Controlling the Dissociation of Peptide Ions Using Laser Desorption Chemical Ionization Fourier-Transform Mass-Spectrometry

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classified as both chemical (preferential sputtering) and physical (surface roughness) effects.

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Registry No. $Fe(vbpy)_2(CN)_2$ (homopolymer), 119058-92-3; vbpy (homopolymer), 82441-96-1; silver nitrate, 7783-99-5.

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Controlling the Dissociation of Peptide Ions Using Laser Desorption/Chemical Ionization Fourier Transform Mass Spectrometry

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Chemical ionization is demonstrated for peptide molecules evaporated by an excimer laser, operated at 193 nm. Ammonium ion chemical ionization of Val-Ala and Val-Pro-Leu confirms that peptide molecules are evaporated by the laser as intact neutrals, without any observable fragmentation. Extensive fragmentation is observed when protonated ethylene or protonated nitrous oxide are used as reagent ions. The extent and type of fragmentations observed are correlated with the proton affinity of the reagent ion species.

Chemical ionization mass spectrometry (*1*) has been extensively applied to the analysis of labile molecules. A useful feature of chemical ionization is that the degree of fragmentation of the molecular ion is determined by the exothermicity of the proton-transfer reaction (*2*). The extent of fragmentation can be adjusted by selecting a reagent ion with the appropriate proton affinity relative to that of the analyte. The capability to control ion fragmentation would be advantageous for applying mass spectrometry to the analysis of biopolymers, such as peptides, polysaccharides and nucleotides. However, these molecules are not easily analyzed by using chemical ionization mass spectrometry, because of their low volatility. Chemical ionization can be applied to molecules with low volatility, using laser desorption to vaporize a sample, followed by a proton-transfer reaction from a reagent ion to the desorbed neutral (*3-5*). Recent work has demonstrated that Fourier transform mass spectrometry is uniquely suited for the application of chemical ionization to laser-desorbed neutrals (*6*, *7*).

Here we report the application of laser desorption/chemical ionization (LD/CI) to peptide molecules and demonstrate that their fragmentation behavior is controlled by the choice of the reagent ion. For such studies, it is important that the desorption process ejects the peptide molecules into the gas phase as intact neutral species. Hillenkamp and co-workers, using resonance-enhanced multiphoton ionization (REMPI) to ionize laser-desorbed neutrals, have shown that nonvolatile organic compounds can be desorbed from thin films, by using 193 or 248-nm radiation from an excimer laser (*8*). However, the published spectra show that a significant proportion of fragment ions is also formed. Their study did not resolve whether the observed fragmentation occurred during the desorption process or during the multiphoton ionization step. In the work reported here, we demonstrate laser desorption of intact neutral peptide molecules exclusively. Furthermore, we are able to control the extent of fragmentation and the type of dissociations that occur upon ionization by adjusting the exothermicity of the proton-transfer reaction.

EXPERIMENTAL SECTION

A Fourier transform mass spectrometer has been designed and constructed specifically for the LD/CI experiment. The main vacuum chamber is pumped with a 330 L/s turbomolecular pump to a base pressure of 2×10^{-10} Torr. The chamber is fitted with two pulsed valves (General Valve, Series 9) for admitting reagent gases. A 4.4-cm cubic analyzer cell is mounted in the vacuum chamber, centered between the pole caps of an electromagnet operated at 0.75 T. An antechamber, separated from the main chamber of a gate valve, and pumped by a 1100 L/s cryopump to a pressure of 1×10^{-8} Torr, is used for pumping solid samples prior to their introduction to the main vacuum chamber. Peptide-coated sample stubs are evacuated in the antechamber and

