination, is very well reviewed by Meites.[8] For convenience, the results of controlled-potential electrolyses are subsequently discussed in connection with the polarography of the compounds involved, since the potentials at which the electrolyses were made were almost invariably determined on the basis of the polarographic data. Other polarographically derived electrochemical techniques will be briefly explained where they are first introduced.

Of the numerous reviews of the polarographic behavior of organic compounds, attention may be directed to the recent thorough review of electrode reaction processes by Perrin[9] and to the review[10] of the effect of pH, which is frequently the most important experimental variable on such processes.

In any discussion of the electrochemistry of organic compounds, reference must be made to Clark's book[11] on the oxidation–reduction potentials of organic compounds, which is primarily concerned with their po-

(3) I. M. Kolthoff and J. J. Lingane, "Polarography," 2 vols., 2nd ed, Interscience Publishers, New York, N. Y., 1952.
(4) L. Meites, "Polarographic Techniques," 2nd ed, Interscience Publishers, New York, N. Y., 1965.
(5) L. Meites in "Treatise on Analytical Chemistry," Part I, Vol. 4, I. M. Kolthoff and P. J. Elving, Ed., Interscience Publishers, New York, N. Y., 1963, pp 2303–2379.
(6) O. H. Müller in "Physical Methods of Organic Chemistry," Part IV, 3rd ed, A. Weissberger, Ed., Interscience Publishers, New York, N. Y., 1960, pp 3155–3379.
(7) C. L. Rulfs and P. J. Elving in "Encyclopedia of Electrochemistry," C. A. Hampel, Ed., Reinhold Publishing Co., New York, N. Y., 1964, pp 944–950.
(8) L. Meites in ref 6, pp 3281–3333.
(9) C. L. Perrin, *Progr. Phys. Org. Chem.*, **3**, 165 (1966).
(10) P. J. Elving, *Pure Appl. Chem.*, **7**, 423 (1963).
(11) W. M. Clark, "Oxidation-Reduction Potentials of Organic Systems," Williams & Wilkins, Baltimore, Md., 1960.

tentiometric measurement. Volke[12,13] has reviewed the electrochemical properties and polarography of heterocyclic compounds.

1. *The Half-Wave Potential*

The most important characteristic of the compound, when examined electrochemically, will be, for the purposes of the present review, the half-wave potential, $E_{1/2}$, which in the case of a thermodynamically reversible redox half-reaction system would correspond to the formal potential for the specific experimental conditions involved, corrected for diffusion coefficient differences, but in the case of an irreversible system would correspond to the formal potential plus terms involving the diffusion coefficient difference, the activation energy for the heterogeneous electron-transfer process, certain experimental conditions, and perhaps the rate constants for chemical steps in the over-all electrode reaction process (*cf.* ref 10 for a summary of the nature of $E_{1/2}$) and the factors which enter into its measured value). In general, where reference is made to such behavior as "the wave is strongly pH-dependent," the phenomenological fact will be that the $E_{1/2}$ of the wave shifts considerably in magnitude with change in pH, *e.g.*, 60 mv per unit pH change.

Signs of the potentials for the half-reactions discussed are determined by the fact that the reactions are formulated as reductions with the usual thermodynamic conventions. The numerical values of potential data reported are on the basis of comparison with the saturated calomel electrode (sce), which has a potential of 0.242 V against the normal hydrogen electrode (nhe).

2. *Current Behavior*

Slopes of plots of log $[i/(i_d - i)]$ *vs.* E are frequently used to evaluate the reversibility of the process producing a polarographic wave. A value of 0.059 V or near it, *e.g.*, 55–65 mV, is frequently taken to indicate a one-electron (1e) reversible process; a value of *ca.* 0.030 V would then indicate a reversible two-electron (2e) process. Values appreciably greater than 60 mV are usually taken to indicate irreversible electrode processes (*cf.* ref 3–7). These plots are often referred to as log current or log current–potential plots. Unfortunately, the difficulty of measuring the slope to ± 5 mV for many of the polarographic waves obtained for organic compounds often makes this test of only limited diagnostic value.

Another common diagnostic test is used to determine the nature of the process controlling the current at the limiting current portion of the wave obtained at the dme; the test involves examining the variation of the limiting current with change in the hydrostatic mercury height (h) in the capillary. Theory predicts that the current will be proportional to $h^{1/2}$ for a diffusion-controlled or limited electrode process and to h^0 for a kinetic controlled process, *i.e.*, one limited by the rate of a chemical reaction. Where adsorption is involved, the limiting current should vary directly with h. Temperature variation of the limiting current is also used for the same purpose; *i.e.*, a temperature coefficient of *ca.* 2% per degree indicates a diffusion process, one of *ca.* 8–10% kinetic control and an inverse temperature effect adsorption.

3. *Electroactive Sites*

Although electrochemical oxidation and reduction in nucleosides and nucleotides occur primarily in the heterocyclic, *e.g.*, pyrimidine, ring moiety, the sugar or sugar-phosphate group influences diffusion, adsorption, and electron density at reactive sites; the latter effect will generally manifest itself in potential shifts and possibly in alteration of the reaction path for the over-all redox process. More detailed study is necessary for estimation of the influence of the sugar and sugar-phosphate moieties on the electrochemical behavior of the parent base and for meaningful quantitative correlations of potentials with structure, quantum mechanical and other calculated reactivity indices, and biological phenomena; in particular, only negligible data are available for oxidation processes. It would also be desirable to compare the behavior of the monomeric units, *i.e.*, bases, nucleosides, and nucleotides, with their polymers, *i.e.*, oligonucleotides and polynucleotides, in order to determine the influence of the primary and secondary structure of polymers on the behavior of the component monomeric units.

4. *Catalytic Hydrogen Reduction*

The catalytic reduction of hydrogen ion is connected with the large activation energy required for hydrogen ion reduction at most electrodes, especially those of mercury, and with the comparatively less difficult reduction of the adducts formed by hydrogen ion with heterocyclic nitrogen, sulfur, and other species, which, on reduction, evolve hydrogen and regenerate the adduct-forming species, which can again complex with hydrogen ion, resulting in a typical catalytic cyclic process. In some instances, both the oxidized and reduced forms of a compound seem to catalyze hydrogen ion reduction, *e.g.*, adenine, and in other cases, only one form, *e.g.*, the reduced form of purine.

Since all nucleosides and nucleotides contain heterocyclic rings with nitrogen in them, all reduction waves, especially in acidic media, need to be carefully examined for the concomitant presence of a hydrogen ion reduction process. The behavior patterns involved in

(12) J. Volke in "Physical Methods in Heterocyclic Chemistry," Vol. I, A. R. Katritzky, Ed., Academic Press Inc., New York, N. Y., 1963, pp 217–323.
(13) J. Volke, *Talanta*, 12, 1081 (1965).

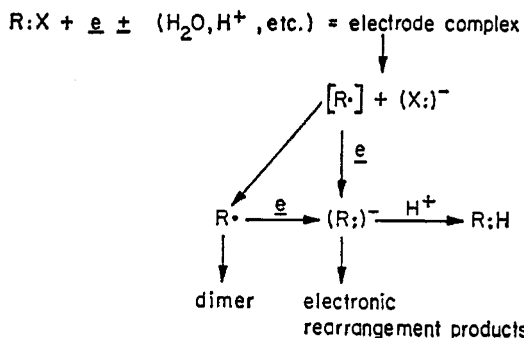


Figure 1.—Generalized mechanistic path for an organic electrode reaction, in which R:X represents a generalized reaction site; the steps in the path are discussed in the text. Allowance may have to be made in the above formulation for (a) the participation of protons, solvent, other solution constituents, and the electrode surface in various steps, (b) the presence of chemical reactions preceding, accompanying, and following charge-transfer processes, and (c) resulting modification of the species shown.

the case of many compounds are not yet clear and further work is needed.

A frequently encountered phenomenon in solution of pH 5 or less is the appearance of the catalytic hydrogen wave as the solution decomposition wave, which thus limits the potential range available and prevents the observation of reduction waves which occur at more negative potential than the hydrogen evolution wave.

B. ORGANIC ELECTRODE REACTION MECHANISMS

Current views regarding the mechanisms of organic electrode processes are critically summarized by Perrin,[9] who emphasizes, as others have done, that, because an electrochemical reaction is a heterogeneous one, a thorough description of its mechanism must await a detailed understanding of phenomena in the electrical double layer and the surrounding solvent layer. At present, descriptions of organic electrode processes frequently still merely involve listing the electrode reaction products with, in some cases, more or less speculative postulation of reaction intermediates. Only in relatively few cases has there been sufficiently detailed experimental evidence to allow the postulation of structures for the transition states involved. Too frequently, even products of organic electrode reactions are postulated on rather indirect evidence.

1. General Mechanistic Path

Electrochemical reduction is illustrated, since reduction, as mentioned, has been studied much more extensively than oxidation.

The fundamental process in an organic electrochemical reduction is bond rupture, which requires only one electron to produce a free-radical species; addition of a second electron completes rupture of the bond to give a carbanion. As a consequence, only one- or two-electron processes need be considered in mechanism discussion, even though polarographic waves involving multiple electron processes are frequently observed, due (a) to the mechanical fusion of waves occurring at only small potential separation from each other and (b), more importantly, to the instability at the potential of its formation of the species produced as a result of the first electron-transfer step and, consequently, its immediate further reduction.

On this basis, Elving and Pullman[14] have postulated a general mechanistic pattern for organic electrode reactions, which rationalizes the general course of such processes as well as the occasional changes in mechanism with experimental conditions or between members of a homologous series. The mechanism is outlined in Figure 1, in which a generalized carbon reaction site, R:X, is assumed, where R represents the reactive carbon center and X represents another carbon, oxygen, nitrogen, halogen, or other atom; there may be more than one bond between R and X, *e.g.*, in a C═N linkage.

In the primary step of the electrode process, the reaction site in the molecule accepts a single electron to form the electrode activated complex, which can either revert to the original species or dissociate to give a free-radical precursor and an anionic species; if a multiple bond connection was originally present between R and X, the two latter species could form a single free-radical anion. The exact nature of these species will be modified by the extent of the participation of protons, solvent molecules, and other solution constituents or even the electrode surface (participation of these species in subsequent steps is not explicitly indicated, but may be involved).

The free-radical precursor can either immediately on formation accept a second electron and be reduced to a carbanion or can exist as a stable free-radical species, which can either dimerize or, at more negative potential, be further reduced to the equivalent of a carbanion. The charge on the carbanion, formed by either path, can be neutralized either by acceptance of a proton from the solution or by electronic rearrangement with the charge being transferred to another part of the molecule where it can be suitably handled.

Chemical reactions preceding, accompanying, or following the charge-transfer process may—and often do—play significant roles in the over-all process, *e.g.*, the possibly profound effect of the kinetics of such chemical reactions upon the observed polarographic pattern.

2. Typical Reaction Paths. Pyrimidine Ring

The reaction scheme outlined can be illustrated by reference to the complex wave pattern observed for the reduction of pyrimidine in aqueous solution at the dme, in which five polarographic waves appear over the nor-

(14) P. J. Elving and B. Pullman, *Advan. Chem. Phys.*, **3**, 1 (1961).

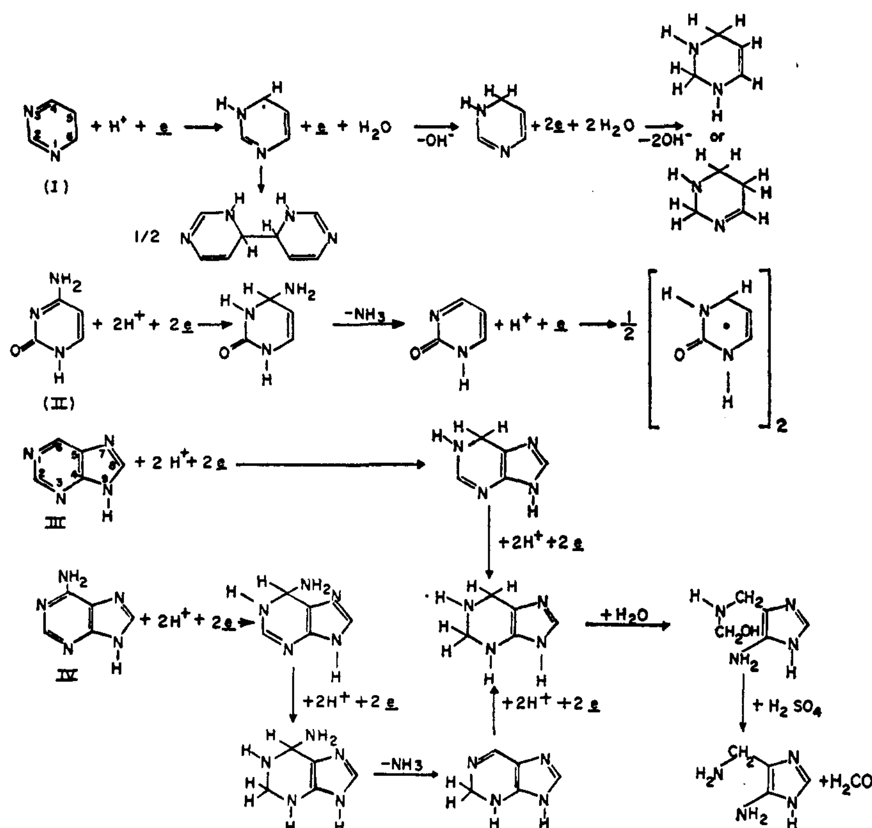


Figure 2. Interpretation of the electrochemical and chemical behavior observed on the polarographic reduction of pyrimidine (I), cycosine (II), purine (III), and adenine (IV). Protonation, dissociation, and other acid-base and keto-enol equilibria are not shown.

mal pH range.[15] The electrode processes producing these waves are as shown in Figure 2. Wave I, visible only in acid solution, is the result of a 1e reduction of the 3,4 bond with the simultaneous acquisition of a proton to form a free radical, which may dimerize to 4,4′-bipyrimidine or may be reduced at more negative potential in a further 1e process (wave II) to 3,4-dihydropyrimidine. Since wave I is strongly pH dependent and wave II shows relatively little pH dependence, the two waves merge with increasing pH at about pH 5 to form pH-dependent 2e wave III. Wave IV, which appears at higher pH and is essentially pH independent, represents a 2e reduction at more negative potential of the dihydropyrimidine to a tetrahydropyrimidine. Again, the difference in pH dependency of waves III and IV results in their merging at about pH 9 to form pH-dependent 4e wave V.

The mechanistic pathways for the reduction of pyrimidine and purine derivatives essentially follow the course outlined for pyrimidine; *e.g.*, the 3,4 N=C bond in the pyrimidine ring is usually the one most readily reduced, with important possible modifying influences due to substituents, *e.g.*, (a) fusing of consecutive electron-transfer steps or the splitting of multiple electron steps, (b) tautomeric shifts due to substituents having divalent sulfur or oxygen bonded to carbon (*e.g.*, replacement of hydrogen by hydroxyl at the 2 or 4 position in pyrimidine results in removal of a double bond from the ring due to ketonization with the concomitant loss of a reduction site), and (c) intramolecular chemical reactions such as deamination, which accompany electrode processes and which may generate electroactive species.

