Electrochemical detection methods for biologically-active molecules

Lawrence E. Welch
Iowa State University

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Electrochemical detection methods for biologically-active molecules

Welch, Lawrence E., Ph.D.
Iowa State University, 1988

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Electrochemical detection methods for biologically-active molecules

by

Lawrence E. Welch

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Chemistry Major: Analytical Chemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

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For the Major/Department

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For the Graduate College

Iowa State University
Ames, Iowa
1988

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GENERAL INTRODUCTION

Literature Review

The quantitative determination of biological compounds has always been a subject of much interest, and its relevance has never been greater than its current status. Large macromolecules such as nucleic acids, proteins, and to a degree, polysaccharides, have been determined to contain informational sequences of different monomeric units. Improved analytical techniques allow these sequences to be elucidated by fractioning the macromolecules and determining the monomeric products. As techniques are improved, less material will be needed for the sequencing procedure, allowing the scientist to focus on minute facets of a biological system. The ultima Thule will be reached when a system can be monitored on the molecular level and perhaps even manipulated to benefit society.

Carbohydrates are polyhydroxy aldehydes and ketones, which make up one of the largest groups of naturally occurring compounds. A single carbohydrate unit is referred to as a monosaccharide which, when polymerized, become polysaccharides. Most carbohydrates are found naturally as polysaccharides, which serve structural and energy storage
functions within an organism. Some polysaccharides are homopolymers, each containing only D-glucose as a building block. Heteropolymeric polysaccharides contain more than one monomeric unit and are believed to contain informational sequences within some organisms. Pertinent reviews of analytical methodology for carbohydrate determination have been made by Hughes (1) and Mead (2).

Proteins are the most abundant components of cells, serving numerous functions and appearing in numerous structural forms. Amino acids (Figure 1) are the monomeric units of proteins, linked together by peptide bonds. There are 20 commonly occurring amino acids, each with its own side chain (Figure 2 (3)). There are many more amino acids that appear in less common proteins or infrequently in common proteins. The discovery that the amino acid sequence controlled the function and form of proteins was a recent one, as earlier this century the composition of proteins was a mystery. Sequencing was greatly aided by the work of Sanger (4-7), who in the early 1950s determined the sequence of the peptide insulin. Once amino acid sequence could be determined, its relationship to protein function was studied and deciphered. Application of x-ray crystallography (8) allowed the three-dimensional structure of proteins to be examined, with the conclusion that this
Figure 1. Amino Acid Structure

(a) Uncharged form

(b) Zwitterion form
Carboxyl end

Amino end

(a)

Basic

Acidic

(b)
Figure 2. Amino Acid Side Chain Structure

Listed as the most prevalent form at pH 7
and categorized as to probable protein position
EXTERNAL

ACIDIC

- Asp (D)
- Glu (E)

NEUTRAL

- Asn (N)
- Gln (Q)

BASE

- Lys (K)
- Arg (R)
- His (H)

AMBIVALENT

- Pro (P)
- Thr (T)
- Ser (S)
- Cys (C)
- Ala (A)
- Gly (G)

INTERNAL

- Tyr (Y)
- Trp (W)
- Phe (F)

- Leu (L)
- Ile (I)
- Met (M)
- Val (V)
was dependent on amino acid sequence as well.

Protein sequencing and/or compositional analysis involve three steps. First, the protein chain must be cleaved. Compositional analysis requires general cleavage of all peptide bonds to produce free amino acids, which is generally accomplished by acid hydrolysis. Sequencing involves more specific cleavage of the chain, often at the carboxy or amino terminus. Enzymatic cleavage is often used because of its inherent specificity of attack. Carboxypeptidase is used to cleave peptides at the carboxy terminus (9), while the Edman Degradation (10-11) allows amino terminus cleavage. Cleavage in the middle of a protein creates smaller fragments that can be sequenced easier than the complete chain. When several different internal cleavages are made, identification of overlapping fragments can allow the sequence to be pieced together. Trypsin (12) and chymotrypsin (13) are commonly used for this type of cleavage.

The second and third steps of the sequencing/compositional analysis process involve separation and detection. High Performance Liquid Chromatography (HPLC) is the most common choice for the separation step, with electrophoresis and other chromatographic methods used as well. The detector chosen is typically sensitive
for all amino acids or derivatives being used, giving quantitative information. The use of a universal detector necessitates a very efficient separation step, so peak identification can be made from retention time (or distance traveled in electrophoretic experiments). If the separation step does not resolve all of the common amino acids, it is of little use for sequence analysis. It would be possible to obtain quantitative data from a limited separation if a multiple array of selective detectors were used, but the availability of simpler methods discourages this practice.

The original sequencing work by Sanger (4-7) employed paper chromatography to separate and identify peaks after they were visualized by making yellow dinitrophenyl (DNP) derivatives. Also examined were derivatives made with ninhydrin, which resulted in purple complexes known as "Ruhemann's Purple" (14-16). Ninhydrin was to become the most common derivatizing agent for amino acids, and is still widely used today.

Moore and Stein (17) improved the derivatization reaction with ninhydrin by limiting side reactions and making the color yield reproducible, allowing precise quantitative analysis based on photometric detection. This led to a landmark development: the first automated amino acid analyzer for quantitative compositional analysis. A
series of papers (18-26) describe the development of this instrument, which employed a cation-exchange separator followed by post-column addition of ninhydrin and photometric detection. The analyzer employed two different cation-exchangers, three different solvents, and took 22 hours to separate a mixture. The limit of detection (LOD) was near 1/16 micromole. The instrument was quickly commercialized by Beckmann (Figure 3), and Moore and Stein won a Nobel Prize for their work in 1972.

The Moore and Stein instrument is still used in its basic form, but it has been improved greatly. The advent of HPLC has reduced analysis time to one hour or less, and LOD values are in the 50-picomole range (27). Less sensitivity is observed for detection of secondary amino acids such as proline and hydroxyproline, which must be monitored at a wavelength of 440 nanometers rather than the standard 570.

Other derivatives have been made in the years following Moore and Stein's work for use with photometric detection. Dabsyl chloride (dimethylaminoazobenzenesulfonyl chloride) derivatives (28) were found to absorb strongly at 436 nanometers after separation on a C-18 reversed-phase HPLC column. The separation took less than one hour, and 2-5 picomoles of each derivative could be quantitated. The pre-column derivatization procedure is more labor-intensive than
Figure 3. Advertisement for an Early Amino Acid Analyzer
Another Advancement in Protein Research

Automatic AMINO ACID ANALYZER using Moore-Stein technique

Quantitative analysis of amino acids with the new Beckman Spinco Amino Acid Analyzer saves as much as 89% of the time required by conventional chromatographic techniques. Analysis of a protein hydrolysate is completed in 24 hours—and because operation is virtually automatic, an operator need be available for only 3½ hours.

The instrument, based on the developments of Drs. Spackman, Stein and Moore of the Rockefeller Institute, uses ion-exchange chromatography to separate a mixture of amino acids. A color is formed continuously with ninhydrin reagent, and a built-in recorder plots color density on a chart. The concentration of each amino acid is then read from the chart. The method is applicable to a variety of ninhydrin-positive compounds, including components in physiological fluids, foods and tissues.

A typical chart from the Spinco Amino Acid Analyzer using the Moore-Stein technique is shown at left. The first run on acid and neutral amino acids required 18 hours; the 4-hour run on a second column recorded basic amino acids. More than twenty amino acids can be measured on the standard instrument, with an accuracy of 3 percent.

For literature on this new instrument, please write Spinco Division, Beckman Instruments, Inc., Stanford Industrial Park, Palo Alto, California. Ask for Bulletin MS-71.

References: D. H. Spackman, W. H. Stein, and S. Moore,

Beckman/Spinco Division
Beckman Instruments, Inc.
current ninhydrin schemes, and dabsyl chloride is not commonly used.

Edman (10-11) described the addition of phenylisothiocyanate to the amino terminus of a protein and the subsequent formation of phenylthiohydantoin (PTH) amino acids, which can be determined by photometric means at 240-270 nanometers. Reversed-phase HPLC can separate all PTH derivatives in less than one hour with LOD values of 1-10 picomoles (29-31). Application of a microbore reversed-phase column showed sub-picomole detection limits (32). A modification of the Edman procedure (33) will produce phenylthiocarbamyl (PTC) amino acids, which can be determined photometrically in a similar manner. Secondary amino acid derivatives can be made and determined quantitatively for both PTH and PTC methods.

Color-forming agents are also commonly used with non-LC separations, including thin-layer chromatography (34), paper chromatography (4-7), and electrophoresis (35). Derivatives are always formed for photometric amino acid detection, because only tyrosine, phenylalanine, and tryptophan can be determined directly. None of the common amino acids will fluoresce, so derivatives are always prepared for fluorescent detection as well.

The most common derivatizing agent used to form
fluorescent derivatives is ortho-phthaldialdehyde (OPA). Roth (36) first described OPA derivatives, finding them to fluoresce strongly at 455 nanometers after excitation at 340 nanometers. The addition of 2-mercaptoethanol as a reducing agent and usage of an alkaline medium promoted the production of a fluorescent product. OPA does not form adducts with secondary amino acids or cysteine, and gives very low response for cystine (27). Post-column addition of OPA after cation-exchange chromatography (37-38) and pre-column derivatization followed by reversed-phase HPLC (39) both are used. Either method should be automated to allow reproducible decay of the unstable OPA adducts. Pre-column OPA derivatization has produced some of the lowest reported LOD values for amino acids, with values of 50 femtomoles for a standard 5-micrometer C-18 column (40) and < 5 femtomoles for a microbore 3-micrometer C-18 column (27).

Fluorescamine (4-phenylspiro-[furan-2(3H), 1'-phthalan]-3,3'-dione) forms amino acid adducts under basic conditions that can be monitored fluorimetrically (41-42). Secondary amines do not react with fluorescamine. The alkaline conditions required are not amenable with silica reversed-phase columns, so this derivatization has mainly been done by post-column reaction.

Dansyl chloride (1-dimethylaminonapthalene-5-sulfonyl
chloride) also will form fluorescent adducts with amino acids (43). Pre-column derivatization followed by separation on a reversed-phase HPLC column has proven successful, with detection limits of one picomole (44). Both primary and secondary amino acids can be determined, but this procedure is not commonly used due to lengthy derivatization time.

One recently developed derivatization agent is FMOC (fluoroenylmethyloxycarbonyl chloride), which produces very stable fluorescent amino acid adducts (45). The procedure can determine both primary and secondary amino acids (46) with detection limits of less than 50 femtomoles. Precolumn derivatization is used exclusively since the FMOC reagent displays a nearly identical fluorescence spectrum as its amino acid adducts. The FMOC procedure is becoming the method of choice for applications demanding tremendous sensitivity and the ability to determine secondary amino acids.

Direct determination of amino acids, without the need for derivatization procedures, can save greatly both in terms of time and equipment. One drawback is that chromatographic methods for free amino acids have been based on ion-exchange processes, which have proven to be less efficient than the reversed-phase methods used for some
derivatives. Numerous laboratories still employ the Moore and Stein ninhydrin method, so a direct detection scheme that displays similar sensitivity may prove valuable (17-26). Also, the ability to determine both primary and secondary amino acids is preferred.

Reitsma (47) coupled an optical activity detector with cation-exchange chromatography for direct determination of amino acids, but sensitivity was considerably less than the ninhydrin approach. Refractive index detection is even less sensitive than optical activity detection.

Electrochemical detection has been demonstrated to be the best way to determine free amino acids. Amperometric methods have proven much better than potentiometric methods (48-49). One novel amperometric detector can determine amino acids directly by monitoring the concentration of denone (2,3-dihydroxy-1-peri-naphthindenone) formed upon conversion of trione (1,2,3-peri-naphthindantrione hydrate) when an amino acid is present (50). A detection limit of 1 nanogram for glycine was found.

An oxide-covered Ni electrode was found to amperometrically monitor amino acids in alkaline solutions (51). Specifically, anodic current was measured at a constant potential of 0.5 Volts vs. SCE. This detector was coupled with anion-exchange (52) and reversed-phase ion-pair
chromatography (53), neither of which demonstrated the ability to separate a mixture of all common amino acids. A LOD of 2.5 picomoles was determined for glycine, but values for most of the amino acids were found to be considerably higher.

Pulsed Amperometric Detection (PAD) has been demonstrated to oxidize a number of organic species at noble metal electrodes through a mechanism catalyzed by simultaneous formation of a surface oxide layer (54-55). A waveform containing three potential steps was employed to continually regenerate electrode activity, which was lost when constant potential detection was attempted. PAD was applied for amino acid determination (56-57) using a Pt electrode in alkaline solution, with sensitivity demonstrated toward both primary and secondary amino acids. Sub-ppm detection limits were reported when PAD was coupled with anion-exchange chromatography. As with the Ni electrode work, the separation scheme lacked the power to separate a mixture of all common amino acids.

Explanation of Dissertation Format

This document follows the alternate thesis format, with the major focus being electrochemical detection methods for biologically-active molecules. Five sections are
presented, each reviewing a specific subtopic of the general thesis research problem. Section I presents a comparison of Pulsed Amperometric Detection (PAD) and Conductivity Detection (CD) for the determination of carbohydrates. Also investigated was the ability to combine the detectors in tandem to expand the linear dynamic range for quantitative work.

Section II presents a comparison of PAD and CD for amino acid determination following anion-exchange separation. A Au electrode was employed for PAD, and the waveform was optimized to obtain low LOD values.

Section III reviews work to improve the determination of amino acids by preparing phenylthiohydantoin (PTH) and methylthiohydantoin (MTH) derivatives. PAD and DC amperometry were compared as detectors for these derivatives, and an effort was made to develop a separation to couple with these detectors.

