Anal. Chem. **1999,** *71,* 5335−5339

A Microcoil NMR Probe for Coupling Microscale HPLC with On-Line NMR Spectroscopy

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An HPLC NMR system is presented that integrates a commercial microbore HPLC system using a 0.5-mm column with a 500-MHz proton NMR spectrometer using a custom NMR probe with an observe volume of 1.1 μL and a coil fill factor of 68%. Careful attention to capillary connections and NMR flow cell design allows on-line NMR detection with no significant loss in separation efficiency when compared with a UV chromatogram. HPLC NMR is performed on mixtures of amino acids and small peptides with analyte injection amounts as small as 750 ng; the separations are accomplished in less than 10 min and individual NMR spectra are acquired with 12 s time resolution. Stopped-flow NMR is achieved by diversion of the chromatographic flow after observation of the beginning of the analyte band within the NMR flow cell. Isolation of the compound of interest within the NMR detection cell allows multidimensional experiments to be performed. A stopped-flow COSY spectrum of the peptide Phe-Ala is acquired in 3.5 h with an injected amount of 5 μg.

Nuclear magnetic resonance (NMR) spectroscopy is unsurpassed as an analytical tool to determine structures of small molecules but requires relatively pure samples. High-performance liquid chromatography (HPLC) is a powerful tool to separate complex mixtures into their individual components. The on-line combination of HPLC separations with NMR detection represents a convenient and practical union that can harness the individual strengths of both techniques.

Stopped-flow HPLC NMR was first demonstrated in 1978 by Watanabe and Niki,[1] with on-line HPLC NMR soon demonstrated by Bayer et al.[2,3] Several of the early applications included the analysis of aromatic fuel samples[4] and the characterization of biomolecules.[5] With recent advances in specialized NMR flow probes, solvent suppression techniques, and the availability of higher field strength magnets, HPLC using standard 4.6-mm-diameter columns with on-line NMR has been applied to a wide variety of analytes. For example, natural products,[6−8] drug metabolites,[9−15] and drug impurities[16] have been assayed using this hyphenated technique. Most commercial NMR flow cells have volumes between 60 and 240 μL using 3−5-mm radio frequency (rf) coils around the detection cell. When >30-μg amounts of an analyte are present, on-line NMR acquisition becomes practical, and for smaller amounts, stopped-flow acquisitions are possible. However, to achieve these detection levels, elution times must be greatly increased compared to that of typical HPLC separations, thereby improving NMR residence times and thus the effective NMR sensitivity. As one example, increasing the residence time of the analyte in the NMR detection cell 10-fold increases the effective NMR sensitivity 3-fold. Stopped-flow approaches can be used to further increase the residence time of a selected peak to achieve even greater increases in signal-to-noise ratios. Most current HPLC NMR methods use low flow rates/long analysis times to increase signal-to-noise ratios but do so at the expense of chromatographic performance.[17−19]

By scaling down the HPLC system from a standard column diameter (4.6-mm i.d.) to microbore or smaller scale (≤1-mm i.d.),

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10.1021/ac990876s CCC: $18.00
Published on Web 10/26/1999

improved separation efficiencies can be obtained, thus providing higher analyte concentrations in the eluted bands. In addition, the solvent consumption is decreased by a factor of 100 or more, thereby making the use of deuterated solvents economically feasible. For an effective reduction to 0.5-mm columns, the NMR detection cell must have an improved mass sensitivity as the volume of the separated analyte band passing through the cell is considerably reduced. Solenoidal microcoil NMR probes with 5-nL to 1-μL detection cells offer substantially greater mass sensitivity compared to conventional NMR probes[20] and thus are well suited for detection of microscale separations.