Thus, cytosine shows a single 3e polarographic wave, which occurs at a potential more negative than that of waves I, II, and III of pyrimidine, but less negative than that of pyrimidine wave IV; the wave is due to three successive processes (Figure 2): (a) 2e reduction of the 3,4 N=C bond (this is a combination of the first two 1e processes seen in pyrimidine), (b) rapid deamination of the product to 2-hydroxypyrimidine, and (c) 1e reduction of the 2-hydroxypyrimidine, similar to pyrimidine and the 2-aminopyrimidines, to a free radical, which dimerizes under the experimental conditions before it can be further reduced.[15]

Purine exhibits two pH-dependent waves in acidic solution at the dme, but none in neutral or alkaline solution.[16] Wave I is due to the 2e reduction of the 1,6 N=C double bond to 1,6-dihydropurine; wave II is due to further 2e reduction, probably to 1,2,3,6-tetrahydropurine, which hydrolyzes to a 4-aminoimidazole.

(15) D. L. Smith and P. J. Elving, *J. Am. Chem. Soc.*, **84**, 2741 (1962).

(16) D. L. Smith and P. J. Elving, *ibid.*, **84**, 1412 (1962).

Behavior similar to that of cytosine is shown by adenine, which undergoes an even more complex reaction sequence of reduction, deamination, and further reduction of a regenerated double bond involving an over-all 6e reduction; 2e hydrogenation of the 1,6 N=C bond is followed by 2e reduction of the 2,3 C=N bond, slow deamination at the 6 position, further 2e reduction of the consequently regenerated 1,6 N=C bond, and hydrolytic cleavage at the hydrogenated 2,3 position to give the same product as the over-all 4e purine reduction.[16]

At the dme, deamination of the reduced adenine is negligible, resulting in a single pH-dependent 4e wave in acidic solution.

The polarographic patterns and data for purine and adenine have to be evaluated with some caution since adenine and the completely reduced forms of it and of purine lower the overpotential for hydrogen ion reduction.[16]

C. SIGNIFICANCE OF ELECTROCHEMICAL STUDIES OF BIOLOGICAL COMPOUNDS

Since energy transfer and other processes in biological systems are frequently intimately associated with electron-transfer or redox processes, information which can be obtained on the nature of the latter processes, *e.g.*, in respect to the energy levels (electrical potentials) and the reaction pathways involved, should be helpful in the understanding, control, and utilization of such processes in biological and clinical situations.

Although actual biological redox systems may be of considerable complexity due, for example, to enzyme interactions, much potentially useful information can be obtained from examination by electrochemical techniques of the redox behavior of the compounds of interest in aqueous solution containing inorganic salts with respect to the nature of the factors controlling the electron-transfer process, *e.g.*, pH. While the results would need to be carefully evaluated in relation to their significance for biological systems, there is some indication that there may be analogous reaction paths in electrochemical and biological, *e.g.*, enzymatic, redox processes.

For example, the chemical oxidation of uric acid, depending on experimental conditions, produces as the principal product either allantoin or alloxan, but not both. However, the electrochemical oxidation of uric acid involves a 2e oxidation to a primary short-lived dicarbonium ion, which undergoes simultaneous transformations including hydrolysis leading to an allantoin precursor and hydrolysis leading to alloxan and urea.[17] The plausibility of postulating an intermediate which can undergo transformation to either allantoin or alloxan suggests that the oxidation of uric acid by any method produces a common intermediate whose subsequent transformations—and consequently the ultimate products obtained—are determined by experimental conditions. An analogous reaction path has been postulated[18] to explain the results for the enzymatic uricase oxidation of uric acid, reflecting an interesting parallelism between the two types of oxidation. The electrochemical oxidation of adenine initially follows the same path as the enzymatic oxidation with xanthine oxidase, but further oxidation and fragmentation of the purine occur.[19]

It is pertinent to note in this connection that electrolytic reductions and oxidations often occur under conditions resembling those of enzymatic transformations, *i.e.*, electron transfer at a heterogeneous interface, previous occurrence of adsorption and/or formation of an adduct, dilute aqueous solution, pH between 2 and 9, and moderate temperature. Postulation of the degree of correlation that may exist between electrolytic and enzymatic redox processes must at present be sheer speculation, but, nevertheless, there is a similarity of conditions under which the two kinds of processes occur. To this extent, knowledge gained in the investigation of the electrochemical behavior of species of biological interest may help to clarify and explain enzymatic and other biological processes, and might even lead to the preparation of compounds of interesting biological activity.

1. *Correlations Involving Potentials*

There is increasing interest in the extent to which the potentials and reaction pathways for the electrochemical oxidation and reduction of biologically significant compounds can be correlated with their chemical, biological, and clinical activity. The matter of pathways has just been briefly considered.

Correlation of polarographic $E_{1/2}$ values with numerical structural and reactivity characteristics is usually based on the postulation that the characteristic $E_{1/2}$ of a compound is a function of electron density and other factors, which, in turn, are also relatively simply related to some biological, physical, or chemical property. The frequently resulting linear relationship between $E_{1/2}$ for a series of more or less closely related compounds and a suitably selected mathematical function of the values of the given property for that series of compounds permits (a) prediction of the magnitude of the property of a compound from its readily measured $E_{1/2}$ and (b) the rapid comparative evaluation of a property based on comparison of $E_{1/2}$ values.

The large variety of experimental and theoretical properties and phenomena, which have been compared to polarographically determined potential data,

(17) W. A. Struck and P. J. Elving, *Biochemistry*, **4**, 1343 (1965).

(18) G. Soberon and P. P. Cohen, *Arch. Biochem. Biophys.*, **103**, 331 (1963).

(19) G. Dryhurst and P. J. Elving, work in progress.

include photoionization potentials, degree of carcinogenesis, transition energies of charge-transfer complexes, wavelengths of spectrophotometric absorption maxima, antioxidant ability, quantum mechanically calculated parameters, and structural summation characteristics. The best known and most extensively used correlations have involved various forms of the Hammett $\sigma-\rho$ equation based on polar substituent quantities and the Taft modification (*cf.* reviews by Perrin[9] and Zuman[20]).

Correlations of molecular orbital (MO) energy level calculations with polarographic data generally involve the quantum mechanically calculated energies for adding an electron to the lowest empty MO (LEMO) or removing one from the highest occupied MO (HOMO). *A priori*, the optimum approach would seem to involve the use of electrochemical data based on initial 1e processes, since the MO data apply to such processes. While such 1e processes can be observed for some organic compounds in aqueous media, in the case of other compounds, measurements in nonaqueous media are necessary. The validity of this approach is shown by the work of Streitwieser and others on the correlation of potentials for the oxidation of organic compounds in nonaqueous media at platinum electrodes with a variety of calculated values based on MO theory (*cf.* reviews by Pullman and Pullman[21] and Zahradník and Parkanyi[22]).

Since MO calculations have been correlated with various types of chemical and biochemical activity for a variety of molecules, a successful correlation with polarographic data will facilitate correlation of biochemical and related medical activity with polarographic data, which are usually relatively readily measured experimentally. The need for methods, which experimentally measure the electron affinities or related properties of biological compounds, has been emphasized.[21]

The value of reliable experimental data on oxidation and reduction potentials as tests of theoretical approaches to the electronic properties of biological molecules is typified by a recent paper,[23] in which the results of calculations for the four nucleic acid bases (guanine, adenine, cytosine, and uracil) by a semiempirical self-consistent field calculation, as well as by the Hückel MO approximation method, are evaluated by comparing the values obtained for ionization potentials (electron-donor properties) and for electron affinities (electron-acceptor properties) with oxidation potentials determined at the stationary graphite electrode and reduction potentials determined at the dme.

Three important factors, which are frequently overlooked, must be emphasized. The reliability of correlations of the type indicated depends not only on the validity of the mathematical approaches and on the accuracy of the experimental measurements, but also on the calculations and measurements having been made on identical molecular species. It is for this reason among others that experimental measurements need to be made under a variety of conditions and that attempts need to be made to elucidate the details of the redox reaction pathway including the location and the exact chemical nature of the sites at which electron transfer occurs, since it is only on the basis of such information that it is possible to assure that theoretical and experimental data are being compared for the same molecular species.

A second factor is the influence of the degree of reversibility of the electron-transfer process upon observed potential. While the potential observed for a completely reversible electrode process is directly relatable to the standard free energy of the process, the potential observed for one which is not so reversible contains a component due to the necessary energy of activation as well as, perhaps, components due to adsorption and other accompanying phenomena; *e.g.*, *cf.* ref 10.

Finally, account must be taken of the solvation energy contribution to the half-wave potential. Theoretical calculations, *e.g.*, those of LEMO and HOMO, are generally based on an idealized gas-phase type molecule. In many past correlations, there has been the implicit assumption that the solvation energy term is constant or varies in a regular fashion for the series of compounds studied. It appears unlikely that the latter assumption is tenable for different bases, nucleosides, and nucleotides because of the variation in the nature, number, and strength of the hydrogen-bonding donor and acceptor groups in the different compounds and in their reduction and oxidation products. A possibility which should be explored is that of considering the diffusion coefficients of the compounds, as estimated from the diffusion currents, as measures of the solvation sphere around the compound.[24]

II. PURINE AND PYRIMIDINE NUCLEOSIDES AND NUCLEOTIDES

A. NOMENCLATURE

Purines and pyrimidines are found in fairly large amounts in most living cells. Occasionally, they occur in the free state, but those which predominate in normal cells are usually present as glycosides, *e.g.*, *nucleosides*. The latter, in turn, mostly occur in cells as their phosphoric esters or *nucleotides*, which play a

(20) (a) P. Zuman, "Organic Polarographic Analysis," Pergamon Press, London, 1964; (b) "Substituent Effects in Organic Polarography," Plenum Press, New York, N. Y., 1967.

(21) B. Pullman and A. Pullman, "Quantum Biochemistry," Interscience Publishers, New York, N. Y., 1963.

(22) R. Zahradník and C. Parkanyi, *Talanta*, **12**, 1289 (1965).

(23) H. Berthod, C. Giessner-Prettre, and A. Pullman, *Theoret. Chim. Acta*, **5**, 53 (1966).

(24) G. Dryhurst and P. J. Elving, work in progress.

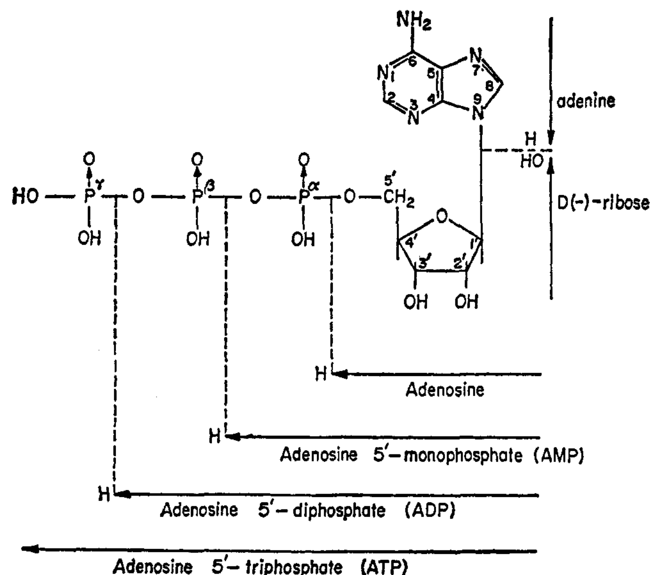


Figure 3.—Formulas for adenine and derived nucleoside and nucleotide species. Configuration, dissociation equilibria, and tautomeric shifts are not shown. Taken with slight modification from ref 26.

vital role in all cells as group-transferring coenzymes and in highly polymerized condition as nucleic acids.

Numbering, nomenclature, and the writing of formulas for purine and, more particularly, for pyrimidine derivatives are not uniform.[25] The trivial names common in biochemical literature and the numbering used in *Chemical Abstracts* will be used in the present review (*cf.* Figure 3).

The purine and pyrimidine nucleosides are, with very few exceptions, either β-D-ribofuranosides (ribonucleosides) or β-D-(2′-deoxy)ribofuranosides (deoxyribonucleosides), in which the sugar moiety is attached to N(9) of a purine or to N(1) of a pyrimidine. Since the ribonucleosides possess three hydroxyl groups on the sugar (positions 2′, 3′, and 5′) and deoxyribonucleotides possess two such hydroxyl groups (positions 3′ and 5′), three and two isomeric monophosphorylated nucleosides, *i.e.*, nucleotides, are, respectively, possible (Figure 3).

The commonly used nomenclature for nucleotides is derived from the trivial name of the nucleoside plus the position and number of attached phosphate groups, *e.g.*, adenosine 5′-monophosphate (Figure 3). The ribonucleoside 5′-monophosphates are generally called adenylic, guanylic, cytidylic, and uridylic acids, and, correspondingly, the deoxyribonucleoside 5′-monophosphates are called deoxyadenylic, deoxyguanylic, deoxycytidylic, and thymidylic acids.

Phillips[26] has reviewed and summarized a considerable amount of information about the ionization, conformation, and metal complex formation of adenosine and the adenine nucleotides in solution; he gives no references to the use of polarographic techniques.

B. POLAROGRAPHY

While the polarographic reduction of the parent purine and pyrimidine bases has been moderately extensively studied (*cf.* review by Elving, Struck, and Smith,[27] the literature on the electrochemical behavior of nucleosides and nucleotides is quite meager. The majority of papers dealing with the polarography of purines does not mention nucleosides or nucleotides; when the latter are mentioned, it is generally only in terms of a qualitative statement of the type that "the behavior of the nucleosides and nucleotides is very similar to that of the corresponding bases."

The electrolytic reductions of nucleosides and nucleotides occur in the purine or pyrimidine moiety, whose polarographic reduction pattern has been indicated. However, as was pointed out, the sugar or sugar-phosphate moiety can influence the adsorbability, diffusion coefficient, electron density, and other characteristics in polarographic behavior, *e.g.*, shift of $E_{1/2}$. No mention of the polarographic reducibility of the sugars involved, ribose and deoxyribose, was found in the literature, except for the reduction of ribose in alkaline media,[28] in which media the purine and pyrimidine moieties are not reduced. Uracil, thymine, guanine, and their nucleosides and nucleotides give no indication of a polarographic reduction wave[16,27,29–34] (however, see subsequent discussion).

The oscillographic polarography of the purine and pyrimidine nucleosides and nucleotides is discussed in a separate section to facilitate comparison of the data for the different compounds.

Some purine and pyrimidine nucleotides have been electrolyzed at a large mercury pool cathode;[35] unfortunately, the potentials were not controlled. The over-all rates of reduction of adenylic and cytidylic acids, based on spectrophotometric measurement, were the same but the rate of deamination in the course of reduction, as measured by ammonia evolution, proceeded much more slowly for cytidylic acid. The re-

(25) D. J. Brown, "The Pyrimidines," Interscience Publishers, New York, N. Y., 1962.

(26) R. Phillips, *Chem. Rev.*, **66**, 502 (1966).

(27) P. J. Elving, W. A. Struck, and D. L. Smith, *Mises Point Chim. Anal. Org. Pharm. Bromatol.*, **14**, 141 (1965).

(28) T. Tsukamoto and T. Tono, International Congress of Polarography, Kyoto, 1966.

(29) E. Chargaff and J. N. Davidson, "The Nucleic Acids," Vol. I, Academic Press Inc., New York, N. Y., 1955.

(30) D. Hamer, E. M. Waldron, and D. L. Woodhouse, *Arch. Biochem. Biophys.*, **47**, 272 (1953).

(31) J. Heath, *Nature*, **158**, 23 (1946).

(32) B. Janík and E. Paleček, *Arch. Biochem. Biophys.*, **105**, 225 (1964).