Section IV discusses new electrochemical waveforms to apply to the direct detection of underivatized amino acids. Pulsed Coulometric Detection (PCD) and Indirect Coulometric Adsorption Detection (ICAD) were applied following anion-exchange separation. Modifications of PCD were made to find a scheme that could be used during gradient elution anion-exchange chromatography.
Section V presents a discussion of current instrumentation available for PCD, and reviews methods for PCD waveform optimization using oxidation of amino acids as a model system.
SECTION I.
A COMPARISON OF PULSED AMPEROMETRIC DETECTION AND CONDUCTIVITY DETECTION FOR CARBOHYDRATES
ABSTRACT

Pulsed amperometric detection at a gold electrode and conductivity detection are compared for glucose. Glucose was chosen as the model compound, representing carbohydrates, since it has a response typical of monosaccharides. In alkaline solutions of barium hydroxide useful for anion-exchange separations of carbohydrates, pulsed amperometric detection is significantly more sensitive than conductivity detection. However, conductivity detection has a linear response to higher concentrations than pulsed amperometric detection. The use of conductivity detection in combination with pulsed amperometric detection yields a linear response for glucose over the range $6 \times 10^{-7}$ to $1 \times 10^{-2}$ M (ca. 6 ng - 10 mg per 50-uL sample injection; i.e., 0.12ppm - 200ppt).
INTRODUCTION

Carbohydrates are of great significance to human health. There is an increasing demand to improve techniques for separation and detection in the analysis of complex samples in research laboratories as well as for quality control in food and beverage industries. Various liquid chromatographic methods have proven useful for separations of carbohydrate mixtures. Included are separations on non-polar C-18 bonded phases (1), polar bonded phases (2), cation-exchange phases (3,4), and anion-exchange phases (5-9).

Photometric detection of carbohydrates by ultraviolet and visible absorption or by fluorescence can only be used after derivatization (10,11). Direct detection of underivatized carbohydrates has most commonly utilized refractive index detection (12,13). Refractive index detection suffers from low sensitivity but has found application for cation-exchange separations of carbohydrates where pure water can be used as the mobile phase.

Recently, pulsed amperometric detection has shown significant promise for detection of underivatized carbohydrates (3-9,14). Pulsed amperometric detection requires an alkaline electrolyte for maximum sensitivity and is well suited for anion-exchange separations of
carbohydrates using alkaline mobile phases. Detection of sub-ppm levels are possible without post-column addition of buffer solution (14).

Conductivity detection has been utilized very successfully in Ion Chromatography for the determination of both inorganic and organic cations and anions (15,16). Conductivity detection of carbohydrates has been reported after derivatization (17), and recently underivatized carbohydrate detection was reported (18). This work compares pulsed amperometric detection with conductivity detection for glucose in an alkaline mobile phase useful for carbohydrate separations by anion-exchange chromatography (7-9,19).
EXPERIMENTAL

Reagents

All chemicals were reagent grade. Water was from either a Millipore Milli-Q purification system or a Barnstead Nanopure II system followed by filtration through a 0.45 um filter. Carbohydrate solutions were prepared immediately prior to use to minimize degradation in alkaline solution.

Apparatus

Chromatographic separations were performed with an AS-6 anion separator column in a CHB-1 chromatography module (Dionex Corp., Sunnyvale, CA). The sample injection loop had a volume of 50-uL. The pump was a GPM-1 (Dionex Corp.) gradient system. Eluent and sample were filtered, after the injection port, with a Rheodyne 7335 column inlet filter (Alltech Associate, Inc., Deerfield, IL).

Conductivity detection was performed with a conductivity module from a Model-10 Ion Chromatograph (Dionex Corp.). The conductivity cell and the amperometric cell were placed in series, with the conductivity cell preceding the amperometric cell. Pulsed amperometric detection was performed with a Model PAD-2 (Dionex Corp.) potentiostat and a homemade gold wire flow-through cell (20). The reference electrode was a miniature saturated
calomel electrode (SCE; Fisher Scientific, Springfield, NJ). Data were collected and processed with an Apple IIe computer (Apple Computer, Inc., Carrollton, TX) and an ADALAB interface (Interactive Microware, State College, PA).
RESULTS AND DISCUSSION

Anion-exchange separation of carbohydrates has been proposed to occur because of the weakly acidic properties of carbohydrates (6,7). The pK_a's reported for glucose and sucrose are 12.35 and 12.51, respectively (21). In the presence of alkaline eluent, carbohydrates are ionized sufficiently to make possible their separation by an anion-exchange mechanism. Sucrose, having a higher pK_a, is ionized to a lesser extent and spends less time on the column; it has a lower capacity factor. Therefore, sucrose elutes before glucose when ion-exchange is the major interaction for retention on the column. This is not true at pHs where both glucose and sucrose are more completely ionized, and other interactions become important for retention on the column. Acetate ion can be added to the mobile phase as a "pusher" ion to adjust retention times of carbohydrates (6-9,19,22).

Solutions of sodium hydroxide have been used as the mobile phase (5,6); however, recently barium hydroxide solutions have been used (7-9,19,21) to eliminate the presence of carbonate from the eluent. Carbonate acts as a "pusher" ion and reduces the resolution of carbohydrate separations. Precipitation of carbonate, as barium carbonate, followed by filtration minimizes this problem.
When the mobile phase is 2 mM barium hydroxide (ca. pH 11.6), elution order follows $pK_a$. However, when the mobile phase is 12 mM barium hydroxide (ca. pH 12.4), elution order is reversed for glucose and sucrose (7-9,9,21). Alkaline conditions used for anion-exchange separations are useful for pulsed amperometric detection of carbohydrates because maximum amperometric sensitivity occurs above pH 11.

The limiting equivalent ionic conductances of $\text{OH}^-$ and $\text{Ba}^{+2}$ are 198 and 63.36 umhos cm$^2$ equiv$^{-1}$, respectively (23). The limiting equivalent ionic conductance values for the carbohydrate anions could not be found but are expected to be much less than the value for hydroxide. The elution of adsorbed carbohydrates with subsequent adsorption of hydroxide, produces a decrease in the conductance from the baseline level, yielding a "negative" peak. Thus, the conductivity technique involves the indirect detection of carbohydrates.

Concentrations of barium hydroxide (ca. 12 mM) most commonly useful for carbohydrate separations (7-9,19,21) produced a background conductance too great to be offset with the baseline compensation adjustment of the Dionex conductivity module. Therefore, lower concentrations of barium hydroxide were tested as eluents. The background conductance of 2 mM solutions could be offset sufficiently
and the pH was still high enough to facilitate ion-exchange separation of low molecular weight carbohydrates. Although resolution and pulsed amperometric sensitivity were sacrificed in order to perform the conductivity measurements, peaks for glucose and sucrose were baseline resolved and the amperometric signal was still significant. A representative chromatogram for the separation of glucose and sucrose using 2 mM barium hydroxide is shown in Figure 1A.

A glucose calibration curve was constructed for a series of standards, beginning near the limit of detection for pulsed amperometric detection to the range where pulsed amperometric detection was observed to be nonlinear. The estimated limit of detection ($S/N = 3$) for glucose by conductivity detection was ca. $1 \times 10^{-5}$ M (90 ng per 50-uL injection, i.e., 1.8 ppm). Peak areas were determined by computer integration. A linear calibration curve was obtained by plotting peak area versus glucose concentration in the range 0.1 mM to 10 mM.

A modified linear regression analysis of the conductivity data for glucose, based on the assumption that the variance in the signal is proportional to the concentration (24), produced the following statistical
parameters: \[ a = 15.63 \, \text{umho sec} \]
\[ b = 3.6 \times 10^{+5} \, \text{umho sec M}^{-1} \]
\[ S_y = 0.007 \times 10^{+5}. \]

Pulsed amperometric detection was performed under the same conditions used for conductivity detection for the sake of comparison. The waveform for detection (Figure 1) was selected on the basis of the cyclic voltammogram at gold using 2 mM barium hydroxide as the solvent. A chromatogram for the separation of glucose and sucrose is shown in Figure 1B. The estimated limit of detection for glucose is ca. 5 \( \times 10^{-7} \) M (5 ng per 50-uL injection, i.e., 90ppb), which is 20 times lower than for conductivity detection. Also, when the conditions are optimized for pulsed amperometric detection, even lower limits of detection are observed.

The calibration curve for pulsed amperometric detection deviated from linearity above 5 \( \times 10^{-5} \) M. Previous work has reported nonlinear response for pulsed amperometric detection of carbohydrates at concentrations above 1 \( \times 10^{-4} \) M (3). In this work, for a 10-fold increase in concentration from 0.1mM to 1.0 mM glucose, the peak area increased by only a factor of 3. Detection limits, for sucrose, by conductivity detection and pulsed amperometric detection can be estimated by multiplying the values given above by the peak ratio (sucrose/glucose) determined from
Figure 1. Chromatograms for the separation of sucrose and glucose with sequential conductivity detection and pulsed amperometric detection.

Conditions: column - Dionex AS-6
eluent - 2 mM barium hydroxide
sample - 50-uL of 1 mM sucrose and glucose

Peaks: 1 - sucrose, 2 - glucose

Inset: waveform (E - t) for pulsed amperometric detection

Chromatograms: A - conductivity detection
B - pulsed amperometric detection
A. inject

3 μmho

B. inject

100 nA

6 min

Potential (V vs. SCE)

E1

E2

E3

current sampled

120 240 360 480

time (ms)
Figure 1. The statistical parameters obtained with the modified linear regression analysis of the amperometric data in the range 1.0 μM - 50 μM are listed below:

- Intercept (a) = -0.062 μcoul
- Slope (b) = 1.7 x 10^{+5} μcoul M^{-1}
- Sy = 0.00004 x 10^{+5}.

The calibration data for conductivity detection and pulsed amperometric detection are compared directly in Figure 2, as plots of

\[ \log \left[ \frac{(A_p - a)}{(b \cdot C)} \right] \text{ vs. } \log C, \]

where \( A_p \) is the peak area corresponding to an injection of concentration \( C \), and \( a \) and \( b \) are the intercept and slope respectively, as reported above. When \( A_p \) is the predicted value (i.e., \( \hat{A}_p = a + b \cdot C \)), the response is represented in Figure 2 by the solid line corresponding to \( \log[\frac{(A_p - a)}{(b \cdot C)}] = 0 \). The limits of detection (LOD) stated previously, which were estimated from the chromatographic peaks for concentrations near the limit of detection, are indicated by arrows in Figure 2. The limits of linearity (LOL) are indicated by arrows at concentration values that correspond to ca. ± 10% relative deviation from the predicted value, i.e., \( \frac{((\hat{S} - a) - (S - a))}{(\hat{S} - a)} = ± 0.1 \).
Figure 2. Log-log plots of the normalized peak area versus the concentration of glucose

DATA:  ● - pulsed amperometric detection
      ◆ - conductivity detection
CONCLUSIONS

The range of linearity for pulsed amperometric detection, using the conditions optimized for conductivity detection, overlaps the lower end of the linear range for conductivity detection. Therefore, the use of the two detectors in series give a combined linear dynamic range of ca. 4 decades for glucose. Band broadening for the pulsed amperometric detection peaks did not increase significantly by the presence of the conductivity cell placed, in series, before the amperometric cell. Hence, a dual detector system can be employed for carbohydrate separations in which a wide range of concentrations is expected. A major disadvantage encountered is that chromatographic resolution is limited when using anion-exchange with conductivity detection because the total ionic strength is restricted due to the high background signal. Therefore, proper pHs and ionic strengths required for some carbohydrate separations are not allowed using conductivity detection.
REFERENCES CITED


ACKNOWLEDGMENTS

I would like to thank Dionex Corporation (Sunnyvale, CA) for providing the conductivity detector used for this research and also for funding this research project.
SECTION II.
PULSED AMPEROMETRIC DETECTION OF UNDERIVATIZED AMINO ACIDS IN LIQUID CHROMATOGRAPHY
ABSTRACT

Pulsed Amperometric Detection (PAD) at a Au electrode is demonstrated for the direct anodic detection of amino acids based on surface-catalyzed reactions occurring simultaneously with formation of surface oxide. The detection limit for lysine in a flow-injection determination was ca. $1.5 \times 10^{-6}$ M, which corresponds to ca. 75 pmol in the 50-μl sample injected (i.e., 11 ng, 0.2 ppm). The PAD method gives a linear response over nearly two decades in concentration. Conductivity detection (CD) was also applied for detection of amino acids. The response of CD is not as sensitive as PAD but is linear at higher concentrations. The calibration curve for the tandem application of PAD and CD was linear over four decades. A brief comparison is made with PAD at a Pt electrode.
INTRODUCTION

Amino acids are the building blocks of protein molecules, their sequence customizing each protein for its specific function. Determinations of the amino acid composition of proteins have become increasingly important with the rapid increase in biotechnological research. According to Lehninger in 1975 (1): "Twenty-five years ago the quantitative analysis of but one amino acid in a mixture might have taken months of work. It was not until chromatographic methods were systematically applied to the analysis of amino acid mixtures that any significant progress was achieved...". Technological advances in amino acid analysis since 1975 undoubtedly have been just as great as perceived by Lehninger.

Separations of amino acids and their derivatives in liquid chromatography (LC) are readily achieved on stationary phases ranging from C-18 and C-8 nonpolar resins (2-5), to cation (6-7) and anion (8) exchangers. Fluorescence and UV-vis absorbance are the most popular detection schemes; however, both require derivatization since none of the common amino acids fluoresce and only tyrosine, phenylalanine, and tryptophan have significant UV-vis absorbance. Orthophthalaldehyde (OPA) is the most common of the fluorescent derivatizing agents (4), resulting
in tremendous sensitivity and sub-pmole detection limits (9). OPA is limited in application in that it does not form adducts with the secondary amino acids such as proline and hydroxyproline. Common derivatizing agents for UV-vis detection include ninhydrin (10) and phenylisothiocyanate (11-13). Sensitivity for these reagents is significantly less than for OPA.