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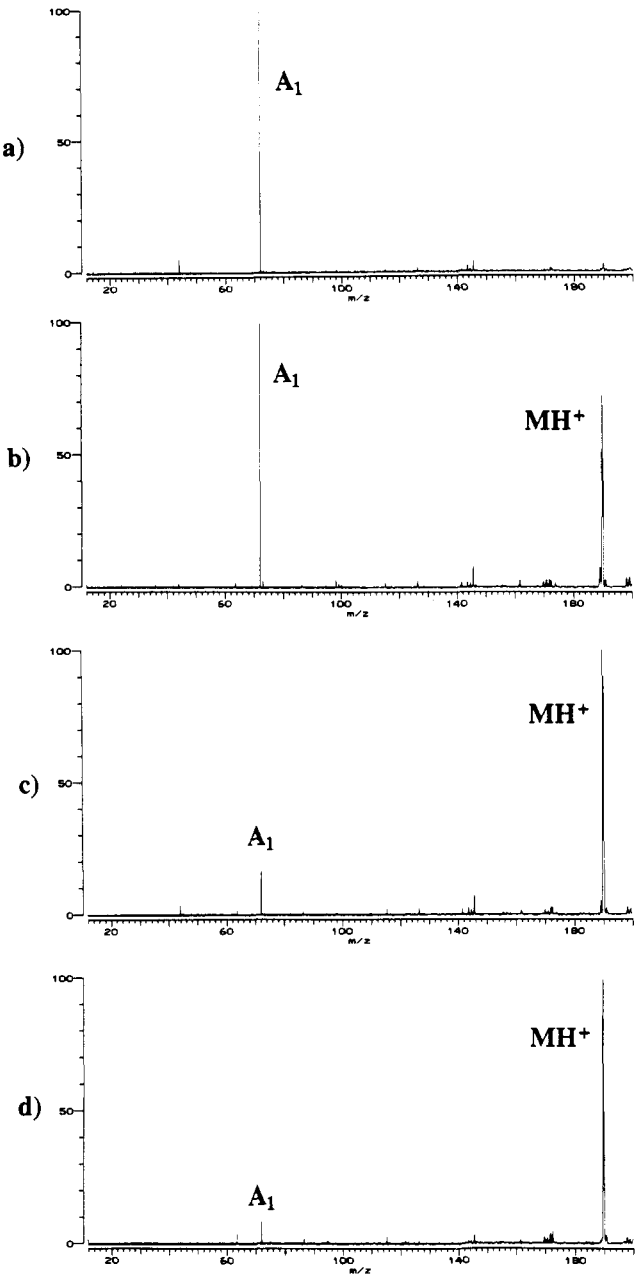


Figure 1. Laser desorption/ionization mass spectra of the dipeptide, Val-Ala, taken at four values of laser irradiance: (a) threshold irradiance (I_t) for observing the direct formation of ions; (b) 1.1 × I_t; (c) 1.2 × I_t; (d) 1.3 × I_t. The principal ions found in the spectra are the protonated molecule, m/z 189, and the m/z 72 fragment ion, A_1.

then transferred to a sample mount attached to the cubic analyzer cell in the main vacuum chamber. After sample transfer, the two chambers are isolated from each other. The main chamber pressure is ca. 5×10^{-10} Torr when a sample is mounted on the cell.

The sample stubs are Macor posts that are sputter coated with a 25-nm film of a gold/palladium (60:40) mixture. Ten to fifty microliters of a 10^{-3}–10^{-4} M solution of a peptide dissolved in methanol are applied to a sample stub, and the methanol evaporated to leave 10–1000 monolayers of the sample distributed across the surface, which has an area of 1 cm^2.

Peptides are desorbed by using the 193-nm (ArF*) output of an excimer laser (Questek, Model 2110). By use of an iris aperture, beam splitters, and neutral filters, a small fraction of the laser output (ca. 0.2 mJ) is focused to a spot with an area of approximately 1 mm^2, yielding an irradiance of ca. 10^6 W/cm^2. Fine tuning the laser power density in this regime allows neutrals to be desorbed without forming ions directly, e.g. by multiphoton ionization or cation attachment. The range of values of the laser