(33) N. G. Luthy and B. Lamb, *J. Pharm. Pharmacol.*, **8**, 410 (1956).

(34) (a) E. Paleček and B. Janík, *Arch. Biochem. Biophys.*, **98**, 527 (1962); (b) V. P. Skulachev and L. I. Denisovich, *Biokhimiya*, **31**, 132 (1966).

(35) A. N. Kotelnikova and V. V. Solomatina, *ibid.*, **30**, 816 (1965).

TABLE I

HALF-WAVE POTENTIALS OF ADENINE AND ITS NUCLEOSIDES AND NUCLEOTIDES

Compound	pK_a	$-E_{1/2}$,[a] V	$-E_{1/2}$,[b] V	$-E_{1/2}$,[c] V	Variation with pH[d]	
					pH range	$-E_{1/2}$, V
Adenine	4.20	1.1	1.46	0.95	1.0–6.5	0.975 + 0.084pH
Adenosine	3.60	1.17	1.42	0.99	2.0–4.5	1.040 + 0.070pH
					4.5–6.0	1.180 + 0.041pH
Deoxyadenosine	(3.7)				2.5–4.6	1.060 + 0.069pH
					4.6–6.5	1.205 + 0.037pH
Adenylic acid	3.80		1.34	0.97	1.0–4.3	1.015 + 0.083pH
					4.3–5.5	1.115 + 0.060pH
Deoxyadenylic acid	4.4				2.0–6.5	0.985 + 0.080pH
Adenosine diphosphate				0.98		
Adenosine triphosphate	4.05			0.98	2.5–4.5	1.035 + 0.083pH
					4.5–5.5	1.175 + 0.052pH
2′,3′,5′-Triacetyladenosine				0.99		
2′,3′,5′,N_6′-Tetraacetyladenosine				(I) 0.78		
				(II) 0.89		
Adenine 1-N-oxide		(I) 0.87				
		(II) 1.1				
Adenosine 1-N-oxide		(I) 0.84				
		(II) 1.14				

[a] Potentials *vs.* sce; 0.5 mM adenine derivative in 0.05 M $HClO_4$.[36] Under the same conditions, $E_{1/2}$ of −0.84 (I) and −1.11 V(II) are reported for adenine 1-N-oxide: C. R. Warner and P. J. Elving, *Collection Czech. Chem. Commun.*, **30**, 4210 (1965). [b] Potentials *vs.* mercury pool anode; 0.1 mM adenine derivative in 0.1 M $HClO_4$.[33] [c] Potentials *vs.* sce; 0.2 mM adenine derivative in unspecified media. $E_{1/2}$ extrapolated to pH 0.0; in pH range 0.65–5.3, $d(E_{1/2})/d(\text{pH}) = -0.080$ V for both tetraacetyladenosine waves and −0.110 V for all other compounds.[34b] [d] Potentials *vs.* sce; average data for 0.125 and 0.5 mM adenine derivative in chloride, acetate, and McIlvaine buffers of ionic strength 0.5.[37]

duction of uridylic acid was also indicated, but not that of guanylic acid. In the case of nicotinamide–adenine dinucleotide (NAD^+), reduction of the adenine moiety, accompanied by stoichiometric elimination of ammonia, occurs in acidic medium and of the pyridine ring in slightly alkaline medium.

1. Adenine Species

The three early papers[31,33,36] dealing with adenine and its nucleosides and nucleotides provide only limited data; *e.g.*, $E_{1/2}$ data (Table I) could be obtained from only two.[33,36] Unfortunately, even these values are contradictory and consequently do not allow any conclusions to be drawn. The current–concentration ratio increases in the order of adenine, adenosine, adenylic acid, and adenine–cytosine dinucleotide with i_d being proportional to concentration in the range of 0.05 to 0.20 mM.[31] Plots of log $[i/(i_d - i)]$ *vs.* E and $(i_d - i)/i^2$ *vs.* E for adenine, adenosine, and adenylic acid are reasonably linear for the greater part of the wave; the slopes of the latter plot range from 0.026 to 0.029,[31] but the implications of these relations were not explored. $E_{1/2}$ and temperature coefficients of both $E_{1/2}$ and i_d are also said to be similar for these three compounds.[31] $E_{1/2}$ for 0.05 mM adenine in perchlorate buffer at 25° shifts from −1.046 V at pH 1.30 to −1.129 V at pH 2.24 and is concentration dependent; *e.g.*, it shifts at pH 1.30 from −1.046 V for 0.05 mM to −1.073 V for 0.196 mM. Similar pH and concentration dependencies of $E_{1/2}$ were found for adenosine and adenylic acid.[31] These data and those of a recent study of compounds containing the adenine moiety[34b] (Table I) suggest that the polarographic behavior of adenine does not change drastically on attachment of the sugar or sugar phosphate moiety and that the current-controlling factors and the reduction mechanisms are common, in principle, for the whole series.

The latter conclusions are reinforced and made more explicit by a more recent systematic study of adenine nucleosides and nucleotides.[37] The remainder of the discussion of the adenine series involves ref 16, 37, and 38.

Adenosine and the adenosine derivatives examined (deoxyadenosine, adenosine 5′-monophosphate (AMP) or adenylic acid, deoxyadenosine 5′-monophosphate (dAMP) or deoxyadenylic acid, and adenosine 5′-triphosphate (ATP)) all show one generally well-defined cathodic polarographic wave, which may be distorted in the case of some compounds, *i.e.*, AMP, dAMP, and ATP, by the appearance of a second wave at more negative potential. The latter wave is probably due to the catalytic reduction of hydrogen. The wave pattern is also generally characterized by the shift of the background discharge wave to more positive potential with increasing adenosine derivative concentration[37] and increasing pH.[34b,37] This over-all behavior pattern is similar in its fundamental aspects to that observed for adenine itself.[16,38]

The normal reduction wave for all compounds is relatively constant in height up to pH 4 or 5, when it begins to decrease sharply with increasing pH and disappears by pH 6 or 7.[37] $E_{1/2}$ becomes more negative with in-

(36) F. A. McGinn and G. B. Brown, *J. Am. Chem. Soc.*, **82**, 3193 (1960).

(37) B. Janík and P. J. Elving, work in progress.

(38) B. Janík and P. J. Elving, work in progress.

creasing pH (Table I) with $d(E_{1/2})/d(pH)$ varying from 0.04 to 0.08 V per pH unit, compared to a theoretical value of 0.06 for a reduction involving the same number of electrons and protons in the potential-determining step of the electrode process.

The calculated diffusion current constants for the normal reduction wave of the adenine nucleosides and nucleotides studied agree within 10% with that of the corresponding wave of adenine itself (a slight simplification is involved even though the waves are largely diffusion controlled).[37,38] Since the electrochemical reduction of adenine under polarographic conditions has been shown to be a 4e process,[16] the same number of electrons may be assumed for each normal wave process.

The current, when measured at pH below that of the decreasing region, is linearly proportional to concentration, but the apparent diffusion current constant decreases with increasing concentration similar to adenine.[16,38,39] The latter effect is likely due to the presence of the catalytic hydrogen wave, which appears as an increased slope to the limiting portion of the normal wave at low concentration and as a merging wave at high concentrations. Partial deamination of the electrolytic reduction product resulting in reduction of the regenerated 1,6 N=C bond may be a complicating factor.

a. Reducible species

The presence of the protonated species (with the proton most probably being on the N(1) as indicated by nmr[40] and X-ray crystallography[41]) as the only polarographically reducible form is supported by the following observations: (a) inflections occur in the straight-line $E_{1/2}$–pH plots for most compounds at pH values, which are 0.5 to 0.7 higher than pK_a (Table I; such inflections frequently indicate a change in protonation of the polarographically active species[10]); (b) inflections in the changing portions of the S-shaped limiting current–pH plots occur at pH values of 1.0–1.7 higher than pK_a; and (c) the current begins to show partial kinetic control in the pH region, where it decreases, indicating recombination of the nonreducible nonprotonated form with protons to give a reducible form in a pH region where the protonated form does not exist in appreciable concentration in the bulk solution.

b. Ease of reduction

Attachment of a sugar or sugar-phosphate moiety to the adenine nucleus, with the exception of dAMP, decreases the ease of reducibility of the parent compound; the exact order of reducibility is slightly pH and concentration dependent; *e.g.*, $E_{1/2}$ in pH 3.7 McIlvaine buffer becomes more negative in the order dAMP $\leqslant$ adenine $<$ adenosine $<$ deoxyadenosine $<$ AMP $\ll$ ATP. The alternating current polarographic behavior of these compounds suggests a generally parallel order of increasing adsorbability of uncharged species, *i.e.*, adenine $<$ dAMP $<$ deoxyadenosine $<$ adenosine $<$ ATP $<$ AMP, which agrees with that based on differential capacity measurement for the first three compounds (only ones in the series measured)[42] (*cf.* section II.C). The general pattern is thus consistent with a frequently observed inhibition of the electrolytic process due to strong adsorption of the depolarizer (or its precursor) with a consequent negative shift of the wave.[13]

Adsorbability, however, is obviously not the only factor influencing reducibility in the adenine series. The electron-withdrawing effect of the ribose ring in nucleosides[40,43] should promote nucleophilic attack on the heterocyclic ring, *i.e.*, should increase its ease of reducibility. This prediction seems to be fulfilled only in the case of the cytosine series, where the reducibility increases in the order base $<$ nucleotide $<$ nucleoside[32,34a] (*cf.* next section). In the adenine series, the distance between the sugar ring and the reduction sites in the pyrimidine ring is great enough to weaken the withdrawal of electrons from the reaction sites; other factors may also contribute to the decreased reducibility of adenine nucleosides and nucleotides. For example, the fact that ATP is the most difficultly reducible of the group is partially due to the repulsion of the negatively charged phosphate groups from the similarly charged dme surface and partially to the remarkable stability of energy-rich ATP in water medium;[21] ATP assumes a more condensed structure than the other compounds, in which two terminal phosphate groups approach the relatively positive amino group and N(7), which are thus surrounded by a protective cloud of negatively charged phosphate groups.

The relatively greater effect of the sugar and sugar-phosphate moieties in the pyrimidine than in the purine series is also evident from the oscillopolarographic results (*cf.* section II.D).

c. Correlations

Published correlations of $E_{1/2}$ with LEMO data are limited to a relatively small group of purine and pyrimidine derivatives for which the calculated quantum-chemical indices are available.

Adenine and ATP fit the straight-line relation between $E_{1/2}$ and k_i (energy of LEMO) obtained for purine and some of its 6-substituted derivatives,[44] while

(39) D. L. Smith and P. J. Elving, *Anal. Chem.*, **34**, 930 (1962).
(40) C. D. Jardetzky and D. Jardetzky, *J. Am. Chem. Soc.*, **82**, 222 (1960).
(41) W. Cochran, *Acta Cryst.*, **4**, 81 (1951).
(42) V. Vetterl, *Collection Czech. Chem. Commun.*, **31**, 2105 (1966).
(43) A. D. Broom, M. P. Schweizer, and P. O. P. Ts'o, *J. Am. Chem. Soc.*, **89**, 3612 (1967).
(44) B. Janík and P. J. Elving, work in progress.

AMP deviates significantly. $E_{1/2}$ for the cytosine series (bases, nucleosides, and nucleotides) are more negative than the $E_{1/2}$ values for the corresponding adenine derivatives, while the LEMO calculations predict the opposite.

To a first approximation, pK_a values of organic compounds are proportional to the electron density at the protonation site; in the adenine series, where the protonation site, *i.e.*, N(1), is the reduction site as well, a decrease in the ease of reducibility with increasing pK_a may consequently be expected. Such behavior is observed for adenosine, deoxyadenosine, AMP, and ATP, while adenine and dAMP strongly deviate in being more easily reduced than expected for an inverse pK_a–$E_{1/2}$ correlation for the series.

The correlation of $E_{1/2}$ with structure in a series of purine or pyrimidine base and corresponding nucleosides and nucleotides is more complex than in the case of simple derivatives; *e.g.*, $E_{1/2}$ for purines or pyrimidines substituted only with alkyl, alkylamino, or amino groups has been, on the whole, well correlated with electronic density at the reduction site and pK_a.[44] This is probably due in large part, if not entirely, to the more complex structure in the nucleosides and nucleotides, resulting in increased adsorption and greater possibility for intramolecular association.

2. *Cytosine Species*

Detailed studies have been reported for cytosine and its nucleosides and nucleotides.[32,34a] $E_{1/2}$ for their single reduction wave becomes more positive in the order base < nucleotide < nucleoside and is linearly dependent on pH (Table II); the $E_{1/2}$–pH dependencies for deoxycytidylic acid and 5-methyldeoxycytidine show two linear segments intersecting at *ca.* pH 7. The kinetic character of the polarographic wave at high pH for all

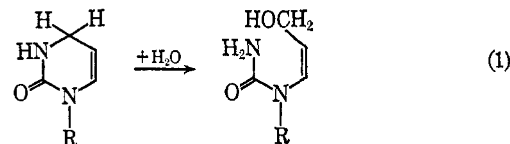
TABLE II
VARIATION WITH pH OF HALF-WAVE POTENTIALS OF CYTOSINE DERIVATIVES[a]

Compound	pH range	$-E_{1/2}$, V	pK_a[c]	pK_a'[d]
Cytosine	2.5–7	1.070 + 0.084pH	4.60	
Cytidine	2.5–7	1.105 + 0.072pH	4.22	8.55
Deoxycytidine	2.5–7	1.154 + 0.068pH	4.60	7.95
Cytidylic acid	6.5	1.68		
Deoxycytidylic acid	3.5–7.3	0.908 + 0.110pH		
	7.3–8.7	1.350 + 0.050pH		
5-Methylcytosine	5.6–6.5	(1.65 to 1.73)		
5-Methyldeoxy-cytidine	4–7.3	0.775 + 0.118pH	4.4	8.86
	7.3–10	1.325 + 0.042pH		
5-Hydroxy-methylcytosine[b]	5.6–6.5	(1.61 to 1.70)		

[a] Data taken from ref 32 and 34a; potentials *vs.* sce. [b] Ill-defined inflection on the background discharge reported as wave. [c] Dissociation constant taken from literature (*cf.* ref 34a). [d] Apparent polarographic dissociation constant, defined as midpoint of the descending segment of the S-shaped limiting current–pH plot.

cytosine derivatives and the good agreement of the experimentally observed S-shaped limiting current–pH relation with the theoretical polarographic dissociation curve, calculated from Koutecký's equation[45] assuming recombination of the species with protons, led to the conclusion that the protonated form of the pyrimidine nucleus was probably the electroactive species.

The products of macroscale electroreduction of cytosine and cytidine at a mercury cathode were identical except for the sugar moiety; consequently, the same mechanism of electrolytic reduction was proposed for both cytosine and its nucleosides and nucleotides;[34a] *i.e.*, hydrogenation of the 3,4 double bond and the splitting off of the C(4) amino group results in formation of 2-hydroxy-3,4-dihydropyrimidine (*cf.* eq 1). The reduction products are very unstable, particularly in acidic medium, being hydrolyzed at the 3,4 bond to give N(1)-substituted 3-ureidoallyl alcohol. The rate constants for hydrolysis of the cytosine and cytidine reduction products were measured.[32]



(1)

(R = H, ribosyl, or ribosyl phosphate)

3. *Azauracil Species*

6-Azauracil (3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine) and its riboside (6-azauridine) each exhibit one reduction wave, which shows kinetic character at high pH; the limiting current–pH plot is S-shaped.[46] Hence, it was suggested that the protonized forms of 6-azauracil and 6-azauridine undergo reduction at the dme. The more negative $E_{1/2}$ and the shift of the i–pH curve of azauridine to lower pH as compared to azauracil are considered to be due to formation of a complex between azauridine (presumably involving the sugar hydroxyl groups) and boric acid present in the background electrolyte.