Sensitive detection methodology that does not require derivatization is desirable for convenience in many applications. Laser-based optical activity detection is applicable to underivatized amino acids (14), but sensitivity is limited. Refractive index detection can also be applied directly to amino acids, but is less sensitive than optical activity detection (14). In the past, amino acids generally have not been considered to be electroactive (15-17). However, it has been shown that direct anodic detection at constant applied potential can occur by catalytic mechanisms on certain metal oxides (18,19).

The development of Pulsed Amperometric Detection (PAD) in this laboratory and elsewhere has been directed to the anodic detection of so-called "electroinactive" aliphatic compounds at noble metal electrodes (Pt,Au). Initial success resulted for determinations of carbohydrates, both reducing and nonreducing, using Pt (20) and Au electrodes
Detection mechanisms rely on transient, surface catalytic dehydrogenation processes. The applicability of PAD at Pt electrodes in alkaline media was illustrated for underivatized amino acids by Polta and Johnson (8) following anion-exchange separations. Applications of LC-PAD for sulfur compounds are forthcoming (24). PAD does not require elevated temperatures, lengthy electrode pretreatment, nor pre- or post-column derivatizations. Since sensitivity in PAD is greatest in alkaline media (pH > ca. 10), it is desirable to design LC strategies around robust separation phases using alkaline mobile phases. However, post-column addition of alkaline buffer can be acceptable.

Conductivity detection (CD) has been utilized successfully in Ion Chromatography for the determination of inorganic and organic ions (25,26). CD of amino acids has been examined (27), but no quantitative results or separations were reported. Recently, Welch, Mead and Johnson (28) applied CD in tandem with PAD for carbohydrate determinations. The dual detection provided linear calibration over a wide dynamic range. In this work, PAD and CD are compared for direct determination of several underivatized amino acids following anion-exchange separation using alkaline mobile phases.
EXPERIMENTAL

Reagents

Solvents and standards were prepared from reagent grade chemicals. L-Lysine and Amino Acid Complex dietary supplements were from American Dietary Laboratories (Pasadena, CA). Water was purified either in a Millipore MILLI-Q system or a Barnstead NANOpure II system, followed by filtration (0.2 um).

Apparatus

Chromatographic separations were performed with an AS-6 anion separator column preceded by an AG-6 guard column in a CHB-1 chromatography module with a GPM-1 pump (Dionex Corp., Sunnyvale, CA). The injection volume was 50 ul.

Conductance detection was made with the CD module from a Model-10 Ion Chromatograph (Dionex). Pulsed amperometric detection was performed with the PAD-2 (Dionex Corp.) and homemade Au and Pt flow-through cells (29). In experiments comparing the calibration of CD and PAD, the detector cells were placed in series (CD first).

Voltammetric data were obtained with a Au rotated disc electrode (AFDTO7, 0.196 cm²) in a PIR rotator under potentiostatic control by a RDE3 (Pine Instrument Co., Grove City, PA). Voltammetric data were traced with a
RE0074 X-Y recorder (EG & G Princeton Applied Research, Princeton, NJ). Current decay was studied with a Model 2230 digital storage oscilloscope and plotted on the X-Y recorder using a GPIB interface (Tektronix Corp.).
RESULTS AND DISCUSSION

Voltammetric Basis of PAD

The previous application of PAD to amino acids made use of a Pt working electrode (8). Since Au electrodes are now recommended over Pt for carbohydrate detection (21-23,30), and can be used successfully for detection of S-compounds (24), it was desired to test PAD for amino acids at Au. As was observed for the PAD response at Pt electrodes, the sensitivity for amino acids at Au electrodes is highest in alkaline media (pH > ca. 11). The current-potential response (I-E) for lysine at the Au rotated disc electrode (RDE) is shown in Figure 1 for 0.05 M NaOH. Additions of lysine enhance the anodic current for the positive potential scan in the range of ca. 0.1 - 0.7 V vs. SCE. This potential region corresponds to the anodic formation of surface oxide on the Au RDE. For the negative scan in the range 0.7 - 0.3 V, growth of surface oxide ceases and no response for lysine is observed. The surface oxide is cathodically dissolved to produce the large peak at +0.2 to -0.2 V. All other essential amino acids displayed nearly identical voltammetric behavior to that for lysine.

The net anodic current for lysine increased markedly with increases in the potential scan rate, yet little change resulted from variations in electrode rotation speed. This
Figure 1. Voltammetric response (I-E) for lysine at a Au rotating disc electrode

Conditions: 0.05 M NaOH supporting electrolyte
900 rev min$^{-1}$ rotation speed
6.0 V min$^{-1}$ scan rate

Lysine concentration (mM): (a) 0  (b) 0.065  
(c) 0.12  (d) 0.26
behavior is typical of processes in which the reaction rate
is under the control of electrode surface processes (8). We
conclude that the anodic detection mechanism for amino
acids is electrocatalytic, requiring simultaneous formation
of surface oxide. Clearly, there is insignificant catalytic
reactivity for the mature oxide-covered surface (negative
scan) and detection of amino acids at Au electrodes is only
possible by way of the transient electrocatalytic mechanism.

PAD for amino acids at Au electrodes in 0.05 M NaOH
solutions was successful based on a three-step potential
waveform. Sampling of the anodic current occurred following
a potential step to the value \( E_1 = 0.5 \) V. Since the
electrode activity is transient, the activity was renewed
following the measurement of the electrode current by
subsequent potential steps to \( E_2 = 1.05 \) V and \( E_3 = -0.55 \) V.
The duration of each potential in the waveform was: \( t_1 = 540 \)
ms, \( t_2 = 180 \) ms and \( t_3 = 240 \) ms. Adsorption of analyte can
occur on the oxide-free surface during the period \( t_3 \) at
potential \( E_3 \) prior to the next step to the detection
potential \( E_1 \). The transient amperometric signal following
the step \( E_3 \) to \( E_1 \) is shown in Figure 2 for the Au RDE as a
function of lysine concentration. For application of PAD
for liquid chromatography, the baseline response corresponds
to the value of current given in Curve a at the designated
Figure 2. Amperometric response (I-t) for lysine following the potential step $E_3 \rightarrow E_1$ at a Au rotating disc electrode

Conditions: 0.05 M NaOH supporting electrolyte
900 rev min$^{-1}$ rotation speed

Lysine concentration (mM): (a) 0 (b) 0.044 (c) 0.28 (d) 2.0
Anodic Signal
delay time (t₂) in period t₁. For lysine, the maximum signal-to-noise ratio (S/N) was obtained for t₂ = ca. 540 ms; current sampling required 16.7 ms (i.e., 1/60 Hz).

LC-PAD/CD

Amino acids can be separated via anion-exchange chromatography, provided the pH is high enough so that the amine functionalities are deprotonated. The pKₐ of the amine group of glycine is 9.57 (31) and the other amino acids have similar values. Solutions of NaOH (8) can be used as eluents and they provide the desired high pH and ionic strength necessary for satisfactory amperometric detection. Recently, Ba(OH)₂ has been used in place of NaOH as the eluent for carbohydrate separations (28,32) to reduce carbonate interference. Both buffers were tested as mobile phases in this work for amino acid separations on the AS-6 column; 2 mM Ba(OH)₂ was found to be preferable for CD because of a smaller requirement for baseline offset. The AS-6 column is a high efficiency column designed for carbohydrate separations. Improved columns for amino acid separations with alkaline mobile phases are under development (33).

The feasibility of CD for amino acids is due to the difference in limiting equivalent ionic conductance (LEIC)
between the eluting anion and the analyte anion. Hydroxide ion has a very high LEIC (198 umhos cm$^2$, 34), whereas the values for amino acid anions undoubtedly are much lower. The elution of the anion of an amino acid, with simultaneous adsorption of OH$, results in a decrease in CD response to give a "negative" peak. The separation of a mixture of lysine, methionine, asparagine and glycine is shown in Figure 3 with CD and PAD in series, using 2 mM Ba(OH)$_2$ as the mobile phase.

Analytical Response

The limit of detection ($S/N = 3$) estimated for lysine by CD was ca. $1.5 \times 10^{-5}$ M which corresponds to 750 pmole per 50-ul injection (i.e., 0.11 ug or 2.2 ppm). Calibration of CD peak area response ($A_p$) was performed using lysine concentrations ($C$) in the range 0.1 - 10 mM. Statistics were produced using modified regression analysis based on the assumption that variance in the signal is proportional to concentration (35). The resulting plot was linear, with regression statistics as follows: intercept ($a$) = $-0.872$ umhos sec, slope ($b$) = $2.71 \times 10^5$ umhos sec M$^{-1}$, and $S_y = 0.0002 \times 10^5$.

The estimated limit of detection ($S/N = 3$) for lysine by PAD at a Au electrode was $1.5 \times 10^{-6}$ M, corresponding to
Figure 3. Simultaneous chromatograms obtained by CD and PAD

Conditions: 2 mM Ba(OH)$_2$ at 1.0 ml min$^{-1}$

Detection: (A) conductivity (B) pulsed amperometry

Peaks: (a) sample matrix (b) 2.2 ug lysine
(c) 2.5 ug asparagine (d) 2.9 ug glycine
(e) 2.8 ug methionine
Conductance →

10 min

1 µmho

inject

A

Time →

Anodic Signal →

Time →

10 min

0.3 µA

inject

B

0.3 µA

b
c
d
e
ca. 75 pmol per 50-μl injection (i.e., 11 ng or 0.22 ppm). Response for other amino acids was within ±3X that for lysine. The secondary amino acids, hydroxyproline and proline, have reasonable PAD detection limits also (3.2 × 10^{-6} M and 4.3 × 10^{-6} M, respectively). PAD response for lysine was linear over nearly two decades with significant deviation from linearity for concentrations greater than 1.10 × 10^{-4} M. For the region of linear response, the modified regression analysis produced the following statistics: \( a = 1.77 \mu\text{coul} \), \( b = 6.79 \times 10^{4} \mu\text{coul} \, \text{M}^{-1} \), and \( S_y = 1.6 \times 10^{-3} \). Polta (8) suggested that calibration of PAD for surface adsorbed species can be linearized by plotting \( 1/A_p \) vs. \( 1/C \). However, a plot of this type was made for lysine and found to be unsatisfactory.

Johnson (36) has proposed that calibration data be presented as plots of \( \log[(A_p - a)/bC] \) vs. \( \log C \), where \( a \) and \( b \) are obtained from the modified regression analysis of the linear region of the plot of \( A_p \) vs. \( C \). Accordingly for data which is predicted exactly by the regression statistics applied, \( \log[(A_p - a/bC) = 0.0 \). This plotting scheme has several advantages over the traditional plotting of \( A_p \) vs. \( C \), including: relative deviations from the regression line are quantitatively depicted throughout the concentration range; the upper (and lower) limits of linearity are readily
apparent; and calibration data for two or more detection techniques can be compared easily on the same relative scale.

The calibration data for lysine obtained by PAD and CD are shown in Figure 4 plotted in the manner described above. The solid horizontal line represents the value \( \log[(A_p - a)/bC] = 0 \). The parallel dashed lines delineate relative errors of 10, 30, and 50%. It can be seen that PAD response deviated significantly from linearity as concentration approaches millimolar values. The CD response was linear throughout the range studied with a higher limit of linearity (LOL) but a poorer limit of detection (LOD), as compared to PAD. The two detectors used in tandem offer linear detectability over more than four decades of concentration.

Two dietary supplement pills were analyzed to illustrate the application of PAD of LC for amino acids. Contents of an L-Lysine capsule were dissolved, and the solution diluted and injected without further sample pretreatment, along with a series of lysine standards. The calibration statistics used for Figure 4 were applied, and the lysine content was found to be within 6% of the listed value (625 mg). Contents of a capsule of Amino Acid Complex were analyzed in a similar manner; the chromatogram is shown
Figure 4. Calibration plots for lysine

Conditions: 2.0 mM Ba(OH)$_2$ at 1.0 ml min$^{-1}$

Symbols: ◆ conductivity detection

○ pulsed amperometric detection
in Figure 5 for PAD. No sample pretreatment was applied other than dissolution. The mobile phase was 50 mM NaOH. Attempts were made to substitute anodic detection at a fixed potential (DC) for the PAD waveform, but response to lysine decayed rapidly to the level of uselessness, as shown in Figure 6. Long term stability of the PAD response was checked by injecting a lysine solution every hour for 6 hrs. The measured peak response showed only a 1.3% relative standard deviation over this time span.