The first example of a microbore HPLC NMR coupling was demonstrated by Wu et al.[21] A 1.0 mm i.d. × 150 mm column in a self-constructed system was used for separating a mixture of peptides and amino acids with analyte injection amounts of 50−60 μg for each component. Although the mass sensitivity of the NMR detection cell was excellent, this system had a 100-fold mismatch between the estimated analyte peak volume of ~5 μL and the NMR observe volume (V_{obs}) of 50 nL, a condition far from ideal. This mismatch was partially alleviated by a rapid pulse repetition time, as new spins flowed into the NMR detection cell for each scan. However, due to the design of the microcoil and short analyte residence time, the line widths were greater than 10 Hz, which masked the *J*-couplings that provide important information in structural elucidation.

Capillary HPLC (cHPLC), with column diameters below 0.5 mm, offers improved separation efficiencies but places more stringent demands on the detector. cHPLC NMR was first demonstrated in 1996,[22] and, since then, has been used in a number of studies.[17,18,23−25] These experiments used a custom-built cHPLC system interfaced to a custom Bruker probe with either 2- or 2.5-mm-diameter rf saddle coils operating inside a 600-MHz NMR spectrometer. A capillary insert for the NMR probe was constructed to hyphenate the cHPLC effluent to the NMR probe. NMR line widths between 1.2 and 2.5 Hz were reported with an insert observe volume between 240 and 400 nL, depending on the particular study. While the bubble-cell capillary insert is an ingenious method for interfacing the Bruker probe to the cHPLC system, its integration with the 2-mm saddle coil provides a filling factor of less than 1%, resulting in reduced NMR mass sensitivity.

Recently, solenoidal microcoils have been constructed that can obtain NMR linewidths of less than 1 Hz[26−31] as well as perform two-dimensional homo-[26] and heteronuclear experiments.[32,33] These microcoils have been created for detection volumes between 5 and 5000 nL, which allows for the optimization of the NMR detector flow cell to the eluted analyte volume for a variety of microseparation techniques.

The goal of this study is to design an optimized NMR probe operated at 500 MHz that interfaces with a commercial microscale HPLC system using columns between 0.3- and 1-mm diameter. Also paramount is the optimization of the NMR detection sensitivity to allow for faster experimental times. The custom probe consists of an rf coil with an observe volume of 1.1 μL and a 1.4-Hz static linewidth. A mixture containing an amino acid and small peptides was separated on a 0.5 mm × 150 mm C_{18} column with injection amounts of less than 5 μg. Continuous-flow NMR spectra were collected every 12 s with the separation requiring less than 10 min; the resulting proton spectra allowed unambiguous identification of each constituent. An on-line two-dimensional COSY spectrum of Phe-Ala was also collected in stopped-flow mode in 3.5 h with all cross-peaks readily observable.

EXPERIMENTAL SECTION

Chemicals. Alanine (Ala), glycine-tyrosine (Gly-Tyr), and phenylalanine-alanine (Phe-Ala) were obtained from Sigma Chemical Co. (St. Louis, MO) and were of reagent quality or better. Deuterium oxide (D_2O, 99.9% D) was obtained from Cambridge Isotope Labs (Andover, MA).

HPLC. A commercial microscale HPLC system with a dual-wavelength UV detector (Magic 2000, Michrom BioResources, Auburn, CA) was used in all separations. The separation column was a 0.5 × 150 mm Reliasil C_{18} with 5-μm packing, and a 500-nL PEEK loop was used for all injections. Isocratic separations of the amino acid and peptides mixture were performed using D_2O as the mobile phase-eluent. Separations were performed at ambient temperature and the mobile-phase flow rate for all experiments was 5 μL/min. All separations were monitored at 214 and 254 nm with the integrated UV absorbance detector on the HPLC system.

