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Laser-based optical activity detection of amino acids and proteins

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Laser-based optical activity detection of amino acids and proteins

by

Barbara Hollenbeck Reitsma

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GENERAL INTRODUCTION

Introduction to Amino Acids and Proteins

Amino acids are the building blocks of life. Many amino acids are known but only about 22 of them are consistently found as products of protein hydrolysis. All amino acids have an amino group and an acid moiety on a carbon atom which is designated as the α-carbon. The amino acids differ by the other two groups which complete the tetrahedral arrangement of substituents around the α-carbon. There are two secondary amino acids, proline and hydroxyproline, in which the amino nitrogen is present in a pyrrolidine ring. Table 1 lists 22 of the most common naturally occurring amino acids, abbreviations and molecular weights. The properties of amino acids vary widely because of their diverse structures. Because of this diversity, several methods are used to classify amino acids. Two common methods are based on the composition of the R group or the acid/base characteristics of the amino acid as a whole. These catagories are listed in Table 2.

The first amino acids were discovered in the early 19th century as chemists explored samples such as gelatin, fermentation products of cheese making, kidney stones and animal horn. Wollaston isolated the first amino acid, cystine, in 1810, followed nine years later by Proust with the isolation of leucine (1). Both proline and alanine were synthesized before being identified as naturally occurring amino acids. The last of the common amino acids to be discovered, threonine, was isolated and characterized in 1935 by Rose cited in (2).

Table 1. Naturally occurring amino acids

Amino acid	Abbreviation	Molecular weight (g/mol)
Alanine	Ala	89.10
Arginine	Arg	174.21
Asparagine	Asn	132.12
Aspartic acid	Asp	132.11
Cysteine	Cys	121.16
Cystine	Cys-Cys	240.31
Glutamic acid	Glu	147.14
Glutamine	Gln	146.15
Glycine	Gly	75.07
Histidine	His	155.16
Hydroxyproline	Hyp	131.14
Isoleucine	Ile	131.18
Leucine	Leu	131.18
Lysine	Lys	146.19
Methionine	Met	149.22
Phenylalanine	Phe	165.20
Proline	Pro	115.14
Serine	Ser	105.10
Threonine	Thr	119.12
Tryptophan	Trp	204.23
Tyrosine	Tyr	181.20
Valine	Val	117.15

Table 2. Amino acid classifications

R group characteristics	
	Aliphatic - glycine, alanine, valine, leucine, isoleucine
	hydroxyl - serine, threonine
	sulfur-containing - cystine, cysteine, methionine
	aromatic - phenylalanine, tyrosine, tryptophan
	acidic - aspartic acid, glutamic acid
	basic - lysine, histidine, arginine
	secondary - proline, hydroxyproline
Acid/base properties	
	acidic - glutamic acid, aspartic acid
	basic - lysine, arginine
	weakly basic - histidine, tyrosine, cysteine

The majority of amino acids found in nature possess only one of the two possible spacial configurations of substituent groups around the alpha (or chiral) carbon. As a result, all amino acids, except glycine, are optically active. This configuration is designated the L configuration because of the structural relationship to L-glyceraldehyde. Amino acids of the D configuration have been found in a few living systems (3).

Table 3 lists the specific rotations, $[\alpha]$, of amino acids at different conditions. As is seen, the rotation differs in direction and magnitude for all amino acids and also for individual amino acids at different conditions. The degree of rotation strongly depends on the pH of the solution. Lutz and Jirgensons (4) observed that increasing acidity caused the optical activity of an L-amino acid to become more positive. But the original specific rotation of the amino acid was either positive or negative so acidity had no common effect on all amino acids. Specific rotation is defined in Equation 1

$$[\alpha] = \frac{\alpha \times 100}{c \times \ell} \qquad (1)$$

where α is the amount of rotation measured, c is the concentration in g/100 ml solution and ℓ is the path length in dm. Because specific rotation is also a function of wavelength, temperature and solvent, these conditions are also reported.

The optical rotation of amino acids was identified in 1851 by Louis Pasteur when he determined that the optical rotation of synthetic amino acids and those isolated from natural sources differed (5). Optical

Table 3. Specific rotations of amino acids[a]

Amino acid	$[\alpha]_D$ in water[b]	$[\alpha]_D$ in 5N HCl[b]
Alanine	+1.8	+14.6
Arginine	+12.5	+27.6
Asparagine	-5.6	+28.6[c]
Aspartic acid	+5.0	+25.4
Cysteine	-16.5	+6.5
Cystine	-211.9[c]	-231.9
Glutamic acid	+12.0	+31.8
Glutamine	+6.3	+31.8[c]
Histidine	-38.5	+11.8
Hydroxyproline	-75.9	-50.5
Isoleucine	+12.4	+39.5
Leucine	-11.0	+16.0
Lysine	+13.5	+25.9
Methionine	-10.0	+23.2
Phenylalanine	-34.5	-4.5
Proline	-86.2	-60.4
Serine	-7.5	+15.1[c]
Threonine	-28.5	-15.0
Tryptophan	-33.7	-2.8[c]
Tyrosine		-10.0
Valine	+5.6	+28.3

[a]Reference 5.

[b]1-2 g/100 ml concentration.

[c]1N HCl.

rotation also led to the discovery of isoleucine (2). Emil Fischer discovered that the specific rotation of leucine varied with each subfraction but could not isolate the isomer. The isolation of isoleucine was accomplished in 1903 by Felix Ehrlich cited in (1).

Proteins consist of amino acids bound together through peptide linkages. Proteins are involved in all phases of chemical and physical activity in plants and animals. There are proteins involved in structure, muscular action, chemical transformations, immune response, respiration and many other functions. The identification of proteins as a special class of compounds predates the discovery of amino acids (5). At first the thrust of research centered on the identification, isolation and purification of new proteins. Later, the research emphasis shifted to the determination of the amino acid sequence, or primary structure. After 1945, according to Jakubke and Jeschkeit (5), research on the elucidation of the three dimensional shape of proteins began.

This three dimensional shape of a protein is called secondary and tertiary structure. Secondary structure refers to the "conformation of the chains resulting from the formation of hydrogen bonds between the oxygen of the carbonyl group and the nitrogen of the amide group of the polypeptide backbone" (5). Tertiary structure denotes the spacial structure of a polypeptide chain resulting from the hydrogen bonds, ionic bonds, and hydrophobic bonds which form between side chains of the amino acids.

Because the spacial configuration is held together by relatively weak bonds, the structure can undergo denaturation. Various definitions

of denaturation have been put forth over the years. Some require loss of biological activity to accompany denaturation (5). The term denaturation as used in this dissertation is less restrictive. Denaturation refers to any intramolecular rearrangement which changes the spacial configuration of the protein without rupture of primary covalent bonds (6). The hydrogen, ionic, and hydrophobic bonds which hold the secondary and tertiary structure together may be broken in denaturation without disturbing the covalent bonds (7). This definition allows for denaturation of different magnitudes which is consistent with experimental results. Most proteins consist of a compact arrangement of amino acid chains arranged in helices, sheets, and random coils held together by noncovalent bonds. Because of the compact arrangement, denaturation of proteins often involves unfolding of this compact structure. Unfolding is a more descriptive term and will be used interchangably with denaturation.

Proteins are optically active as a result of the chirality of the amino acids which make up the protein backbone, in addition to the three dimensional arrangement of the amino acids in relation to each other. Because of the contribution of the three dimensional structure to the optical activity of the protein, any changes in secondary and tertiary structure can result in a change in optical rotation.

Prior to the advent of chromatography, amino acids and proteins were analyzed by a variety of methods. Calvery (8) gives an extensive list of methods used to detect the presence of individual amino acids, as well as general tests for amino acids or proteins. Many methods involved

spectroscopy or colorimetry, but isotope dilution (9), enzymatic methods (10), and microbiological assays (11) were also used. Some of these reactions are still in use today in chromatographic methods.

Chromatography of Amino Acids and Proteins

In searching for methods of amino acid and protein analysis, researchers turned to chromatography. Methods were needed to separate a mixture of amino acids from an amino acid sample, protein hydrolysate or sequencing reaction products so the amount of individual amino acids could be determined. Early work on chromatography of amino acids involved partition, paper, and ion-exchange chromatography. Martin and Synge (12) separated N-acetyl amino acids on a silica column. Paper chromatography of free amino acids was first described by Consden et al. (13). Partition chromatography using starch columns for the separation of amino acids was first suggested by Elsden and Synge (14) and used by Synge (15). Stein and Moore illustrated this separation in a series of reports (16-18) that described the application of starch column chromatography in the separation of amino acids from protein hydrolysates. Ion-exchange chromatography of amino acids was demonstrated by Moore and Stein (19). These workers illustrated the separation of amino acids using sulfonated polystyrene ion-exchange columns. Quantitation of the basic amino acids required elution from a second, shorter column. Spackman, Stein, and Moore (20) introduced the first automated amino acid analyzer. The system involved dual ion-exchange columns and automated recording of results. Single

column systems were introduced by Piez and Morris (21) using a continuous gradient buffer solution, and Hamilton (22) employing discrete buffer solutions for all amino acids.

According to Snyder and Kirkland (23), the late 1960s marked the transition from classical liquid chromatography to modern HPLC. This change involved the introduction of new columns, rigid column materials, forced liquid flow, and improved detection methods. These advances increased the convenience of LC as an analytical technique, thus leading to widespread use.

Presently, amino acids are analyzed by one of two general methods. The first method is cation-exchange separation of free amino acids followed by derivatization to facilitate detection. In the second method, amino acids are derivatized prior to separation by reversed-phase chromatography. These post- and pre-column methods use a variety of derivatives, some of which are used in both methods. Derivatization is necessary since amino acids possess no chromophores in the visible region of the spectrum and none in the UV region free of solvent interferences, except for the three aromatic amino acids. In the early papers ninhydrin was most often the method of detection (13-22,24) either by spraying the reagent onto paper chromatograms, reacting the reagent with collected fractions, or adding the reagent to the column effluent.

Ninhydrin (triketohydrindene hydrate) reacts with primary amines to form Ruhemann's purple, which is detected at 570 nm. Secondary amino acids form another complex which is detected at 440 nm. The reaction requires a high temperature and the presence of a reducing agent.

A variety of reducing reagents is used (25-27). Fluorescamine, which forms a fluorescent product with primary amines, was introduced by Udenfriend et al. (28). σ-Phthalaldehyde (OPA) is another derivitizing agent used to detect amino acids by fluorescence. In the presence of a thiol, OPA forms highly fluorescent compounds with primary amines (29).

Ninhydrin, fluorescamine, and OPA are commonly used post-column derivatization agents for amino acids separated by ion-exchange. Each method has advantages and disadvantages, but OPA is frequently the preferred method (30,31). OPA is also among the common pre-column derivatization methods. Others include dansyl, phenylthiohydantion, and dabsyl.

Dansyl chloride, 5-(dimethylamino)naphthalene-1-sulfonyl chloride, forms highly fluorescent derivatives with amino acids. Detection is accomplished using fluorescence (32), UV absorbance (33), or chemiluminescence (34). Phenylthiohydantion (PTH) derivatives of amino acids are best known for use in the Edman degradation method for peptide sequencing. But PTH amino acids have also been separated by RP-HPLC (35). Another pre-column derivatization method for amino acid analysis involves the labeling of amino acids with 4-dimethylamino azobenzene-4'-sulfonyl chloride (dabsyl chloride) (36). Chang and co-workers (37) illustrated the RP chromatography of dabsyl derivatives. Several other pre-column derivatization methods exist (30) and new methods continue to be introduced (38).

Two nonspectroscopic methods of amino acid detection are worthy of note. The pulsed amperometric detector (PAD) has been demonstrated as a

detector for amino acids separated by anion-exchange chromatography (39). Many types of organic molecules are adsorbed on noble metal electrodes. This usually interfering phenomenon is the basis of the PAD. The measurement step of the PAD determines the Faradaic current resulting from the oxidative removal of the organic species adsorbed onto the electrode. Another electrochemical detector for amino acids was reported by Allison et al. (40), using the OPA/β-mercaptoethanol derivatives of amino acids. This method is based on the rapid, irreversible oxidation of the isoindole. Other less used separation schemes for amino acids include anion-exchange chromatography (39,41) and gas chromatography (42). The separation of amino acid enantiomers, a very active field of investigation, is discussed in Section I. This dissertation illustrates both ion-exchange chromatography of free amino acids and reversed-phase chromatography of derivatized amino acids.

Early work in the isolation and purification of peptides and proteins involved many different techniques. These methods include paper (43), ion-exchange (44,45), chromatography on silica gel (43), gel chromatography (the forerunner of size exclusion chromatography) using starch (46,47) and polymers (48), reversed-phase (49,50), hydrophobic interaction (51,52), and affinity (53,54).

There are five major chromatographic methods of protein separation used today: ion-exchange, reversed-phase, hydrophobic interaction, size exclusion and affinity. These methods exploit different properties of proteins to produce a separation. Each method has advantages and disadvantages which steer the technique into specific applications.

Because proteins can exist as anions and cations depending on pH, separations by ion-exchange chromatography (IEC) are possible based on the electrostatic interactions between the protein and the ion-exchange matrix (55). Elution of proteins from an ion-exchange column is brought about by increasing ionic strength, changing pH, or a combination of both. Ionic strength gradients are used most frequently. IEC can be performed at close to physiological conditions and, as a consequence, can give a high recovery of biological activity.

Reversed-phase (RP) chromatography is a very popular method for peptide and protein separation and has been explored and reviewed extensively (56-58). Protein retention in RP-HPLC is based on the interaction of the hydrophobic (nonpolar) groups of the protein with similar groups on the stationary phase. The protein is eluted by increasing the concentration of organic phase in the eluent. Section III illustrates the RP chromatography of soybean trypsin inhibitor.

Hydrophobic interaction chromatography (HIC) is based on the same protein/stationary phase interactions as RP chromatography. HIC uses stationary phases with much lower ligand densities resulting in weaker protein adsorption. Because the retention mechanism is weaker, organic solvents are not required, and elution is accomplished using decreasing salt concentrations (57). These conditions lead to protein separation with retention of biological activity (59).

Size exclusion chromatography (SEC) separates molecules according to size using a packing material with a given pore size. Large molecules, which cannot enter the pores, are eluted first. Small molecules, which

are retained in the pores, elute last. Under the proper conditions, elution occurs based only on molecular size without contribution from other retention mechanisms (23). SEC, like IEC and HIC, provides good recovery of biological activity because of the conditions under which the technique is performed (60).

Affinity chromatography utilizes selective adsorbants to purify a biochemical. Adsorbants, or affinity ligands, are immobilized to a stationary phase. The sample is applied to the column under conditions which promote strong binding between the affinity ligand and the analyte. Once the nonretained and weakly retained components are washed from the column, the analyte is eluted by an increase in solvent strength. A change in pH is frequently used which dissociates the affinity ligand/analyte complex. Diol-bonded silica is used to immobilize affinity ligands producing high performance affinity chromatography (HPAC) columns which can be used to purify proteins in a few minutes (61).

Laser-based Optical Activity Detector

The optical activity detector (OAD), a detector for high performance liquid chromatography (HPLC), was developed in this research group (62). This laser-based detector is capable of detecting rotations two orders of magnitude smaller than commercially available systems (63,64).

Optically active molecules rotate plane polarized light. A molecule which is not superimposable on its mirror image is optically active. Since carbon atoms form the backbone of all biological compounds and at

least one chiral carbon is present in most biological compounds, this detector possesses the capability to be a detector for biological activity, past or present. Uses already documented include sugars in urine (65), cholesterol in blood (66), optical activity in coals and shale oil (67,68), and indirect polarimetry as a universal detector (69).

Optical activity has been an active area of interest to chemists ever since Louis Pasteur was able to physically separate l- and d-tartaric acid crystals in 1848. But it was not until 30 years later that van't Hoff and Le Bel independently determined that all optically active organic compounds then known contained at least one chiral carbon. Instruments to measure specific rotation quickly followed the discovery of optical activity. Improvements in polarimeters paralleled advances in optics, light sources, and methods of detection. Specific rotation is most commonly measured at the sodium D line because it is a readily available monochromatic line. Other instruments used white light sources and monochrometers to measure specific rotation over many wavelengths. This is the basis of optical rotatory dispersion (ORD).