The limit of detection for lysine at the Pt electrode was determined to be ca. $5 \times 10^{-7}$ M under the conditions used above, i.e., ca. 3X lower than for the Au electrode. Nevertheless, the Au electrode is preferred over Pt because of better reproducibility at low ionic strengths.
Figure 5. Chromatogram for Amino Acid Complex

Conditions: contents of single capsule diluted to 5 liters, pulsed amperometric detection

Peaks: (a) arginine  (b) lysine
      (c) leucine   (d) phenylalanine
Anodic Signal

Time

injection

5 min

0.2 μA

a

b

c

d
Figure 6. Comparison of DC and pulsed detection

Conditions: 50 ul injections of 0.1 mM lysine
50 mM NaOH at 1.0 ml min\(^{-1}\)

Detection: (A) pulsed amperometric detection waveform as described in text
(B) DC detection at 0.50 V vs. SCE
Anodic Signal

A

0.5 μA

5 min

B

5 nA

Time

3
CONCLUSIONS

Determination of amino acids using an anion-exchange separation with pulsed amperometric detection is a direct and simple procedure. PAD is much more sensitive than CD but has a response which deviates from linearity above ca. 0.3 mM. Use of the two detectors in tandem can give a combined linear dynamic range of ca. four decades for lysine.
REFERENCES CITED


ACKNOWLEDGMENTS

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SECTION III.
LIQUID CHROMATOGRAPHIC SEPARATION WITH
ELECTROCHEMICAL DETECTION OF THE PHENYLTHIOHYDANTOIN
AND METHYLTHIOHYDANTOIN DERIVATIVES OF AMINO ACIDS
ABSTRACT

Phenylthiohydantoin (PTH) and methylthiohydantoin (MTH) derivatives of the common amino acids were examined for electroactivity on platinum and gold electrodes. All of the derivatives could be electrocatalytically oxidized at both electrodes. Constant-potential and pulsed amperometry were examined as possible detectors for thiohydantoins following reversed-phase liquid chromatography. Amperometric detection at a fixed potential of 1.2 V vs. SCE resulted in detection limits of less than 10 pmol for lysine and glycine derivatives (50-μl injection). Use of a gradient elution program with a C-18 reversed-phase column allowed separation of nearly all common amino acids as MTH derivatives.
INTRODUCTION

The degradation method of Edman (1) has been responsible chiefly for analytical information regarding amino acid sequences in proteins and peptides for nearly 40 years. While the basic chemistry of the Edman procedure has not changed, advances in analytical techniques have resulted in improved ability to identify and quantitate the phenylthiohydantoin (PTH) adducts of amino acids produced in the reaction. Current methods (2-7) allow separation and determination of most of the common PTH amino acids by HPLC with photometric detection (UV-vis) in a single experiment of less than one hour duration. A slight modification of the Edman procedure (8-9) produces the methylthiohydantoin (MTH) adducts of amino acids, which can be determined rapidly through HPLC techniques (10) in a similar manner as for the PTH derivatives.

The PTH amino acids have been made in situations where sequencing was not needed, serving simply as derivatives for enhanced detectability of the amino acids. A simplification of the Edman procedure (11) will produce the phenylthiocarbamyl (PTC) adducts of amino acids which also can be quantitatively determined to lower concentrations than the free amino acids.

Amperometric detection of organic compounds
traditionally has suffered because of problems caused by the loss of electrode surface activity, especially in the case of aliphatic compounds. The introduction of pulsed amperometric detection (PAD) has eliminated this problem and has been demonstrated to be useful for the direct detection of a wide range of analytes (12-15) including amino acids (16-17). While PAD ranks as one of the most sensitive methods for the direct detection of free amino acids, users of microsequencing techniques for sub-nanomole quantities of protein require even lower detection limits than available from PAD and typically will employ derivatization techniques to form the OPA (18), PTH, or PTC adducts.

No report was found in the literature of a thorough examination of the electrochemical detection of PTH and MTH amino acids. Amperometric detection of PTC amino acids has been reported (19); however, the electrode material was not specified and very little information on the electrochemical response was provided. The purpose of this work was to determine if amperometric detection can provide sufficiently high sensitivity for the Edman products to support microsequencing work in conjunction with a commercially available HPLC column.
EXPERIMENTAL

Materials

Standard MTH and PTH adducts of amino acids were obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals were reagent grade from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Water was deionized followed by purification with a MILLI-Q system from Millipore, (Bedford, MA, U.S.A.) and filtration (0.2 mm). All chromatography solvents were filtered through a 0.45-mm Nylon-66 filter from Rainin, (Woburn, MA, U.S.A.) prior to use.

Procedures

Voltammetric data were obtained with Au and Pt rotated disc electrodes (RDE; Model AFDT07, 0.196 cm²) in a PIR rotator under potentiostatic control by a RDE3 from Pine Instrument Co. (Grove City, PA, U.S.A.). Voltammetric data were traced with a RE0074 X-Y recorder from EG & G Princeton Applied Research (Princeton, NJ, U.S.A.). Amperometric detection was performed with a PAD-2 from Dionex Corp., (Sunnyvale, CA, U.S.A.) and homemade Au and Pt flow-through cells (20). A saturated calomel electrode (SCE) provided the reference potential.

Chromatographic separations were performed in a NuBondapak C-18 reversed-phase column (3.9-mm i.d. x 30 cm)
from Waters Associates (Milford, MA, U.S.A.). The injection loop volume was 50 ul. Isocratic separations and flow injection analysis were performed with a CMA-1 chromatography module and an APM-1 solvent pump from Dionex Corp. Gradient separations were performed using a CHB-1 chromatography module and a GPM-1 pump and GM-2 gradient mixer from Dionex Corp.

A flow rate of 0.9 ml min⁻¹ was used for all work in flowing streams, and all experiments were done at ambient laboratory temperature (i.e., ca. 27 ± 3 C). Voltammetric and isocratic chromatographic experiments employed Solvent A [0.1 M acetate buffer (pH 4.5)/ 0.1 M KNO₃/ 30% acetonitrile]. Gradient elution separations of MTH amino acids mixed Solvent B [0.1 M acetate buffer (pH 4.5)/ 0.05 M KNO₃/ 8.5% acetonitrile] and Solvent C [0.1 M acetate buffer (pH 4.5)/ 0.05 M KNO₃/ 45% acetonitrile]. The gradient is described in Table I.
TABLE I. Composition of the mobile phase in gradient elution chromatography

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solvent B (%)</th>
<th>Solvent C (%)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>95</td>
<td>5</td>
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<tr>
<td>5</td>
<td>70</td>
<td>30</td>
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<tr>
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<tr>
<td>60</td>
<td>45</td>
<td>55</td>
</tr>
</tbody>
</table>

Solvent composition: (B) 0.1 M acetate buffer (pH 4.5) / 0.05M KNO₃ / 8.5% acetonitrile.
(C) 0.1 M acetate buffer (pH 4.5) / 0.05 M KNO₃ / 45% acetonitrile.
RESULTS AND DISCUSSION

Voltammetry

Initial investigation of the electrochemical properties of PTH and MTH amino acids was by cyclic voltammetry (CV) at rotating disc electrodes (RDE). The response for a Pt RDE in 0.5 M H₂SO₄ is shown in Fig. 1A for MTH alanine and in Fig. 1B for PTH alanine in comparison to the response for the blank solution. It is concluded, based on the marked similarity of the voltammetric data, that the substitution of a methyl group for a phenyl on the adduct does not affect the electrochemical response for these amino acid adducts. Furthermore, all corresponding MTH and PTH adducts were observed to produce virtually equivalent voltammetric response. Therefore, results for only one example of each adduct are presented here.

The addition of the PTH and MTH amino acids to the supporting electrolyte results in the elimination of the cathodic and anodic peaks for hydrogen adsorption and desorption obtained for the supporting electrolyte on the negative and positive potential sweep, respectively, in the potential range 0.1 to -0.3 V vs. SCE. This is strong evidence that the PTH and MTH amino acids are adsorbed and, thereby, block the hydrogen adsorption sites. The onset of the anodic wave corresponding to oxide formation obtained on
Figure 1. Cyclic voltammetry of thiohydantoin amino acids: concentration dependence on Pt

Electrode: Pt RDE, 900 rpm, 6 V min$^{-1}$
Solution: 0.5 M H$_2$SO$_4$

(A) Samples: a) residual
b) 2.54 x 10$^{-5}$ M MTH alanine
c) 6.01 x 10$^{-5}$ M MTH alanine

(B) Samples: a) residual
b) 1.13 x 10$^{-5}$ M PTH alanine
c) 3.23 x 10$^{-5}$ M PTH alanine
the positive potential sweep in the region 0.55 - 1.00 V is suppressed also because of adsorption of the adducts.

In acidic solution, the MTH and PTH adducts are oxidized at the Pt electrode in the region of 1.10 - 1.30 V on the positive potential sweep simultaneously with the anodic formation of surface oxide (Figure 1). This anodic signal was observed to increase for higher rotation speeds of the electrode. However, the increase in signal for a change from 900 to 1600 rev min⁻¹ was only ca. 50% of the value expected on the basis of the Levich equation for a mass transport-limited reaction. The current in the region 1.10 - 1.30 V on the positive sweep also was observed to increase nearly as a linear function of the rate of potential sweep. This is diagnostic evidence for a surface-controlled reaction mechanism. Hence, it is concluded that the detection process is under mixed control by kinetic and mass-transport processes, and probably involves the simultaneous oxide-catalyzed anodic reaction of adducts in the adsorbed as well as the dissolved states.

When a Au RDE is employed (Figure 2), it is evident that PTH glycine can be oxidized in both acidic and basic media. Current is also rotation speed dependent for the oxidative region in both media. A very favorable result of these voltammetric results is the discovery that the
Figure 2. Concentration dependence of PTH glycine on a Au RDE

Electrode: Au RDE, 900 rpm, 6 V min\(^{-1}\)

(A) Solution: 0.5 M H\(_2\)SO\(_4\)
Samples:  
  a) residual  
  b) 6.94 \(\times\) 10\(^{-6}\) M PTH glycine  
  c) 1.73 \(\times\) 10\(^{-5}\) M PTH glycine

(B) Solution: 0.25 M NaOH
  a) residual  
  b) 5.20 \(\times\) 10\(^{-6}\) M PTH glycine  
  c) 1.21 \(\times\) 10\(^{-5}\) M PTH glycine
thiohydantoins can be oxidized throughout a wide pH range, contrary to free amino acids which can only be oxidized in basic solution. The freedom to choose pH for oxidation allows good flexibility for the choice of elution conditions for optimum HPLC separation without the need for post-column addition of pH buffer.

Much literature published on HPLC separation of PTH amino acids describe acetate buffer eluents with acetonitrile and/or methanol as eluent modifiers. Methanol is known to be oxidized at noble metal electrodes under certain conditions. Therefore, to avoid background interference, only acetonitrile was used as an organic modifier. Some problems were encountered as the fraction of acetonitrile was increased in early trials due to high cell resistance (IR loss). Consequently, the eluent was supplemented with KNO₃ electrolyte to reach the composition of solvent A. The CV data of MTH lysine in solvent A on a Au electrode is shown in Figure 3A and 3B as a function of analyte concentration and rotation speed. Mixed mass transport - surface control is present in this system as well, although it appears that mass transport is more predominant then for Pt in acid.
**Figure 3.** Cyclic voltammetry of MTH lysine on a Au electrode

Electrode: Au RDE, 6 V min\(^{-1}\)
Solution: Solvent A

(A) Samples: 
- a) residual, 900 rpm
- b) \(1.62 \times 10^{-6}\) M MTH lysine
- c) \(3.24 \times 10^{-6}\) M MTH lysine
- d) \(8.10 \times 10^{-6}\) M MTH lysine
- e) \(2.11 \times 10^{-5}\) M MTH lysine
- f) \(3.89 \times 10^{-5}\) M MTH lysine
- g) \(6.80 \times 10^{-5}\) M MTH lysine

(B) Samples: 
- a) 400 rpm, \(2.60 \times 10^{-6}\) M MTH lys
- b) 900 rpm
- c) 1600 rpm
Waveform Development

From experience with applications of PAD to the determination of other compounds, it was evident that a PAD waveform could be developed to determine thiohydantoin amino acids in solvent A. It had originally been assumed that DC amperometry would be unacceptable due to loss of electrode surface activity, which has been observed with other organic compounds (12,13,15,16), including amino acids (17).

However, the voltammetric response observed for the thiohydantoins differs considerably from that for free amino acids, and the CV data in acidic solutions appears to be consistent with the prediction that DC amperometry might be suitable for detection. In particular it was noted that current does not decay to zero immediately on the reversal from positive to negative scan during a cyclic sweep (Figure 3A), as observed typically for underivatized amino acids. The presence of anodic current on the negative scan is evidence that the oxidation products formed on the positive scan have not irreversibly adsorbed to the electrode surface, and electrode activity is still intact. This phenomenon appears limited to more acidic solutions for Au (Figure 2), where the thiohydantoins can be oxidized, in contrast with free amino acids, which require basic solutions.
A current decay experiment was performed in solvent A to explore the possibility of DC amperometric detection. The potential was swept in cyclic manner to pretreat the electrode, followed by a pulse from the negative sweep limit to 1.2 V, where oxidation of MTH lysine will take place. The potential was held at 1.2 V and the anodic current was plotted vs. time. Some decay was seen in the first 5 min (Figure 4), but steady-state current is reached for MTH lysine solutions at values considerably greater than the residual value. Since electrode activity is not seen to decay to the residual value, DC amperometry should be successful under these conditions.

DC detection was tested by repetitive injections of PTH glycine into a flowing stream of solvent A. The Au electrode was potentiostated at 1.2 V and anodic current measured vs. time in Figure 5. No loss of sensitivity (< 1 %) was noted over several hours and dozens of injections. The optimum S/N ratio was found to exist for an electrode potential in the range 1.15 to 1.25 V vs. SCE, and 1.20 V was used for chromatographic work. DC detection also was determined to be acceptable with stable and reproducible signals at a Pt electrode. PAD was found also to work for both Pt and Au electrodes; the optimum waveforms on each are listed in Table II.
Figure 4. Current decay with time at a fixed potential of 1.2 V vs. SCE

Electrode: Au RDE, 900 rpm

Solution: Solvent A
10-

H

Z

U

(E

tr

3

O

4.52 \times 10^{-5} \text{ M MTH-Lys}

residual

7.29 \times 10^{-6} \text{ M MTH-Lys}

ANODIC CURRENT (\mu A)

TIME (min)
Figure 5. Reproducible DC anodic detection of PTH glycine

Electrode: Au, 1.2 V vs. SCE
TABLE II. Optimum waveforms for pulsed amperometric detection of amino acid adducts

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>Au</th>
<th>Pt</th>
</tr>
</thead>
<tbody>
<tr>
<td>E₁ (t₁)</td>
<td>1.35 V (420 ms)</td>
<td>0.90 V (540 ms)</td>
</tr>
<tr>
<td>E₂ (t₂)</td>
<td>-0.35 V (240 ms)</td>
<td>1.10 V (120 ms)</td>
</tr>
<tr>
<td>E₃ (t₃)</td>
<td>1.55 V (60 ms)</td>
<td>-0.30 V (240 ms)</td>
</tr>
</tbody>
</table>
The limit of detection (LOD) was measured for four model compounds using flow injection analysis. Results using a Au electrode are summarized in Table III. PAD detection limits using a Pt electrode were in general 3-5 times worse than for Au, while DC results were considerably worse on Pt.