4. *Guanine Species*

Guanine, guanosine, and guanylic acid, which give no indication of a polarographic reduction wave at the dme,[16,30,31,33] have been studied by other electrochemical methods.[47–50] The original explanation[50] of the anodic indentation on the oscillopolarographic dE/dt *vs.* E curves of guanine, its nucleosides, and nucleotides

(45) J. Koutecký, *Collection Czech. Chem. Commun.*, **18**, 597 (1953).
(46) J. Krupička and J. Gut, *ibid.*, **25**, 592 (1960).
(47) B. Janík, work in progress.
(48) B. Janík and E. Paleček, *Z. Naturforsch.*, **21b**, 1118 (1966).
(49) B. Janík and E. Paleček, *Abhandl. Deut. Akad. Wiss. Berlin, Kl. Med.*, 513 (1966).
(50) E. Paleček and B. Janík, *Chem. Zvesti*, **16**, 406 (1962).

(*cf.* subsequent discussion of oscillographic polarography), as being due to the formation of a product at potentials very close to those of background discharge, has been confirmed by means of Kalousek switching technique, voltammetry at a hanging mercury drop electrode, and macroscale electrolysis.[47,48,50]

At a mercury electrode polarized to sufficiently negative potentials, *i.e.*, in the region of background electrolyte reduction, guanine, guanosine, and guanylic acid are reduced to products, which, in each case, give an anodic wave and are unstable, disproportionating, and being oxidized to regenerate the original compound. The decomposition of the reduction product in the presence of oxygen is rapid; the oxygen is reduced to hydrogen peroxide.[47] The position of the reduction sites has not yet been ascertained with certainty; it is suggested that hydrogens are attached on reduction to N(7) and C(8) of the imidazole ring.

The reduction of guanosine could not be observed at platinum, silver, and carbon electrodes.[47]

C. CAPACITY AT DROPPING MERCURY ELECTRODES

The differential capacity of the dme in solutions of nucleic acid bases, deoxyribonucleosides, deoxyribonucleotides, and some purine derivatives in 1 M sodium chloride was measured with a low-frequency capacitance bridge (1000 Hz; 10-mV peak-to-peak voltage).[42] The data suggest that the surface activity of the bases increases in the order cytosine < thymine < adenine < guanine. The surface activity of deoxyribonucleosides and deoxyribonucleotides increases in the order deoxycytidine < thymidine < deoxyadenosine < deoxyguanosine, and deoxycytidylic acid < thymidylic acid < deoxyadenylic acid < deoxyguanylic acid. The parallelism between the three series of compounds would indicate that the base, which forms the hydrophobic part of the nucleoside or nucleotide and is thus the portion of the molecule directed to the mercury electrode surface, plays a decisive role in determining the surface activity of nucleosides and nucleotides. The surface activity increases in the order base < nucleotide < nucleoside, with the exception of the guanine series, which exhibits the reverse order.

D. OSCILLOPOLAROGRAPHY

Oscillographic polarography (oscillopolarography),[51] in which the dme is polarized with a constant magnitude alternating current, generally about 50 Hz, and the variation of dE/dt with E is recorded, has been a useful tool for the study of nucleic acids and their constituents, *e.g.*, for the estimation of the guanine plus cytosine content of DNA,[52] for the detection and estimation of denatured DNA,[53] and for following the course of thermal denaturation of DNA[53,54] and genetic relationships of bacterial DNA.[55]

Characteristic electrochemical phenomena, such as faradaic redox processes and variation in double-layer capacity due to adsorption–desorption processes, manifest themselves as notches in the roughly circular plot usually obtained; these notches are commonly referred to as incisions or indentations. The qualitative characteristic, *i.e.*, relative position on the potential axis of an incision is usually expressed as the Q value, which is defined as Q = (linear distance of the incision peak from the potential of anodic mercury dissolution)/(linear distance between the latter potential and that of background electrolyte discharge).

All nucleic acid constituents, *i.e.*, purine and pyrimidine bases and their nucleosides and nucleotides, were studied oscillopolarographically in various media.[56–62] Q values (Table III) were reported only for incisions of analytical significance,[56,58,62] which makes comparison of the data for groups of compounds difficult.

In the pyrimidine series, differences in Q seem to be significant. Q values are lower for nucleosides and nucleotides; *i.e.*, the potentials shift to more positive values in agreement with the polarographically measured $E_{1/2}$ values, indicating an increased ease of reducibility. The relatively low resolution of oscillopolarography does not allow a similar comparison in the purine series; differences in Q less than 0.02, *i.e.*, *ca.* 35 mV, are not reported (Table III), while corresponding $E_{1/2}$ differences in the adenine series do not exceed this general magnitude. However, it is evident that, on the basis of oscillopolarography, the sugar or sugar-phosphate moiety influences the behavior of the parent compound more in the pyrimidine series than in the purine one.

Besides incisions, which are due to faradaic processes and consequently correspond to dc polarographic waves, the purine and pyrimidine derivatives also exhibit capacity incisions as well as those due to electrolysis of chemically or electrochemically altered species, *e.g.*, formation of mercury compounds. Various criteria, proposed for distinguishing the nature of the incisions,[63] *e.g.*, dependence of an incision depth on tem-

(51) R. Kalvoda, "Techniques of Oscillographic Polarography," 2nd ed, Elsevier Publishing Co., Amsterdam, 1965.

(52) J. Boháček and E. Paleček, *Collection Czech. Chem. Commun.*, **30**, 3456 (1965).
(53) E. Paleček, *Biochim. Biophys. Acta*, **94**, 293 (1965).
(54) E. Paleček, *J. Mol. Biol.*, **11**, 839 (1965).
(55) E. Paleček, *Collection Czech. Chem. Commun.*, **31**, 2360 (1966).
(56) A. Humlová, *ibid.*, **29**, 18 (1964).
(57) E. Paleček, *Naturwissenschaften*, **45**, 186 (1958).
(58) E. Paleček, *Collection Czech. Chem. Commun.*, **25**, 2283 (1960).
(59) E. Paleček, *Chem. Zvesti*, **14**, 798 (1960).
(60) E. Paleček, *Biokhimiya*, **25**, 803 (1960).
(61) E. Paleček, *Biochim. Biophys. Acta*, **51**, 1 (1961).
(62) E. Paleček and D. Kaláb, *Chem. Listy*, **57**, 13 (1963).
(63) R. Kalvoda in "Progress in Polarography," Vol. 2, P. Zuman and I. M. Kolthoff, Ed., Interscience Publishers, New York, N. Y., 1962, p 449.

TABLE III

INCISIONS ON OSCILLOPOLAROGRAPHIC CURVES SUITABLE FOR DETERMINATION OF PURINE AND PYRIMIDINE DERIVATIVES[a]

	Background electrolyte[b]				
Compound	1 N H_2SO_4	1 N $HCOONH_4$	2 N $HCOONH_4$	1 N NaOH	KCl
Adenine	Q_c 0.85	...	...	R	C
Adenosine	Q_c 0.85[c]	...	...	R	C
Deoxyadenosine	Q_c 0.85[c]	...	...	R	C
Adenylic acid	Q_c 0.85[c]	C	C	R	C
Deoxyadenylic acid	Q_c 0.85[c]	C	C	R	C
Guanine	...	Q_a 0.17	Q_a 0.17, C	R	C
Guanosine	...	Q_a 0.17[c]	Q_a 0.17, C[c]	R	C
Deoxyguanosine	...	Q_a 0.17[c]	Q_a 0.17, C[c]	R	C
Guanylic acid	A, C	Q_a 0.17[c]	Q_a 0.17, C[c]	R	C
Deoxyguanylic acid	A, C	Q_a 0.17[c]	Q_a 0.17, C[c]	R	C
Xanthine	...	Q_a 0.22	...		
Xanthosine	...	Q_a 0.22[c]	...		
Cytosine	C[d]	Q_c 0.93	Q_c 0.92	R	C
Cytidine	Q_c 0.91	C[e]	C[e]	R	C
Deoxycytidine	Q_c 0.91	C[e]	C[e]	R	C
Cytidylic acid	Q_c 0.90	C[e]	Q_c 0.85	R	C
Deoxycytidylic acid	Q_c 0.90	C[e]	C[e]	R	C
5-Methylcytosine	Q_c 0.97	Q_c 0.93	Q_c 0.90	R	C
5-Methylcytidine		Q_c 0.88	C[e]		
Uracil	X	...	C	Q_c 0.12	C
Uridine	X	...	C	Q_c 0.12[c]	C
Deoxyuridine	X		C	X	C
Uridylic acid	X	...		X	
Thymine	X	...	Q_c 0.11	X	
Thymidine	X	...		X	
Thymidylic acid	X	...	X	X	
6-Azauridine	Q_c 0.67	Q_c 0.73		Q_c 0.14	

[a] All data are for $(dE/dt) - E$ curves and are taken from ref 58 and 62, except for those on 6-azauridine, which are from ref 56. [b] Definition of symbols: Q = (distance of the incision from potential of anodic mercury dissolution)/(distance between latter potential and that of background discharge); Q_c = Q of a cathodic incision; Q_a = Q of an anodic incision; C = a cathodic incision; A = an anodic incision; R = a reversible incision or deformation very close to anodic mercury dissolution; X = no incision. Incisions denoted by C, A, or R are not suitable or have not been used for analytical purposes in the given media. Dots indicate no incision or one not suitable for analysis; a blank indicates that data are not available. [c] Q value does not differ from that of the corresponding base by more than 0.02. [d] Incision is very close to background discharge. [e] Well-developed incision, but Q values are not available.

perature, frequency of alternating current, and concentration and behavior of individual nucleic acid components at the streaming mercury and hanging mercury drop electrodes and at the dme polarized with a single sweep ("first curve" technique) and with various shapes of alternating current, were applied to explain the incisions.[58,59]

Cathodic incisions for adenine, cytosine, and their derivatives in sulfuric acid and ammonium formate media (Table III) are due to faradic reduction; incisions (mostly reversible) in sodium hydroxide solution are probably due to mercury salt formations. The anodic incision for guanosine is due to oxidation of the product formed at potentials near background discharge; at equal concentrations, the less soluble guanosine gives a deeper incision than guanylic acid; this plus the decrease of the incision depth with increasing temperature indicate that adsorption processes are involved. The incisions for deoxycytidylic (cathodic), guanylic (anodic), and adenylic (cathodic) acids are not capacity controlled, since their depth decreases with the frequency of the alternating current. The cathodic incisions of deoxycytidylic and adenylic acids become deeper with increasing temperature, indicating a diffusion-controlled process.

E. ANALYTICAL APPLICATION

Polarography has been applied for determination of nucleosides and nucleotides in various sources: 6-azauracil and 6-azauridine mixtures,[46] 6-azauridine in blood,[64] 5′-O-stearyl-6-azauridine in hydrolyzates of 2′,3′-O-isopropylidene-5′-O-stearyl-6-azauridine,[65] purine and pyrimidine nucleosides and nucleotides in growing cultures of *Escherichia coli*,[66,67] purine nucleosides in blood,[68] and adenylic acid in hydrolyzates of RNA[69] and in some tissues.[70]

The oscillopolarographic activity of nucleic acid bases and nucleosides has been utilized for the analysis of nucleic acids and their constituents,[56–59,62] and as an analytical method in connection with the study of the photochemical behavior of these compounds.[67,71]

(64) V. Bulant, M. Urks, and H. Pařízková, *Antibiotiki*, **9**, 545 (1964).

(65) F. Šorm, J. Beránek, J. Smrt, J. Krupička, and J. Škoda, *Collection Czech. Chem. Commun.*, **27**, 575 (1962).

(66) D. Kaláb, *Sb. Ved. Praci Biovet.*, 56 (1960–1).

(67) D. Kaláb, *Experientia*, **17**, 275 (1961).

(68) S. Eguchi, *Nichidai Igaku Zasshi*, **20**, 2368 (1961); *Chem. Abstr.*, **61**, 3496e (1964).

(69) S. Matsushita, F. Ibuki, and A. Aoki, *J. Agr. Chem. Soc. Japan*, **37**, 67 (1963).

(70) S. Fiala and H. E. Kasinski, *J. Natl. Cancer Inst.*, **26**, 1059 (1961).

(71) D. Kaláb, *Experientia*, **19**, 392 (1963).

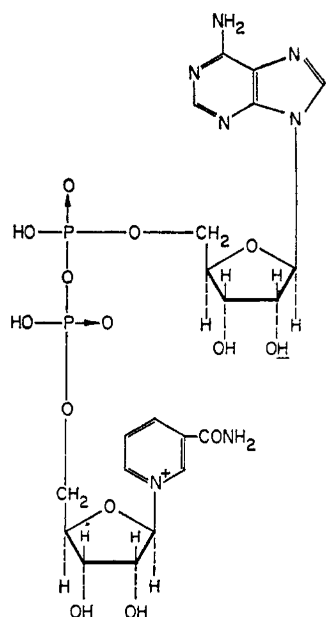


Figure 4. Formula for NAD^+, *i.e.*, nicotinamide–adenine dinucleotide. Synonyms: diphosphopyridine nucleotide (DPN^+) or coenzyme I. In $NADP^+$ (nicotinamide–adenine dinucleotide phosphate, triphosphopyridine nucleotide (TPN^+), or coenzyme II), the underlined H is replaced by a $-PO(OH)_2$ group. Configuration, dissociation equilibria, and tautomeric shifts are not shown.

III. Pyridine Nucleotides

A. Nomenclature

The essential component of the pyridine nucleotides is nicotinamide (3-carbamidopyridine), which is itself widely distributed in plant and animal tissues; the amide and the corresponding acid are also known as the vitamin niacin. Two pyridine nucleotides, *i.e.*, nicotinamide–adenine dinucleotide (NAD^+) and nicotinamide–adenine dinucleotide phosphate ($NADP^+$) (Figure 4), are hydrogen-transferring coenzymes for the class of enzymes known as dehydrogenases, which catalyze oxidation–reduction reactions; a third coenzyme, nicotinamide mononucleotide (NMN^+), is less well known.

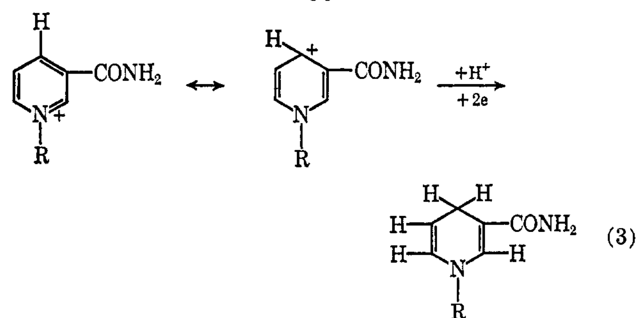
The names for NAD^+ and $NADP^+$ were proposed in 1961 by the Commission on Enzymes of the International Union of Biochemistry; however, much of current literature refers to NAD^+ as diphosphopyridine nucleotide $(DPN)^+$ or coenzyme I and to $NADP^+$ as triphosphopyridine nucleotide (TPN^+) or coenzyme II. The names DPN^+ and TPN^+ were originally proposed by Wartburg.[72]

B. Redox Activity

Both NAD^+ and $NADP^+$ participate in reactions, which they catalyze and in which they are simultaneously reduced, *e.g.*

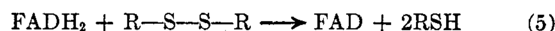
$$RCH_2OH + NAD^+ \rightleftharpoons RCHO + NADH + H^+ \quad (2)$$

(NADH and NADPH are the reduced forms of NAD^+ and $NADP^+$, respectively.) The reduction of NAD^+ and $NADP^+$ occurs at the pyridine nucleus.