Instrumental requirements for all polarimeters are the same: a light source, polarizer and analyzer prisms, a sample cell, and a method to read amount of rotation. Early methods read rotaton by eye using the half-shade method, which was an improvement over determining the point of extinction by eye. Later instruments used a photoelectric detector to measure light intensity. The first recording spectropolarimeters reestablished null by rotating the analyzer (70) or using the Faraday effect (71).

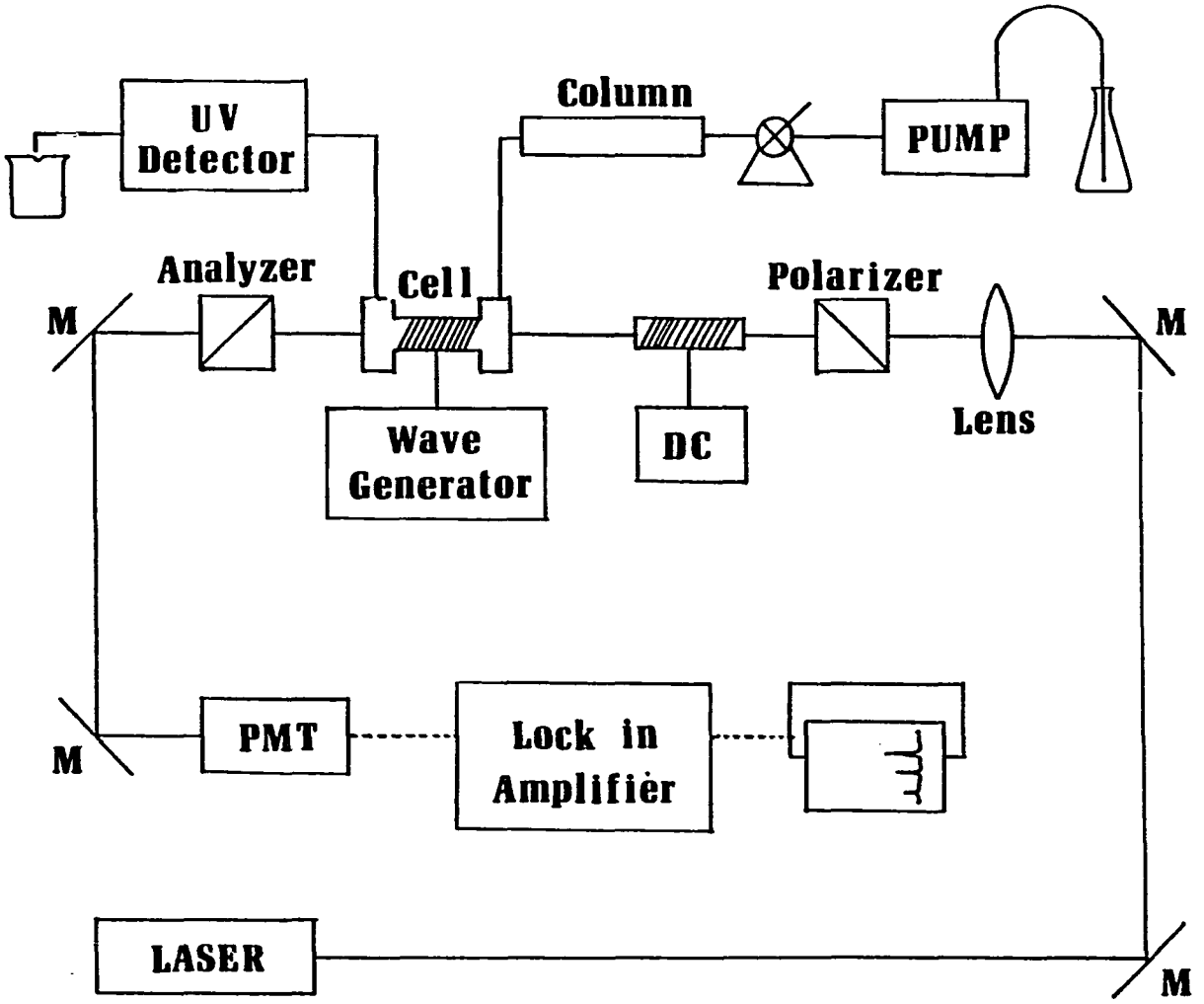
The detection system for HPLC used in these experiments is capable of routinely detecting 10^{-5}° of rotation. The present system has the same general configuration of all polarimeters, but certain components are responsible for the decreased detection limits. These are the polarizers, the modulation, and the laser. Excellent quality Glan-Thompson prisms are required in the OAD, which are capable of an extinction ratio of 10^{-6} or better. The extinction ratio is defined as the fraction of light transmitted when the polarization of the light incident on the polarizer is perpendicular to the polarization which the polarizer passes. By judicious choice of locations for the laser beam to pass through, an extinction ratio of 10^{-10} is obtainable. Since the locations are fairly small, the position of the laser beam is important. During operation the polarizers are set in the crossed position (extinction point) with no sample in the cell. When an optically active substance passes through the cell, the intensity of the light emerging from the analyzer increases. Because measuring an AC signal is preferred over a DC signal in order to discriminate against noise, modulation is introduced into the detection system. Modulation can be accomplished by a variety of means but this system uses the Faraday effect in which a solenoid placed around the light beam produces a magnetic field which in turn produces a rotation of the plane of polarization of the polarized light. Modulation is introduced in the flow cell causing the plane of polarization of the beam to oscillate between points equidistant from, but on opposite sides of, the plane which produces extinction of the beam. Consequently, the light emerging from the analyzer is intensity

modulated. Air-based modulation by the Faraday effect was originally used in this system (62), but liquid-based modulation requires a smaller magnetic field (and less current) to produce the same amount of rotation than in air-based modulation. In the liquid-based system, a commercially available wave generator is sufficient to drive the modulation.

The laser is important to this system for several reasons. First, since $[\alpha]$ is dependent on wavelength, a monochromatic light source is desired for accurate measurements (72). Second, laser beams are collimated which allows focusing without loss of power. Also, a collimated beam can remain focused for long distances without dispersion. This aspect is useful for passing the beam through flow cells and through specific regions of the polarizers. Third, the polarization of the laser is also important. Since argon ion lasers are typically polarized to 1 part in 1000, less light is needed to be rejected by the polarizers to achieve a 10^{-10} extinction ratio. Rejected light can heat up the polarizers and mounts and add instability to the polarization of the light that degrades the system performance. Finally, the laser possesses pointing stability which means the beam does not significantly change position with time. This is important because as the beam wanders on the polarizer face, the extinction ratio degrades.

A schematic diagram of the present configuration of the OAD is given in Figure 1. An argon ion laser (488 nm) or a helium neon laser (632.8 nm) is used. Mirrors direct the beam and the lens focuses the light at the center of the flow cell. The DC coil is an air-based Faraday rotator which produces a constant rotation of 2.5×10^{-4}°. This

Figure 1. Schematic diagram of the laser-based optical activity detector. See text for details


UV
Detector
Column
PUMP
Analyzer
Cell
Polarizer
M
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Wave
Generator
DC
Lens
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PMT
Lock in
Amplifier
LASER
M

rotation is used to standardize chromatograms and check system performance. The flow cell is constructed by drilling a hole 1/16" in diameter into an aluminum cylinder 5 cm in length and 5 cm in diameter. Microscope cover slides, which are glued to the ends of the cell, serve as windows. The column effluent flows in and out at the ends of the cell through stainless steel tubing 1/16" o.d. by 0.02" i.d. which is glued into channels intersecting the bore of the cell. The cell is held in place by a mount which has spring-loaded positioners at each end for optical alignment. A new cell made of stainless steel was developed during this work and used in Section III. The tubing for liquid flow was soldered to the stainless steel cell to form a connection that would not leak. The cell and tubing were then mounted into an aluminum holder and placed into the cell mount. This new cell is less susceptible to corrosion from salts and acids than the original aluminum cells. In addition, the soldered connections between the cell and the HPLC tubing eliminate leaks caused by the cracking of glue which occurs with manipulation of connections to the original cell. The photomultiplier tube (PMT) detects light passing through the analyzer, which is demodulated by the lock-in amplifier. The data are displayed on a chart recorder and collected by the computer. The HPLC system consists of a pump (or pumps for gradient elution), an injector for sample introduction, and a column where separation occurs. In addition to the OAD, detection by RI or UV is used. For a detailed discussion of the intricacies of this detection system refer, to References 62, 73 and 74.

This dissertation describes the application of the OAD to three

different problems encountered in the detection and analysis of amino acids and proteins. Section I describes the detection of amino acids separated by ion-exchange chromatography. Enantiomeric ratios of D- and L-amino acid mixtures can be calculated if two detectors are used. One of these detectors must have different responses to the enantiomers while the other must respond identically to the different conformations. OA/RI and OA/UV used for this purpose are illustrated. Since amino acid detection by RI is not sensitive and only three amino acids are detectable by UV absorbance, another detection method must be identified before the procedure described in Section I can be widely applicable. Derivatization of amino acids to make them detectable by UV absorbance enhances the applicability of OA/UV for the determination of enantiomeric ratios. Such an application is described in Section II using dansyl amino acids. Section II also illustrates the first successful coupling of the OAD to gradient elution. Section III demonstrates the RP-HPLC separation of conformers of soybean trypsin inhibitor. Detection by OA/UV provides information from the chromatogram unavailable with UV absorbance detection alone.

SECTION I.

HPLC DETERMINATION OF ENANTIOMERIC RATIOS OF AMINO ACIDS WITHOUT CHIRAL SEPARATION

INTRODUCTION

The analysis of enantiomers by high performance liquid chromatography (HPLC) of a wide variety of compounds is presently an area of active research. The traditional method of diastereomer preparation followed by chromatographic separation is still widely used (1). More recently, researchers have turned to methods which are less cumbersome. These methods involve a chiral stationary phase, ligand exchange, or a chiral mobile phase additive. Many reviews and papers have been published in this area (2-5).

The first chiral stationary phase to be widely applicable was introduced by Pirkle et al. (6). This stationary phase consisted of (R)-N-(3,5-dinitrobenzoyl)phenylglycine attached to gamma-aminopropyl silanized silica gel and was useful in normal-phase chromatography. Very popular chiral stationary phases for use in reversed-phase systems were developed by Armstrong and DeMond (7). In this work, α-, β-, and gamma-cyclodextrin were bonded to 5 μm silica gel and used to demonstrate chiral separations of enantiomeric dansyl amino acids and barbituates using a methanol/water mobile phase.

A second use of a chiral stationary phase is in chiral ligand exchange. This method involves a chiral stationary phase which forms a chelate with the analyte. The first successful stationary phase of this type consists of L-proline affixed to a resin (8). The L-proline forms ternary complexes with Cu(II) ions and the racemic amino acid anions present in the sample to be separated. The enantioselectivity of the

ternary complex is the mechanism by which the enantiomeric separation is accomplished. Many variations on this original scheme have been reported since the introduction of chiral ligand exchange. These developments have been reviewed extensively (2,4).

The separation of enantiomers using achiral stationary phases and chiral additives to the eluent provides a more easily accessible technique for enantiomeric separations. This form of ligand exchange was initially applied to the resolution of amino acids (9,5). This work demonstrated ligand exchange resolution on reversed-phase and cation-exchange stationary phases, respectively. As in ligand exchange chromatography involving a chiral stationary phase, this method involving an achiral stationary phase, derives its enantioselectivity from the formation of a ternary complex, which is retained on the stationary phase. The eluent contains a metal ion and the resolving chiral reagent.

The determination of the amount of each enantiomer present in a sample is important in many disciplines. Several examples are: mechanistic studies, catalysis, chiral synthesis, kinetics, biology, geochronology, biochemistry, pharmacology, and medicine (10). In the pharmaceutical industry enantiomer analysis is often required for quality control and in the study of drugs in biological fluids (11). Furthermore, "numerous examples of chiral (asymmetric) drugs with large enantiomeric differences in their actions are known" (1). An example of this phenomenon is S-3,4-dihydroxyphenylalanine (L-DOPA). L-DOPA is used in the treatment of Parkinson's disease but only the S isomer is active. The R isomer needs to be removed to reduce side reactions (12).

As previously mentioned, there are many separation schemes available for the resolution of enantiomers. And while the scope of applications of these methods are continually broadening, chiral separation is still a very specialized field. A suitable configuration of stationary phase, eluent and elution conditions must be determined for each new application. These difficulties must be overcome if the physical separation is required, which is often the case when the enantiomeric separation is part of a synthesis or purification procedure. But if only the amounts of each enantiomer present is to be determined, an alternative to chiral separations is available. This chapter illustrates the determination of enantiomeric ratios of amino acids at low concentrations without derivatization or the physical separation of enantiomers. This method is applicable to many systems in addition to amino acids.

The idea of using an ultraviolet detector in series with an optical activity detector (OA/UV) for the determination of enantiomeric ratios in HPLC without physical separation of the enantiomers was first demonstrated in 1982 by Boehme et al. (13) with their work on permethrinic acid pentafluorobenzyl ester (PBE). PBE exists in both the cis and trans configuration and each configuration has enantiomers: R and S. It is important to study the two pairs of enantiomers because of differing biological activities as insecticides. In this technique, chromatographic separation of the enantiomers is not necessary because the UV detector responds to the total amount of sample present and the OA response additionally depends on the ratio of the enantiomers. Using

these two detectors in series allows the determination of the total amount of the sample as well as the D/L ratio of the enantiomers. Linear response was established up to 600 μg injected for both detectors. The limit of detection is 50 μg due to the limits of the Perkin-Elmer 241 LC polarimeter. A second application of this dual system for the determination of enantiomeric ratios was reported in 1985 by Scott and Dunn (14). These workers were interested in the determination of the D/L ratio of epinephrine in opthalmic solutions used to treat glaucoma. This is important since the D isomer is inactive. Scott and Dunn used a procedure similar to that of Boehme et al. (13). L-Epinephrine solutions ranging from 6.0 to 14.0 mg/ml, with an injection volume of 50 μl, were used to check linearity. The authors note that this method, although adequate for opthalmic solutions containing 1% epinephrine, does not possess enough sensitivity to determine epinephrine in biological samples.

In addition to the use of enantiomeric ratios for analysis of drugs and pesticides, another area of interest is the determination of amino acid ratios for use in dating. Geochronology involving amino acid racemization is based on the fact that nearly all amino acids present in living matter are in the L configuration. When an organism dies, racemization begins. Given enough time, a racemic mixture of the amino acids is formed. The increase in D/L ratio which occurs over time can be used to measure the amount of time that passed since the death of the organism. Many reviews have been published on this subject (15-17). In theory, all amino acids can be used for dating purposes but a few have

been favored. Aspartic acid is frequently used because it possesses a large racemization rate constant (18). The conversion of L-isoleucine to D-alloisoleucine is also popular because these epimers are readily separated using conventional amino acid analyzers. In contrast, diastereomers of all other amino acids must be prepared prior to chromatographic separation.

A recent report (19) of interlaboratory comparison of amino acid enantiomeric ratios shows that there is an increase in interest in this dating method, but the methods of analysis have remained the same as when amino acid dating was first introduced. The most popular procedure was ion-exchange for the determination of D-alloisoleucine/L-isoleucine. Gas chromatography, after diastereomeric preparation, was the other method used. Derivatization, however, is tedious and has the potential to introduce racemization. Separation of amino acid enantiomers is possible but has yet to be used routinely. It is obvious that an ion-exchange separation of amino acids followed by optical activity/refractive index (OA/RI) or OA/UV detection would increase the amount of information available from a sample while simplifying the sample preparation.