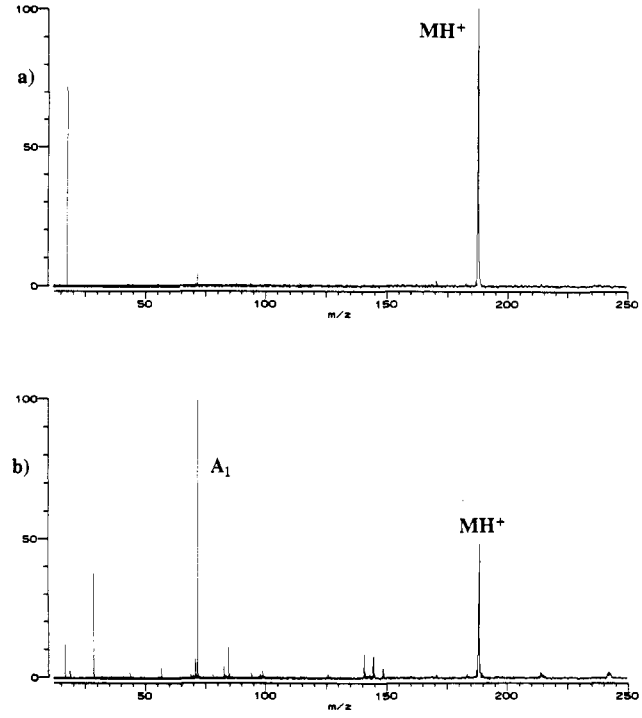


Figure 2. (a) LD/CI mass spectrum of Val-Ala (a) using NH_4^+ as a reagent ion. The base peak is the protonated molecule, and the abundant ion at m/z 18 is residual NH_4^+. (b) LD/CI mass spectrum of Val-Ala using $C_2H_5^+$ as the reagent ion. The base peak is the immonium fragment, A_1.

power density that allows neutrals to be desorbed without forming ions directly is observed to be very narrow.

The experimental procedure for LD/CI has been described (*6*). Briefly, a pulsed valve admits the reagent gas into the main chamber in a 10-ms burst. The pressure in the region of the analyzer cell rises to 10^{-4} Torr after 100 ms and then exponentially decreases to 10^{-8} Torr after 1 s. During the pressure pulse, the gas is ionized by 70-eV electron bombardment for 100 ms, and reagent ions are formed in bimolecular reactions between the initially formed ions and the neutral reagent molecules. After the formation of reagent ions and after the reagent gas has pumped away (1 s), the laser is fired at the peptide sample. Desorbed peptide molecules pass through the cloud of reagent ions stored in the analyzer cell. A fraction of the neutrals undergoes chemical ionization and is trapped by the magnetic and electric fields present in the cell. The trapped ions are detected by using standard FTMS (Fourier transform mass spectrometry) detection methods. An IonSpec Omega 2000 data system controls the experimental sequence and data collection. Mass spectra are obtained from a single laser shot, without the need for signal averaging.

RESULTS AND DISCUSSION

LD vs LD/CI. For the di- and tripeptide molecules studied to date, the threshold laser power density (irradiance) necessary for observing neutral desorption is less than that required for observing the formation of ions directly and is on the order of 10^5–10^6 W/cm^2. At the threshold irradiance for forming ions directly, fragment ions are observed in the mass spectrum, and the molecular ion species is absent. At increased laser irradiance, a protonated molecule is observed. The relative intensity of the molecular ion species increases as the irradiance is raised. This behavior is illustrated for the dipeptide Val-Ala in Figure 1. At the threshold laser irradiance required to form ions directly, a m/z 72 fragment ion dominates the mass spectrum. As the irradiance is increased in steps of 10%, the protonated molecule increases and then dominates the mass spectrum. LD/CI of Val-Ala using ammonium as the reagent ion yields the mass spectrum shown in Figure 2a, in which the protonated molecule represents

Scheme I

more than 95% of the observed ion signal. Assuming that fragment species are protonated at the same rate as the molecule, the neutrals being desorbed are almost entirely intact molecules.