In biological systems, reduced forms of the pyridine nucleotides may reduce, for example, flavin–adenine dinucleotide (FAD), whose reduced form, in turn, reduces the substrate, *e.g.*

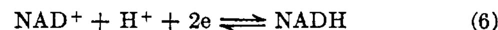
$$NADH + H^+ + FAD \longrightarrow NAD^+ + FADH_2 \quad (4)$$



$$FADH_2 + R{-}S{-}S{-}R \longrightarrow FAD + 2RSH \quad (5)$$

Flavin nucleotides (discussed in section IV) belong to the same group of coenzymes, *i.e.*, hydrogen-transferring coenzymes, as the pyridine nucleotides.

The existence of the reversible redox reaction shown by the pyridine nucleotides under physiological conditions



$$NAD^+ + H^+ + 2e \rightleftharpoons NADH \quad (6)$$

has prompted extensive study of the reaction by electrochemists, using polarographic technics.

The collection of potential data and its evaluation in Clark[11] are very helpful to anyone concerned with NAD^+ and related species and redox systems. Unfortunately, pK_a data could be located only for nicotinamide, whose pK_a is 3.61 at 20°.

C. Polarography

1. *Model Compounds*

A number of papers deal with the polarographic behavior of NAD^+ model compounds, *i.e.*, nicotinamide with various substituents on the ring nitrogen,[73–82] and

(72) E. E. Conn and P. K. Stumpf, "Outlines in Biochemistry," John Wiley and Sons, Inc., New York, N. Y., 1963, p 130.

(73) I. Bergmann in "Polarography 1964," G. J. Hills, Ed., Interscience Publishers, New York, N. Y., 1966, p 985.

(74) M. Březina and P. Zuman, "Polarography in Medicine, Biochemistry, and Pharmacy," Interscience Publishers, New York, N. Y., 1958.

(75) J. N. Burnett and A. L. Underwood, *J. Org. Chem.*, **30**, 1154 (1965).

(76) W. Ciusa, P. M. Strocchi, and G. Adamo, *Gazz. Chim. Ital.*, **80**, 604 (1950).

(77) H. Hanschmann, IVth International Congress on Polarography, Prague, 1966, personal communication.

(78) S. J. Leach, J. H. Baxendale, and M. G. Evans, *Australian J. Chem.*, **6**, 395 (1953).

(79) J. Nakaya, *Nippon Kagaku Zasshi*, **81**, 1459 (1960).

(80) F. Šorm and Z. Šormová, *Chem. Listy*, **42**, 82 (1948).

(81) P. C. Tompkins and C. L. A. Schmidt, *Univ. Calif.* (Berkeley) *Publ. Physiol.*, **8**, 237 (1943).

(82) H. Yasuda and S. Kitagawa, *Yakugaku Kenkyu*, **27**, 779 (1955).

TABLE IV
HALF-WAVE POTENTIALS OF NAD+ MODEL COMPOUNDS. N-METHYLNICOTINAMIDE SALTS

Anion[a]	Medium[b]	pH	$-E_{1/2}$, V[c] Wave I	Wave II	Ref
Cl	1	2.0	0.92	...	78
	6	5.2	0.94	1.32	78
	6	7.0	0.94	1.50[d]	78
	14	9.0	0.94	1.51	78
	16	9.7	1.08	1.65	76
	2,3,11	1.0–5.3	(1.0)[d]	...	81
	Varied	7.1–12	1.02–1.03	1.58–1.69[e]	81
	4,7,18	4–7	1.10–1.12	...	75
	7,10	7.5–8.8	1.11	1.68–1.70	75
	18	9	1.12	...	75
	7	9	1.10	1.72	75
	15	9.9	1.09	1.78	75
Br	12	Acidic	1.09	...	80
	12	Alkaline	1.09	1.66	80
	16	9.7	1.07	1.65	76
I	16	9.7	1.07	1.65	76

[a] Anion of salt added. [b] Media are listed in Table VII. [c] Dots indicate that no wave was observed or reported. [d] Ill-defined wave merging with background decomposition. [e] Potential varied irregularly over the pH range with no clear trend.

TABLE V
HALF-WAVE POTENTIALS OF NAD+ MODEL COMPOUNDS. N-SUBSTITUTED NICOTINAMIDE SALTS

N-Substituent	Anion[a]	Medium[b]	pH[d]	$-E_{1/2}$, V[c] Wave I	Wave II	Ref
$-C_2H_5$	Cl	16	9.65	1.065	1.650	76
	Br	16	9.65	1.070	1.654	76
	I	16	9.65	1.060	1.650	76
$-C_3H_7$	Cl	16	9.65	1.055	1.650	76
	Br	16	9.65	1.055	1.655	76
	I	16	9.65	1.055	1.653	76
$-C_4H_9$	Cl	16	9.65	1.048	1.655	76
	Br	16	9.65	1.050	1.655	76
	I	16	9.65	1.05	1.655	76
$-CH_2C_6H_5$	Cl	16	9.65	1.003	1.673	76
	Br	16	9.65	1.00	1.65	76
	I	16	9.65	...	1.62	76
	Cl	21	7–9	1.00	...	73
$-CH_2C_6H_4SO_3^-$		21	7–9	0.96	...	73
$-CH_2CH_2SO_3^-$		21	7–9	1.02	...	73
D-Glucopyranosidyl	Br	22		0.871	...	74, 89
D-Glucopyranosidyl tetraacetate	Br	22		0.991	...	74, 89
	Br	12		e		80

[a] Anion of salt added. [b] Media are listed in Table VII. [c] Dots indicate that no wave was observed or reported. [d] A blank indicates that the pH was not specified. [e] One wave was observed in acidic solution and two were observed in alkaline solution; numerical data were not given.

other related compounds, *e.g.*, N-substituted nicotinic acid and nicotinic acid nitrile.[73,74,83,84]

Most of the model compounds exhibit two reduction waves of about equal height, which vary directly with the square-root of the mercury height at the dme, indicating diffusion-controlled current.[73,75,81] Tables IV and V summarize the data for N-substituted nicotinamide model compounds; other model compounds are included in some of the references listed in the tables. In general, $E_{1/2}$ of the first wave (and of the single wave if only one wave is observed) is independent of pH, background electrolyte composition, and concentration of the model compound.[73,75,78,81] A linear log current plot, *i.e.*, of log $[i/(i_d - i)]$ *vs.* E, with the slope close to 0.059 V, as expected for a reversible 1e redox couple, has been reported[81] for the N-methyl model compound between pH 7 and 12; however, it has been suggested that the first wave process is irreversible under polarographic conditions.[75]

$E_{1/2}$ and slope for the second wave vary with pH and background electrolyte (details regarding the manner of variation are not given).[75,78,81] Only in the case of 1-methyl-3-carbamidopyridinium chloride are more details reported; *e.g.*, a well-defined second wave is observed[75,81] in buffered solution above pH 8, which at lower pH merges with the background discharge. In strongly basic solution, *e.g.*, pH 13, the 1-methyl-3-carbamidopyridinium ion is apparently altered, since the two waves gradually merge as the solution stands, finally giving one wave at -1.52 V.[75] $E_{1/2}$ for the second wave is independent of compound concentration in buffered solution but becomes more negative with increasing concentration in nonbuffered solution;[81] this effect could be due to pH change at the electrode surface due to hydrogen ion consumption in the electrode reaction ($E_{1/2}$ of organic compounds in buffered aqueous media generally becomes more negative with increasing pH).[10] The log current–potential plots are straight lines, whose slopes vary from 0.053 to 0.099 V;[81] the second wave process is considered to be irreversible under polarographic conditions.[75]

a. Controlled-potential electrolysis

The products obtained on reducing N-alkyl- and N-aralkyl-substituted pyridines with a $-CONH_2$, $-CN$, or $-COCH_3$ group in the 3 position by controlled-potential electrolysis, at a mercury pool cathode at a potential about 0.2 V more negative than $E_{1/2}$ of the single wave,[73] exhibit an anodic wave, whose $E_{1/2}$ is about 0.6 V more positive than $E_{1/2}$ of the cathodic wave of the original compound.

The solution obtained on exhaustive electrolysis of N-methylnicotinamide at a potential on the limiting-current portion of the first wave exhibits a reduction wave, which is near in potential but not identical with that of the second wave of the starting compound,[75] thus supporting the assumption that the second wave is not due to reduction of a stable first wave product.

b. Reduction mechanism

Based on analysis of the polarographic data and the results of controlled-potential electrolysis, the follow-

(83) M. Fedoronko, O. Manouˇek, and P. Zuman, *Chem. Listy*, 49, 1494 (1955).
(84) M. Fedoronko, O. Manoušek, and P. Zuman, *Collection Czech. Chem. Commun.*, 21, 678 (1956).

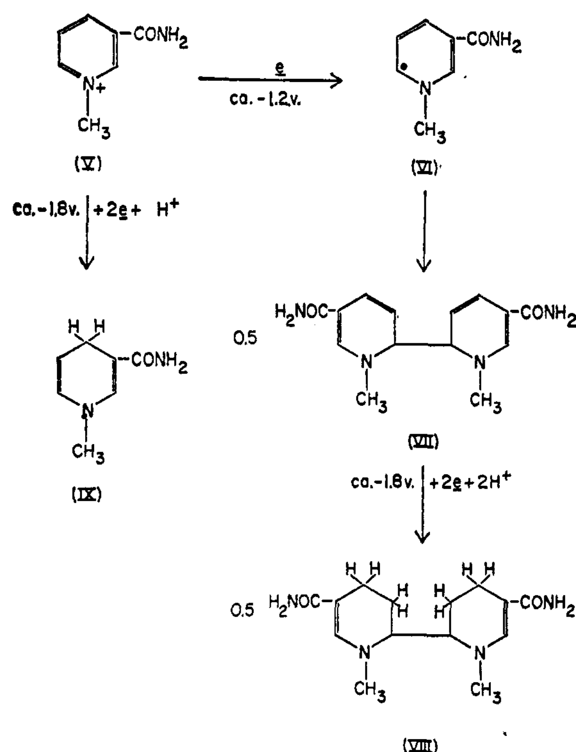


Figure 5.—Electrochemical reduction of N-methylnicotinamide (1-methyl-3-carbamidopyridinium chloride) at the dme.[75] Protonation, tautomeric equilibria, and chloride anions are not shown. See text for detailed explanation.

ing electrochemical reduction scheme was proposed[75] for N-methylnicotinamide (V in Figure 5). A reversible 1e addition to produce a free radical (VI) (source of the first wave) is followed by irreversible dimerization of the free radical in the 6 position. At the potential of the second wave, reduction both of the dimer (VII) to reduced dimer (VIII) and of the original compound (V) to N-methyl-1,4-dihydropyridine (IX) occur (the latter is equivalent to further reduction of the free radical (VI)).

Similar dimerization in the 6 position is also reported for the electrolytic and photochemical reductions of N-ethylnicotinamide under specific conditions.[77] The free radical produced on 1e reduction dimerizes to the enzymatically inactive dimer, which yields an anodic wave and which may be reoxidized by oxygen; in the latter case, the products are N-ethylnicotinamide and hydrogen peroxide.

On the other hand, model compounds with more bulky substituents at the 1 position, *e.g.*, 1-N-propyl-3-carbamidopyridinium salts, appeared to yield a 4,4′ dimer on electrolytic reduction,[85] based on spectrophotometric absorption and some chemical behavior; however, the proof is not conclusive. Obviously, the formation of the 4,4′ dimer for large substituents in the 1 position has important implications for the reduction of the pyridine nucleotides.

(85) Y. Paiss and G. Stein, *J. Chem. Soc.*, 2905 (1958).

2. Pyridine Nucleotides

The NAD^+ redox system has been extensively studied both potentiometrically[86,87] and polarographically,[73,74,77,80,88–96] and will be discussed in detail.

The one polarographic wave observed for NMN^+ and the first of the two waves observed for $NADP^+$ show the same behavior as the first wave of NAD^+.[90] Contrary to the behavior of NAD^+, $NADP^+$ is almost quantitatively reduced electrochemically to NADPH.[97,98]

a. NAD^+. Wave I

Most investigators[34b,73,74,80,89–91] report one cathodic polarographic wave for NAD^+ in both acidic and alkaline buffered solutions, whose $E_{1/2}$ does not vary significantly with pH (Table VI). However, under certain conditions, *e.g.*, in tetramethylammonium chloride[92] and tetra-*n*-butylammonium carbonate[88] buf-

Table VI

Half-Wave Potentials of NAD^+ and Related Pyridine Nucleotides[a]

Medium[b]	pH	$-E_{1/2}$, V[c] Wave I	Wave II	Ref
9	3.2–7.1	0.91–0.92	...	92
19	7.3–8.7	0.92–0.94	...	92
19	9.0	9.95	...	92
19	9.2	0.96	...	92
13	5.0	0.97	1.38	92
22	4–8	1.09[f]	...	74
5	4–9	0.93	...	88
17	7–9	0.93	1.72	88
21	7–9	0.91	...	73
6	7.4	0.93	...	91
23	4.2–6.6	1.09	...	89
24	8.3	1.10	...	89
25	10.4	1.18	...	89
	10.6[g]	0.98	...	34b
20	10.3–10.6	0.98	...	90
20[d]	10.3–10.6	1.23	...	90
20[e]	10.3–10.6	1.14	...	90

[a] Data refer to NAD^+ except where otherwise indicated. [b] Media are listed in Table VII. [c] Dots indicate that no wave was observed or reported. [d] Data for $NADP^+$. [e] Data for nicotinamide mononucleotide. [f] Slight shift of $E_{1/2}$ to more negative values with increasing pH. [g] Medium not specified.

(86) F. L. Rodkey, *J. Biol. Chem.*, **213**, 777 (1955).
(87) F. L. Rodkey and J. A. Donovan, Jr., *ibid.*, **234**, 677 (1959).
(88) J. N. Burnett and A. L. Underwood, *Biochemistry*, **4**, 2060 (1965).
(89) C. Carruthers and V. Suntzeff, *Arch. Biochem. Biophys.*, **45**, 140 (1953).
(90) C. Carruthers and J. Tech, *ibid.*, **56**, 441 (1955).
(91) R. C. Kaye and H. I. Stonehill, *J. Chem. Soc.*, 3244 (1952).
(92) B. Ke, *Biochim. Biophys. Acta*, **20**, 547 (1956).
(93) B. Ke, *Arch. Biochem. Biophys.*, **60**, 505 (1956).
(94) B. Ke, *J. Am. Chem. Soc.*, **78**, 3649 (1956).
(95) V. Moret, *Giorn. Biochim.*, **4**, 192 (1955).
(96) V. Moret, *ibid.*, **5**, 318 (1956).
(97) T. Kono and S. Nakamura, *Bull. Agr. Chem. Soc. Japan*, **22**, 399 (1958).
(98) R. F. Powning and C. C. Kratzing, *Arch. Biochem. Biophys.*, **66**, 249 (1951).