If the detectability of the polarimeter is lowered, the applicability of the two studies outlined above can be improved. The laser-based optical activity detector for HPLC described in the General Introduction offers many advantages over the commercially available polarimeters presently in use. Enhanced sensitivity and smaller cell volumes are two such advantages. The latter makes this system compatible with microbore chromatography. This is illustrated by the work of

Bobbitt and Yeung (20) in which an OA/microbore LC system with a 1 μl flow cell was used. The most recent version allows 1 microdegree (1 ng fructose) to be detected (21). Two adaptations of this technique for enantiomeric measurements were utilized in the laboratory. OA/RI was used to study enantiomeric ratios of samples of threonine (Thr) and proline (Pro). Tyrosine (Tyr) and phenylalanine (Phe) were examined using OA/UV.

EXPERIMENTAL

Chromatography

The amino acids D-, L-proline, D-, L-threonine, D-, L-phenylalanine, and D-, L-tyrosine were obtained from Chemical Dynamics (South Plainfield, NJ). Deionized water used in these experiments was further purified on a Milli-Q system (Millipore, Bedford, MA). Separation was performed on a 12 X 0.46 cm i.d., 6 micron cation-exchange column (Interaction Chemicals, Inc., Mountain View, CA, Model AA511) heated to 60° C with heat tape (Thermolyne BriskHeat, Dubuque, IA). Thr and Pro were eluted at a flow rate of 0.5 ml/min with a 0.2 M sodium citrate (Pierce, Rockford, IL, pH buffer grade) buffer, adjusted to pH 3.25 with hydrochloric acid (Captree, Farmingdale, NY, electronic grade). A volume of 1 ml phenol (Fisher Scientific, Fair Lawn, NJ) per liter of buffer was added as a preservative. Tyr and Phe were eluted with a buffer consisting of 0.2 M sodium citrate and 1.4 M sodium formate (Fisher Scientific, reagent grade). Formic acid (Fisher Scientific, reagent grade) was used to adjust the pH to 3.6. Flow rate was 0.33 ml/min. All eluents were filtered through a 0.2 μm filter and degassed under vacuum using ultrasonic agitation to minimize degassing in the cell. To reduce any pressure fluctuations caused by the pump (Micromeritics, Norcross, GA, Model 750), we used a commercial pulse dampener (Handy and Harman, Norristown, PA, Model Li-Chroma-Damp III) in conjunction with a pressure gauge (Alltech, Deerfield, IL, Model 9299).

Injections were made through a conventional injection valve

(Rheodyne, Berkeley, CA, Model 7010) with a 20 μl loop for the Thr/Pro analysis and 100 μl for Tyr/Phe. Since the OA flow cell was essentially at room temperature, the eluent must be cooled so that turbulence would not exist in the polarimeter cell. A 50 cm length of standard chromatographic stainless-steel tubing placed after the column was used for this purpose. For the Thr/Pro analysis, a refractive index detector (Waters Assoc., Milford, MA, Model R401) was used in series after the flow cell. UV detection (Rainin, Woburn, MA, Model 153) was used prior to the flow cell in the Tyr/Phe experiments.

Optical Activity Detector

The arrangement of an OA detector for LC was discussed in the General Introduction. For the Thr/Pro work, 20 mW of the 488 nm line from an argon ion laser was used with a photomultiplier tube (Amperex, Hicksville, NY, Model XP2020) operated at 1.7 kV. A 5 mW helium neon laser at 632.8 nm (Uniphase, Sunnyvale, CA, Model 1202-1) was used for the Tyr/Phe studies with a R928 phototube (Hamamatsu Corp., Middlesex, NJ) operated at 1.0 kV. The flow cell for the Thr/Pro work was 10 cm long with an internal volume of 200 μl. A second cell, 5 cm in length and 100 μl in volume was used for Tyr/Phe. Modulation of the detection cell was driven by a waveform generator (Wavetek, San Diego, CA, Model 184) at a frequency of 500 Hz. Using an independent air-based Faraday rotator to provide a standard optical rotation, laser power drift and overall system performance were monitored. A one second time constant was used. The outputs of the OA detector and either the RI or the UV

were connected to two voltmeters (Keithley, Cleveland, OH, Models 155 and 160B), the analog outputs of which were connected to a computer (Digital Equipment, Maynard, MA, Model PDP 11/10 with a LPS-11 laboratory interface). The computer took readings every 0.1 s and averaged a set of 10 before storing the information.

Procedure

In the first set of experiments, OA/RI detection was used to determine the enantiomeric ratio of samples of Thr and Pro. In a second experiment, a UV absorbance detector replaced the RI detector. Tyr and Phe which contain phenyl groups were detected at 254 nm. The samples examined ranged from 100% D, 0% L to 0% D, 100% L of each amino acid. Between 1 and 50 μg of each amino acid were injected.

Calculations

The area of each peak from the OA detector was determined by summation of adjusted values above a chosen baseline for each peak. These are then normalized against the signal obtained from the DC coil (which produces a constant 0.25 millidegree rotation) for each chromatographic trial. In this way, laser power fluctuations are properly accounted for. These normalized values were then divided by either the RI or UV area of the corresponding peak from the second detector. The values of (OA/DC)/UV and (OA/DC)/RI were plotted versus the fraction of L-amino acid present in the sample.

RESULTS AND DISCUSSION

Figures 1a and 2a show optical activity chromatograms of the amino acids examined in this experiment. Baseline separation is achieved in all cases. The limits of detection (LOD) for Thr, Pro, Tyr and Phe are 0.12 μg, 50 ng, 2 μg and 2 μg injected, respectively (signal-to-noise ratio, S/N = 3). The LOD naturally depends on the specific rotation of the material. When this is taken into account, these LODs are 2-3 orders of magnitude better than the 50 μg LOD reported for the analysis of PBE (13). The corresponding RI and UV chromatograms are shown in Figures 1b and 2b. Specific rotation [α] is a function of wavelength, optical path length, temperature, concentration, and solvent. Furthermore, the specific rotations of amino acids vary widely with pH (22), thus literature values for the conditions used were not available. Because of this, it is interesting to calculate the specific rotation of these four amino acids under the conditions used in this study. The concentration at the apex of the peak can be calculated from the peak area, the area of a one second interval at the peak maximum, the amount injected, and the flow-rate. Using Equation 1, where α is the actual rotation measured, c

$$[\alpha] = \frac{\alpha \times 100}{c \times \ell} \qquad (1)$$

is the concentration in grams solute per ml solution, and ℓ is the path length in decimeters, the specific rotation can be calculated. Results are given in Table 1 along with literature values for comparison. Solvent conditions are given in the Experimental section.

Figure 1. Chromatograms of a mixture of 5.3 μg of L-threonine (7 min) and 2.3 μg of L-proline (10 min). (a) Optical activity, and (b) refractive index

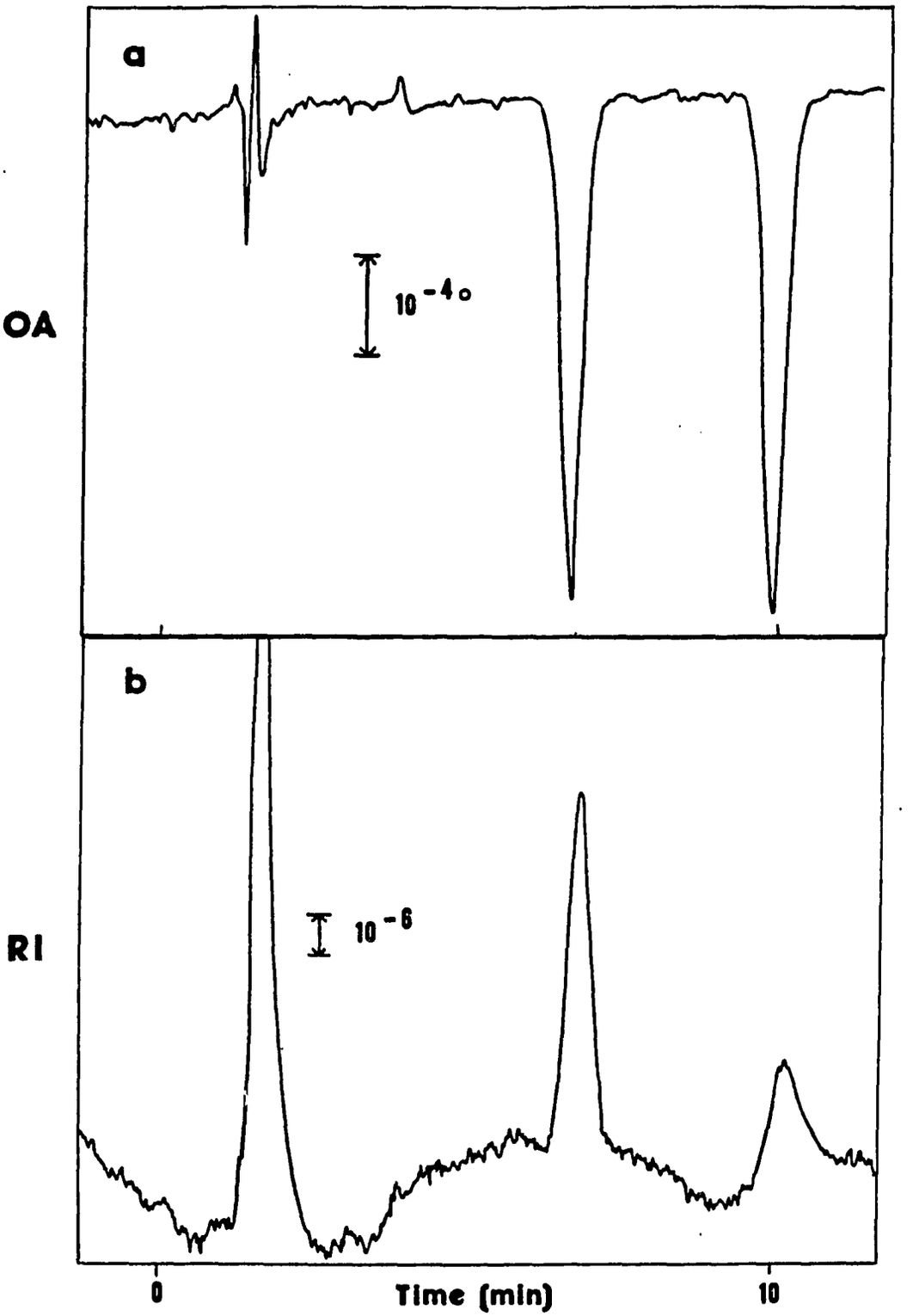
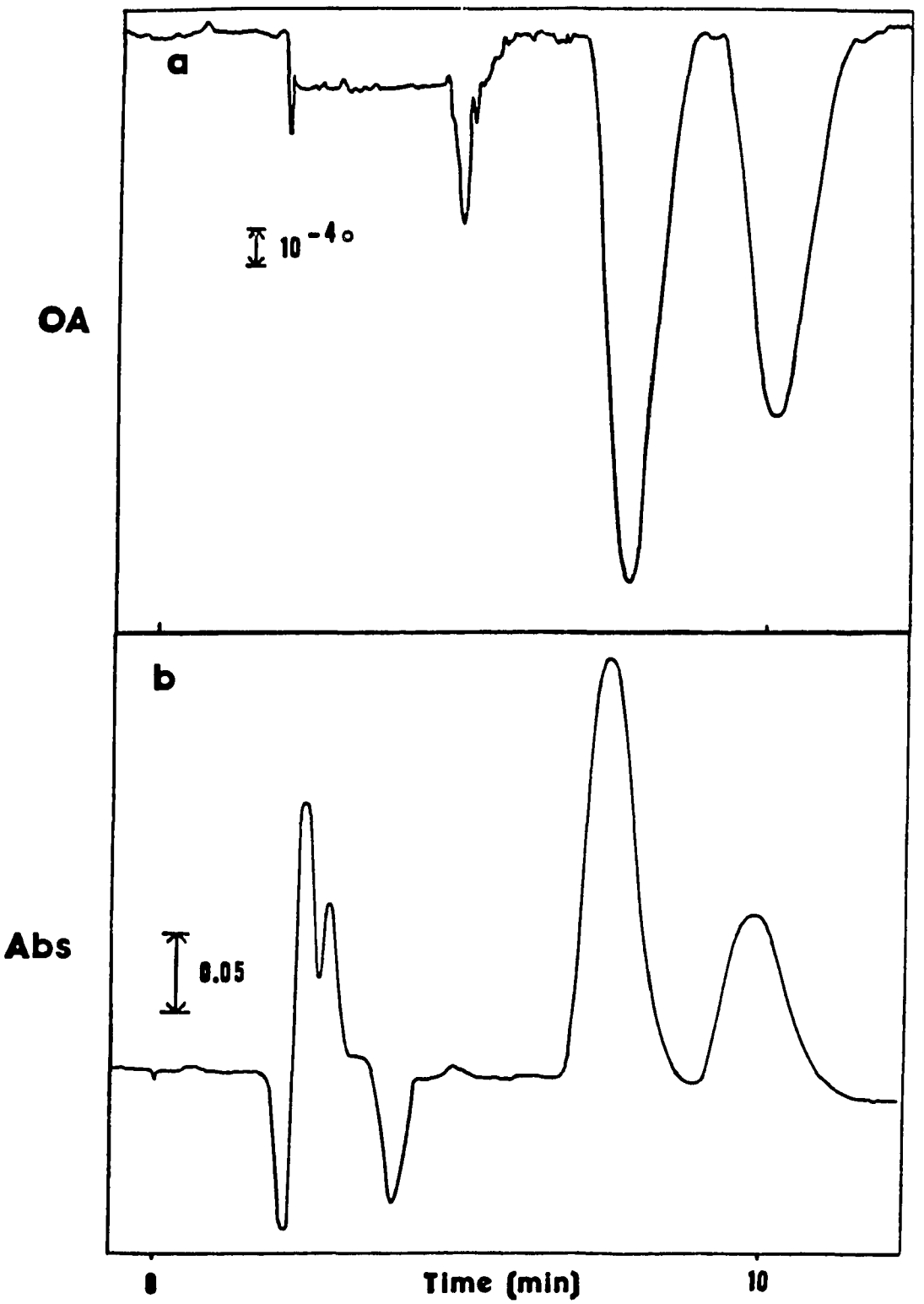

a
OA
10 -4 o
b
RI
10 -6
0
Time (min)
10

Figure 2. Chromatograms of a mixture of 50 μg of L-tyrosine (7 min) and 50 μg of L-phenylalanine (10 min). (a) Optical activity, and (b) absorption at 254 nm


a
10 -4 o
OA
b
Abs
0.05
8
Time (min)
10

To test the utility of the dual detector scheme for determining enantiomeric ratios, one can compare the measured values of [α] for a series of samples of known D/L ratios with the expected values. This is done for each of the four amino acids in Figures 3-6. Each data point is the average of 2-4 chromatograms. The fraction of the L enantiomer in each sample is used as the abscissa. Since the UV and the RI detectors respond linearly with concentration (at these low concentration levels), Equation 1 shows that the OA/RI and the OA/UV responses should be proportional to [α]. These responses then represent the ordinate. Each set of responses should fall on a straight line with intercepts (at 1.0 and 0.0 fractional concentrations) of equal magnitudes but opposite signs. Also, the response at 0.5 fractional concentration (a racemic mixture) should be zero. The ordinates are in arbitrary units, so that the conversion between the chromatographic peak areas and actual concentrations need not be separately determined. For routine applications, Figures 3-6 can be used directly as calibration curves for determining enantiomeric ratios without chiral separation. In this mode of operation, the LOD for the OA detector depends on the enantiomeric ratio, but the LODs for the UV or RI detectors do not. So, the useful concentration range for this mode of operation is limited by either the usual LODs of the UV or RI detector, or the LOD of the OA detector, whichever is higher.

Table 2 summarizes the results in Figures 3-6. The racemic response (RR) indicates the overall accuracy for determining enantiomeric ratios.

Table 1. Specific rotations of amino acids

L-amino acid	$[\alpha]^a$	conc.[b]	$[\alpha]_D$ in water[c]	$[\alpha]_D$ in 5M HCl[c]	conc.
Thr[d]	-20.6	16mg	-28.5	-15.0	1-2g
Pro[d]	-53.3	13mg	-86.2	-60.4	1-2g
Tyr[e]	-19.5	2mg	-----	-10.0	2g
Phe[e]	-17.4	0.1mg	-34.5	-4.5	1-2g

[a] This work 25° C.