Chromatography

Separations of the thiohydantoin adducts of amino acids have been described and these conditions served as the starting point for this work. However, the KNO$_3$ added as an electrolyte to the mobile phase was found to alter the solvent polarity and, therefore, the chromatograms obtained differed greatly from those published. Solutions of Solvent A were made without KNO$_3$ and S/N values compared to previous results with KNO$_3$ present. DC amperometric detection was only slightly worse in the absence of KNO$_3$, but PAD results suffered markedly. It was decided to develop a separation with KNO$_3$ present, so either detection scheme could be applied.

For practical use with the Edman degradation procedure, it is desired that all the common thiohydantoin derivatives should be separated in a single chromatographic run. The capacity factors ($k'$) for the PTH amino acids were
TABLE III. Representative values of detection limits

<table>
<thead>
<tr>
<th>ADDUCT</th>
<th>PAD (picomoles)</th>
<th>DCD (picomoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTH Glycine</td>
<td>25</td>
<td>5.6</td>
</tr>
<tr>
<td>PTH Lysine</td>
<td>17</td>
<td>2.4</td>
</tr>
<tr>
<td>MTH Glycine</td>
<td>35</td>
<td>5.8</td>
</tr>
<tr>
<td>MTH Lysine</td>
<td>19</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Sample injections: 50 ul
determined to have a very wide range of values with KNO$_3$ present in the eluent, making isocratic separation impossible within a reasonable time. The nonpolar amino acids eluted very late using pure solvent A; and the higher percentage of organic modifier (acetonitrile) needed to decrease retention time caused co-elution of many of the more polar derivatives. For a limited separation of PTH derivatives with similar polarities, isocratic elution can be useful, as illustrated in Figure 6. The MTH amino acids are more polar than the PTH derivatives, yet they also had $k'$ values distributed over a wide range. Isocratic elution again lacked resolving power to separate all of these compounds; a partial separation can be seen in Figure 7. The MTH amino acids appear to have a smaller range of $k'$ values than the PTH adducts, and application of a more efficient column may permit an acceptable isocratic separation.

Small variations in the acetonitrile fraction were found to effect large changes in the $k'$ values of the MTH amino acids, suggesting application of an acetonitrile gradient in lieu of a pH gradient. Acetonitrile is not electroactive under the experimental conditions used, but its apparent adsorption at a Au electrode surface will affect the kinetics of the detection response. This
Figure 6. Separation and DC anodic detection of MTH amino acid derivatives

Electrode: Au, 1.2 V vs. SCE
Column: uBondapak C-18
Solution: Solvent A, 0.9 ml min$^{-1}$

Samples:
(a) PTH aspartic acid (4.33 nmol)
(b) PTH asparagine (4.35 nmol)
(c) PTH arginine (3.30 nmol)
(d) PTH glycine (6.07 nmol)
(e) PTH alanine (8.08 nmol)
(f) PTH tyrosine (3.07 nmol)
Figure 7. Separation and DC anodic detection of MTH amino acid derivatives

Electrode: Au, 1.2 V vs. SCE
Column: uBondapak C-18
Solution: Solution A, 0.9 ml min\(^{-1}\)

Samples:
(a) 5.91 \times 10^{-5} M MTH glutamic acid
(b) 1.15 \times 10^{-4} M MTH glycine
(c) 1.14 \times 10^{-4} M MTH alanine
(d) 7.07 \times 10^{-5} M MTH lysine
(e) 6.95 \times 10^{-5} M MTH tyrosine
(f) 9.53 \times 10^{-5} M MTH valine
(g) 1.12 \times 10^{-4} M MTH isoleucine
(h) 1.28 \times 10^{-4} M MTH leucine
adsorption causes the oxidation wave of the analyte to be shifted slightly to more positive potentials. The major portion of the shift occurs during the addition of the first few percent of acetonitrile (14), so a gradient was attempted avoiding this composition range. Experimental data confirmed that attempts to span a wide range of acetonitrile concentration would result in a large baseline shift. Gradients covering a smaller range were found to produce only a slight baseline drift and were deemed to be acceptable as long as this range was covered slowly. The gradient selected varied the acetonitrile fraction from 10.3% to 28.6% over 60 min (see Table I). A sample containing the adducts of 19 amino acids at ca. 20 ppm each was chromatographed using this gradient and the resultant chromatogram is shown in Figure 8. MTH glycine and MTH glutamine coelute, but all other compounds are resolved.

PAD response to the gradient used in Figure 8 is similar to that for the DC amperometry. Improvements in the separation are expected if a more efficient column were utilized. Application of a faster gradient to allow more rapid analysis is possible if background subtraction methods are available.
Figure 8. Separation of MTH amino acids using gradient elution HPLC

Electrode: Au, 1.2 V vs. SCE (DC)
Column: uBondapak C-18
Solution: described in Table I

Samples:
(a) aspartic acid  (g) norleucine
(b) asparagine   (r) phenylalanine
(c) histidine    (s) tryptophan
(d) glycine
(e) glutamine
(f) glutamic acid
(g) arginine
(h) alanine
(i) aminoisobutyric acid
(j) aminobutyric acid
(k) lysine
(l) tyrosine
(m) valine
(n) methionine
(o) isoleucine
(p) norleucine
CONCLUSIONS

The electrochemistry of MTH and PTH amino acids differs greatly from that of the free amino acids, and it is concluded that the thiocarbonyl group is oxidized rather than the amino acid nitrogen. Theoretical treatment of thiocarbonyl oxidations has been reviewed by Polta (20).

Gradient elution chromatography can be performed with amperometric detection, and its application allows a single-injection analysis of an Edman Degradation sample with sensitivity to concentrations less than the 10-pmol value desired by modern microsequencing techniques (7). No problems with reproducibility were encountered during the gradient work (4,21). The application of amperometric detection to thiohydantoins is not limited to the pH range used, and can be employed with other stationary phases should a separation be developed.

It should be emphasized that although PAD results are inferior to DC amperometry, PAD should not be discarded automatically for separations of the thiohydantoin adducts of amino acids. Relatively "clean" samples were used for this work, and DC amperometry may suffer surface poisoning from extraneous material within a more complex sample matrix. PAD has great resistance to electrode fouling and
may prove to be the best detector choice for laboratory applications.
REFERENCES CITED


ACKNOWLEDGMENTS

The assistance of Dave Mead and Bill LaCourse has aided the completion of this work, and the financial support of Dionex Corp. is greatly appreciated.
SECTION IV.
ADVANCES IN ELECTROCHEMICAL DETECTION OF AMINO ACIDS
BY APPLICATION OF COULOMETRIC WAVEFORMS
ABSTRACT

Pulsed coulometric detection was applied at a Au electrode to the determination of amino acids. Original work employed anion-exchange chromatography in 0.05 M NaOH, and waveform parameters were optimized and detection limits were found to be lower than those seen for pulsed amperometric detection. The reductive current for dissolved oxygen was monitored as an indirect procedure for determining adsorbate concentration. Presence of the adsorbate would suppress the cathodic current, which could be monitored within a pulsing waveform. A glass reference electrode was used to allow application of PCD to a gradient elution program without severe baseline perturbation. This system allowed the separation and sensitive detection of 20 amino acids in less than 1 hour.
INTRODUCTION

The increasing interest in biotechnology has rendered the determination of amino acids, both as protein components and free species, as a very important procedure. Technological advances in HPLC have allowed the entire spectrum of common amino acids to be separated as free species or derivatives in periods of less than 1 hour (1-3). Decreased LOD values for amino acids have resulted from improved detector instrumentation, and the synthesis of better derivatization agents.

Electrochemical systems have proven useful as the only class of detector that can combine great sensitivity toward amino acids with the ability to determine them as underivatized species (4-5). Pulsed amperometric detection (PAD, 6-7), the most promising of the electrochemical detectors, has allowed sensitive determination of all amino acids, including the secondary species, following anion-exchange chromatography (8-9). The major limitation of the PAD method has been the lack of an anion-exchange separator with the ability to resolve a complex amino acid mixture during a single isocratic HPLC experiment.

Pulsed coulometric detection (PCD, 10) has shown S/N improvements when compared to PAD, resulting in lower LOD values. Noise is limited by integration during a
triangular potential sweep, which automatically rejects the background due to surface oxide formation while summing the analytical current due to surface oxide catalysis. PCD also has been shown to resist baseline drift during pH changes of less than 2 units, in contrast to PAD, which is so sensitive to this shift that it can be used as a pH sensor (11). The difficulty of complex amino acid mixture separation has demonstrated the need for a gradient separation, and the ability of PCD to withstand pH gradients without change makes it an ideal detector choice.

The software for control of the PCD waveform (12) is very flexible, and allows for waveforms that differ from the traditional PCD principles. One particular software application attempted to determine amino acids indirectly by monitoring current due to the reduction of dissolved O₂. Previous applications of PAD for this indirect method (13) have encountered problems with peak tailing and baseline noise. Improved waveforms were tested with the new software to limit these problems, resulting in the development of indirect coulometric adsorption detection (ICAD).
EXPERIMENTAL

Reagents

Amino acid standards were reagent grade from Aldrich (Milwaukee, WI), Fisher (Springfield, NJ), and Pierce (Rockford, IL). The protein hydrolyzate was Amino Acid Standard H from Pierce. All other chemicals were reagent grade. Water was deionized followed by treatment with either a Millipore MILLI-Q system or a Barnstead NANOpure II system, followed by filtration (0.2 um). All chromatography solvents were filtered with 0.45 um Nylon-66 filters from Rainin (Woburn, MA) prior to use.

Apparatus

Voltammetric data were obtained with Au and Pt rotated disc electrodes (APDT07, 0.196 cm²) in a PIR rotator under potentiostatic control by a RDE3 (Pine Instrument Co., Grove City, PA). Voltammetric data were traced with a RE0074 X-Y recorder (EG & G Princeton Applied Research, Princeton, NJ).

Pulsed amperometric detection was performed with the PAD-2 (Dionex Corp., Sunnyvale, CA). Pulsed coulometric detection and indirect coulometric adsorption detection were performed by a modification of the Computer Aided Electroanalysis System using the so-called Johnson Pulse Amperometry software option (Cypress Systems, Lawrence, KS).
The potentiostat was interfaced via a 12-bit A/D converter to a System 1800 (Everex Systems Inc., Fremont, CA) IBM-AT compatible personal computer with a 20 MB hard disk drive and EGA color monitor. Software control was benefited by usage of a Logitech (Fremont, CA) Bus Mouse and hard copy was obtained with a Hewlett Packard (San Diego, CA) model 7440A ColorPro plotter. Electrochemical cells used were commercial thin-layer cells from Dionex and customized cells produced in these laboratories and described by Polta (14). Reference electrodes used were a saturated calomel electrode (SCE, Fisher) and a universal glass electrode (Fisher).

Chromatographic separations were done using AS-4, AS-6, and AS-8 anion-exchange columns (Dionex). Flowing stream work employed either a CMA-1 Advanced Chromatography Module and an APM-1 pump or a CHB-1 chromatography module and a GPM-1 pump (Dionex). A GM-2 gradient mixer (Dionex) was placed after the pump outlet to enhance mixing. The sample loop had a volume of 50 ul.
RESULTS AND DISCUSSION
Voltammetry and Amperometry

Cyclic voltammetry and PAD of amino acids on both Au and Pt have been examined previously within this laboratory (8-9). It was determined that the oxidation of amino acids was electrocatalytic, requiring the simultaneous formation of surface oxide for anodic detection. LOD values of 75 pmol and 25 pmol were determined for lysine on Au and Pt respectively using PAD. The limiting factor in these experiments was the noise associated with the background current due to oxide formation.

PCD

The optimum PCD waveform (Figure 1) was designed to totally reject the background by summing the charge due to oxide formation and oxide dissolution, which are of equivalent magnitude and opposite polarity. On Au, a cyclic voltammogram was traced using the PCD potentiostat in Figure 2 to allow potential selection. The solvent was 0.05 M NaOH and the AS-6 separator was employed, similar to conditions used with PAD (9). The reductive wave seen for negative potentials was due to reduction of dissolved oxygen. This region, as well as the region of anodic solvent breakdown (> 700 mv), were avoided during the integration period to
Figure 1. Controllable parameters for designing a PCD waveform
CYPRESS PCD OPTIONS

E1

E2

E3

E4

E5

integrate during this interval

start next cycle

Ta

Tb

Te

Td

Tf

Th

Tg

Ti
Figure 2. Residual Au CV in basic solution

Cyclic Voltammetry
Init E (mV): 0          High E: 800
Scan Rate (mV/sec): 50
Solution: .05 M NaOH residual (flow)

File: 3-15a
Low E: -1000          Final E: 0
Number of Scan: 2
Working Electrode: AU wire
minimize background noise. The integration zone was chosen to start at 0 mv, scan to 600 mv, and back to 0 mv. Oxidative cleaning and adsorption potentials were assigned similar values to those used in PAD waveforms.