TABLE VII

MEDIA SPECIFIED IN TABLES IV, V, AND VI

No.	Medium[a]
1	Hydrochloric acid
2	0.1 *M* hydrochloric acid
3	0.2 *M* acetate buffer
4	0.2 *M* acetate and citrate buffers
5	Acetate, citrate, phosphate, and pyrophosphate buffers
6	Phosphate buffer
7	0.02 *M* phosphate buffer
8	0.1 *M* phosphate buffer
9	0.1 *M* phosphate–citrate buffer
10	0.02 *M* pyrophosphate buffer
11	0.1 *M* phthalate buffer
12	Robinson buffer
13	0.3 *M* tetramethylammonium chloride
14	Glycine buffer
15	0.02 *M* ammonia
16	Tetramethylammonium borate buffer
17	Tetra-*n*-butylammonium carbonate buffer (ionic strength = 0.1)
18	0.02 *M* Tris buffer
19	0.1 *M* Tris buffer
20	0.5 *M* Tris buffer
21	Tris and phenolsulfonate buffers
22	50% dioxane with 0.1 *M* tetra-*n*-butylammonium iodide (TBAI)
23	Citrate buffer in 50% dioxane with 0.1 *M* TBAI
24	Phosphate buffer in 50% dioxane with 0.1 *M* TBAI
25	Borate buffer in 50% dioxane with 0.1 *M* TBAI

[a] Concentrations of electrolyte or principal buffer component are given, where they could be located in the original paper. Cations, such as quaternary ammonium ions, are indicated where they may have had a significant effect, *e.g.*, on surface activity.

fers, two reduction waves have been reported.[77,88,92,95,96] Under some (not specified) conditions, a small concentration-independent prewave (about 20 mV, before the first wave) was observed, but was not studied.[73,88]

The first (or only) reduction wave is diffusion-controlled[73,88] with a constant i_d/C ratio from low pH to about pH 10.4, where it drops somewhat;[88–90,92] the log current–potential plot exhibits the straight line and slope[80,89,90] expected of a polarographically reversible 1e reduction. It has been suggested[73,92] that this first wave is irreversible because of the pH independency of $E_{1/2}$ below pH 7 and of the differences between $E_{1/2}$ and the oxidation–reduction (potentiometric?) potential and between $E_{1/2}$ of the cathodic wave of the compound and that of the anodic wave of its reduced form; however, these arguments are not in themselves conclusive.

In buffered 50% dioxane solutions, NAD^+ was polarographically reducible only in the presence of tetrabutylammonium iodide;[89] it is possible that the buffer systems tried (not specified) gave background discharge waves prior to the NAD^+ wave.

b. NAD^+. Wave II

The second NAD^+ reduction wave, where observed,[88,92] more or less completely merges in most buffered solutions with background discharge, whereas the second wave of related model compounds is well defined at the same potential. This effect may be due to the shift of background discharge to more positive potential, *e.g.*, by 0.18 V at pH 1.6 and 0.3 V at pH 10.3,[34b] because of the lowering of the hydrogen overpotential by NAD^+ or its reduction products as a result of the presence in the NAD^+ molecule of the adenine moiety, which does exhibit the same effect.[16]

Even under the most favorable conditions, the reproducibility of wave heights and $E_{1/2}$ is rather poor; however, $E_{1/2}$ is apparently independent of pH over the pH range of 6–9.[88] This may be contrasted with the behavior of N-methylnicotinamide, which exhibits nearly the pH dependence expected for a 2e process involving one hydrogen ion, *i.e.*, 30 mV per pH unit.[75]

The second wave height is about the same as that of the first wave[92] and shows a dependence on the mercury height (h)[88] intermediate between those expected for diffusion (proportional to $h^{1/2}$) and kinetic (proportional to h^0) controlled currents; *i.e.*, the current is proportional to $h^{1/4}$.

Slopes of log current–potential plots for the second wave vary from about 0.025 to 0.1, which was taken to indicate the irreversibility of the electrode process.[88]

c. NAD^+. Controlled-potential electrolysis

Attempts to prepare the reduced nicotinamide–adenine dinucleotide (NADH) by controlled-potential electrolysis of NAD^+ at mercury electrodes[73,77,88,92,93,97–100] and at various solid metal electrodes[94] have led to reduction products (not isolated), the coenzyme activity of whose solutions varied from 0 to 76% of the expected NADH activity (*cf.* subsequent discussion).

The dimer frequently postulated as resulting from the free radical produced on 1e reduction of NAD^+ has never actually been isolated and unequivocally identified. Its existence has been inferred on the basis that solutions supposed to contain it gave an ultraviolet absorption spectrum similar to that of NADH but showed no coenzyme activity. Work is now in process[101] in the attempt to isolate and characterize the postulated dimer, whose nature as a 4,4′ or 6,6′ species is even uncertain.

The solution obtained after elimination of the first wave by macroscale electrolysis at a mercury cathode at a potential corresponding to the limiting portion of the wave exhibits an anodic wave about 0.6 V more positive[73,77,78] than $E_{1/2}$ for the original cathodic wave I, *i.e.*, at *ca.* −0.25 V. The product in this solution is reoxidized to NAD^+ at a mercury pool electrode at the anodic wave potential as well as by an enzyme obtained from mung beans.[88]

(99) T. Kono, *Bull. Agr. Chem. Soc. Japan*, **21**, 115 (1957).
(100) M. B. Yarmolinsky and S. P. Colowick, *Biochim. Biophys. Acta*, **20**, 177 (1956).
(101) C. Schmakel and P. J. Elving, work in progress.

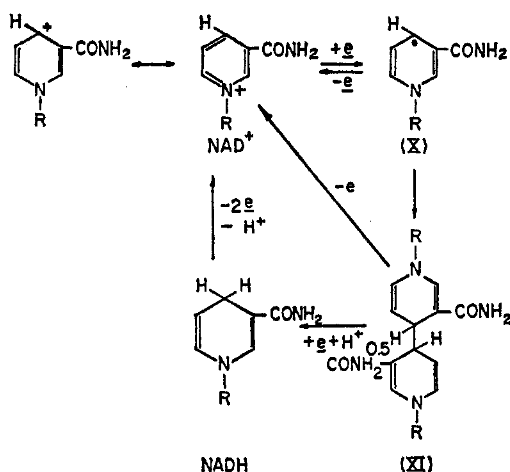


Figure 6.—Electrochemical redox pattern for NAD^+, NADH, and postulated reduction products of NAD^+ at the dme. See text for detailed explanation.

The first wave product solution exhibits no NADH enzymatic activity and is believed to be a 4,4′ dimer,[75] similar to that obtained on reduction of N-*n*-propylnicotinamide,[75,85] but in contrast to the N-methyl- (20) and N-ethyl- (21) nicotinamides, which form 6,6′ dimers. Also contrary to the behavior of N-methylnicotinamide,[75] solutions of the first wave product exhibit a very small cathodic wave ($E_{1/2}$ = −1.7 V) which is ascribed to reduction of the dimer to the 1,4-dihydro compound, *i.e.*, NADH.[88]

Results of electrolysis at a potential on the second NAD^+ wave-limiting portion are equivocal. When NAD^+ solutions were electrolyzed at −1.85 V, NADH enzymatic activity developed very slowly[88] (it is not clear from the original paper whether the electrolysis rate was equally slow); *e.g.*, conversion of NAD^+ to NADH was 35% complete in 3 hr but after 54 hr was still only 42%. This is again in marked contrast to N-methylnicotinamide whose reduction to the 1,4-dihydro product was complete in 2–3 hr.[75] Addition of cationic surfactant and vigorous ultrasonic agitation of the mercury–solution interface increased the rate of NADH formation considerably.[88] The low yields of enzymatically active NADH were explained as due to the concurrent formation of dimer.[88]

d. NAD^+. Mechanistic interpretation

The interpretations in individual papers of the polarographic data and of the results of macroscale electrolysis of NAD^+ vary even if the results are similar. However, the following mechanism (*cf.* Figure 6) seems now to be the most probable, based on all of the available data, if the postulation of formation of the 4,4′ dimer is accepted.

The first reversible 1e reduction step (wave I), in which the free radical (X) is formed, is followed by the irreversible dimerization of X to dimer XI. The second polarographic wave for NAD^+ solutions then represents the further reduction of the free radical or of the dimer to NADH, which can be electrolytically and enzymatically reoxidized to NAD^+.

The anodic wave observed for the solution of the first-wave product would then be due to the oxidation of the dimer to the original NAD^+; the cathodic wave observed for the same solution would be due to reduction of the dimer to NADH.

On the basis of this mechanism, it would appear that previous workers, who attempted the electrolytic preparation of NADH at a potential on the first wave, actually obtained the dimer.

A number of questions are raised by this mechanism and other postulations made in the consideration of the electrochemical data on NAD^+ and its reduction products. One is the failure to detect any electrochemical reduction of the adenine moiety of the NAD^+, which should be reducible in the potential range covered, except in one report of electrolysis at uncontrolled potential[35] (*cf.* section II.B); this may be due to the manner in which the NAD^+ orients itself at the electrode; *e.g.*, *cf.* discussion of effect of hydrophobic groups in section II.C. Other questions concern the ease of reducibility and oxidizibility of the carbon–carbon single bond, which is the basis of the postulated electroactivity of the dimer; electrochemical reduction or oxidation of carbon–carbon single bonds under polarographic conditions is virtually unknown, except in semiquinone type dimers, such as alloxantin,[102] where the carbon atoms forming the single bond each carry a hydroxyl group. Studies attempting to answer these questions are now in progress.[101,103]

D. ANALYTICAL APPLICATION

Both NAD^+ and $NADP^+$ have been polarographically determined in various tissues and organs.[89,90,104–106] The polarographic determination of nicotinic acid and its various derivatives has been well reviewed.[74]

IV. FLAVIN NUCLEOTIDES

A. NOMENCLATURE

Flavin is the trivial name of the redox-active prosthetic group[107] of a class of respiratory enzymes, *i.e.*, the flavin enzymes or flavoproteins, which occur widely in animals and plants. Many of these flavoproteins

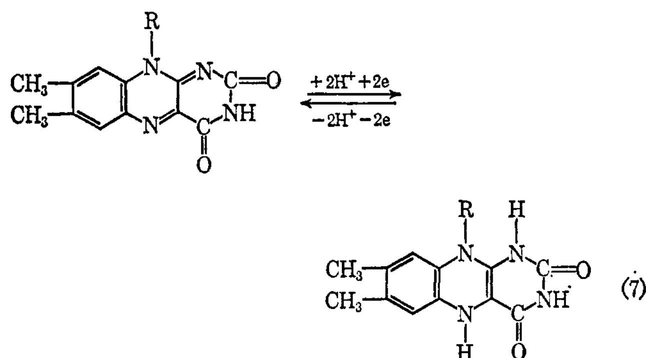
(102) W. A. Struck and P. J. Elving, *J. Am. Chem. Soc.*, **86**, 1229 (1964).
(103) B. Janík and P. J. Elving, work in progress.
(104) C. Carruthers and V. Suntzeff, *Cancer Res.*, **12**, 253 (1952).
(105) C. Carruthers and V. Suntzeff, *ibid.*, **14**, 29 (1954).
(106) C. Carruthers, V. Suntzeff, and P. N. Harris, *ibid.*, **14**, 845 (1954).
(107) A prosthetic group is usually considered to be a cofactor firmly bound to the enzyme molecule. The general term *cofactors* refers to factors, which, together with an inactive protein (apoprotein), form an active enzyme. Cofactors may be divided rather loosely into three types: (a) prosthetic groups, (b) coenzymes, and (c) metal activators.

work directly on the substrates, while others act as intermediates in the respiratory chain by bridging the electron-transport gap between the pyridine nucleotides, which they dehydrogenate, and the cytochromes, to which they transfer the hydrogen. Two main groups of flavoproteins can be distinguished; one contains iron (and sometimes also molybdenum) as an essential cofactor, while the other is free of metals.

The flavin group constitutes a part of hydrogen-transferring coenzymes, *i.e.*, flavocoenzymes, which are linked with the apoproteins. Two flavocoenzymes (flavin mononucleotide (FMN) and flavin–adenine dinucleotide (FAD)) are called flavin nucleotides, although this term is inapt, since these flavins are not N-glycosides of ribose but derivatives of ribitol, *i.e.*, 6,7-dimethyl-9-D-ribitylisoalloxazine (Figure 7). Their essential part is riboflavin (vitamin B_2, 6,7-dimethyl-9-ribitylisoalloxazine), which occurs in nature almost exclusively as a constituent of FMN and FAD.

B. REDOX ACTIVITY

The isoalloxazine moiety forms a reversible redox system (eq 7) which has been studied polarographi-



cally.[108] A recent review[109] contains much valuable information on the chemistry and molecular biology of flavins and flavocoenzymes.

Flavins form thermodynamically reversible redox systems, irrespective of whether one or two electrons are being transferred per flavin molecule, thus fulfilling the criteria of a quinoid redox system (Figure 8). Depending on pH, one cationic, one neutral, and one anionic species can be distinguished for each redox state. The flavohydroquinone to flavoquinone conversion can be easily produced by oxygen.

Flavins are very sensitive to light. On absorption of light, hydrogen is transferred from the hydroxylic side chain to the isoalloxazine ring with the side chain being subsequently split off. This irreversible photolysis can be prevented by the addition of external elec-

(108) Y. Mori and K. Murata, *Vitamins* (Kyoto), 2, 24 (1949).
(109) P. Hemmerich, C. Veeger, and H. C. S. Wood, *Angew. Chem. Intern. Ed. Engl.*, 4, 671 (1965).

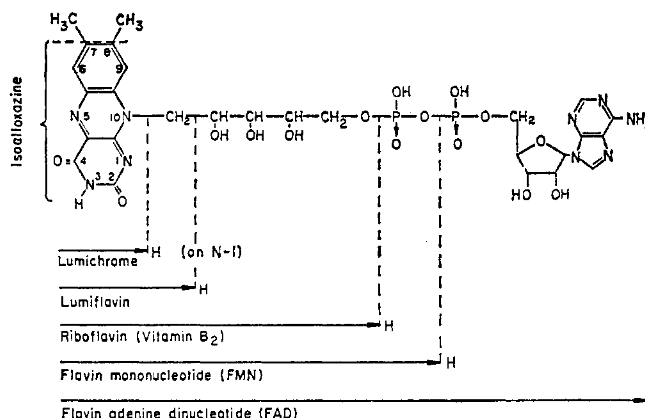


Figure 7.—Flavin nomenclature (adapted from ref 109).

tron donors, that are themselves more easily dehydrogenated by the light-activated flavin in its triplet state, so that merely a photoreduction occurs, which is reversible on addition of oxygen.

A pK_a could be located only for riboflavin, whose pK_a is 9.69 at 25°.

C. POLAROGRAPHY

All compounds derived from flavin, *e.g.*, lumiflavin, riboflavin, and the flavin nucleotides, which are in general N(10)-substituted isoalloxazines, form reversible redox systems, whose half-wave potentials are identical with the potentiometrically determined formal potentials (*cf.* ref 11).