[b] per 100 ml.

[c] Reference 23.

[d] 488 nm; solvent see text.

[e] 632.8 nm; solvent see text.

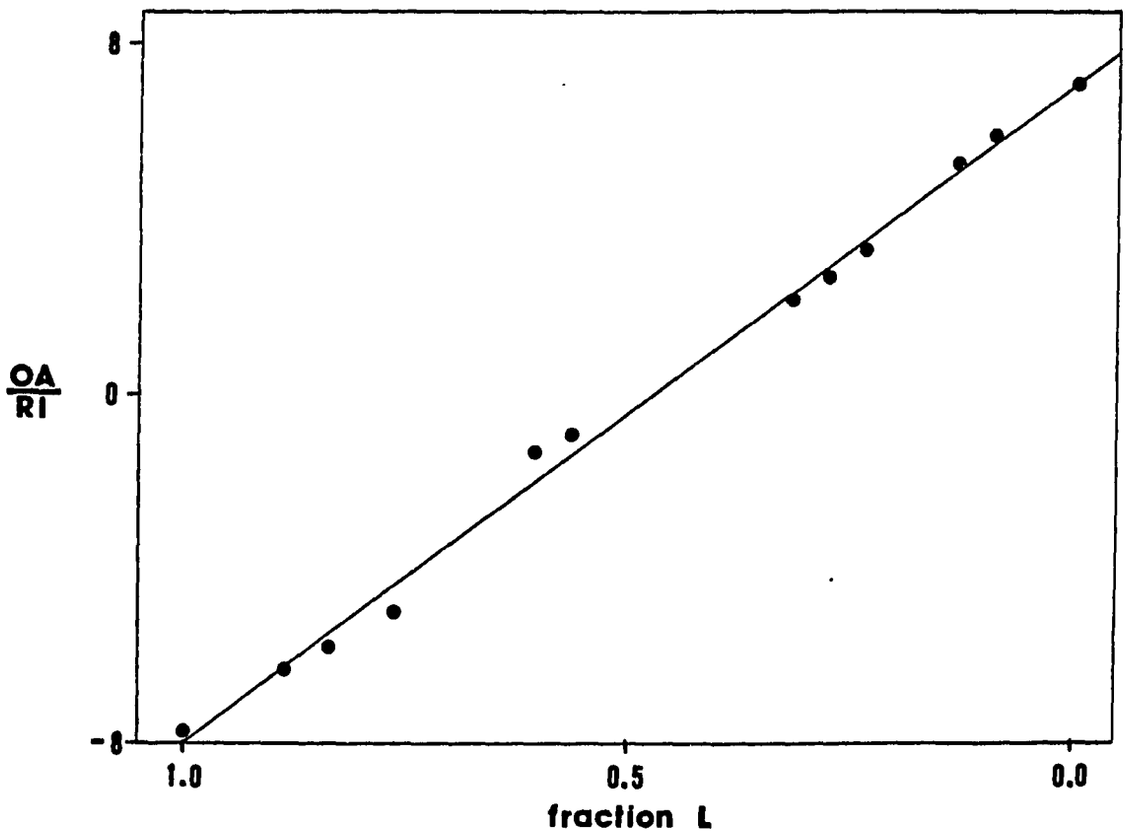


Figure 3. Enantiomeric ratio calibration curve for threonine. Total amount of threonine injected is about 5 μg

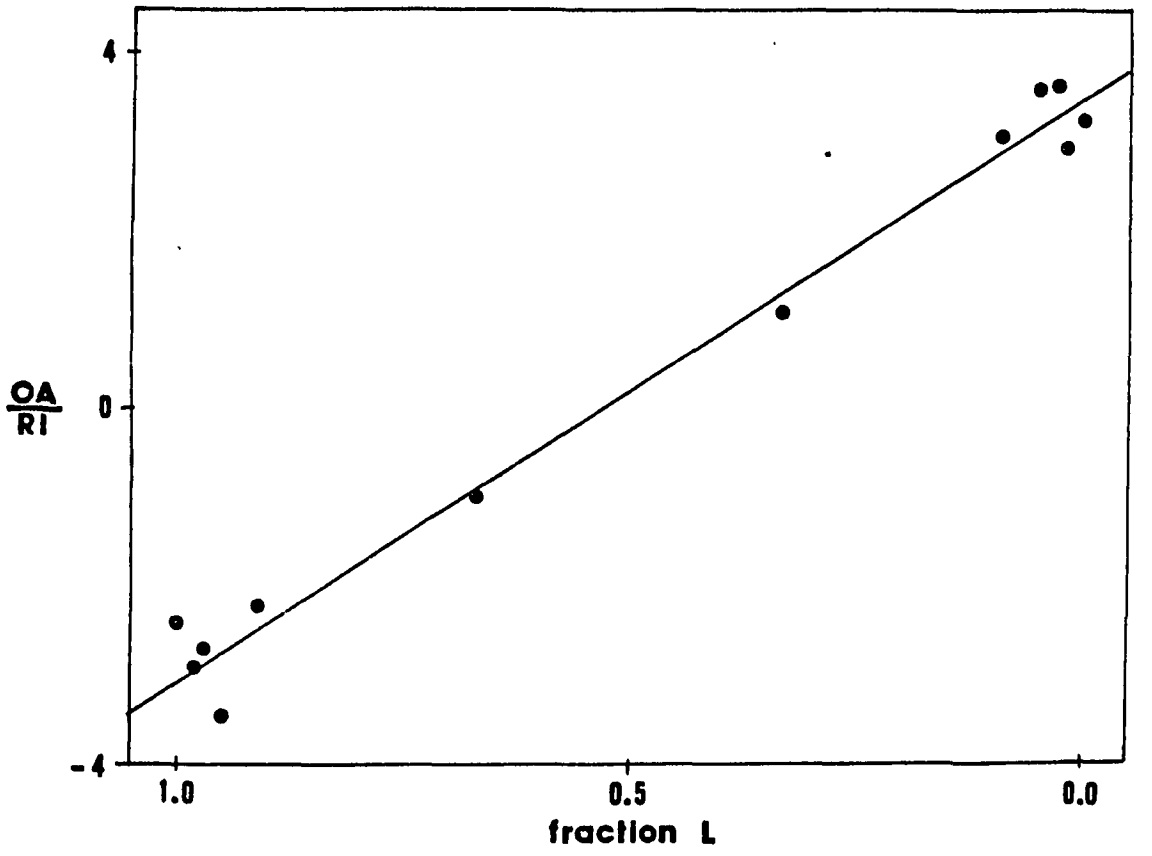


Figure 4. Enantiomeric ratio calibration curve for proline. Total amount of threonine injected is about 2 μg.

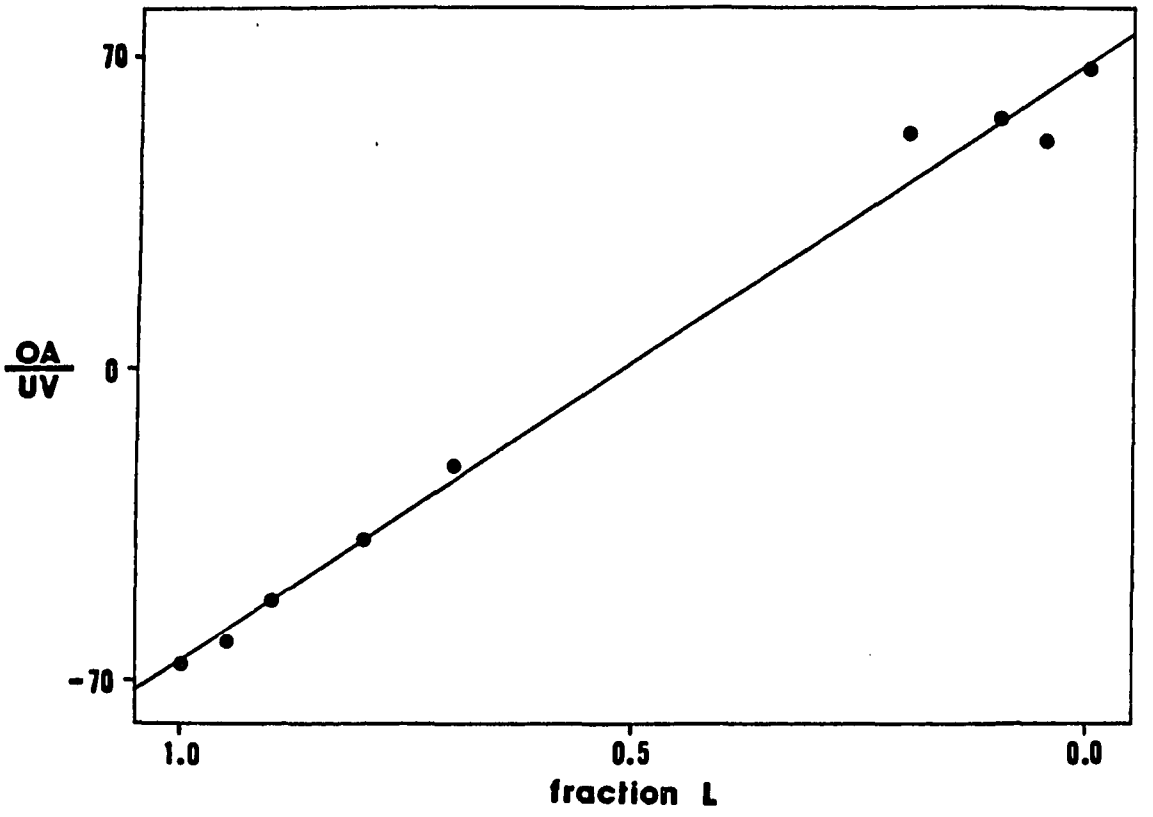


Figure 5. Enantiomeric ratio calibration curve for tyrosine. Total amount of threonine injected is about 50 μg

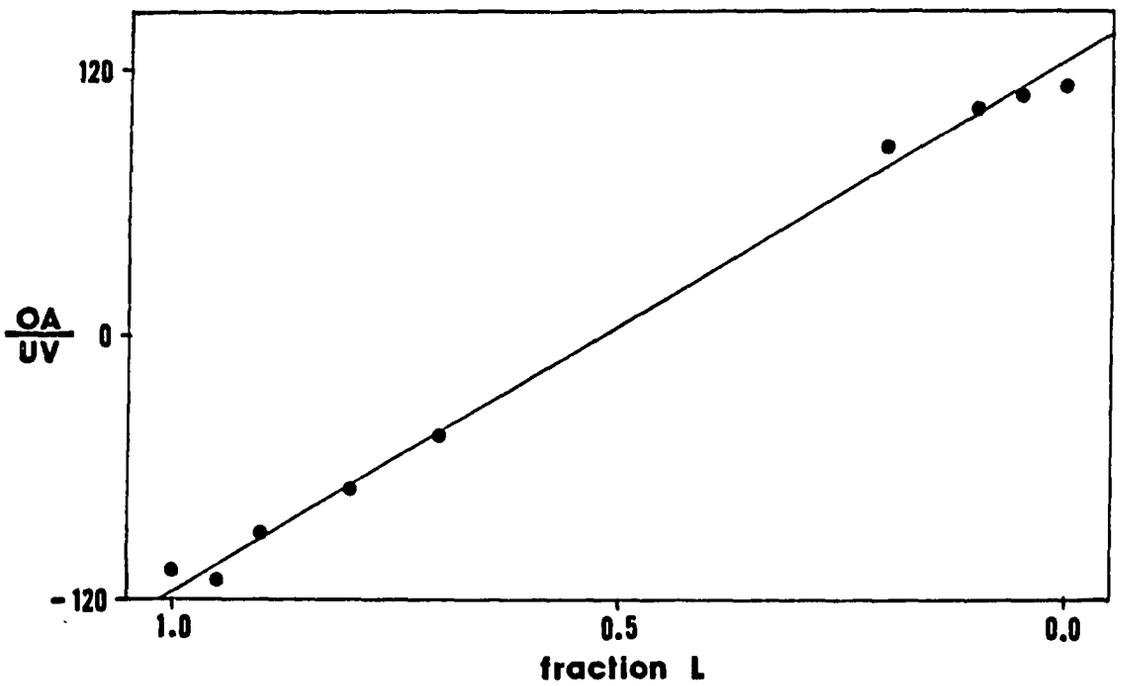


Figure 6. Enantiomeric ratio calibration curve for phenylalanine. Total amount of threonine injected is about 50 μg

Any deviation from zero is a measure of the bias of the method. Since the ordinates are in arbitrary units, it is useful to use the value of the slope (S) for normalization. So, the last column in Table 2 (RR/S) reflects the percentage accuracy that is achieved. The precision of any individual measurement naturally depends on the number of replicate injections and on the noise level in the chromatograms. The correlation coefficients in Table 2 and the scatter of the data points in Figures 3-6 show the precision that can be expected for triplicate injections and at the noise levels in Figures 1 and 2.

The implications of Table 2 are as follows. First, there is no obvious bias (last column) in the results in all cases, so that good quantitative accuracy can be achieved in either detector combination. This is in fact a confirmation that the micropolarimeter used provides reliable quantitative data at these low concentrations. Second, the precision of the dual detector method is good, judging from the correlation coefficients and the scatter of the data in Figures 3-6. Third, for the OA/RI combination, the LOD for the OA is better than the RI detector. Precision is therefore limited by the RI chromatogram. We note in Figure 1 that the concentration of Pro is smaller than that of Thr for about the same response in the OA detector. This means that the S/N for the RI peak of Pro is worse than that for Thr. The precision is thus also worse for Pro (Figure 4) than for Thr (Figure 3). If an alternate method is used to determine their individual concentrations, then the precision for Pro should be better than that for Thr by a factor of 2.6, which is the ratio of their respective [α] values.

Table 2. Determination of enantiomeric ratios using dual detectors

Species	Mode	No. points	correlation coefficient	Slope (S)	Racemic[a] response (RR)	RR/S (%)
Thr	OA/RI	12	0.998	15.0	-0.40	-2.7
Pro	OA/RI	12	0.991	6.54	0.17	2.6
Tyr	OA/UV	9	0.995	135	1.0	0.7
Phe	OA/UV	9	0.998	241	3.5	1.5

[a]Racemic response is defined as the value for a sample with a fractional concentration of 0.5 L.

Unfortunately, there are no convenient absorption bands for these two amino acids to allow the use of an absorption detector. Fourth, for the OA/UV combination, the LOD for the OA detector is worse than the UV. Precision is therefore limited by the OA chromatogram, particularly since the [α] values are small for these two amino acids. The high salt content of the buffer required for the separation in Figure 2 also degraded the S/N of the OA chromatograms by a factor of 2 compared to Figure 1. The decrease in S/N caused by the high salt concentration is the result of two events. First, the increase in particulates in the eluent caused scatter of the laser beam. Second, the weakening of the glue which attaches the windows to the cell produced instability of the windows. Bacterial contamination may have been another source of uncertainty because of the omission of the preservative, which is UV absorbing. Still, Table 2 shows that the UV results are better than the RI results. Naturally, UV works well only for the aromatic amino acids. Recently, the LOD of the OA detector has been improved to 1 ng for materials with [α] = 100° in conjunction with microbore columns (21). The OA/UV combination is thus expected to be useful at the 1 ng level for ideal cases.

Even though the results here are for amino acids, one can expect similar accuracy when other species (e.g., pharmaceuticals) are of interest. The best results are obtained for species that are highly absorbing and have high specific rotations. Phenylthiohydantoin derivatives of amino acids are frequently used for reversed-phase separation followed by UV detection (24,25). This procedure renders all

amino acids UV detectable and makes OA/UV a viable technique for the determination of enantiomeric ratios of all amino acids. An added bonus is that these derivatives all show a larger [α] than the amino acids themselves to further enhance S/N. It can also be expected that the other common derivatization methods for amino acids (e.g., dansyl or σ-phthalaldehyde derivatives) will enhance detection for both OA and UV to extend the scope of optical purity determinations. Naturally, the important consideration is whether racemization occurs in the derivatization reaction.