The observation of fragment ions at low laser irradiance, and formation of a protonated molecule at higher irradiance are interesting features of laser desorption at this wavelength. This trend in fragmentation behavior as a function of laser irradiance has been reported previously for the UV laser desorption of dipeptides, although no explanation was presented (*9*). At the laser irradiance for which the m/z 72 fragment is the only ion observed, LD/CI using ammonium ion shows that the desorbed neutrals are unfragmented molecules. The data suggest that at the threshold irradiance, the desorbed molecular neutral of Val-Ala is photoionized, producing an odd electron molecular ion which rapidly decomposes to form the observed fragment. A mechanism for the formation of the m/z 72 ion, an immonium ion derived from the valine residue, from the odd electron molecular ion of the peptide is proposed in Scheme I. The suggested α-cleavage mechanism is frequently observed in the electron ionization mass spectra of peptides (*10*). As the irradiance increases, the number of desorbed neutrals also grows, so that their density in the vicinity of the laser spot is large enough to allow the initially formed odd electron peptide molecular ion to undergo a reactive collision with a peptide molecule before it can decompose. Hydrogen abstraction to form a stable, even electron, protonated molecule, that is, self chemical ionization (*11*), is expected if the collision rate of the odd electron ion with a peptide molecule is competitive with the rate of ion dissociation. Hillenkamp and workers have proposed a similar mechanism for the formation of ions by laser desorption, in which photoionization followed by condensed-phase ion–molecule reactions yields the observed ions (*12*).

A rough estimate of the density of desorbed molecules can be made to determine if the bimolecular collision frequency is large enough to compete with ion dissociation. At high irradiance, all the sample under the laser spot is evaporated. For a thin sample, this would correspond to the desorption of 10 monolayers of material or 10^{19} molecules/m^2. Assuming a thermal temperature of 1000 K for the desorbed neutrals, their velocity, v, is given by

$$V = (8kT/\pi m)^{1/2} \quad (1)$$

The velocity of desorbed Val-Ala is approximately 300 m/s. During the 10-ns laser pulse, desorbed species travel 3×10^{-6} m from the sample stub. The density of molecules in the small volume above the surface of the sample stub at the end of the laser pulse is therefore 3×10^{24} molecules/m^3. The collision frequency, z, is given by

$$z = 2\sigma v d \quad (2)$$

where σ is a collision cross section, taken to be 10^{-18} m^2, typical for an ion–molecule reaction, and d is the density of the vaporized peptide. From the values of velocity and density calculated above, the collision frequency is estimated to be 10^9 s^{-1}, or 1 collision/ns. The rate constant for the competing process, dissociation of the odd electron peptide molecular ion, will depend on the ion internal energy, which in turn depends on the number of photons it has absorbed and the amount of heat transferred to the molecule before desorption occurred. However, the calculated collision rate predicts that hydrogen abstraction by the photoionized molecular ion should be competitive with dissociation for all but the most rapid of reactions. This model predicts that the ionic products of direct laser ionization are determined by sample thickness and laser irradiance as well as by molecular structure. These factors contribute to the difficulty of applying direct laser ionization to structure determination problems, due to the complex relationship between laser irradiance and the degree and type of fragmentation observed. Techniques which allow control of the fragmentation of a molecule, such as chemical ionization, should be better suited to structure analysis.

Table I. Proton Affinities of Selected Reagent Ions

reagent ion	reagent gas	proton affinity,[a] eV
NH_4^+	NH_3	8.9
H_3O^+	H_2O	7.5
$C_2H_5^+$	CH_4	7.1
N_2OH^+	N_2O/CH_4	5.9
CH_5^+	CH_4	5.7
N_2H^+	N_2/H_2	5.1
H_3^+	H_2	4.4

[a] Proton affinity values are from ref 2 and are for the conjugate base of the reagent ion. Proton affinity is defined as $-\Delta H$ for the reaction $B + H^+ \rightarrow BH^+$.