Variations in the length of the PCD time periods caused large changes in peak shape and sensitivity, so three different waveforms (A-C, Table I) were designed. Waveform A was the Best Skew waveform, which was designed to minimize peak tailing while retaining near-maximum sensitivity. Waveforms B and C are the Best S/N waveforms for total waveform periods of one and two seconds respectively. Peak shapes obtained with waveforms B and C were considerably worse than with waveform A. Waveforms were limited to a total of two seconds to minimize peak distortion from inability to rapidly monitor change in detector response.

A separation of three amino acids using the AS-6 column is shown for both waveform B and C in Figure 3; note the inclusion of hydroxyproline, a secondary amino acid. Detection limits were determined for lysine due to the convenience of its early elution time, and values are summarized in Table II. Due to current limitations in the prototype instrument, it is expected that future improvements can result in a 3-5X decrease in the LOD for PCD.
Table I. PCD waveforms for amino acids in 0.05 M NaOH

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Best Skew</strong></td>
<td><strong>Best S/N (1 sec)</strong></td>
<td><strong>Best S/N (2 sec)</strong></td>
<td></td>
</tr>
<tr>
<td>$E_1$: 0</td>
<td>$E_1$: 0</td>
<td>$E_1$: 0</td>
<td></td>
</tr>
<tr>
<td>$E_2$: 600</td>
<td>$E_2$: 600</td>
<td>$E_2$: 600</td>
<td></td>
</tr>
<tr>
<td>$E_3$: 0</td>
<td>$E_3$: 0</td>
<td>$E_3$: 0</td>
<td></td>
</tr>
<tr>
<td>$E_4$: 800</td>
<td>$E_4$: 800</td>
<td>$E_4$: 800</td>
<td></td>
</tr>
<tr>
<td>$E_5$: 0</td>
<td>$E_5$: -300</td>
<td>$E_5$: -300</td>
<td></td>
</tr>
<tr>
<td>$T_a$: 350</td>
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<td>$T_a$: 400</td>
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<td>$T_b$: 50</td>
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<td>$T_f$: 50</td>
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<td>$T_g$: 10</td>
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<td>$T_h$: 100</td>
<td>$T_h$: 5</td>
<td>$T_h$: 100</td>
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<tr>
<td>$T_i$: 90</td>
<td>$T_i$: 235</td>
<td>$T_i$: 600</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3. A comparison of 2 PCD waveforms for amino acid determination

Electrode: Au

Column: AS-6

Solution: 0.5 M NaOH, 1.0 ml min$^{-1}$

Samples: a) solvent
          b) $1.10 \times 10^{-4}$ M lysine
          c) $9.99 \times 10^{-5}$ M asparagine
          d) $2.67 \times 10^{-4}$ M hydroxyproline

(A) Waveform: waveform B, Table I

(B) Waveform: waveform C, Table I
Table II. LOD values for lysine using PCD

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Best Skew waveform</td>
<td>48 pmoles</td>
</tr>
<tr>
<td>Best S/N waveform (1 sec)</td>
<td>19 pmoles</td>
</tr>
<tr>
<td>Best S/N waveform (2 sec)</td>
<td>13 pmoles</td>
</tr>
</tbody>
</table>

sample injection: 50 ul
Indirect Adsorption Detection

While the PCD detection limits have provided a new state-of-the-art value for direct amino acid determination, other waveforms were examined for further improvements. Adsorption detection originated from a serendipitous discovery of an apparent anodic signal from amino acids at a very negative detection potential in a PAD waveform. Closer examination of the results revealed that the peak was not the result of oxidation of the amino acids, but rather a reduction in a very large cathodic background current. The cathodic current was due to the reduction of dissolved O$_2$, the mechanism of which had apparently been suppressed by the presence of the amino acid, perhaps in an adsorbed state.

One of the dogmatic principles of electrochemistry is that dissolved oxygen is detrimental to electrochemical experiments. Tremendous amounts of time and money are spent purging solvents or entire instruments of oxygen. When oxygen is not removed, interference from simultaneous oxygen signals is avoided by not using potential regions where oxygen can be reduced. Completely new waveforms have been developed to allow cathodic determinations when normal methodology encounters interference due to dissolved oxygen (15). Rather than being detrimental, dissolved oxygen is
actually necessary for this indirect method of amino acid
detection (ICAD).

Demonstration of the necessity of dissolved oxygen for
ICAD was done using cyclic voltammetry with and without N₂
purging of oxygen. Figure 4A shows the response to glycine
in a deaerated alkaline solution at a Au electrode. While
electrocatalytic oxidation is seen at positive potentials,
no anodic wave or cathodic suppression wave is evident at
more negative potentials. Under similar conditions without
N₂ purging (Figure 4B), glycine additions suppress the
residual cathodic wave from 0 to -700 mv.

The electrochemical reduction of oxygen has been
examined by a number of researchers (16-25), with much of
the interest spurred by potential fuel-cell applications.
No single equation can describe this reaction, rather a
network of pathway options exist for each particular set of
reaction conditions (electrode material, solvent,
electrolyte, ... ). A general diagram of typical pathway
options is given in Figure 5 (20). O₂ can either be
directly reduced to H₂O, or reduced to H₂O₂, which may or
may not further react to form H₂O.

In aqueous solutions, Pt predominantly catalyzes the
direct 4-electron reduction to H₂O, while Au
predominantly produces a pair of 2-electron reduction
Figure 4. Cyclic voltammetry of glycine with and without deaeration with $N_2$

Electrode: Au RDE, 900 rpm, 6 V min$^{-1}$

(A) Solution: 10 mM NaOH, deaerated with $N_2$
Samples: a) residual
          b) glycine added

(B) Solution: 2.5 mM NaOH, no deaeration
          a) residual
          b) glycine added
Figure 5. Pathway options for the electrochemical reduction of $O_2$.
steps through the H$_2$O$_2$ intermediate (16,17,20,21). The difference is due to the bonding energy of surface sites toward molecular oxygen adsorption, stronger bonding being necessary for direct oxidation to H$_2$O. The presence of impurities on the electrode surface can greatly alter the catalysis of the reduction reaction.

The presence of metal ad-atoms on Au can produce stronger adsorption of O$_2$, catalyzing its reduction by promoting the 4-electron pathway to H$_2$O. The presence of organic or other non-metal adsorbates has been shown to inhibit oxygen reduction (16,23-25). Platinum is affected more strongly than Au, because the 4-electron reduction is hindered more than the H$_2$O$_2$ pathway for these adsorbates. The formation of H$_2$O$_2$ is also inhibited, and both Au and Pt show a drop in cathodic current. This phenomenon is the basis for the observation that amino acids were suppressing the cathodic background signal, the amino acid nitrogen being adsorbed strongly under alkaline conditions. This catalytic suppression is not limited to amino acids, and upon examination carbohydrates, sulfur compounds, and inorganic anions were found to exhibit this behavior as well.

The analytical usefulness of this indirect detection mechanism for liquid chromatography is dependent on several
factors. First, the residual current is large due to unsuppressed oxygen reduction and the potentiostat used for the determination must have the ability to electronically offset the baseline by a large amount for proper viewing of the response. The background current should ideally be very stable, because a small relative variation for currents of this magnitude will manifest itself as a large amount of noise. LOD values will inevitably be limited by baseline noise. Peak shape is also a major consideration. Incorporation of this indirect determination into a pulsed waveform appears ideal, because an adsorbate must be cleaned from the electrode surface before its successor is eluted from the column. Potential steps will aid this desorption process and reduce peak broadening. Nevertheless, slow change of O₂ reduction kinetics upon adsorption/desorption of adsorbates may broaden peak profiles despite efficient cleaning. A final consideration is the extent of the dynamic range of detection, which should ideally span a wide range of concentration. The lower concentration range will be limited by the aforementioned background noise, and the higher concentration range will be limited to analyte concentrations that do not completely suppress the reduction of oxygen.

This indirect adsorption detection was examined as a
detector for a series of anions commonly separated by anion chromatography. All were known to adsorb to a Pt electrode in acidic solution, for they could be indirectly determined using the method demonstrated by Polta (26) and a PAD potentiostat. Current decay of a model anion, Br\(^-\), is shown in Figure 6 on a Pt rotating disc electrode in acidic solution. Current was cycled, then pulsed to the low potential used for indirect detection. It is clear that additions of the adsorbate suppress the cathodic signal due to \(\text{O}_2\) reduction, and no decay of the suppressive effect occurs for times up to 30 seconds. When oxygen was purged from the cell, all three scans were identical.

A separation of four anions was done using the AS-4 column and \(\text{H}_2\text{SO}_4\) eluent. Indirect adsorption detection on Pt was done using a two step waveform with a detection potential of -100 mv (Figure 7A). A comparison with indirect anodic detection using a PAD waveform (Figure 7B) reveals the sensitivity enhancement of the adsorption detection. Both chromatograms exhibit significant tailing of the later eluting peaks. Calibration experiments for adsorption detection showed a nonlinear current vs. concentration response; when examined for a wide concentration range similar response has been observed for DC amperometry (27) and PAD (9,26), leading to the
Figure 6. Current vs. time response for NaBr suppression of cathodic $O_2$ reduction

Electrode: Pt RDE, 900 rpm

Waveform: a) cycled from +1280 mv to -320 mv  
           b) held at positive limit  
           c) pulsed to +80 mv

Solution: 0.025 M $H_2SO_4$
Figure 7. Comparison of indirect adsorption detection and indirect PAD

Electrode: Pt
Column: AS-4
Solution: 25 mM H₂SO₄, 0.8 ml min⁻¹
Samples: a) Cl⁻  40 ppm
b) Br⁻  18 ppm
c) I⁻  44 ppm
d) SCN⁻  66 ppm

(A) Waveform:  \( E₁ = -100 \text{ mv, 120 ms} \)
\( E₂ = 1400 \text{ mv, 100 ms} \)

(B) Waveform:  \( E₁ = 1300 \text{ mv, 350 ms} \)
\( E₂ = -280 \text{ mv, 200 ms} \)
\( E₃ = 1400 \text{ mv, 50 ms} \)
development of alternative calibration methods (28). Dynamic range for the determination of a test compound (Cl\(^-\)) spanned the range from 1*10\(^{-6}\) M to 1*10\(^{-2}\) M, more than sufficient for analytical usage.

**ICAD**

The flexibility of the potentiostat used for the amino acid PCD work (12) allowed the creation of indirect coulometric adsorption detection (ICAD). Previously, detection was limited to a single potential and a 16.7-millisecond integration period. ICAD allows extension of the integration time, and potential steps or sweeps within that time. The extended integration time promised improved S/N for ICAD, and the problem of peak tailing could be combated with greater waveform flexibility. The adsorption of amino acids on Au in basic solution was chosen as a model system, to examine for possible improvements over PCD. The zone of integration chosen has been superimposed on a cyclic voltammogram in Figure 8. ICAD application over this range resulted in excellent sensitivity, but peak width and tailing was still a concern. The optimized waveform was designed to minimize peak skew, but resolving power was still considerably poorer than seen with PCD. This waveform and a sample chromatogram on the AS-6 column are included in
Figure 8. Superimposition of the ICAD integration range on a cyclic voltammogram of glycine

Electrode: Au RDE, 900 rpm, 6 V min$^{-1}$
Solution: 2.5 mM NaOH, no deaeration
Samples:  a) residual
         b) glycine added
Figure 9. Anion-exchange separation of amino acids with ICAD

Electrode: Au
Column: AS-6
Solution: 0.05 M NaOH, 1 ml min$^{-1}$
Samples: a) solvent
          b) $1.10 \times 10^{-4}$ M lysine
          c) $9.99 \times 10^{-5}$ M asparagine
          d) $2.67 \times 10^{-4}$ M hydroxyproline

Waveform:

E1: -300   E2: 0   E3: -300   E4: 800   E5: -300
Ta: 240    Tb: 100  Tc: 220    Td: 10    Te: 180
Tf: 50     Tg: 10   Th: 90     Ti: 90
Figure 9. Kinetic limitations during the adsorption/desorption processes appear inherently to produce some peak broadening with ICAD.

The detection limit for lysine under the conditions used in Figure 9 was determined to be 14 picomoles for a 50-microliter injection, as compared to 19 picomoles for PCD. Despite the better LOD for ICAD vs. PCD, the improved PCD peak shape will outweigh the slightly poorer S/N for cases when either could be applied. The advantage of ICAD is its ability to function as a universal detector for adsorbed species. For amperometric monitoring of oxidation reactions, different analytes require waveform changes for optimum response in each case. Some oxidizable analytes may be completely invisible when viewed with a PAD waveform for another oxidizable species. PCD waveforms can be made more universal than PAD, yet there still exists a large dependence between length of waveform time periods and anodic response. ICAD monitors the potential region of oxygen reduction, which will not be greatly affected by a change in adsorbate.

Gradient Chromatography

The major limitation toward application of pulsed electrochemical detection for underivatized amino acid
determination had been the inability to couple the detector with an LC separation scheme able to separate all the common amino acids from a single injection. The AS-6 column previously used had been designed for carbohydrate separations, and lacked the ability to separate amino acids with similar functionality. The AS-8 column has been developed recently for specific use as an amino acid separator. Application of a four-solvent gradient has been described for separation of a 17 component protein hydrolyzate without severe baseline shift using UV-vis detection following post-column addition of ninhydrin (29).