Much of the literature up to *ca.* 1954 on the polarographic behavior of flavin nucleotides and the analytical application of such behavior has been reviewed by Brezina and Zuman.[74] The polarography of isoalloxazine derivatives, riboflavin, and flavin nucleotides has been briefly and not too completely reviewed in Japanese.[110]

1. *Riboflavin and Related Compounds*

Riboflavin (vitamin B_2, lactoflavin), which is an important growth factor and an essential component of a number of flavin-containing enzymes, has been extensively studied potentiometrically[111–119] and polarographically, *e.g.*, by classical polarography at the

(110) Y. Asahi, *Rev. Polarog.* (Kyoto), 8, 1 (1960).
(111) E. S. G. Baron and A. B. Hastings, *J. Biol. Chem.*, 105, Proceedings vii (1934).
(112) R. Kuhn and P. Boulanger, *Chem. Ber.*, 69B, 1557 (1936).
(113) R. Kuhn and G. Moruzzi, *ibid.*, 67, 120 (1934).
(114) L. Michaelis, *Chem. Rev.*, 16, 243 (1935).
(115) L. Michaelis and G. Schwarzenbach, *J. Biol. Chem.*, 123, 527 (1938).
(116) L. Michaelis, M. D. Schubert, and S. V. Smythe, *ibid.*, 116, 587 (1936).
(117) F. J. Stare, *ibid.*, 112, 223 (1935–6).
(118) K. G. Stern, *Biochem. J.*, 28, 949 (1934).
(119) M. Valentinuzzi, L. E. Cotino, and M. Portnoy, *Anales Soc. Cient. Arg.*, 147, 45 (1949).

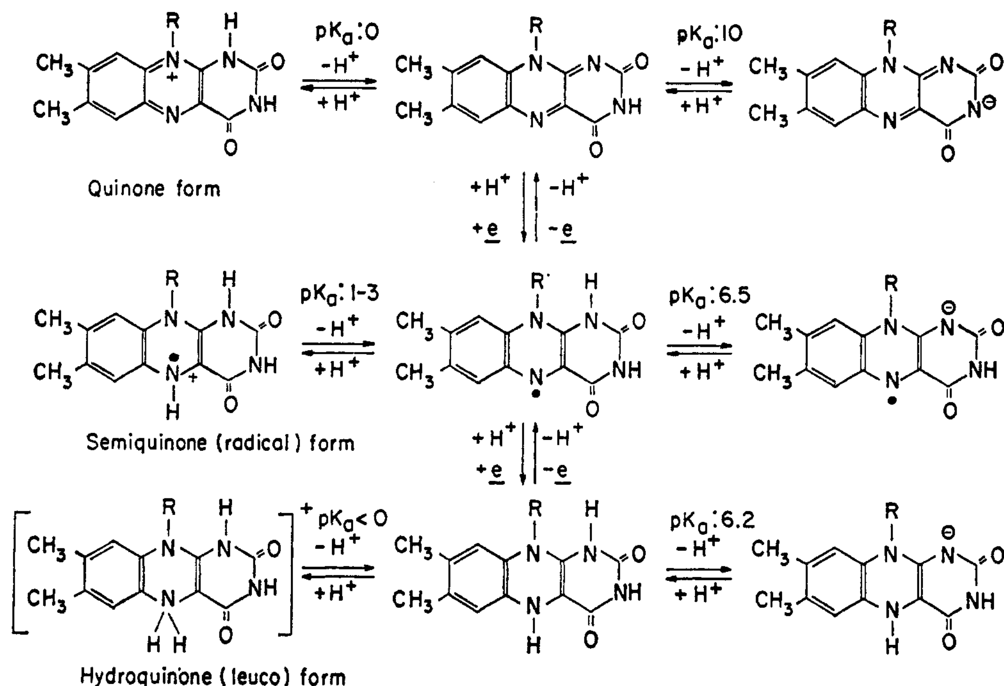


Figure 8.—The flavin redox system: flavin species are shown as a function of redox state and pH (adapted from ref 109).

dropping mercury electrode,[91,110,120–130] alternating-current polarography,[131] and chronopotentiometry.[132,133]

Potentiometric studies[111,112,114–116,118] have shown that riboflavin is reduced in two overlapping 1e steps. Formation constants for the intermediate semiquinone were calculated from potentiometric[112,115–117] and polarographic data[125] using Michaelis' index potential method.[116,134] Standard redox potentials for riboflavin and compounds related to riboflavin[112,113,115–118] and for a number of flavin derivatives, *i.e.*, 10-substituted isoalloxazines,[112,116] have been determined in solutions of various pH, and critical summaries of these data are available.[11,12]

Riboflavin[91,117,121–130] and compounds[110,128,130] related to it exhibit a reversible 2e reduction wave over the whole pH range in conventional dme polarography. An analysis of this wave has indicated,[91,121,125,127,135] similar to the potentiometric studies, that polarographic reduction of these compounds involves two overlapping 1e steps with the intermediate formation of a semiquinone (Figure 8).

The $E_{1/2}$–pH dependence of the riboflavin reduction wave (Figure 9) is linear, but with two changes in slope[120,124,125,128] at pH values, which correspond to the acid dissociation constants of the reduced and oxidized forms.[120,125] The equation for $E_{1/2}$ *vs.* nce (eq 8), which

$$E_{1/2} = 0.188 + 0.029 \log \frac{5.01 \times 10^{-7} + [\mathrm{H}^+]}{6.31 \times 10^{-10} + [\mathrm{H}^+]} - 0.058\mathrm{pH} \quad (8)$$

was calculated from dc polarographic data,[125] fits fairly well the potentiometrically estimated formal potentials.[111–118] The apparent semiquinone formation constant at 25° calculated from dc polarographic data[91] (K = 1.49 at pH 7.4) also agrees well with potentiometric data. The diffusion coefficient, $D \times 10^6$ cm²/sec, was calculated to be 7.41 and 5.64 for 0.0475 and 0.38 mM solutions of riboflavin, respectively[121] (units in the original paper were erroneously given as "mm/sec").

In connection with Figure 9, it should be noted that the slope of each linear segment of the $E_{1/2}$–pH curve is related to the ratio of protons to electrons in the controlling-electrode process in that pH region, *i.e.*, the slope = $0.059p/n$ for a reversible reaction at 25°, where p = number of protons involved and n = number

(120) Y. Asahi, *J. Pharm. Soc. Japan*, **76**, 378 (1956).
(121) R. Brdička, *Z. Elektrochem.*, **48**, 686 (1942).
(122) R. Brdička, *Chem. Listy*, **36**, 286 (1943).
(123) R. Brdička, *ibid.*, **36**, 299 (1943).
(124) R. Brdička, *Collection Czech. Chem. Commun.*, **14**, 130 (1949).
(125) R. Brdička and E. Knobloch, *Z. Elektrochem.*, **47**, 721 (1941).
(126) J. J. Lingane and O. L. Davis, *J. Biol. Chem.*, **137**, 567 (1941).
(127) J. R. Merkel and W. J. Nickerson, *Biochim. Biophys. Acta*, **14**, 303 (1954).
(128) Y. Mori and K. Murata, *Vitamins* (Kyoto), **2**, 14, 24 (1949).
(129) K. Murata and Y. Mori, *ibid.*, **2**, 68 (1949).
(130) K. Schwabe, "Polarographie und Chemische Konstitution Organischer Verbindungen," Akademie-Verlag, Berlin, 1957.
(131) B. Breyer and T. Biegler, *Collection Czech. Chem. Commun.*, **25**, 3348 (1960).
(132) H. Herman, S. V. Tatwawadi, and A. J. Bard, *Anal. Chem.*, **35**, 2210 (1963).
(133) S. V. Tatwawadi and A. J. Bard, *ibid.*, **36**, 2 (1964).
(134) R. Brdička, *Z. Elektrochem.*, **47**, 314 (1941).
(135) R. Brdička, *Collection Czech. Chem. Commun.*, **12**, 522 (1947).

of electrons (*cf.* ref 4, 10, 11, and 136 for further treatment, including that of the irreversible electrode process). After an inflection point, the change of the slope to a smaller value with increasing pH indicates that the pH at the inflection point pertains to the pK_a of the reduced form; if the slope changes to a higher value, the pK_a pertains to the oxidized form.

While free riboflavin is readily reducible, the protein-bound riboflavin is not reduced at the dme.[137] From the titration curve of riboflavin with protein, where the riboflavin concentration was estimated polarographically, a molar ratio of 1:1 was calculated for the protein–riboflavin complex.

a. Adsorption of riboflavin and leucoriboflavin

In addition to the reduction wave mentioned, riboflavin and a number of other flavin derivatives exhibit in acidic solution a reduction prewave.[91,120–125,127–129,138,139] In very dilute riboflavin solutions (below about 0.05 mM), only one wave is seen, whose height reaches a limiting value with increasing concentration[121] (limiting concentration not specified). At higher concentration, a second, more negative wave appears, whose height is directly proportional to concentration, while the height of the original more positive prewave remains unchanged. To interpret the riboflavin prewave, as well as that shown by methylene blue,[140] an adsorption theory was developed by Brdička[121,135] (and subsequently generalized), in which it is assumed that the reduction product of the riboflavin (leucoriboflavin or flavohydroquinone) is more strongly adsorbed than riboflavin on the mercury electrode; this results in the appearance of the prewave, since the reversible reduction of a compound to an adsorbed species requires less energy than its reduction to the same species in solution. The pK_a value of leucoriboflavin is 6.3, so that in solutions of higher pH, the anion of leucoriboflavin is the form mainly present (Figure 8); since the anion is more soluble and hence less strongly adsorbed than the unchanged molecule, no dc polarographic prewave is found under conditions where no uncharged species is present,[127] *i.e.*, above pH 7.3. The kinetics of the adsorption were investigated by means of *i–t* curves;[121] *i–t* curves obtained in the potential region of the prewave exhibit two maxima, one being assigned to the adsorption of the semiquinone and the other to that of the leuco form. From the shapes of the *i–t* curves, delayed establishment of adsorption equilibrium was assumed for the leuco form and was explained as due to the change of an adsorption-active form of the reduction product into an adsorbable form (the difference between the two forms was not made clear); this change was considered to be probably autocatalytic. The adsorption prewave is not suppressed by the addition of urea, but disappears on the addition of pyridine[138] (presumably due to the stronger adsorption of pyridine on the electrode).

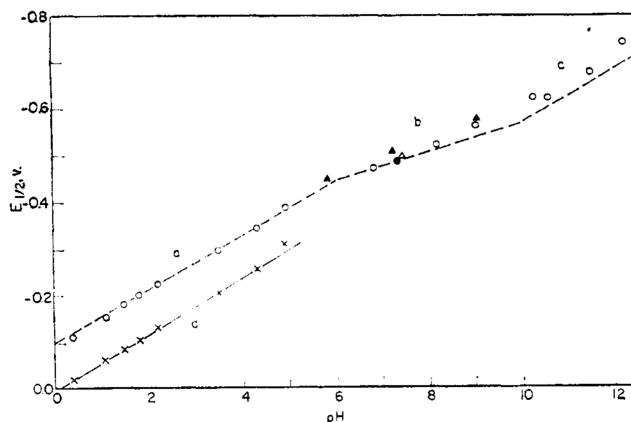


Figure 9.—Variation with pH of $E_{1/2}$ for riboflavin. Potentials are *vs.* nce (add 0.037 V to get potential *vs.* sce). Reduction (main) wave: dashed line (concentration, 0.3 mM);[125,135] empty circes (0.475 mM);[120] full circles;[127] empty triangles (1 mM);[91] full triangles.[126] Adsorption prewave: crosses (0.475 mM).[120] Calculated[125,135] $d(E_{1/2})/d(\text{pH})$: a, 0.0582; b, 0.0304; c, 0.0590; d, 0.0165. First inflection point is at pH 6.05 and second at pH 9.95.

Adsorption of leucoriboflavin is largely confirmed by the use of alternating current polarography.[131] A depression of the base current on one or both sides of the ac reduction wave of riboflavin (summit potential, E_s: about −0.05 and −0.25 V in 0.1 M $HClO_4$ and pH 4.7 acetate buffer, respectively) is observed up to pH 9.5. These results were interpreted to indicate that slight adsorption of leucoriboflavin on the dropping mercury electrode persists even at pH as high as 9.5 and, moreover, that both the uncharged and anionic forms of riboflavin are appreciably adsorbed. Adsorption of riboflavin is accompanied by the appearance of an ac polarographic wave at pH 3 and above, whose E_s shifts from 0.28 V at pH 3 to −0.1 V in 0.1 M NaOH and whose height increases with increasing riboflavin concentration to a limiting value at about 0.06 mM. No dc polarographic wave corresponding to the ac wave was found, although the residual current in the presence of riboflavin, at potentials more positive than that of the latter's reduction wave, is slightly below that observed for supporting electrolyte alone. Moreover, in the potential region corresponding to this ac wave, a slight kink could often be observed on the dc polarograms. The behavior of the ac wave was interpreted as due to formation of an insoluble covalent compound with mercury by the adsorbed riboflavin molecules.

(136) H. J. Gardner and L. E. Lyons, *Rev. Pure Appl. Chem.*, **3**, 134 (1953).
(137) Z. Ostrowski and A. Krawczyk, *Acta Chem. Scand.*, **17** (Suppl), 241 (1963).
(138) S. Fowler and R. C. Kaye, *J. Pharm. Pharmacol.*, **4**, 748 (1952).
(139) G. A. Tedoraze, E. Yu. Khmelnitskaya, and Ya. M. Zolotovitskii, *Electrokhimiya*, **3**, 200 (1967); *Chem. Abstr.*, **66**, 10351 (1967).
(140) R. Brdička, *Z. Elektrochem.*, **48**, 278 (1942).

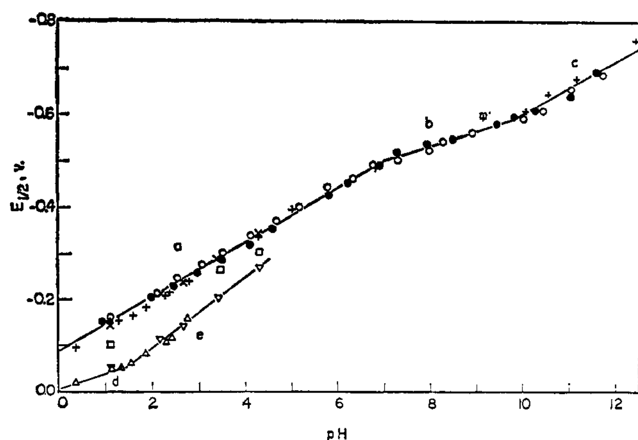


Figure 10.—Variation of $E_{1/2}$ for FMN and FAD with pH. Potentials are *vs.* nce (add 0.037 V to get potential *vs.* sce). Reduction (main) wave of FAD: full circles;[147] pluses (concentration, 0.46 mM);[120] crosses (0.092 mM);[120] squares (0.0184 mM).[120] Adsorption prewave of FAD: triangles (0.46 mM);[120] reversed triangles (0.092 mM).[120] Calculated d($E_{1/2}$)/d(pH): a, 0.0597; b, 0.0310; c, 0.0568; d, 0.0346; e, 0.0772. First inflection point in upper curve is at pH 7.0 and second at pH 9.75; inflection point in lower curve is at pH 1.5.