In summary, two dual detector schemes for determining enantiomeric ratios without derivatization and without chiral separation have been shown. Although amino acids were used to demonstrate the capability of this instrumental configuration, the method is not limited in scope. The D/L ratios of other types of compounds (e.g., sugars and pharmaceuticals) could be easily determined. Furthermore, the determination of enantiomeric ratios by this method has applicability in many areas including chiral synthesis, mechanistic studies, and medicine.

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SECTION II.

OPTICAL ACTIVITY AND ULTRAVIOLET ABSORBANCE DETECTION OF DANSYL-L-AMINO ACIDS SEPARATED BY GRADIENT HPLC

INTRODUCTION

Dansyl (1-dimethylaminonaphthalene-5-sulfonyl) chloride derivatization of amino acids was first used in peptide sequencing (1) and determination of proteins by fluorescence polarization (2). Recently, dansylation has become a popular pre-column derivatization method for fluorescence or UV absorbance detection of amino acids. Reversed-phase (RP) or ion pair RP-HPLC is employed in the separation of the product mixture. Much work has been done with dansyl derivatives including determination of reaction by-products and optimum conditions for the reaction (3,4). DeJong et al. (5) determined that quantitative data result if stringent reaction and chromatography conditions are used. Other workers (6) have found pre-column dansyl derivatization to be highly reliable and reproducible, producing variations of less than 5%. The separation of D- and L- isomers of dansyl amino acids has also been accomplished using chiral β-cyclodextrin in the mobile phase (7) and by mixed chelate complexation (8).

Many scientific investigations (e.g., geochronology, pharmaceuticals) have the need to determine enantiomeric ratios of amino acids and other compounds. It was shown in Section I that OA/UV or OA/RI are ideal methods for the determination of enantiomeric ratios without the need for chiral columns, chiral eluents, or diastereomer preparation. Unfortunately, only three amino acids are naturally UV absorbing (254 nm), and RI sensitivity for amino acids is low. Derivatization by several methods (o-phthalaldehyde, dansyl, phenylisothiocyanate,

fluorescamine, 2,4-dinitrofluorobenzene, and phenylthiohydantoin) renders all amino acids UV absorbing and makes OA/UV a viable technique for amino acid analysis. This section describes the detection of a mixture of 17 dansyl amino acids by UV absorbance and optical activity.

Previously, the optical activity detector has been used only with isocratic HPLC. Unfortunately, the OAD is not perfectly selective and responds to refractive index changes. This is most evident at the void volume when eluent and sample solvent are not identical. The change in refractive index displaces the laser beam which is interpreted by the detector as a change in rotation. Thus, it was assumed that gradient HPLC could not be coupled to the OAD without severe limitations. By using a previously reported mixture of 0.13 M ammonium acetate and acetonitrile as eluent (9) in conjunction with a small change in solvent composition per unit of time, it was determined that the OAD detection of dansyl amino acids separated by gradient elution was possible.

EXPERIMENTAL

Reagents

The dansyl-L-amino acids were obtained from Sigma (St. Louis, MO). Dansyl-L-aspartic acid (Asp), Nα-dansyl-L-asparagine (Asn), dansylglycine (Gly), dansyl-L-proline (Pro), dansyl-L-phenylalanine (Phe), N,N'-didansyl-L-cystine (Cys), dansyl-L-glutamine (Gln), dansyl-L-cysteic acid (Cya), and Nε-dansyl-L-lysine (Lys), were obtained as the free acid; N-dansyl-L-serine (Ser), dansyl-L-alanine (Ala), dansyl-L-valine (Val), dansyl-L-methionine (Met), dansyl-L-isoleucine (Ile), and dansyl-L-α-amino-n-butyric acid (Abu), as the cyclohexylamine salt; N-dansyl-trans-4-hydroxy-L-proline (Hyp), dansyl-L-threonine (Thr), and Nα-dansyl-L-tryptophan (Trp), as the cyclohexylammonium salt; dansyl-L-norvaline (Nval) and dansyl-L-leucine (Leu) as the monocyclohexylammonium salt; dansyl-L-glutamic acid (Gln) as the di(cyclohexylammonium) salt; and α-dansyl-L-arginine (Arg) as the hydrochloride. Deionized water used in these experiments was further purified on a Milli-Q system (Millipore, Bedford, MA).

Chromatography

The gradient system consisted of two Beckman 110A pumps, a mixer and a Model 420 gradient controller (Beckman, Berkeley, CA). Additional pulse dampening was accomplished with a commercial pulse dampener (Handy and Harman, Norristown, PA, Model Li-Chroma-Damp III), and monitored with a pressure gauge (Alltech, Deerfield, IL, Model 9228). Separation was

performed on a 25 X 0.46 cm, 5 micron Hypersil ODS column (Alltech, Deerfield, IL). The separation was carried out using an ammonium acetate (0.13 M, pH 6.8)/acetonitrile eluent. Eluent A was 50/50 ammonium acetate/acetonitrile, B was acetonitrile. This configuration eliminated bubble formation upon mixing of the aqueous and organic phases. The gradient used was initially 30% B and changed to 70% B in 70 minutes. Injections were made through a conventional injection valve (Rheodyne, Berkeley, CA, Model 7010) with a 10 μl loop. Between 4.1 μg and 24 μg of each amino acid was injected. Injection was made 20 minutes after the start of the gradient to adjust for the dead volume of the system. A flow rate of 0.6 ml/min was used. All eluents were filtered through a 0.2 micron filter and degassed under vacuum using ultrasonic agitation to minimize degassing in the cell.

Detection

The optical activity detector for HPLC was described in the General Introduction. The work described in this section used the configuration described in Section I. The 488 nm line from an argon ion laser (Laser Ionics, Orlando, FL) was used at a power of 5-10 mW. The flow cell, 5 cm in length, had a volume of 100 μl.

The outputs of the OA and the UV absorbance detector were connected to two voltmeters (Keithley, Cleveland, OH, Models 155 and 160B), the analog outputs of which were connected to the I/O ports of the computer (Digital Equipment Corporation, Maynard, MA, Model PDP 11/10 with a LPS-11 laboratory interface). The computer took readings every 0.1 s,

averaged a set of 10 measurements and then stored the information. Ultraviolet detection at 254 nm (Altex, Berkekey, CA, Model 153) was used prior to the OA flow cell.

Calculations

The area of each peak from the OAD was determined by summation of the baseline corrected measurements. These values were then normalized against the signal obtained from the DC Faraday rotator for each chromatographic trial. The specific rotation of each compound can be calculated from the peak area, the amount injected and the flow rate as in the method explained in Section I. Specific rotations for the dansyl amino acids in the 17 component mixture were calculated at the conditions of gradient elution (except for Hyp and Asn). The remaining seven were eluted isocratically with solvent conditions as follows: Abu and Lys at 25% acetonitrile, Hyp, Asn, and Arg at 20% acetonitrile, Cya and Gln at 15% acetonitrile.

RESULTS AND DISCUSSION

Figures 1 and 2 show the OA and UV chromatograms of a 17 component mixture of dansyl-L-amino acids. Since Hyp and Asn co-elute, only 16 amino acids can be resolved in this sample. Table 1 lists the retention times of dansyl amino acids not included in Figures 1 and 2. Based on these results, Gln and Lys would not co-elute with any peaks in the mixture. Therefore, 18 dansyl amino acids could be separated in one chromatogram. A slight baseline drift is often observed during the gradient. This artifact is not reproducible and is the only difference between gradient and isocratic elution. A disturbance is always observed by the OA detector for glycine, which is not optically active. The shape of this disturbance is not the same as observed for optically active compounds. It has the general shape of the first derivative of a gaussian peak and is attributed to the change in refractive index upon elution of glycine.

Table 2 lists the specific rotations calculated from this work as well as literature values for comparison. Since this work was done at pH 6.8, comparison to [α] values in water is appropriate. In most cases the specific rotation for the dansyl amino acids is different from the free form, although no change is observed for Glu, Hyp, Gln and Lys. A property of L-amino acids frequently used to determine absolute configuration is that L-amino acids show positive shifts in the sign of [α] as acidity is increased (10). But the changes in specific rotation

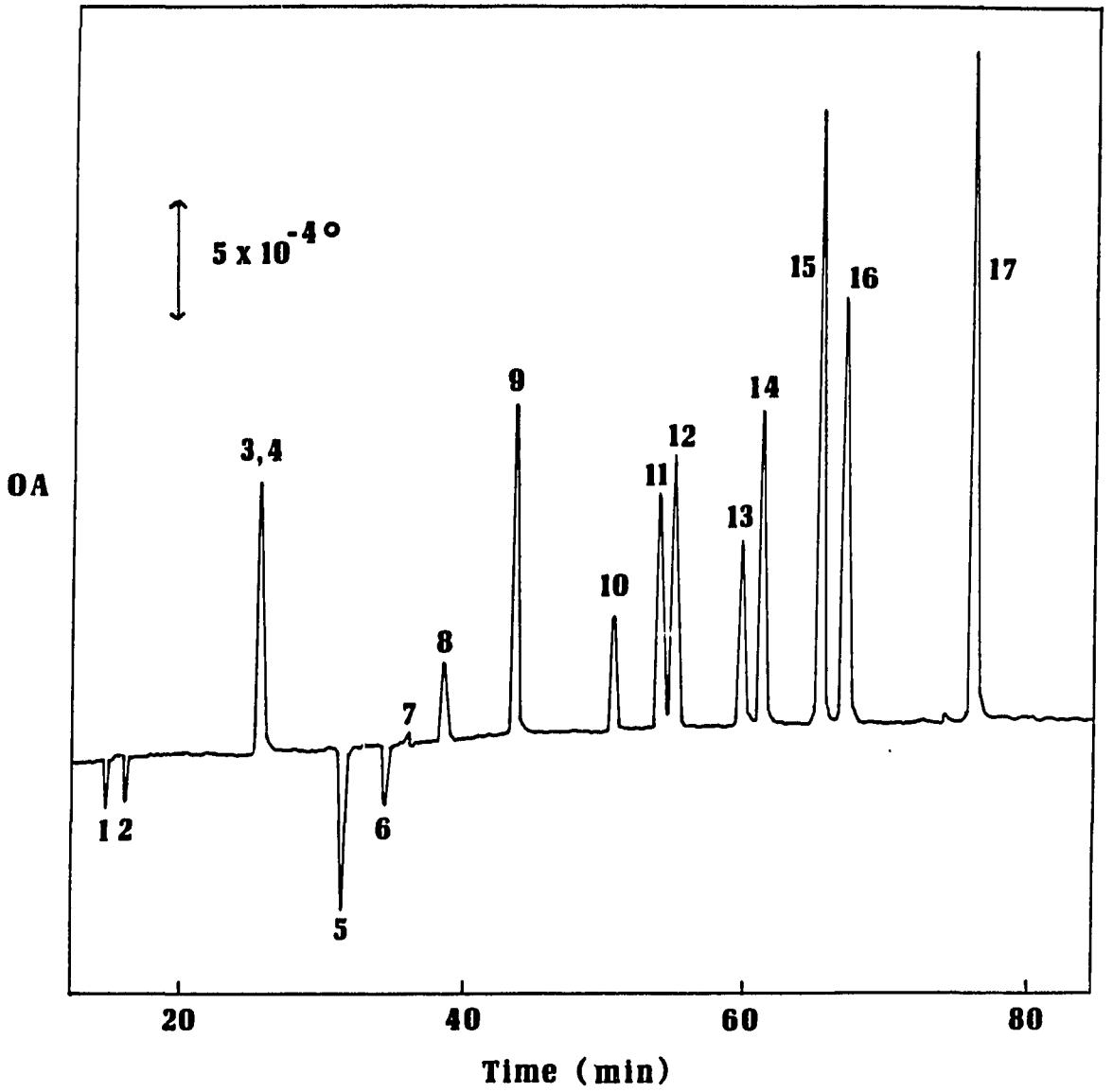

Figure 1. Optical activity chromatogram of dansyl-L-amino acid mixture. 1. Asp, 2. Glu, 3. Hyp, 4. Asn, 5. Ser, 6. Thr, 7. Gly, 8. Ala, 9. Pro, 10. Val, 11. Nval, 12. Met, 13. Ile, 14. Leu, 15. Trp, 16. Phe, 17. Cys. Amount injected ranges from 4.8 μg (Val) to 8.4 μg (Ala). Gradient conditions: A = 50% 0.13 M ammonium acetate (pH 6.8), 50% acetonitrile, B = acetonitrile. 30% B to 70% B in 75 min. Isocratic hold at 70% B for 10 min. Flow rate 0.6 ml/min

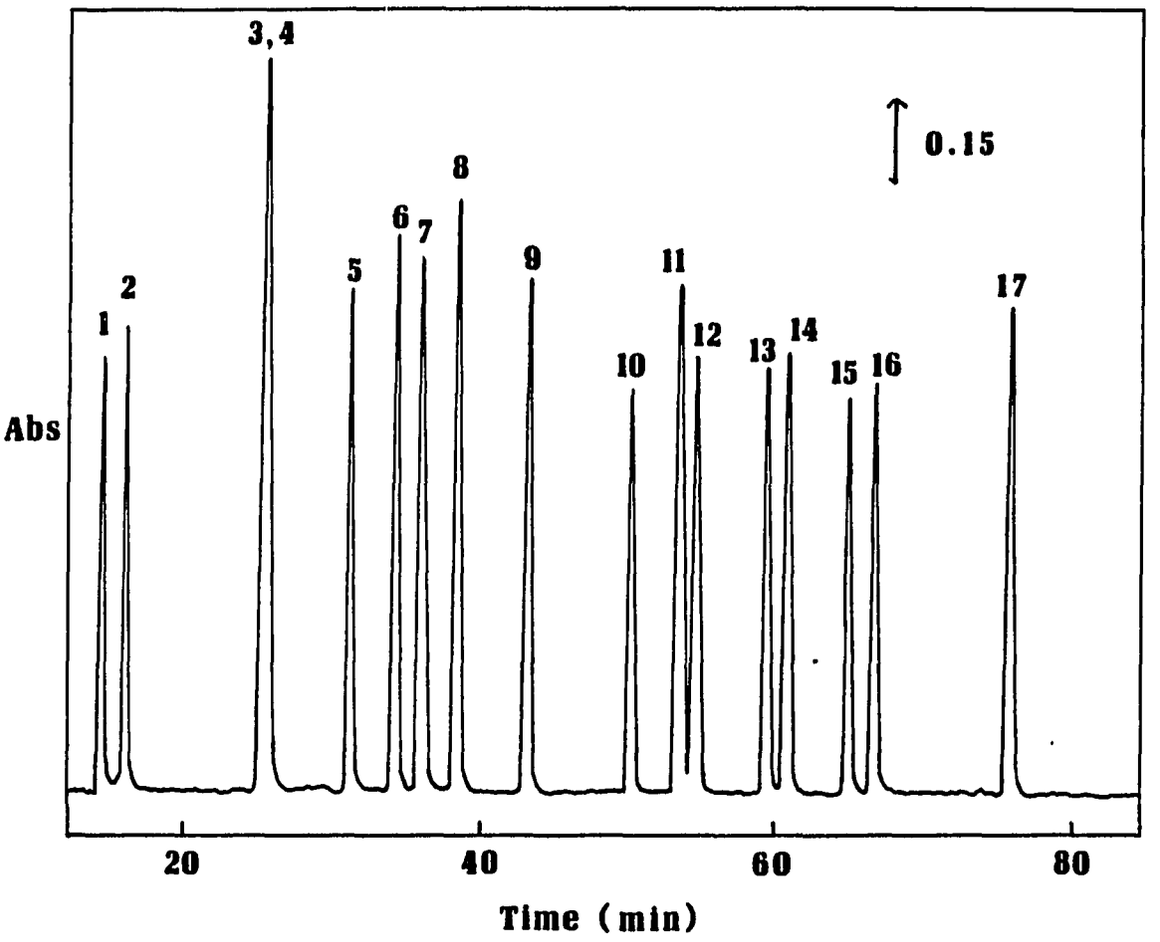

Figure 2. UV absorbance chromatogram of dansyl-L-amino acid mixture. Conditions as in Figure 1