Controlled Fragmentation by LD/CI. The internal energy of a peptide ion formed by chemical ionization is approximately equal to the enthalpy of the protonation reaction and can be calculated from the proton affinities of the reagent gas and the sample (*2*) as follows:

$$\Delta H = \text{PA(reagent)} - \text{PA(peptide)} \quad (3)$$

The proton affinity of a peptide molecule is estimated to be approximately 9.2 ± 0.3 eV, similar to that of an amino acid (*13*, *14*) or an amide (*N*,*N*-dimethylacetamide, 9.2 eV). The proton affinities of a variety of reagent ions are shown in Table I. As can be seen, ammonium chemical ionization of peptides is almost thermoneutral, and so the protonated molecule formed should have very little excess internal energy and therefore should be stable. A reagent ion such as N_2OH^+ will create a protonated peptide with ca. 3.3 eV of excess energy, which is large enough to cause bond dissociation. The reagent species selected determines the amount of excess internal energy present in the protonated peptide. For a peptide with a proton affinity of 9.2 eV, the internal energy can be adjusted over the range 0.3–4.8 eV, by using the reagent ion listed in Table I. Figure 2 compares chemical ionization of Val-Ala using NH_4^+ versus $C_2H_5^+$. Ionization using $C_2H_5^+$ is 1.8 eV more exothermic than with NH_4^+. Accordingly, fragmentation is absent in the case of NH_4^+ chemical ionization, and extensive for $C_2H_5^+$ ionization, leading primarily to the formation of the m/z 72 immonium ion of valine.

Figure 3 shows LD/CI mass spectra of the tripeptide Val-Pro-Leu protonated by NH_4^+, $C_2H_5^+$, and N_2OH^+. Two trends are found in this series of mass spectra. The first is an increase in the extent of fragmentation as the protonation reaction becomes more exothermic. By changing the proton affinity of the reagent gas over a range of 3.3 eV, the extent of fragmentation of the protonated molecule changes from 0% to 100%. The LD/CI mass spectrum using ammonium ion (Figure 3a) contains only the protonated molecule, establishing that no fragmentation of the tripeptide occurs during the laser desorption process. The fragmentations observed in the spectra shown in Figure 3b,c are controlled by the thermochemistry of the proton-transfer reaction. A second trend present in this series of mass spectra is the change of the identity of the most abundant fragment ion as the internal energy of the protonated precursor is increased. The two major types of fragment ions found in the spectra are B_2 (m/z

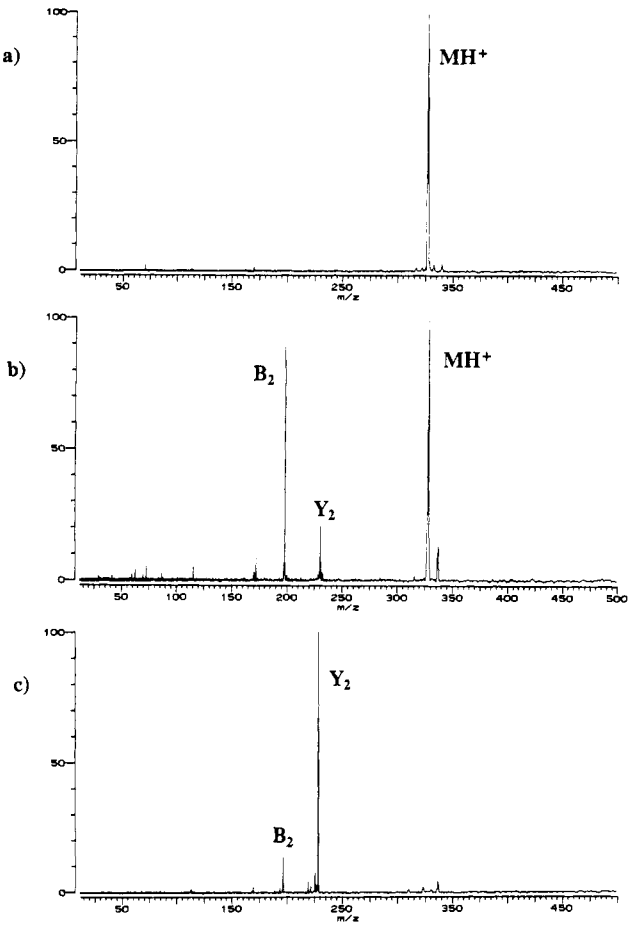
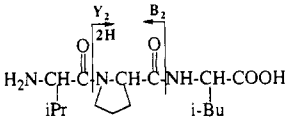