LOD values for PCD of underivatized amino acids quoted in Table II are better than for ninhydrin adducts (30), with no need for post-column addition of reagent. Clearly, the ability to apply PAD and PCD with the AS-8 would be advantageous. The solvent system designed for the AS-8 (Table III) is sufficiently alkaline for electrochemical oxidation of the amino acids without post-column pH adjustment. However, the solvent gradient is a severe one, with changes in pH, ionic strength, and organic modifier content. PAD appeared unlikely as a good detector for this gradient, due primarily to the variation in pH. PCD looked like a better choice since it had been proven to resist baseline fluctuations for pH changes of less than two units.
Table III. Solvent system for amino acid separation with the Dionex AS-8 column

| Solution #1 (regenerant): | 0.56 M NaOH/ 0.64 M boric acid |
| Solution #2:              | 0.023 M NaOH/ 0.007 M Na$_2$B$_4$O$_7$ |
|                         | (0.005 M Na$_2$B$_4$O$_7$ substituted for LC-PAD and LC-PCD) |
| Solution #3:             | 0.08 M NaOH/ 0.018 M Na$_2$B$_4$O$_7$/ 2% MeOH |
| Solution #4:             | 0.4 M NaOAc/ 0.001 M NaOH/ 2% MeOH |

Flow rate = 1.0 ml min$^{-1}$

<table>
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<th>Sol #2</th>
<th>Sol #3</th>
<th>Sol #4</th>
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</table>
The effect of ionic strength and organic modifier change on PCD could not be predicted, but the addition of the first few percent of methanol to an aqueous system was known to exert a major influence on PAD.

Gold electrodes were selected for use in the detection due to previous problems applying PCD for Pt electrodes. Cyclic voltammograms were traced for each of the three gradient solvents on the same set of axes (Figure 10). No potential in the anodic electrocatalysis region can be selected such that the three scans overlap, illustrating the expected difficulty of PAD with this gradient. PCD, which is based on potential scan through a detection region rather than a single potential, is more difficult to assess. The major problem appeared to be selection of a negative integration limit such that oxide reduction current can be summed for all three solvents without interference from reduction of dissolved oxygen. The large differences seen in this region (0 to -200 mv) suggest PCD may also suffer from baseline fluctuations with this gradient.

An attempt to use PAD with the separation of the Pierce protein hydrolyzate resulted in severe baseline shift, as predicted from the cyclic voltammograms. Sensitivity to the amino acids was excellent, suggesting its application with a less severe gradient. It was also found that substitution
Figure 10. Solvent comparison for the AS-8 gradient

Electrode: Au, SCE reference
Waveform: CV, 6 V min$^{-1}$
Solutions: a) solution #2 (table III)  
b) solution #3  
c) solution #4
of 4.5 to 5 mM Na$_2$B$_4$O$_7$ in solution #2 of Table III enabled superior resolution of the threonine/alanine and glycine/serine doublets over the standard gradient.

Application of potential sweep PCD also gave excellent sensitivity toward amino acids, but suffered from baseline drift during the prescribed AS-8 gradient as well. By varying the potential limits of the integration window the magnitude of the baseline shift could be varied, but not eliminated. A chromatogram of the protein hydrolyzate with integration between -100 mv and 600 mv is shown in Figure 11. A large baseline shift 15-18 minutes after injection largely obliterates peaks for isoleucine and leucine. This shift corresponds to the change from solution #2 to solution #3 within the gradient, and the shift from solution #3 to solution #4 causes baseline changes too. Minor changes in gradient composition were made to reduce baseline shift, but none were successful and most caused peaks to coelute.

Pulsed electrochemical detectors have typically been avoided when pH gradient chromatography was required, for detector response would change with pH. Half-wave potentials for reactions being monitored shift with pH, but the reference potential obtained from standard Ag/AgCl and SCE reference electrodes will not, so response at the detection potential vs. reference will change. Mead (31)
Figure 11. Anion-exchange amino acid separation followed by PCD

Electrode: Au, SCE reference
Column: AS-8
Solutions: see Table III
Samples: Pierce protein hydrolyzate, 5 x 10^{-4} M each, except cystine at 2.5 x 10^{-4} M

1) arginine 10) leucine
2) lysine 11) methionine
3) threonine 12) histidine
4) alanine 13) phenylalanine
5) glycine 14) glutamic acid
6) serine 15) aspartic acid
7) valine 16) cystine
8) proline 17) tyrosine
9) isoleucine

Waveform:

| E1: -100 | E2: 600 | E3: -100 | E4: 900 | E5: -650 |
| Ta: 350  | Tb: 50  | Tc: 5    | Td: 1   | Ts: 344  |
| Tf: 50   | Tg: 10  | Th: 100  | Ti: 90  |
substituted a glass pH electrode for the SCE reference, and demonstrated that residual current response to pH changes was considerably reduced. The half-wave potentials still shifted, but the reference potential shifted in unison, allowing detection at the same relative potential throughout the gradient. Some increase in noise was seen, but it was limited by placing a Faraday cage around the cell and shortening the reference electrode cable.

A major portion of the baseline shift seen in Figure 11 was suspected to be due to pH shift within the AS-8 gradient. Examination of the potential value of a glass reference using cyclic voltammetry is depicted in Figure 12. A residual scan using a Au electrode of each gradient solvent was made and the group superimposed. Differences are still evident, but they are smaller than with the SCE. Mead's glass electrode work employed detection potentials for carbohydrate detection, which differ greatly from amino acid detection potentials. Examination of the amino acid detection range (500 to 950 mv in Figure 12) suggests that the glass electrode will not alleviate baseline drift for PAD with the AS-8. PCD with the glass electrode is more promising, mainly due to the smaller overlap between the surface oxide reduction and dissolved O₂ reduction than seen with the SCE.
Figure 12. Solvent comparison for the AS-8 gradient using a glass reference electrode

Electrode: Au, glass reference

Waveform: CV, 6 V min\(^{-1}\)

Solutions: a) solution #2 (table III)
            b) solution #3
            c) solution #4
A Dionex flow cell with a modified top (31) to allow usage of a glass reference electrode was added to the system and tested using AS-8 separations of the Pierce protein hydrolyzate. As expected, PAD gave large baseline shifts (Figure 13). Only the shift from solution #2 to solution #3 perturbed the baseline, but the magnitude of the perturbation was larger than any of the amino acid peaks. PCD results were much better, with a zone of current integration of 200 to 800 mv chosen. Only slight baseline perturbations were visible (Figure 14), and all 17 amino acids were resolved and detected with excellent sensitivity. Repetitive trials showed reproducibility of the separation, and no decay of glass electrode performance, as seen in other solvents (31), was noted. Asparagine, glutamine, and cysteine were added to a hydrolyzate in Figure 15, allowing separation of 20 amino acids.

Other than selection of different potentials due to the reference electrode change, waveform parameters are the same as the Best Skew waveform used with the AS-6. Modifications to resemble the Best S/N waveforms of one and two seconds resolve all peaks in the protein hydrolyzate, and offer improved sensitivity over the Best Skew waveform. However, they also suffered from greater peak overlap, greater peak skew, and worse baselines. The LOD for lysine using an AS-8
Figure 13. Anion-exchange LC-PAD of the Pierce protein hydrolyzate

Electrode: Au, glass reference
Column: AS-8
Solutions: see Table III
Samples: Pierce protein hydrolyzate,
5 x 10^{-4} M each except cystine

Waveform:

1) arginine 10) leucine \( E_1 = 750 \text{ mV, 300 ms} \)
2) lysine 11) methionine \( E_2 = 1000 \text{ mV, 100 ms} \)
3) threonine 12) histidine \( E_3 = -350 \text{ mV, 100 ms} \)
4) alanine 13) phenylalanine
5) glycine 14) glutamic acid
6) serine 15) aspartic acid
7) valine 16) cystine
8) proline 17) tyrosine
9) isoleucine
LC-PAD OF AMINO ACID HYDROLYZATE

0 5 10 15 20 25 30 35 40
TIME (min.)

inject

1 2 3 4 5 6 7 8
1 μA

9 10 11 12 13 14 15 16 17
Figure 14. LC-PCD of the Pierce protein hydrolyzate using a glass reference electrode

Electrode: Au, glass reference
Column: AS-8
Solutions: see Table III
Samples: Pierce protein hydrolyzate,
5 x 10^{-4} M each, except cystine
at 2.5 x 10^{-4} M
1) arginine  10) leucine
2) lysine  11) methionine
3) threonine  12) histidine
4) alanine  13) phenylalanine
5) glycine  14) glutamic acid
6) serine  15) aspartic acid
7) valine  16) cystine
8) proline  17) tyrosine
9) isoleucine

Waveform:

Ta: 353  Tb: 50  Tc: 5  Td: 1  Te: 344
Tf: 50  Tg: 10  Th: 100  Ti: 90
Figure 15. LC-PCD of 20 amino acids

Electrode: Au, glass reference
Column: AS-8
Solutions: see Table III

Samples:
1) arginine
2) lysine
3) glutamine
4) asparagine
5) threonine
6) alanine
7) glycine
8) serine
9) valine
10) proline
11) isoleucine
12) leucine
13) methionine
14) histidine
15) phenylalanine
16) glutamic acid
17) aspartic acid
18) cysteine
19) cystine
20) tyrosine

Waveform:

Ta: 350   Tb: 50   Tc: 5    Td: 1     Te: 344
Tf: 50    Tg: 10   Th: 100  Ti: 90
column and the waveform in Figure 15 (Best Skew waveform) was 38 picomoles (50-ul injection). Improvements in PCD equipment should allow a 3-5X improvement in the near future.
CONCLUSIONS

PCD can be applied for the direct determination of amino acids; detection limits are superior to PAD and UV-vis detection of ninhydrin adducts. Indirect detection of amino acids by suppression of oxygen reduction upon their adsorption to Au and Pt can also be employed, but peak shapes are inferior to PCD results. Other adsorbates can be detected as well, with ICAD being postulated as a universal adsorption detector. Application of an AS-8 separator column and its gradient gave less baseline shift when a glass reference electrode was substituted for an SCE for PCD. This allowed separation and sensitive detection of a 20 amino acid mixture using a single injection.
REFERENCES CITED


ACKNOWLEDGMENTS

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SECTION V.

PULSED COULOMETRIC DETECTION FOR LIQUID CHROMATOGRAPHY
ABSTRACT

Pulsed coulometric detection (PCD) is a relatively new technique that promises improved performance over pulsed amperometric detection (PAD) in most cases. Current PCD instrumentation is reviewed, using the electrocatalytic oxidation of amino acids as a model system. The complex PCD waveform (5 potentials, 9 time periods) makes optimization difficult; suggestions are made for selecting potentials and times when designing a waveform. The PCD potentiostat was evaluated for its veracity toward the selected potentials and the noise associated with current/charge measurement.
INTRODUCTION

The development of pulsed amperometric detection (PAD, 1-2) has allowed direct electrochemical detection for a number of compounds using electrocatalysis at noble metal electrode surfaces (3-7). Potential steps within the PAD waveform (Figure 1A) allow surface cleaning of the electrode such that response remains constant, in contrast to DC amperometry, where electrode surface fouling occurs with a concomittant loss in sensitivity for many organic analytes. Current is integrated for 16.7 ms at the end of the detection potential (E₁) and an average value for the period stored in a lock-in amplifier. This period was chosen to minimize contributions from 60 Hz sinusoidal sources of environmental noise.

It has been determined that increasing the integration time to a higher multiple of a 60 Hz cycle period can result in improved S/N for certain analytes (8). When this extended integration period is employed within E₁ of a PAD waveform, it is referred to as constant potential pulsed coulometric detection (PCD). Dionex Corp. (Sunnyvale, CA) now manufactures an instrument, the PAD-2, with this capability. Constant potential PCD has proven most valuable for detection potentials that yield low background noise, such as potentials used for carbohydrate detection (2,8,9).
In the case of amino acid determination by PAD (4,5), the detection potential chosen has an inherently large background component due to the formation of a catalytic surface oxide (on both Pt and Au) necessary for oxidation. Under these conditions minimization of 60 Hz environmental noise by application of constant potential PCD has little to no effect on S/N, suggesting that most of the background noise is related to oxide formation rather than environmental 60 Hz signals.

Improvement of S/N over PAD for analytes with detection potentials in the region of large background oxide currents can be achieved with potential-sweep PCD (10). In this waveform the integration period can be deployed during a triangular potential sweep instead of a fixed potential. With proper selection of scan limits, the charge due to both oxide formation and reduction can be integrated during this sweep. The anodic charge due to oxide formation is equivalent to the cathodic charge resulting from oxide reduction, so integration of these opposing charges will sum the background to zero. Electrocatalytically oxidized species are ideal analytes for potential-sweep PCD, because their oxidative charge is summed during the triangular sweep, but due to the irreversible nature of these reactions the anodic charge is not canceled by its corresponding
Figure 1. Previous waveforms used for pulsed electrochemical detection

A) PAD waveform
B) and C) early PCD waveforms, with integration from the start until the step to $E_2$
reduction reaction. This charge is then superimposed on a much smaller background due to cancellation of oxide current, resulting in improved S/N.

Initial work with potential-sweep PCD was done on "homemade" instruments, which limited performance in several ways. Typical waveforms (Figure 1B,1C) were limited by the instrument to simpler forms than desired, often with substitution of a potential step for a potential sweep. Poor electronics within the instrument impaired it from reaching its potential S/N. Better LOD results when compared to PAD using the same potentiostat were obtained, yet potential-sweep PCD still gave higher LOD values than commercially available PAD potentiostats from Dionex Corp.