Adsorption of riboflavin and of leucoriboflavin at the dme was also studied chronopotentiometrically[132,133,139] and by measurement of the double-layer capacity[141] and electrocapillary curves.[133] The adsorption of both was confirmed,[132,133] with the leuco form being the more strongly adsorbed. The surface excess values (Γ) are of the order of 0.2×10^{-9} mole/cm^2 for both,[131,141] which agrees well with the value of 0.14×10^{-9} mole/cm^2 obtained from dc polarographic data.[121] However, only limited physical significance can be given to these values, since the assumption that all of the product remains on the electrode surface is obviously not correct;[141] in addition, the method of calculating[133] the extent of adsorption of electroactive riboflavin onto the mercury electrode from chronopotentiometric data has been found to be generally inaccurate.[142]

A recent chronopotentiometric study[139] indicated that, in the potential region of the riboflavin adsorption prewave, the dme surface is covered by riboflavin and that the adsorption (relative coverage of electrode surface?) of the leucoriboflavin produced on reduction is less than that calculated.

In connection with the latter and preceding discussion, the point has recently been made that chronopotentiometry has only limited applicability to the study of adsorption.[143]

b. Catalytic wave

In addition to the waves mentioned, solutions of riboflavin,[144,145] FMN,[145] and FAD[144] have been reported to exhibit up to pH 8 a catalytic hydrogen wave between the normal reduction wave and background discharge. The catalytic wave height at equimolar depolarizer concentration decreases in the following order (relative wave heights shown in parentheses): FAD (6) > riboflavin > (2) > FMN (1). The catalytic wave height of isoalloxazine derivatives without the sugar moiety is about one-tenth that of riboflavin: lumichrom, which is the product of photolytic degradation of riboflavin, does not give the catalytic wave.[145]

The height of the riboflavin catalytic wave in phosphate buffers and potassium chloride solution is about a thousand times that of the reduction wave.[144]

A second catalytic wave, which appears (potential region not specified) in riboflavin solutions above 3 mM, is stated not to be dependent on the first catalytic wave.[144]

The protonated reduced forms of riboflavin, FAD, and FMN in their adsorbed state are suggested to be the catalytically active species.[145]

2. *Flavin Nucleotides*

The polarographic behavior of FMN and FAD is quite similar to that of riboflavin; a 2e reduction wave produced on dc polarography is also due to two overlapping 1e steps, indicating semiquinone formation.[120,146–149] The $E_{1/2}$ values for FMN and FAD[147] correspond to the standard redox potentials of the thermodynamically reversible systems determined potentiometrically.[112] The $E_{1/2}$–pH plot is linear with two inflection points (Figure 10), corresponding to the acid dissociation constants of the reduced and oxidized forms, respectively.[120,147] Semiquinone formation constants, calculated from the polarographic data by Michaelis' index potential method,[147,149] increase with concentration[147] and change with pH,[149] *e.g.*, log $K = -2$ in pH 2.0 citrate–phosphate buffer + 0.9 N KNO_3, -0.2 in 0.1 N HNO_3 + 0.9 N KNO_3, and 0.8 in 1 N HNO_3.

a. Adsorption prewave

Similarly to riboflavin, FMN and FAD exhibit an adsorption prewave at the dme in acidic solution,[110,120,146,149] which has been interpreted by Brdička's adsorption theory on the basis that the reduced form is strongly adsorbed (at least more strongly than the oxidized form) on the mercury. The sum of the main reduction wave and the prewave, which is independent of pH, is directly proportional to concentration with

(141) T. Biegler and H. A. Laitinen, *J. Phys. Chem.*, **68**, 2374 (1964).
(142) P. J. Lingane, *Anal. Chem.*, **39**, 541 (1967).
(143) P. J. Lingane, *ibid.*, **39**, 485 (1967).
(144) B. Jambor, *Magy. Kem. Folyoirat*, **73**, 178 (1967); *Chem. Abstr.*, **67**, 3706 (1967).
(145) E. Knobloch, *Collection Czech. Chem. Commun.*, **31**, 4503 (1966).
(146) A. M. Hartly and G. S. Wilson, *Anal. Chem.*, **38**, 681 (1966).
(147) B. Ke, *Arch. Biochem. Biophys.*, **68**, 330 (1957).
(148) V. Moret and S. Pinamonti, *Giorn. Biochim.*, **9**, 223 (1960).
(149) M. Senda, M. Senda, and I. Tachi, *Rev. Polarog.* (Kyoto), **10**, 142 (1962).

the height of the prewave becoming constant at about 0.06 mM.[120,149]

The dc polarographic results have been unambiguously confirmed by alternating current polarography[149–151] and by cyclic voltammetry and chronopotentiometry.[146,152]

Alternating current polarograms of FMN and FAD in acidic solution exhibit two waves, whose summit potentials correspond quite well to the $E_{1/2}$ values of the dc adsorption prewave and main reduction wave[149–151] (Figure 11); the summit potentials and peak heights of the two waves remain constant for different ratios of oxidized to reduced forms of FMN in the bulk solution.[149] The ac adsorption wave of FMN is several times higher than the main ac reduction wave; at very low FMN concentrations, only the adsorption wave is observed with its height being proportional to the concentration even though it is almost ten times that expected for a simple inorganic redox system,[149] presumably for a 1e faradic process. The theoretical equation for an ac polarogram involving a reversible redox system with an intermediate semiquinone step and strong adsorption of the reduced form at the dropping mercury electrode has been derived;[149] on the basis of the degree of conformity of behavior to this equation, the flavin redox system does not seem to be completely reversible by ac polarography.

b. Adsorption postwave

In neutral and alkaline solutions of FMN and FAD, where the prewave is not observed, a dc wave, which appears at more negative potential than the main reduction wave (probably close to background discharge, since this postwave merges with background discharge below pH 5.6), is accompanied by abnormal current oscillations.[147] The FAD postwave has a slightly more positive $E_{1/2}$ and a slightly smaller height than the FMN postwave at identical concentrations.[147] The postwave appearing in basic solution of FMN has also been described as ill-defined; a corresponding ac postwave is seen at 15 mV more negative potential than the main ac wave.[149]

At very low concentrations, only the postwave is observed. When both waves appear, their total height is proportional to concentration; the height of the postwave is independent of concentration (apparently above a certain limit) and directly proportional to the mercury height, indicating its adsorption character. The diffusion current constants for the total wave heights of FMN and FAD (main wave plus postwave) are reported to be 1.22 and 1.60, respectively;[147] the ratio of 1.31 for the square roots of the diffusion coefficients agrees only moderately well with the value of

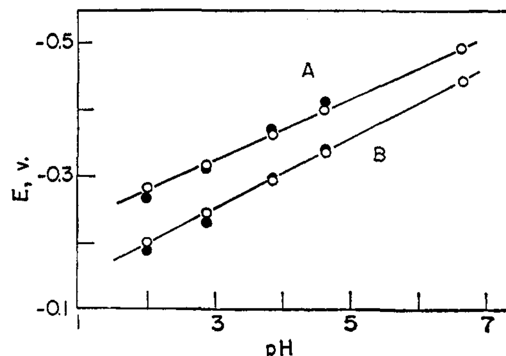


Figure 11.—Comparison of dc and ac polarographic data for 0.2 mM FMN.[149] Potentials given are *vs.* nce (add 0.037 V to get potential *vs.* sce). A, reduction (main) wave. B, adsorption prewave. Empty circles, $E_{1/2}$; full circles, E_s. Calculated d($E_{1/2}$)/d(pH): A, 0.0458; B, 0.0520.

1.10 for the similar ratio calculated from the Stokes–Einstein equation.[4]

On raising the temperature from 25 to 45°, the postwave almost disappears while the height of the main wave increases by about 50% (this is equivalent to a 2% increase per degree) and its $E_{1/2}$ shifts by 25 mV to more negative potential.

The appearance and behavior of the postwave were explained as due to the strong adsorption of the oxidized form, *i.e.*, FMN or FAD, at the dme.[147,149]

Alternating current polarography of FMN in neutral and slightly basic solutions[149] revealed, in agreement with the behavior of riboflavin,[153] not only the adsorption of the reduced form but also of FMN itself. In addition to the ac reduction wave, an anodic wave observed at about 0.0 V is probably due to the formation of an insoluble compound of mercury with the nucleotide, involving oxidation of the mercury.[149]

FMN exhibits in pH 7 phosphate buffer a very high wave, accompanied at more negative potential by a small wave (about one-tenth of the former wave), on both the cathodic and anodic sweeps on oscillographic square-wave polarography.[154] (In the latter technique, cathodic and anodic processes can be observed continuously as the applied ac voltage sweeps in triangular pattern between 0 and −2 V while synchronized to the mercury drop.[155]) The summit potentials of the two waves almost coincide with those of their counterparts on the cathodic and anodic sweeps and correspond, respectively, to the summit potentials of the adsorption and main reduction waves seen on ac polarography.

3. *Electrochemically Calculated Molecular Areas*

The areas occupied by the adsorbed molecules of riboflavin and FAD on mercury electrodes were calcu-

(150) T. Tachi and Y. Takemori, *Vitamins* (Kyoto), **9**, 441 (1955).
(151) Y. Takemori, M. Senda, and M. Senda, *ibid.*, **16**, 492 (1959).
(152) Y. Takemori, *Rev. Polarog.* (Kyoto), **12**, 63 (1964).
(153) B. Breyer and T. Biegler, *J. Electroanal. Chem.*, **1**, 453, (1959/60).
(154) K. Okamoto, *Rev. Polarog.* (Kyoto), **11**, 225 (1964).
(155) Y. Saito and K. Okamoto, *ibid.*, **10**, 227 (1962).

lated (method not specified) to be 117 and 280 Å^2, respectively;[120] since these values are close to the maximum cross section of these molecules calculated from molecular models, it was suggested[120] that the molecules form a monomolecular layer on the electrode with their planes parallel to the mercury surface. Recent chronopotentiometric and cyclic voltammetric measurements[146,152] indicate that the adsorption of FMN is not a simple process; experiments on the hanging mercury drop, which allow greater time for the attainment of adsorption equilibrium, result in the disappearance of the prewave.[146] This behavior has been attributed[146] to slow adsorption of FMN, implying the reorientation of the originally adsorbed FMN into a stable film in which the ring system is parallel to the electrode. Equilibrium surface coverage calculated[146,152] from chronopotentiometric data, assuming the applicability of several possible adsorption mechanisms and using a method of graphical evaluation,[156] is 0.7×10^{-10} mole/cm^2 for FMN and 1.22×10^{-10} mole/cm^2 for its reduced form. This corresponds to areas of 135 and 409 Å^2, respectively. However, these data must be considered very cautiously, because, as previously mentioned, chronopotentiometry seems to be only of very limited value for the study of adsorption.[143]

4. *Complexation*

Since riboflavin, FMN, and FAD form polarographically reversible redox systems, polarography can be used to study their complexation. Thus, investigation by dc polarography and chronopotentiometry[157] of mixtures of iron(III) and FMN in neutral oxalate medium indicated that two Fe(II) ions combined with one FMN molecule to form a stable complex, which produces a kinetically controlled Fe(III) reduction wave due to its dissociation.

5. *Biological Fuel Cells*

Enzymatic redox systems have been examined in biological fuel cells in conjunction with an oxygen cathode.[158] The redox mechanism involves electron transfer in the case of flavoprotein enzymes and hydrogen transfer in the case of pyridine nucleotide enzymes, so that only the former system produces a voltage in the biological fuel cell. Elemental iron promotes the flavoprotein systems, *i.e.*, the glucose oxidase and the D-amino acid oxidase systems, causing an increase in voltage output from 175–350 to 635–750 mV. The promoting effect of iron was considered to be due to an increased rate of oxidation of FADH to FAD$^+$ coupled with the greater oxidation potential of iron.

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D. ANALYTICAL APPLICATION

The monograph by Březina and Zuman[74] contains considerable useful information on the analytical application of the polarographic behavior of riboflavin.

The photolysis of riboflavin under various conditions has been followed polarographically,[121–124,127,138,159–161] and some of the products have been identified and studied polarographically, *e.g.*, lumichrome,[122,124–160] lumiflavin,[138] rhodoflavin,[160] deuterioriboflavin,[161] and deuteriolumiflavin.[161] Polarographic data were used to establish the reversibility of the photoreduction system involving riboflavin;[127] the photoreduction can be inhibited by oxygen, certain metal ions, dinitrophenol, *p*-nitrobenzaldehyde, and sodium azide. Oxygen also protects riboflavin from photolysis.[127] Photochemical oxidation of EDTA and methionine by riboflavin was also followed polarographically.[162]

A number of procedures have been published for the polarographic determination of riboflavin, mostly in pharmaceutical preparations.[125,126,163–168] The fact that the reduction wave of riboflavin occurs at relatively positive potential in acidic and neutral media, *i.e.*, in the region of -0.1 to -0.5 V, allows it to be used for the determination of riboflavin in mixtures with other polarographically reducible vitamins, *e.g.*, thiamine, nicotinic acid, pantothenic acid and pyridoxine,[126] folic acid,[163,165] and ascorbic acid.[163,167] The polarographic determination of riboflavin is preferable[74,138,166,168] to other analytical methods, because it is quicker than the current fluorimetric, microbiological, and spectrophotometric methods and has approximately the same or better accuracy.

Polarography has been successfully used for the determination of riboflavin in food[169] and in tissues and biological fluids,[170,171] and to follow the formation of riboflavin in butylogenous *Clostridii* cultures[172] and

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the change in riboflavin concentration of fruit juices during storage.[173]

Riboflavin can be determined in pharmaceutical preparations and biological material by means of an ac polarographic method in concentrations as low as 2×10^{-4} m*M*.[153]

The catalytic wave recently observed on dc polarography of riboflavin and FAD solutions can be used to determine them in solutions as dilute as 10^{-5} m*M*;[145] the similar determination of riboflavin down to 10^{-3} m*M* has also been reported.[144]

V. NEEDED STUDIES

The need for more detailed study of the polarographic behavior of nucleosides and nucleotides is evident from the preceding discussion. Among the problems which should be investigated are the following, some of which have been previously mentioned.

(1) The data are still inadequate for satisfactory estimation of the influence of the sugar and sugar-phosphate moieties on the electrochemical behavior of the parent nitrogen heterocyclic base. Even where these data are available as in the case of cytosine derivatives, they have not been adequately used, *e.g.*, to explain why the addition of the sugar or sugar-phosphate moiety enhances the reducibility of the cytosine nucleus.

(2) Suitable experimental data, *i.e.*, $E_{1/2}$ values for both oxidation and reduction processes, are not available for evaluation of the predictions based on quantum mechanical calculations and other structural approaches concerning the ease of electron transfer into and from individual purine and pyrimidine bases, and the corresponding nucleosides and nucleotides.

(3) The electrode reaction mechanism patterns for both the pyridine nucleotides and their model compounds have not been established with satisfactory certainty; *e.g.*, some reported experimental results are not consistent with the reaction paths proposed. For example, the fraction of NAD^+ going to NADH *via* the dimer should be investigated as well as the fact that $NADP^+$ is almost quantitatively electrolytically reduced to NADPH, while NAD^+ is not. The feasibility of carbon–carbon bond fission in the dimer at the potentials involved needs to be investigated, as does the apparent failure to detect the reduction of the adenine moiety.

(4) It would be of considerable interest to have data available to permit comparison of the behavior of the monomeric units, *i.e.*, bases, nucleosides, and nucleotides, with their polymers, *i.e.*, oligonucleotides and polynucleotides, in order to determine the influence of the primary and secondary structure of polymers on the behavior of their component monomeric units.

(5) Helpful information about energy-controlling steps and long- and short-lived intermediates in redox processes could be obtained by more extensive use of available electrochemical investigative techniques, *e.g.*, cyclic voltammetry, supplemented by optical and magnetic measurement, and, examination of solutions during and after electrolysis by absorption spectrophotometry and electron spin resonance.

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