Figure 3. LD/CI mass spectra of the tripeptide, Val-Pro-Leu, using as reagent ions (a) NH_4^+, (b) $C_2H_5^+$, and (c) N_2OH^+. The interpretation of the spectra is found in the text. Ions observed in the range ±12 amu from the mass of the protonated molecule are presumably due to sample impurities.

Scheme II



197) and Y_2 (m/z 229) ions, using the Roepstorff designation (*15*). Scheme II illustrates the origin of the two ions. The Y_2 fragment is formed by a hydrogen rearrangement reaction (*16*), and the B_2 fragment is formed by cleavage of an amide bond, with charge being retained on the N-terminus portion of the molecule. A mechanism for the formation of B-type ions has not been established. The spectra show that B_2 is more abundant than Y_2 for low protonation energy ($C_2H_5^+$, ΔH = −2.1 eV) but that the intensities of the two ions reverse at higher energy (N_2OH^+, ΔH = −3.3 eV). This trend suggests that B_2 has the lower appearance energy of the two ions and that the unimolecular rate constant for the formation of Y_2, a hydrogen rearrangement reaction, escalates more rapidly with increasing internal energy of the parent than does the rate constant for the formation of B_2. This implies that the frequency factor for the formation of B_2 is smaller than that for Y_2. The data are consistent with a mechanism in which fragment B_2 is formed by a rearrangement with a "tight" transition state (*17*), and not by a direct cleavage of the amide bond, as has been suggested previously for the formation of B-type ions (*18–21*). Direct cleavage reactions have larger frequency factors than rearrangement reactions, and are kinetically favored when the reaction energy is increased (*17*). We are currently pursuing isotope-labeling experiments to elucidate the reaction mechanism for the formation of B-type ions.

CONCLUSIONS

There has been great progress recently in the capability of mass spectrometry to analyze large proteins of up to 300 kDa. The main advances have been the development of methods to desorb and ionize such large molecules, such as matrix-assisted laser desorption (*22–24*) and electrospray ionization (*25, 26*). These techniques are useful for the production of a molecular species that can be used to determine molecular weight. Obtaining sequence information still remains a challenge. Laser desorption/chemical ionization experiments can aid the structural characterization of peptides in two ways. First, they can be used to study the mechanisms of the dissociation of protonated peptides and to determine the energy dependence of the fragmentation pathways. Such information can be used to interpret complex spectra obtained from the collisional dissociation of protonated peptides, an important mass spectrometry tool for peptide sequencing (*18, 20, 27*). Secondly, they can be used as an analytical tool for determining peptide structures. As our understanding of the ultraviolet laser desorption of neutrals improves, we hope to apply LD/CI to larger peptides. This seems a reasonable expectation. By use of infrared laser desorption, large peptides neutrals including angiotensin (*28*) and insulin (*29*) have been ejected into the gas phase. The capability to control the extent of fragmentation of a molecule could make LD/CI a powerful tool for peptide sequencing.

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Protocol for Resolving Protein Mixtures in Capillary Zone Electrophoresis

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The separation of protein mixtures by capillary zone electrophoresis can be plagued by wall adsorption of the protein components, causing peak broadening and distortion. A method is presented for overcoming this problem by adding ethylene glycol to the protein sample and by choosing the running buffer and protein sample to be at different pH values and molarities. This protocol appears to work for a wide class of proteins having different molecular weights and p*I* values. The method has been applied to the analysis of proteins in human serum. Compared to the traditional method of agarose gel electrophoresis, the present method is more rapid and offers better resolution, suggesting its potential as a clinical diagnostic of certain disease states.