To expand the scope of PCD, it was felt that an improved PCD potentiostat was necessary to allow an accurate assessment of its predicted benefits. The most important upgrade was hoped to be improved electronics within the potentiostat, such that instrumental noise was no longer the limiting factor in S/N measurements. Greater flexibility in waveform design was also desirable, and it was felt that this could best be achieved via software control using a personal computer interfaced to the PCD potentiostat. This would allow the added benefit of storage and manipulation of the PCD data.
EXPERIMENTAL

Hardware

The customized PCD instrument was produced by Cypress Systems (Lawrence, KS) as a modification of their Computer Aided Electroanalysis System. The potentiostat was interfaced via a 12 bit A/D converter to a System 1800 (Everex Systems Inc., Fremont, CA) IBM-AT compatible personal computer with a 20 MB hard disk drive and EGA color monitor. Software control was benefited by usage of a Logitech (Fremont, CA) Bus Mouse and hard copy was obtained with a Hewlett Packard (San Diego, CA) model 7440A ColorPro plotter.

Flowing stream work was done with AS-6 and AS-8 anion exchange columns (Dionex) using an injection loop with 50-µl volume. A CHB-1 chromatography module and a GPM-1 pump (Dionex) were used to deliver a 1 ml/min flow at room temperature. Electrochemical cells used were commercial thin-layer cells from Dionex and customized cells produced in these laboratories and described by Polta (11), with Au working electrodes.

Waveform integrity was examined with a Model 2230 digital storage oscilloscope with a GPIB interface (Tektronix Corp., Beaverton, OR) to a RE0074 X-Y recorder (EG & G Princeton Applied Research, Princeton, NJ).
Software

Waveform control software was developed by Cypress Systems as the Johnson Pulse Amperometry option for their Computer Aided Electroanalysis menu. Programming was done in machine language to minimize response time.
RESULTS AND DISCUSSION

A summary of waveform options with the Johnson Pulse Amperometry software is shown in Figure 2. The waveform has grown in complexity from the 3 time periods and 3 potentials of PAD to 9 time periods and 5 potentials. Diagnostic work was done using the electrocatalytic oxidation of amino acids as a model system for testing waveform parameters.

Potentials

Voltages vs. the reference electrode could be set between +2048 and -2048 mv to increments of one mv. Potentials of consecutive steps can be equivalent if desired. E₁, E₂, and E₃ are encompassed within the range of integration, while E₄ and E₅ are typically oxidative cleaning and adsorption pulses as in PAD. Reversal of the cleaning and adsorption steps for PAD has proven beneficial for certain analytes (12), and can also be applied for PCD. However, the benefits of this variation for PAD of preforming oxide to minimize background current during sampling will not be seen with potential-sweep PCD.

During the integration period the potential was swept from E₁ to E₂, then back to E₃, which was always equivalent to E₁ to keep the anodic and cathodic sweep ranges equal for maximum background cancellation. E₁ and E₃ were chosen to
Figure 2. Controllable parameters for designing a PCD waveform
CYPRESS PCD OPTIONS

integrate during this interval

start next cycle
be more negative than the oxide reduction wave on the working electrode. \(E_2\) was chosen to be a very positive potential to allow oxide formation and electrocatalytic oxidation of the analyte, yet negative of the onset of solvent breakdown and \(O_2\) evolution. For maximum cancellation of anodic and cathodic charge, it was important to not integrate any irreversible waves other than for the analyte, hence the avoidance of \(O_2\) evolution potentials. Noise increases were observed when integration was extended into this region.

A similar problem exists for \(E_1\) and \(E_3\), which ideally are set more negative than oxide reduction, yet more positive than a wave for the reduction of dissolved \(O_2\). Unfortunately, this is not always possible as these waves often overlap, so potentials are chosen to minimize the net background current. As seen in Figure 3, there is only an overlap for Au electrodes at higher pH values. A successful application under similar conditions (13) proves that a small overlap can be tolerated. The overlap for Pt is much more severe than Au; all PCD work in alkaline media with the presence of dissolved \(O_2\) has shown poor S/N. Detection in acidic media is more promising for Pt, but if an alkaline solvent is needed the removal of dissolved \(O_2\) should improve performance. It was felt that use of a Au working
Figure 3. Cyclic voltammograms on Au for both acidic and basic media

Electrode: Au RDE, 900 rpm, 6 V min$^{-1}$

Samples: residual scans, no deaeration with $N_2$

A) Solution: 0.5 M $H_2SO_4$

B) Solution: 0.25 M $NaOH$
(A) 100 pA

(B) 300 mV

100 μA
300 mV
electrode, when possible, was much more desirable than dealing with the often problematic procedures for O_2 purging in liquid chromatography.

Although not fitting into the framework of traditional PCD, the Johnson Pulse Amperometry software allows a number of other waveform variations. Potential scans have been successfully used without concern for background elimination, although S/N is not expected to be as good.

**Times**

Times are defined as periods of chosen length that begin at the end of the previous period, rather than as points in time after the start of a cycle. Times have to be at least 1 ms, and cannot exceed 10 seconds. Larger time values are avoided because the duration of the entire waveform should be short so concentration profiles within flowing streams can be traced accurately. PAD waveforms are typically kept to less than one second, so attempts were made to limit PCD waveforms to this value as well. The experienced PAD user must grapple with "time inflation" when using the Johnson Pulse software, having three times as many periods in the same 1 second waveform and inevitably having to shorten periods. A closer examination (Figure 4) found that waveforms up to two seconds in length were acceptable,
Figure 4. Examination of the effect of increased waveform period

Electrode:    Au, glass reference
Column:      AS-8
Solutions:   see Table III, section IV
Samples:     Threonine/Alanine doublet with $\Delta T_R < 20$ sec

A) 1 sec waveform

B) 2 sec waveform
but waveforms of greater than two seconds displayed significant loss of peak definition.

Current is integrated from the beginning of $T_b$ until the end of $T_f$, which typically spans 40-60% of the total waveform. When an effort was made to create an integration period that was an exact multiple of a 60 Hz cycle, no improvement in S/N was noted. With the integration time being much larger than a typical PAD value, it is suspected that 60 Hz noise was an insignificant fraction of the total current. As instrumentation for PCD improves, 60 Hz noise may yet emerge as a major concern.

Variations of specific times can not only affect the sensitivity, but baseline noise and peak skew as well. If the oxidative cleaning time (usually $T_h$) is too short, peak tailing is observed. The adsorption time (typically $T_i$) is usually made as large as possible, due to the resultant sensitivity enhancement seen for oxidations involving isotherm adsorption (14). If $E_1$ is similar to $E_5$, adsorption may occur during $T_a$ as well, and net adsorption time becomes $T_a + T_i$.

For PAD, the delay time at the detection potential is typically large (300-500 ms) to allow decay of background current before sampling. In PCD, $E_1$ typically has a much smaller background current due to its more negative
positioning, so $T_a$ need not be as lengthy. Periods $T_b$, $T_f$, and $T_g$ are kept short, usually under 60 ms, to minimize the tailing seen at longer values.

The values of $T_c$, $T_d$, and $T_e$, which control potential sweep during the integration period, exert the largest influence on sensitivity and peak skew. It had been predicted that $T_d$ should be kept as short as possible to minimize accumulation of irreversible anodic charge from solvent breakdown. This was determined to not be a factor, and lengthened $T_d$ actually enhanced performance slightly. Nevertheless, $T_d$ was generally kept very short because it was determined that S/N was augmented by lengthening other periods in lieu of $T_d$.

It was originally thought that $T_c$ and $T_e$ should be equivalent to create a symmetrical integration period to improve the chance of canceling the background current. When $T_c$ and $T_e$ were kept < 50 ms, peak shapes were excellent but sensitivity was poor. When they were > 250 ms sensitivity was excellent but peak shapes poor (Figure 5). When $T_c$ exceeded 400 ms instrumental difficulties, which are described in the following section, were encountered and no data could be obtained. By shortening one of the two to < 50 ms while keeping the other > 250 ms, good current sensitivity was obtained with an acceptable peak shape. $T_c$
Figure 5. Peak shape variance with changes in the PCD integration ramp

Electrode: Au, SCE reference

Column: AS-6

Solution: 0.05 M NaOH, 1 ml min$^{-1}$

Sample: $7.66 \times 10^{-5}$ M lysine

A) Waveform:

<table>
<thead>
<tr>
<th>E1: 0</th>
<th>E2: 600</th>
<th>E3: 0</th>
<th>E4: 800</th>
<th>E5: -300</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ta: 400</td>
<td>Tb: 50</td>
<td>Tc: 50</td>
<td>Td: 150</td>
<td>Te: 50</td>
</tr>
<tr>
<td>Tf: 50</td>
<td>Tg: 10</td>
<td>Th: 100</td>
<td>Ti: 600</td>
<td></td>
</tr>
</tbody>
</table>

B) Waveform:

<table>
<thead>
<tr>
<th>E1: 0</th>
<th>E2: 600</th>
<th>E3: 0</th>
<th>E4: 800</th>
<th>E5: -300</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ta: 400</td>
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<td>Tc: 300</td>
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<td>Te: 300</td>
</tr>
<tr>
<td>Tf: 50</td>
<td>Tg: 10</td>
<td>Th: 100</td>
<td>Ti: 600</td>
<td></td>
</tr>
</tbody>
</table>
was assigned the short period and $T_e$ the long period due to the previously mentioned instrumental difficulty and the fact that short $T_e$ values tended to create a trough-shaped perturbation in the baseline following the peak. Compromise waveforms need not be made, and best peak shape waveforms can be produced at the expense of sensitivity and vice versa.

**Electronics**

Potentiostat performance was evaluated using the Tektronix oscilloscope and a dummy cell. In Figure 6 the potential is displayed vs. time for a PCD waveform, and it can be seen that the potential scan regions are considerably noisier than the regions of fixed potential. Being that all of the potential ramps are in the period of current integration, this is a major concern and improvement will be sought in the future. No major problems were noted from the current response seen in Figure 7. Background noise associated with the current (charge) output of the potentiostat could not be evaluated accurately due to instrumental difficulties. Low sensitivity scales had to be used for all work, and upon application of the zoom function to view noise, the gaps between successive charge increments became so large that the superimposed noise was not seen
Figure 6. Oscilloscope trace of potential vs. time for a PCD waveform

Electrode: Au

Waveform:

Ta: 350  Tb: 50  Tc: 150  Td: 1  Te: 150
Tf: 50  Tg: 10  Th: 100  Ti: 90  End Time: 10
Figure 7. Oscilloscope trace of current vs. time for a PCD waveform

Electrode: Au

Waveform:

Ta: 350   Tb: 50   Tc: 100   Td: 1    Te: 200
Tf: 50    Tg: 10   Th: 100   Ti: 90
Figure 8. Depiction of the inability to view noise due to large gaps between successive charge increments

Electrode: Au
Column: AS-6
Solution: 0.05 M NaOH, 1 ml min\(^{-1}\)
Samples: a) solvent
b) 1.92 x 10\(^{-6}\) M lysine

E1: 0   E2: 600   E3: 0   E4: 800   E5: 0
Ta: 350  Tb: 50   Tc: 5   Td: 1   Te: 344
Tf: 50   Tg: 10   Th: 100  Ti: 90
(Figure 8). Use of higher sensitivity scales would solve this problem, but were not allowed because they resulted in integrator overload. Whereas the output from an integration cycle is expected to be small and overload not a problem, the transient charge at the end of the positive sweep is very large before it is canceled by cathodic current during the negative sweep. Overload occurs in this region not only for high sensitivity scales, but also for the previously mentioned case when $T_C$ was long. It should be emphasized that this instrumentation was vastly superior to its "homemade" forerunner, and the second-generation Cypress Potentiostat and Johnson Pulse software will be designed to improve the performance of these limiting parameters.

The Cypress System with Johnson Pulse software need not be limited to potential-sweep PCD, because the waveform allows other types of electrochemical measurements. DC amperometry, PAD, and constant potential PCD can be programmed, and the system flexibility has allowed new waveforms to be developed (15). Other software options sold by Cypress Systems permit cyclic, square wave, and differential pulse voltammetric and chronoamperometric experiments.
CONCLUSIONS

The Cypress PCD system is a tremendous improvement over the previous generation of PCD instrumentation. Software control of the waveform through the Johnson Pulse Amperometry option allows great flexibility in design, allowing customization for each particular analyte group.

Noise cannot be properly evaluated, due to overload of the integration at high sensitivity scales and the subsequent use of low sensitivity scales. At low sensitivity the zoom function is employed to view small current/charge changes, but the gap between successive charge increments allowed by the software became greater than the superimposed noise.

A PCD waveform for the model system of amino acid oxidation can be optimized for peak shape or for sensitivity. Compromise waveforms emphasizing both peak shape and sensitivity can also be produced. Further instrumental improvements are being incorporated into the next generation of PCD instruments, which will lower detection limits in the near future, and also remove hardware/software limitations as a factor in waveform design.
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GENERAL CONCLUSIONS

Electrochemical detectors for HPLC are becoming viable alternatives to more commonly used methods. The development of PAD was a tremendous step toward this goal, expanding the scope of electrochemical detector application to a number of compounds that had heretofore resisted detection by electrochemical means. Amino acids are one of these compound classes that were not amenable to classical electrochemical detection. As PAD research opened up new compound groupings for electrochemical detection, attention was focused on improving the quantitative response toward these compounds.

The development of PCD was a result of work to improve detection limits seen with PAD, while retaining its broad range of applicability. PCD has not completed the transition from theoretical basis to instrumental reality, but this will occur in the near future. Detection limits for current PCD systems already are better than with PAD, and when the instrumentation has matured, further improvements are expected.

Certainly, PCD will not be the final word in pulsed electrochemical detection. The advent of PAD and PCD has served as an inspiration to those hesitant to delve into the electronic interior of electrochemical instrumentation, and
plans are already in progress for new pulsed waveforms. The lowering of detection limits seen with these new waveforms will still be coupled with a robustness toward electrode fouling due to complex sample matrices.

For amino acid determinations, detection limit improvements combined with the absence of derivatization procedures and minimal sample preparation requirements will make electrochemical detection a viable alternative to any other detector choice.
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