Table 1. Retention times of dansyl amino acids

Amino acid	t_r (min)	located near
Glutamine	28	Asn
Cysteic acid	15	Asp
Arginine	31	Ser
α-Aminobutyric acid	44	Pro
Lysine	45	Pro

Table 2. Specific rotations of free and dansylated amino acids

	Dansyl-L-amino acid	$[\alpha]^a$	Free amino acids $[\alpha]_D^b$ H_2O	$[\alpha]_D^c$ 5N HCl
1.	Dansyl-L-aspartic acid	18 ± 3	5.05	25.4
2.	Dansyl-L-glutamic acid	14 ± 1	12	31.8
3.	N-dansyl-trans-4-hydroxy-L-proline	-77 ± 4	-76.0	-50.5
4.	Nα-dansyl-L-asparagine	-15 ± 1	-5.6	33.2[d]
5.	N-dansyl-L-serine	63 ± 2	-7.5	15.1
6.	Dansyl-L-threonine	20 ± 1	-28	-15
7.	Dansylglycine	-0.2 ± 2	---	---
8.	Dansyl-L-alanine	-24 ± 2	1.8	14.5
9.	Dansyl-L-proline	-149 ± 3	-86.2	-60.4
10.	Dansyl-L-valine	-54 ± 4	5.63	28.3
11.	Dansyl-L-norvaline	-87 ± 2	-23[e]	---
12.	Dansyl-L-methionine	-114 ± 6	-10	23.2
13.	Dansyl-L-isoleucine	-79 ± 3	12.4	39.5
14.	Dansyl-L-leucine	-135 ± 4	-11	16
15.	Nα-dansyl-L-tryptophan	-276 ± 15	-33.7	2.8[f]
16.	Dansyl-L-phenylalanine	-224 ± 6	-34.5	-4.5
17.	N,N'-didansyl-L-cystine	-289 ± 16	-232[c]	---
18.	Dansyl-L-glutamine	9 ± 2	6.3	31.8[f]
19.	Dansyl-L-cysteic acid	-0.7 ± 0	8.66	---
20.	Dansyl-L-arginine	-55 ± 2	12.5	27.6
21.	Dansyl-L-α-amino-n-butyric acid	-49 ± 2	9.3	20.5
22.	Nε-dansyl-L-lysine	-13 ± 1	14.6	---

[a] This work, 488 nm.

[b] 1-2 g/100 ml concentration, H_2O (11).

[c] 1-2 g/100 ml concentration, 5N HCl.

[d] 3N HCl.

[e] 10g/100 ml concentration, 20% HCl (12).

[f] 1N HCl.

observed upon dansylation cannot be attributed to a pH effect since the pH conditions are similar. Ser, Thr, Ala, Val, Nval, Ile, Arg, and Abu are observed to change sign. Of these eight, Ser and Thr derivatives do not follow the acid rule when compared to [α] values in 5 N HCl (i.e., the [α] value in acid is not more positive than the [α] for the dansyl amino acid). One could conclude that the dansylation reaction changes the composition of serine and threonine to such an extent that they no longer behave as amino acids in regard to the acid rule. Large changes in specific rotation are determined for Ser (+70), Thr (+48), Pro (-63), Val (-60), Nval (-112), Met (-104), Ile (-91), Leu (-124), Trp (-242), and Phe (-189). Derivatization makes the already large specific rotation of Cys even larger. It is interesting to note that only five of the 21 dansyl amino acids have positive rotations whereas ten of the free (water solvent) amino acids do.

The refractive indices of the eluents can be calculated using Equation 1.

$$\left[\frac{n^2 - 1}{n^2 + 2}\right] = \sum_i C_i \left[\frac{n_i^2 - 1}{n_i^2 + 2}\right] \qquad (1)$$

The refractive index of the mixture is denoted by n; the volume fraction of component i, C_i; and the refractive index of component i, n_i. The results are listed in Table 3 along with the refractive indices of acetonitrile and water for reference. These results show that the change in refractive index is only 0.002 units over the gradient used in this work. Because the change in refractive index is small, the position of the laser beam on the analyzer does not change significantly. A small

Table 3. Refractive indices of HPLC eluents

Eluent	Refractive index
acetonitrile	1.344
water	1.333
0.13 M ammonium acetate	1.334
15% acetonitrile /85% 0.13 M ammonium acetate	1.336
35% acetonitrile /65% 0.13 M ammonium acetate	1.338

change in the OAD baseline does occur during gradient elution but does not degrade the quality of the the chromatogram.

It has been suggested that there are two approaches to the analytical problem of resolution of D-, L-amino acid pairs. The first requires a column which is able to resolve all the pairs in one chromatogram. This could be with chiral mobile or stationary phases, diastereomer preparation, or ligand formation. A second approach is suggested which involves column switching (13). In this method only the amino acids of interest are directed to a second column and enantiomeric separation is accomplished using a chiral additive in the mobile phase. The existence of an optical activity detector for HPLC necessitates the inclusion of a third alternative. With the OAD coupled to UV, physical separation of D- and L-isomers is not required, simplifying the chromatographic apparatus. For OA/UV determination of enantiomeric ratios, calibration curves must be prepared as in most analyses. Also, it must be determined that racemization does not occur during derivatization. This has been shown by Lindner and co-workers (14). They studied the dansylation of several optically pure free L-amino acids and found little or no racemization during or after the reaction.

The successful separation and detection of dansyl amino acids with the OAD confirms the expectation that derivatization of amino acids allows the determination of enantiomeric ratios using OA/UV following the method described in Section I. This method could be applicable in the field of geochronology which presently uses only a few amino acids in the racemization dating method (15).

In summary, we have successfully coupled gradient elution to the OAD for the separation and detection of dansyl amino acids. Gradient elution was previously thought to be incompatible with detection by the OAD. The change in percent organic phase is small in these experiments. Additional investigation will show how large a change in solvent composition can be tolerated. Reaction with dansyl chloride produces amino acid derivatives which can be detected by UV absorbance. This derivatization increases the specific rotation of many, but not all, of the amino acids examined. Such a procedure decreases the detection limit of these amino acids. The determination of enantiomeric ratios of amino acids is a natural extension of this work.

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SECTION III.

REVERSED-PHASE HPLC OF SOYBEAN TRYPSIN INHIBITOR WITH OPTICAL ACTIVITY AND ULTRAVIOLET ABSORBANCE DETECTION

INTRODUCTION

Proteins have long occupied the minds of scientists. The determination of the amino acid sequence of proteins is presently a well established technique. The identification of protein structure and properties is also an area of active research. In the last decade, the use of high performance liquid chromatography (HPLC) for protein separation and purification has increased dramatically (1). In addition, this use of HPLC has afforded considerable research in the separation mechanisms involved in the HPLC of proteins (2-5). Retention characteristics of many peptides and polypeptides have been studied in order to shed some light on the processes involved in reversed-phase (RP) HPLC of proteins (6). Separation of polypeptides by RP-HPLC is rapidly becoming an established procedure, but the complex secondary equilibria which come into play in protein chromatography are still under intense investigation (7).

An explanation of the several types of HPLC techniques used in protein separation is given in the General Introduction. Of particular interest is reversed-phase chromatography. Reversed-phase separations are popular because of the high resolving power available and widespread use of RP-HPLC in many other areas. But the conditions which produce good separations are known to significantly change the protein structure. Such conditions are low pH and high concentrations of organic solvents. Sadler et al. (8) show evidence from a variety of methods that under chromatographic elution conditions 1-propanol produces conformational

changes in the studied proteins, which may be important in the elution process. Hearn et al. (7) explain that the addition of water miscible organic solvents can rupture the hydrophobic interactions present in a protein in its biological conformation. The resulting change in three-dimensional structure affects the retention characteristics of the protein.

Broadened or multiple peaks resulting from subjecting a single protein to RP-HPLC have been observed by several researchers for a variety of proteins (9-13). It is believed that these multiple peaks result from interaction with the stationary phase when "the overall time constants for the processes of equilibration between native and unfolded forms of a particular protein are of comparable magnitudes to the time of separation" (7). Separation of protein conformers has also been accomplished by electrophoresis (14).

The unfolding which occurs to produce the mutliple peaks is not solely the result of contact with the organic component of the eluent. In studies with soybean trypsin inhibitor (STI), on a C4 RP column, Cohen et al. (9) found no unfolding for up to 2 hours of incubation in 10 mM H_3PO_4 (pH 2.2) or 9% 1-propanol/10 mM H_3PO_4. However, incubation in 30% 1-propanol/10 mM H_3PO_4 did cause unfolding of STI. The adsorption-facilitated denaturation of STI is further illustrated by observing the increase in unfolding as contact time with the column surface increases.

Multiple peak formation is a function of solvent conditions, temperature, gradient steepness, and flow rates. This phenomenon has been observed in RP-HPLC of STI under two different sets of conditions.

Hearn et al. (7) used an n-alkylphenyl silica column with 15 mM H_3PO_4/acetonitrile eluent. An n-butyl-bonded silica gel with 10 mM H_3PO_4/1-propanol as eluent was used by Cohen et al. (9). Both groups report similar chromatograms which show several early eluting peaks and a single later eluting peak. The early peaks, which are native forms of STI, are biologically active and partially convert to the latter peak upon reinjection. The latter peak is denatured, possesses significantly less biological activity, and does not convert to the native forms upon reinjection.

Trypsin is the most widely used proteolytic enzyme in peptide mapping. This enzyme catalyzes the hydrolysis of peptide bonds in specific locations (15). Trypsin inhibitors are relatively low molecular weight proteins which combine specifically with trypsin to inhibit its activity (16). Soybean trypsin inhibitor was independently discovered in 1944 by Ham and Sandstedt (17) and Bowman (18). Nine different trypsin inhibitors were reviewed by Laskowski and Laskowski, Jr. (19). Wu and Scheraga later studied the physicochemical properties of STI (20) and reported a molecular weight of 21500. Other values ranging from 14300 to 24000 have been reported (21,22). The conformational properties (23) were also studied with optical rotatory dispersion, and the native form of STI was found to contain mostly random coiled regions plus a small amount of helical structure. This non-helical structure of STI was also reported by Ikeda et al. (24). The amino acid composition has been reported by several workers (20,25), and the structure has been identified as that of a normal globular protein (26).

Detection by OA is useful for samples of biological origin since chirality is inherent in life processes. Optical activity has been used for many years as an indicator of structural changes of proteins. Yang and Foster used optical rotation to study acid binding in bovine serum albumin (27), and Kauzmann and Simpson studied the kinetics of urea-induced denaturation of three proteins with optical rotation (28). Circular dicrosim (CD) and optical rotatory dispersion (ORD) have also been extensively used to study proteins. One example involved the use of the Cotton effect in ORD as a probe for conformation of a large group of globular proteins (29). A second example utilizes ORD and CD in the study of solvent denaturation of proteins (30).

The successful coupling of OA/UV to HPLC was described in Section I. As was pointed out in Section II, refractive indices must be closely matched for successful coupling of the OA detector to gradient elution. Such refractive index matching is necessary since the OA detector responds to refractive index changes. Acetonitrile is a good choice for the organic phase in a gradient system for reversed-phase chromatography for that very reason.

Acetonitrile and 1-propanol are the most commonly used RP-HPLC of proteins and have comparable selectivities (15). Acetonitrile is often preferred over frequently used 1-propanol (31) because the viscosity of 1-propanol is large resulting in high column back pressure. Low back pressures are especially desirable if the silica support has a large pore diameter and, as a result, is fragile (32). 1-propanol is preferred for proteins with low solubility in organic solvents (15). Mixtures of

acetonitrile and 1-propanol have been used to produce different selectivities in the purification of subunits of oligomeric membrane proteins (33).

This section illustrates the formation of multiple peaks of soybean trypsin inhibitor under reversed-phase chromatographic conditions with detection by optical activity and ultraviolet absorption (OA/UV).

EXPERIMENTAL

Reagents

The soybean trypsin inhibitor type I-S was obtained from Sigma (St. Louis, MO). HPLC grade acetonitrile, analytical reagent grade phosphoric acid and certified grade 1-propanol were obtained from Fisher Scientific (Fair Lawn, NJ). Deionized water further purified on a Milli-Q system (Millipore, Bedford, MA) was used to prepare eluents and samples.

Chromatography

The gradient system consisted of two syringe pumps (Isco, Lincoln, NE, Models 314 and LC2600) and a mixing tee. The pumps were controlled by a personal computer (IBM, Boca Raton, FL) with an ADALAB-PC interface using Adapt software (Interactive Microware, Inc., State College, PA). The separation was performed on a 10 X 0.41 cm, 6.5 micron Synchropak RP4 with 300Å pores (Synchrom, Inc., Linden, IN) which was pretreated with hemoglobin to remove any irreversibly adsorbing sites. Such pretreatment is known to improve the chromatographic reproducibility (34,35). The separation was carried out using a phosphoric acid (10 mM pH 2.2)/acetonitrile eluent. Eluent A was 10 mM phosphoric acid, B was 45/55 acetonitrile/water with 10 mM phosphoric acid overall. For the incubation studies, a phosphoric acid/1-propanol system (A = 10 mM phosphoric acid, B = 45/55 1-propanol/water with 10 mM H_3PO_4 overall) was used in addition to the phosphoric acid/acetonitrile eluent system. Injections were made through a conventional injection valve (Rheodyne,

Berkeley, CA, Model 7010) with a 10 μl loop. Between 30 μg and 120 μg of STI was injected. All samples were filtered through 0.45 micron syringe filters before injection. A flow rate of 0.6 ml/min was used for all experiments. All eluents were filtered through a 0.2 micron filter and degassed under vacuum using ultrasonic agitation to minimize degassing in the cell.

Detection

The work described in this section used the configuration of the optical activity detector given in Section II with no modifications. Ultraviolet detection at 280 nm in series with the OA flow cell was provided by a Hitachi spectrophotometer (Tokoyo, Japan, Model 100-10) with an Altex flow cell (Berkeley, CA, Model 155-00). The outputs of the OAD and the UV were connected to two voltmeters (Keithley, Cleveland, OH, Models 155 and 160B), the analog outputs of each were connected to the I/O ports of the personal computer. The computer took five readings every second and stored the sum each second.

Procedure

Injection was made into 0% B two minutes before the start of the gradient for all but the incubation studies. After the initial two minutes, the eluent concentration increased from 0% to 80% B in 30 minutes followed by an isocratic hold at 80% B for 20 minutes. For the incubation studies, determination of the extent of denaturation as a

function of time spent on the column was performed by varying the time between injection and start of the gradient at room temperature. Two different organic phases were used.

Calculations

Areas were determined by summation of adjusted values above a chosen baseline for each peak. OA areas were normalized for laser power differences as described in Section I. All figures shown are based on a ten point smooth of the chromatographic data.

RESULTS AND DISCUSSION

Figure 1a and b show OA and UV chromatograms of soybean trypsin inhibitor detected simultaneously. The peaks are labeled I-IV corresponding to an earlier report of STI under similar conditions (9). Through reinjection and inhibition measurements, these workers determined that peaks I and II are native forms and peak IV is denatured. In the work of Cohen et al. (9), the identity of peak III was questioned because it did not convert to peak IV upon reinjection as did peaks I and II.

In order to confirm that the peak assignments were the same as those of Cohen et al. (9), we repeated a series of experiments they reported. Injection into a solvent gradient already in progress changes the relative peak heights in the resulting chromatogram. This is one form of evidence for the early-eluting peaks being in the native conformation and the last peak being denatured. The height of peak IV decreases with increasing percent organic phase in the eluent upon injection. This is explained by fewer sites being available for protein adsorption because of competition by the organic solvent for these sites. Denaturation is known to occur upon adsorption on the nonpolar n-alkyl surface of the reversed-phase column (7,10). In addition, Soderquist and Walton (36) characterized the structural changes of plasma proteins on polymer surfaces including a neutral hydrophobic polymer of ϵ-carbobenzoxylysine and leucine.