INTRODUCTION

The recent technology of capillary zone electrophoresis (CZE) has been shown to separate many types of molecules quickly and efficiently (*1-3*). Jorgenson and Lukacs (*4*) noted in the early stages of (CZE) development that proteins posed a particular problem; i.e., many proteins had a tendency to stick to the walls of the capillary. The resulting slow adsorption and desorption kinetics caused extensive tailing in the protein peaks.

A number of solutions to this problem have been proposed and implemented, among which are the use of buffer pHs higher than the p*I* of the proteins (*5*), the use of low pH buffers (*6*), the coating of the inside walls of the capillary with materials that slow or eliminate electroosmotic flow (*4, 7-9*), and coatings combined with very low pH buffers (*10*).

We report here an alternative method that appears to be quite effective for a wide class of proteins. This procedure circumvents the difficulties of preparing coated capillaries and of using buffers with pH values so extreme that the denaturation of the protein sample may become significant. As one example of the utility of this protocol, we apply it to the separation of human serum proteins.

As early as 1937, the improved moving-boundary electrophoresis apparatus developed by Tiselius was applied to the investigation of serum proteins (*11, 12*). Since that time, numerous applications of electrophoresis to physiological studies of clinical significance have been made by using not only moving-boundary electrophoresis (*13*), but many forms of solid matrix media including paper (*14*), cellulose acetate (*15*), and gels (starch (*16*), agar (*17, 18*), agarose (*19*), and polyacrylamide (*20*)). Agarose gel electrophoresis, as it is now used in most clinical laboratories, is a qualitative screening method useful for the detection of abnormalities of the major proteins. It quickly prompts the pathologist to seek additional confirmation of such diseases as liver cirrhosis, inflammatory response, acute nephritis, biliary obstruction, membranoproliferative glomerulonephritis, systemic lupus erythematosis, hypogammaglobulinemia, and myeloma (*19*). In the case of myeloma, it is necessary, at present, to perform additional immunologic procedures in order to determine the classification, e.g., to see whether the κ chain or the λ chain of the IgG is involved. If resolution of the γ region could be improved so that the various components could be separated, it might add confirmation to immunological procedures. We use a commercially available, automated capillary electrophoresis instrument. Our method provides rapid analysis time, has high sensitivity and good reproducibility, and requires very small sample volumes.

EXPERIMENTAL SECTION

Instrumentation. The CZE instrument used in this work is the automated PACE 2000 (Beckman Instruments, Inc., Palo Alto, CA) controlled by an IBM PS/2 50SX computer fitted with PACE software (Beckman Instruments, Inc.), running in a WINDOWS (Microsoft, Redmond, WA) environment, and System Gold (Beckman Instruments, Inc.). The System Gold software will integrate the area under each peak so that these area numbers can be printed along with peak heights, retention times, and height/area ratios. The electropherogram can be printed showing retention times at each peak and the names of the peaks if that option is chosen. While quantitation in terms of peak areas is easily accomplished, it would take additional software to provide grams per deciliter in the serum protein runs.

The capillary cassette used was fitted with a 75 μm i.d. fused-silica column, 37.5 cm in length (30.5 cm to the detector). Injection of sample was by pressure for 2 s avoiding any bias problems that may occur with electrokinetic injection (*21*). On-column detection was performed by UV absorption at 200 nm. Electrophoretic runs were made by using an applied voltage of 10 kV, and the temperature was controlled at 20 ± 0.1 °C.

Reagents. α-Lactalbumin, carbonic anhydrase, β-lactoglobulin A, chicken egg albumin, bovine serum albumin, chicken ovalbumin, jack bean urease, α-chymotrypsin (bovine pancreas), and DL-dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, MO). Ribonuclease A was obtained from Cooper Development (Menlo Park, CA). Ethylene glycol was purchased from J. T. Baker (Phillipsberg, NJ). Whole blood was drawn from a willing, male member of this laboratory at various intervals and used to prepare serum. Pooled serum (male) was obtained from Sigma Chemical Co. Frozen serum samples from male and female

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