Figures 2 and 3 show the results of injection of STI into various parts of the gradient as detected by the UV absorbance detector and the

Figure 1. Ultraviolet absorbance (a) and optical activity (b) chromatograms of soybean trypsin inhibitor. 115 μg injected. Conditions given in text

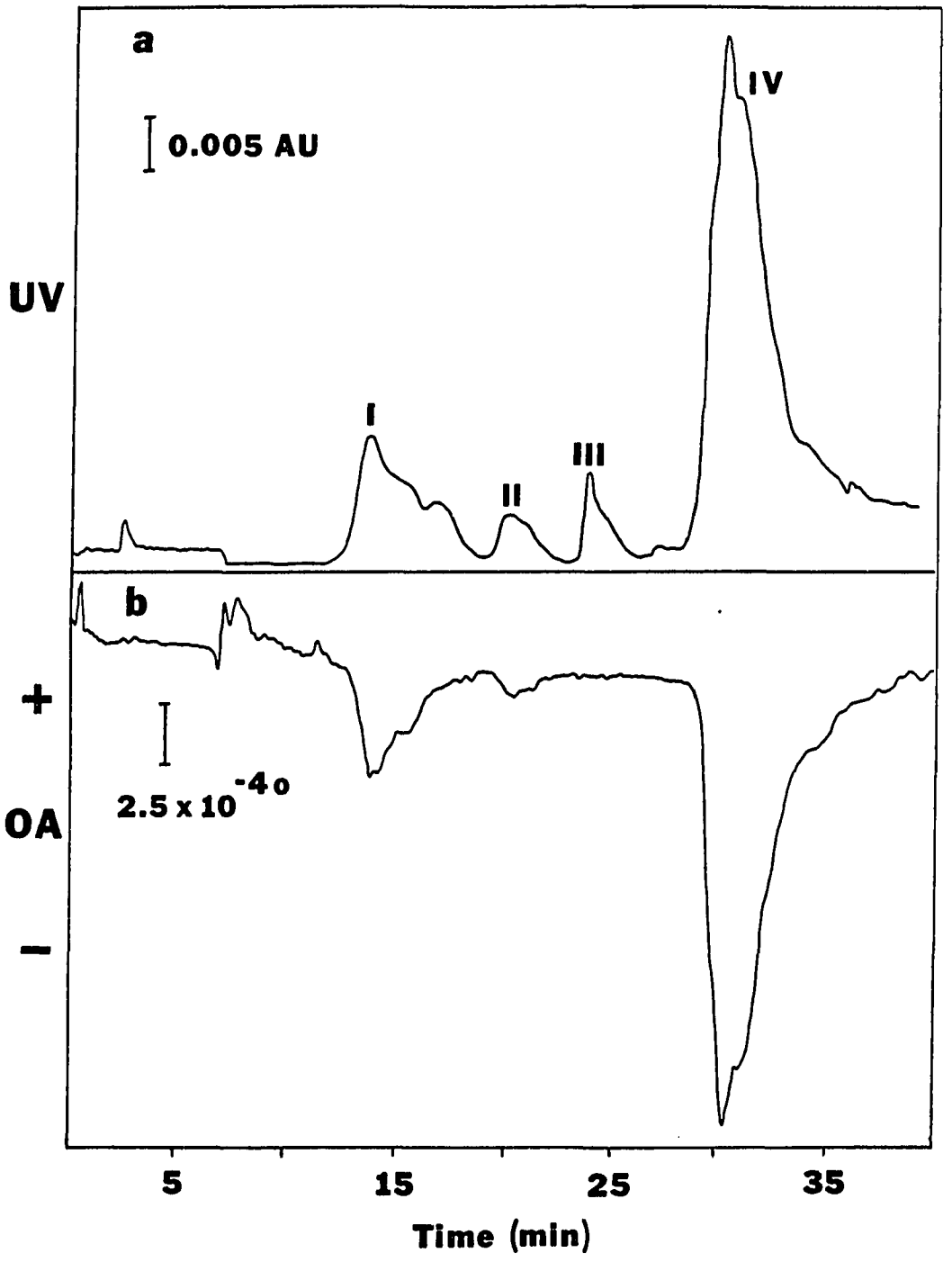

a
0.005 AU
UV
I
II
III
IV
b
+
OA
−
2.5 x 10^-4 o
5
15
25
35
Time (min)

Figure 2. Ultraviolet absorbance chromatograms of STI. (a) injection at start of gradient, (b) injection 6 minutes after start of gradient, and (c) injection 10 minutes after start of gradient. 35 μg injected. Other conditions given in text

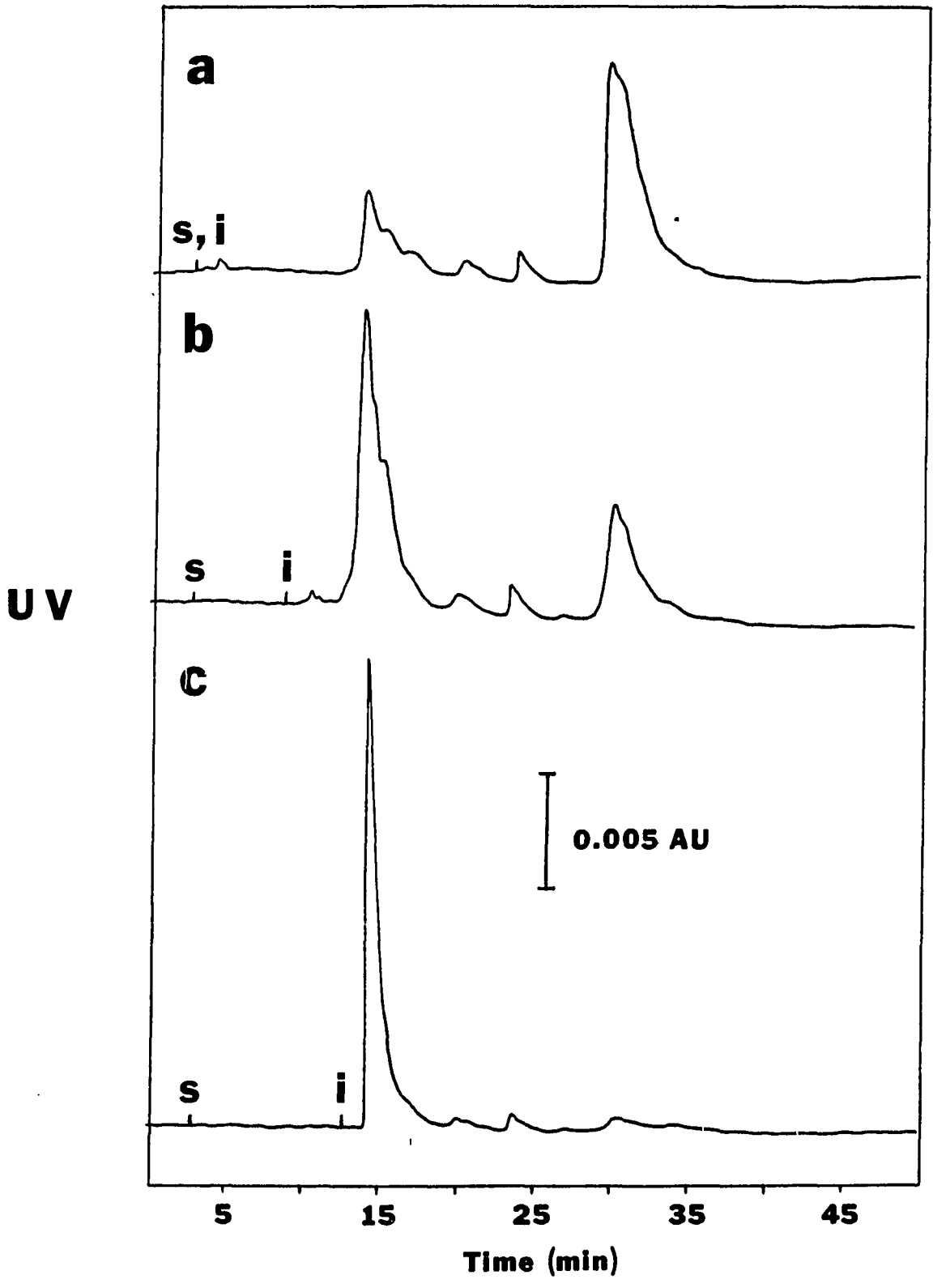
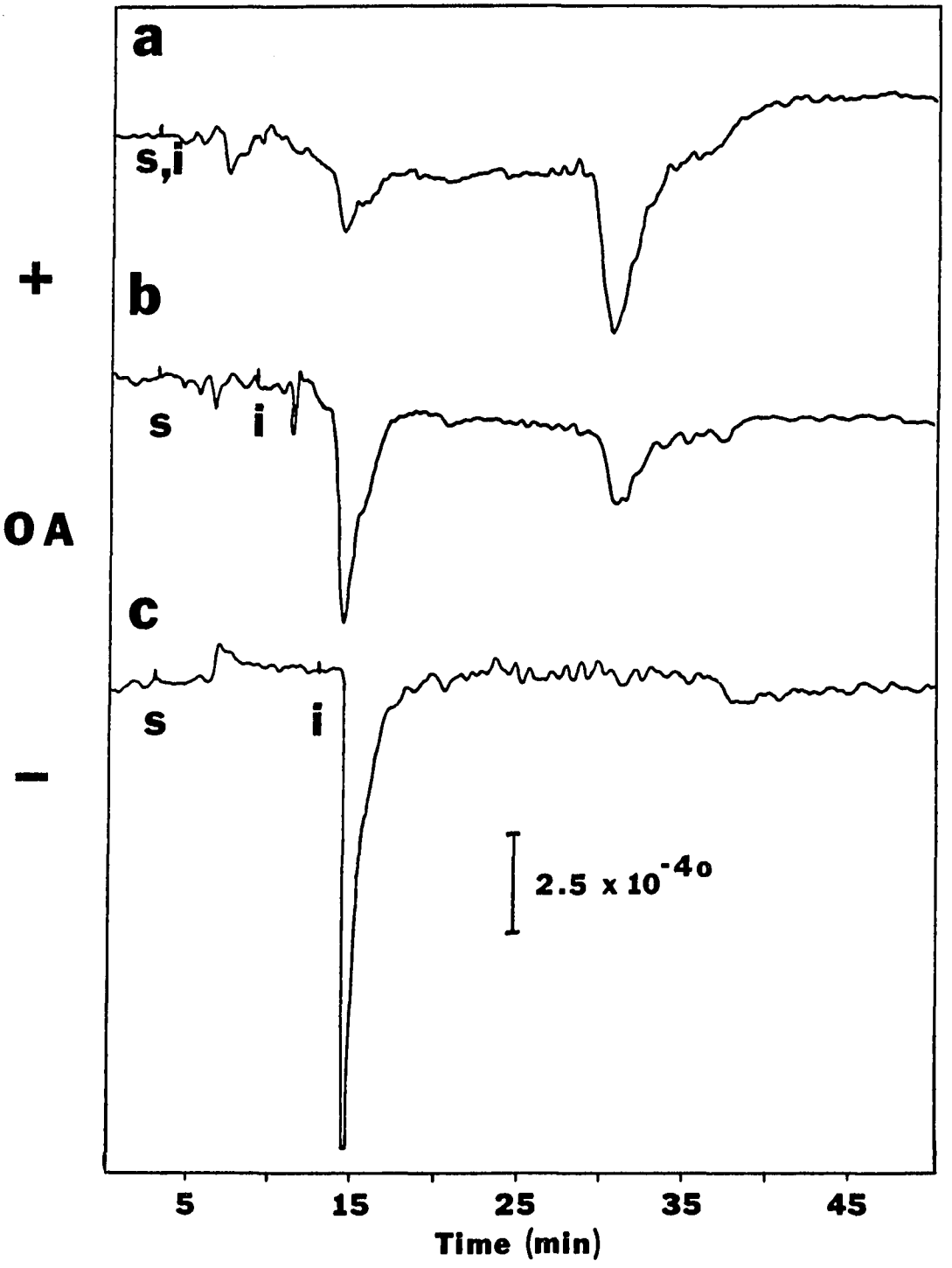

a
s, i
b
s
i
UV
c
0.005 AU
s
i
5
15
25
35
45
Time (min)

Figure 3. Optical activity chromatograms of STI. Conditions as indicated in Figure 2


a
s,i
+
b
s
i
OA
c
s
i
−
2.5 x 10-4o
5
15
25
35
45
Time (min)

OA detector, respectively. Injection occurred at the start of the gradient in Figures 2a and 3a, and 6 minutes after the start of gradient in 2b and 3b. A large early-eluting peak was produced upon injection 10 minutes after the start of the gradient, with only a very small amount eluting at a time corresponding to the denatured peak (Figures 2c and 3c). Based on these results, we can assume that we are observing the same phenomena reported earlier (9).

Several observations can be made upon comparison of the OA and UV chromatograms in Figure 1. Peak I appears to consist of three components when detected with UV absorbance but only two with OA. Peak III is not optically active or else possesses a much smaller specific rotation than the other peaks. This correlates well with the suggestion of Cohen et al. (9) that peak III is an impurity or a form of STI which does not undergo conversion to other forms. Our data suggest that peak III is an impurity since any form of STI, or any protein, should be optically active. Proteins have negative specific rotations which become more negative upon denaturation (37). Assuming component I is the native form of STI, then component III should possess the same or a more negative specific rotation than the native form. Thus, if peak III is a partially denatured form of STI, it should produce a peak in the OA chromatogram.

Another method of comparing the two chromatograms is possible. Ostojic (38) first suggested the use of multiple detectors for solute identification. Ratioing of two or more ultraviolet absorbances was illustrated by Yost et al. (39) using stop-flow liquid chromatography. For a pure component, the detector ratios are constant over the entire

peak because the ratio is not affected by changes in concentration. If the ratio is not constant over the peak, impurities are indicated. Drouen et al. (40) applied this technique to separation optimization schemes for HPLC. In determining optimum elution conditions, peak identity must be confirmed and co-eluting and unresolved peaks are encountered. Peak identities are determined by the value of the ratio and peak purity is indicated by the shape of the ratio plotted against time. For a pure component, a block-shaped ratio plot is obtained. A variety of ratio shapes can occur for unresolved peaks (40). Another application of this technique was shown by Webb et al. (41) in the characterization of a mixture of phenylthiohydantoin derivatives of amino acids.

Clearly, one is not required to use two measurements from the same type of detector (i.e., two wavelengths from a UV absorbance detector) to apply this technique. The ratio of responses from the optical activity detector and an UV absorbance detector (OA/UV) is applicable as well as the frequently used UV/UV. Drouen et al. (40) introduces a method of calculating ratio values which causes the ratios to fall between 0 and 2 producing ratio values which are distributed homogeneously over the whole range of ratio values. The following equations from Drouen et al. were used with slight modification.

$$RAT = A1/A2 \qquad \text{when } A2 \geq A1 > \text{threshold} \qquad (1)$$

$$RAT = 2 - A2/A1 \qquad \text{when } A1 > A2 > \text{threshold} \qquad (2)$$

$$RAT = 0.0 \qquad \text{when } A1 \text{ or } A2 \leq \text{threshold} \qquad (3)$$

A1 and A2 are the OA and UV detector responses, respectively. The threshold value (typically 1-5%) is used to eliminate noise from the baseline and the beginning and the end of the peak producing a block-shaped ratio plot for a pure component. Figure 4 shows the RAT values plotted versus time for the chromatogram shown in Figure 1. Note that the ratio values do not differ significantly between peaks I and IV. But the RAT value of peak II is different.

Aside from comparing RAT values as a characteristic of a peak, the shape of each RAT peak gives an indication of the purity of the peak. It was mentioned previously that the OA and UV chromatograms of peak I do not correspond. This is further illustrated in the RAT plot of this peak. There is a significant change in the RAT values corresponding to the location of the discrepancy between the two detector responses, indicating the presence of an impurity. Conversely, the RAT peak for component IV is block-shaped, indicating only one component in this peak. Since the OA area for peak II is small, the RAT plot suffers, but still indicates that peak II does not contain an impurity. Peak III is not detected by OA and so its RAT value is set to 0.0.

By studying the OA and UV detected chromatograms in conjunction with the RAT plot of a single chromatogram, we can make several observations which cannot be made from the UV chromatogram alone. The most obvious feature is the lack of a response for peak III by the OA detector and consequently also in the RAT plot. These observations immediately suggest that peak III is not protein. The difference in detector response between peaks I and II are more readily discernible using the

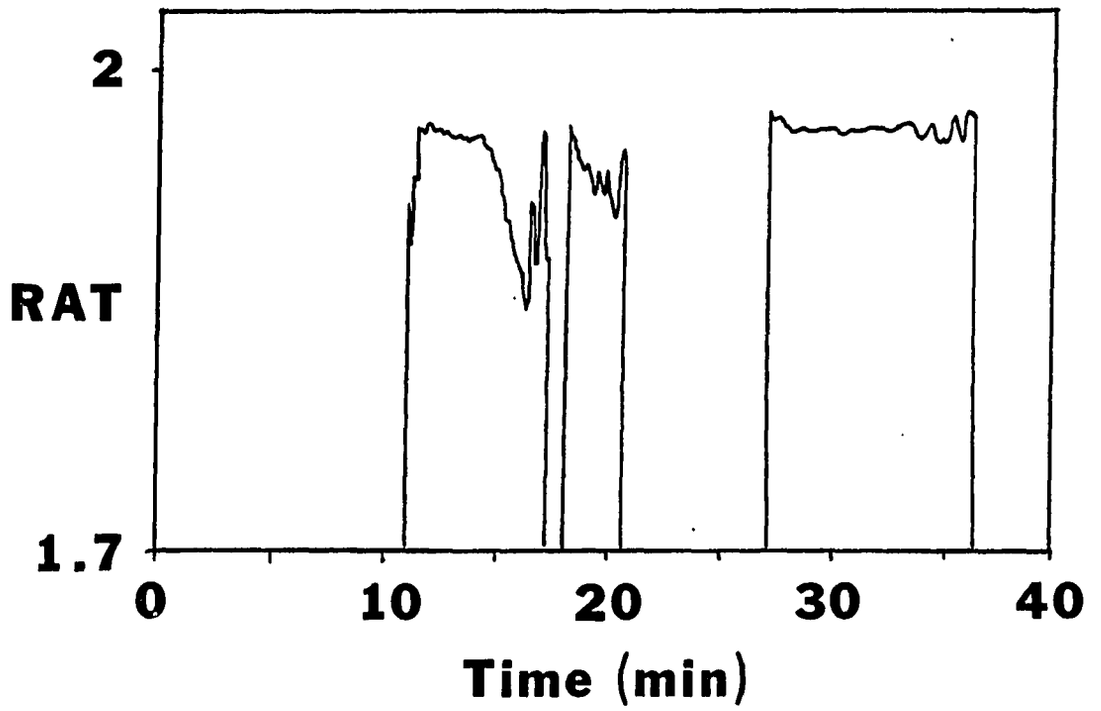


Figure 4. Plot of RAT values versus time for chromatogram shown in Figure 1

RAT plot. Finally, the optically inactive component in peak I can be easily observed in the RAT plot as well as by comparing the UV and OA chromatograms. As in any dual detector method, OA/UV provides more information than a single detector method. This is especially the case since OA is a very suitable detector for proteins: a detector selective for biological origin. Dual detection using OA/UV provides a large amount of information about a separation from only one sample injection.

Molar absorptivity ϵ is known to change with protein conformation (42) but the changes are very small (43). Difference spectroscopy is frequently used to measure the change in absorbance upon denaturation. Wu and Scheraga (23) performed ultraviolet difference spectroscopy on STI under various conditions and their results show only a small change in absorbance at 280 nm upon denaturation. Our data also indicate this change is not significant under the conditions used. Assuming ϵ is a constant for each peak and using the UV peak areas, it is possible to estimate the quantity of protein present in each peak. This also requires the assumption that all protein injected onto the column elutes in one of the four peaks. This is expected because of the pretreatment of the column with protein to remove the irreversibly adsorbing sites (34,35). Having an estimate of the quantity of protein in each peak allows the calculation of specific rotation values for the three optically active peaks. The method for these calculations is explained in Section I. Specific rotations of the three peaks are reported in Table 1. These results are based on data from triplicate injections of a 11.5 mg/ml solution of STI. Once again, the small area of peak II causes

Table 1. Specific rotation of STI conformers

Peak	$[\alpha]_{488}^{26}$
I	-97 ± 11[a]
II	-67 ± 14[a]
III	0 ± 4[a]
IV	-97 ± 4[a]

[a]One standard deviation.

a large uncertainty in the calculated [α] value. But even so, the [α] values for peak II is significantly different than those of peaks I and IV. This corresponds to the differences observed in the RAT plot of these three peaks. Our calculated specific rotations compare well with the results of Wu and Scheraga (23) who report [α] = -97° for STI in 0.069 M HCl (pH 1.22) at 589 nm. Specific rotations of proteins are known to become more negative upon denaturation (37). But as with molar absorptivity, we do not observe a large change in specific rotation between the three peaks. If we assume that peak IV is denatured, we do not observe a change in [α] accompanying denaturation. It is possible that the change in conformation is too small to be manifested by a change in [α]. The lack of change in specific rotation could also be explained by the structure of STI which possesses few helical portions in its structure. Consequently, a large change in [α] upon unfolding is not expected.

Incubation studies were performed under two different solvent conditions. Rate constants for on-column denaturation were calculated following the method of Benedek et al. (11) using

$$A = ae^{-k^* t_G} \qquad (4)$$

with A defined as the area of native peak eluted from the column, a the amount of protein injected onto the column in the native conformation, t_G the time from injection until the native peak elutes and k^* the average rate constant for the given gradient conditions. In this set of

experiments, $k^* t_G$ is assumed to be a constant since the gradient conditions are not changed. For the on-column incubation before the start of the gradient

$$a = Ne^{-kt} \tag{5}$$

with N being the amount of injected material, k the rate constant for denaturation under isocratic conditions, and t equal to the incubation time. Substitution of Equation 5 into Equation 4 results in Equation 6.

$$A = Ne^{-kt} e^{-k^* t_G} \tag{6}$$

Taking the natural log of both sides of Equation 6 and combining the constants yields Equation 7.

$$\ln A = (\ln N - k^* t_G) - kt = K - kt \tag{7}$$

Consequently, the negative slope of the plot of ln A versus incubation time yields the rate constant for the on-column denaturation. The area of peak I was used as the area of the native peak (A). Results are shown in Table 2 with reference values for comparison. Hearn et al. (7) reported rate constants of similar magnitude for STI on an octadecylsilica column with a trifluoroacetic acid/1-propanol eluent. The incubation solvent was identical and the column was thoroughly flushed with eluent A before each incubation experiment in the

Table 2. Rate constants for on-column[a] denaturation of STI

k (sec^{-1})	Temperature (°C)	Organic phase
$(3 \pm 1^{b}) \times 10^{-4}$ [c]	26	acetonitrile
$(9 \pm 5^{b}) \times 10^{-4}$ [d]	26	1-propanol
18.5×10^{-4} [e]	20	1-propanol
42.0×10^{-4} [e]	25	1-propanol
81.0×10^{-4} [e]	30	1-propanol

[a] Incubation mobile phase: 10 mM H_3PO_4 (pH 2.2).

[b] One standard deviation.

[c] Five data points.

[d] Four data points.

[e] Reference 11.

experiments being compared. As a consequence, any contribution of the organic phase to the rate of denaturation occurs during gradient elution and is included in the $k^* t_G$ portion of the constant in Equation 7. Our results agree with this conclusion since there is no significant difference in the rate constants (two standard deviations) obtained with the two different organic phases.

In comparing our results with those reported by Benedek et al. (11), we find our rate of denaturation slower. A possible explanation would be differing column material but a comparison of the column characteristics does not support such a conclusion. The column made by Benedek et al. and used for the STI incubation studies consisted of silica bonded with n-butyldimethylchlorosilane and endcapped with a mixture of hexamethyldisilazane and trimethylchlorosilane. The silica particle size is 10 μm with 500 Å pores. No column dimensions are given but can be assumed to be 10 x 0.46 cm based on a later publication (10). In the present study, a commercial 10 x 0.41 cm C4 column was used. The silica particle size is 6.5 μm with 300 Å pore diameter. STI is a small protein. Sweet et al. reported the diameter of STI to be 35 Å (44). As a result, the difference in pore size between the two stationary phases should not be an important factor when comparing the column characteristics. Consequently, our results can be compared with those of Benedek et al. (11) because the columns are essentially the same. Hence, we suggest that we have conditions which produce less denaturation. This could be explained by the pretreatment of the column resulting in the elimination of irreversibly adsorbing sites and much milder column

conditions, slower denaturation, and larger recovery of native form of the protein. As indicated by Benedek et al. (11), the recovery of the native form of STI would increase at lower temperatures. Thus, performing the separation at lower temperatures on a column pretreated with protein would provide the largest recovery of native STI.

In summary, this section reports the second successful coupling of OAD to gradient HPLC. The conditions used in the two OAD/gradient systems involved an aqueous solution modified with acetonitrile. But in this work a larger total change of organic phase was used. In the separation of the dansyl amino acids presented in Section II, the gradient started at 70% A and was changed to 30% A. Eluent A contained 50% acetonitrile and eluent B was 100% acetonitrile. Consequently, the resulting increase in organic phase was from 65% to 85% acetonitrile. In this work, a change of 36% organic phase was used (0% to 36% acetonitrile). As a result of the larger change in eluent composition, there is an increase in baseline noise (decrease in S/N) and additional baseline instability and drift. These problems are tolerable at the protein concentrations frequently used in RP-HPLC of proteins.

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GENERAL SUMMARY

This dissertation has described three applications of a dual detector (OA/RI, OA/UV) system for HPLC. Dual detection methods obviously provide more information than a single detector, but the utility of dual detection is enhanced with a selective and sensitive detector such as the optical activity detector. Enantiomeric ratios of amino acid mixtures can be obtained either with or without derivatization. OA/RI has the attraction of not requiring a derivatization procedure in the analysis but lacks sensitivity. Sensitivity can be gained by derivatizing the amino acids and detecting by UV absorbance. Dansyl derivatives detected by UV absorbance were used as an example, but other derivatization (e.g., dabsyl, PTH) and detection methods (e.g., fluorescence) could be used. The detectability of some dansyl amino acids is not enhanced by derivatization. Perhaps another derivative would provide a uniform increase in the magnitude of specific rotations. Hydantion derivatives of amino acids have been reported to have large negative rotations (75). Of course, enantiomeric ratio calculations are possible with substances other than amino acids. Additional areas of application might include development and clinical testing of drugs (76), organic synthesis, and the determination of reaction rate constants. Polarimetry has already been used in the study of the racemization of rhenium compounds (77). Optically active organometallics are presently an area of interest (78-80), which could benefit from HPLC/OAD. Aflatoxins separated by HPLC present another

potential area of use for the HPLC/OAD detector system (81,82).

Gradient elution was not previously attempted using the OAD because of the change in refractive index which accompanies gradient elution. The laser beam must pass through specific, small regions of the polarizers, consequently, any movement of the beam caused by a change in refractive index in the flow cell is interpreted as an OA signal. By choosing an organic phase which has a refractive index close to that of water, the refractive index changes were kept to a minimum. Baseline drift and noise increased during gradient elution but was tolerable. An aqueous/acetonitrile (RI of water = 1.333, RI of ACN = 1.344) gradient performed well (Section II). An aqueous/1-propanol (RI of 1-propanol = 1.385) gradient produces a large change in RI causing the beam to distort and deflect making detection by OAD impossible. Bubble formation was serious when mixing the aqueous eluent with acetonitrile but was solved by premixing the aqueous system with a portion of acetonitrile, since 100% organic was never needed. In Section III, an aqueous/acetonitrile gradient was also used but with a larger change in percent organic phase. As a result, an increase in noise was observed. In Section II a 50/50 aqueous/organic solution was used as eluent A and 55/45 in Section III. It is unlikely that the OAD could be used for a 0 to 100% organic gradient because of bubble formation even if the problem with the refractive index change was overcome.

Baseline noise caused by the gradient could be reduced by the use of a shorter cell. The decrease in noise would result because a smaller percent change in solvent composition would exist between the ends of the

cell. A decrease in detectability will also result because of the ℓ term in Equation 1 in the General Introduction. Total drift from start to end of the gradient will not change. Therefore, the only advantage of using a shorter cell would be to decrease the short-term fluctuations in the baseline. It is possible that this noise could decrease enough to offset the loss in detectability because of the shorter path length.

This work illustrates the calculation of specific rotations at different wavelengths and solvent conditions than are reported in the literature. We are limited by the availability of emission lines of the laser, but using an argon ion laser and a helium neon laser, specific rotations at three wavelengths can be determined (514.5, 488, 632.8 nm). Specific rotations become larger with shorter wavelengths. Therefore, using ultraviolet radiation would decrease detection limits using the OAD. Although UV lasers are available, the system optics (lenses and polarizers) would have to be changed for successful work in the UV.

Since most proteins are optically active as a consequence of amino acids and three dimensional structure, OAD is an attractive detector for protein HPLC. In theory, OA detection should work for all popular methods of protein HPLC with the exception of affinity chromatography. The large change in solvent conditions required to elute the analyte would probably limit the application of OAD for detection in affinity separations. HIC might also present a problem because of the high salt concentrations of the eluents. In the work presented in Section I, it was found that large amounts of salt in the eluent caused two problems. First, the tendency of the salt to deposit on the cell walls and windows

produced increased system noise because of scattered radiation. The ability of chloride to corrode stainless steel components is well known. In addition, the corrosion of stainless steel by acetonitrile or methanol in process liquid chromatography has been documented (83). Second, the salt weakens the glue used to affix the windows to the cell. This results in drastically decreased lifetimes of windows (appromixately two days) and resulting problems with air bubbles. In standard reversed-phase eluent conditions (e.g., water, acetonitrile, methanol), windows have been used for up to four months with no problems. It is possible that another glue could be found which is resistant to deterioration by salts and will still provide strain-free attachment of windows. IEC and SEC are performed with moderate salt concentrations and so no problems are anticipated in these separation systems.

Reversed-phase chromatography was chosen to illustrate the detection of proteins with the OAD based on the observations of other researchers. The existence of several peaks corresponding to different conformations resulting from RP-HPLC of a pure protein appeared to be an interesting system to probe with the OAD. Since specific rotations are known to change with conformation, it was thought that a difference could be identified between the different conformers. In the specific system studied, soybean trypsin inhibitor did not exhibit a large change in specific rotation between the two peaks that have been identified as native and denatured using other methods. This lack of change in specific rotation could be explained by the structure of STI, which is almost entirely random. It is assumed that if similar experiments were

performed on a highly ordered protein, a change in specific rotation would be encountered.

Aside from the determination of change in specific rotation with conformation, OA/UV is very useful for detection in protein HPLC. The dual detection method facilitates identification of impurities by examination of the chromatograms. These impurities can be identified more readily if the ratio of the detector responses (RAT) is plotted.

In addition to amino acids and proteins, the OAD has been used for the elucidation of crystal structure of a racemic bicyclooctane obtained from Dr. Wroblewski. Single crystals were dissolved and subjected to RP-HPLC. Results showed that the compound crystallized as a racemic mixture and not in separate crystals of each enantiomer.

In conclusion, the OAD possesses great potential as an HPLC detector for many additional classes of compounds. Such potential exists because the use of HPLC continues to expand into areas previously dominated by conventional chromatographic methods (e.g., paper, thin layer and low pressure column chromatography) and into disciplines which traditionally did not use chromatographic methods of separation and purification.

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