HPLC to NMR Probe Interface. The connections from the HPLC UV absorbance detector outlet to the NMR probe were made using a 360 μm o.d. × 100 μm i.d. fused-silica capillary (Polymicro Technologies, Inc., Phoenix, AZ). A microinjection valve (Upchurch Scientific, Oak Harbor, WA) was placed in the transfer line and was used as a flow diversion switch for stopped-flow experiments. The length of the transfer capillary from the HPLC to the microinjection valve was 100 cm and from the valve to the NMR detection cell was usually 230 cm unless otherwise indicated. The transfer capillary was joined to the 2.3 cm long × 850 μm o.d. × 700 μm i.d. detection capillary with a polyimide resin (PI-2721, HD Microsystems, Parlin, NJ). This process was carried out according to the manufacturer's instructions (Processing guidelines for PI-2700, Du Pont Electronics, Wilmington DE). Briefly, a few coats of the polyimide resin were initially applied on one clean end of the transfer line and the solvent was evaporated by heating on a hot plate at 100 °C for 10 min, followed by repositioning the larger capillary. Additional coats of the

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polyimide resin were applied at the capillary junction followed by repeated heating of the connected ends at 100 °C. The final cure was performed by heating the assembly at 325 °C for 1 h. The opposite end of the detection capillary was attached to a Teflon tube by a shrink-melt Teflon sleeve (Small Parts Inc., Miami Lakes, Fl).

Microcoil Probe. A 2.7-mm-long solenoid, consisting of 10 turns of 50 μm × 225 μm polyurethane-coated flat copper wire (California Fine Wire Co., Grover Beach, CA), was wound on the NMR detection capillary (700 μm i.d., 850 μm o.d.) enclosing a V_{obs} of 1.1 μL. The microcoil was impedance matched to 499.89 MHz using a balanced single resonance circuit. The microcoil was encased within a 10-mL polyethylene bottle filled with a perfluorinated, magnetic susceptibility matching fluid (MF-1, Magnetic Resonance Microsensors Corp., Savoy, IL). A more detailed description of microcoil fabrication was published previously.[26,31] Approximately 3 mm of space was left between the end of the transfer capillary and the rf coil to prevent magnetic susceptibility mismatch-induced line broadening from the transfer line−NMR cell connection. The transfer capillary was fixed in place using a polyimide polymer (as described above) with care taken to prevent any contamination of the detection region.

NMR Spectroscopy. ^{1}H NMR spectra were obtained using a Varian Inova 500 (11.7 T) 89-mm wide-bore NMR spectrometer. Data were processed on a PC platform using the NUTS software package (Acorn NMR, Inc., Fremont, CA). For the on-flow experiments, initiation of NMR data acquisition coincided with the start of the HPLC run. Twelve transients consisting of 3616 complex points over a spectral width of 3616 Hz were coadded for each spectrum in the NMR chromatogram. Typically, a total of 128 spectra were recorded for the entire separation. A pulse tip angle of 50° and a pulse repetition time of 1 s were used; hence spectra were obtained every 12 s. Prior to Fourier transform, the first four points of each free induction decay (FID) were replaced by linear prediction to give a flat baseline, and 4-Hz line broadening was applied to each FID. For signal-to-noise calculations, a line broadening of 1.4 Hz (matched filter) was used. Since the separations were carried out in D_2O, solvent suppression was not used and all spectra were acquired with the spectrometer gain set to its maximum value. Thus, while intense peaks were observed from residual protonated water and acetonitrile, no spectral distortion or clipping resulted from these resonances.

RESULTS AND DISCUSSION

The overall goal of this study involves the development and evaluation of a high-sensitivity NMR probe as an on-line detector for microscale HPLC separations. After insertion into the bore of the magnet, the probe was shimmed on the residual water resonance from the mobile phase. The Lorentzian-shaped peak had a full width-half-maximum of 1.4 Hz. The 90° pulse length was 1.6 μs with 14-dB transmitter attenuation of the 50-W amplifier. The limit of detection (LOD, defined as 3σ) for a single-scan acquisition of alanine for the most intense resonance was 96 ng. This LOD neglects sample loading and LC NMR interfacing issues and reflects the intrinsic sensitivity of the NMR probe described here. Overall, this probe compares favorably with the best microcoil probes and provides better mass sensitivity than previous HPLC NMR probes.

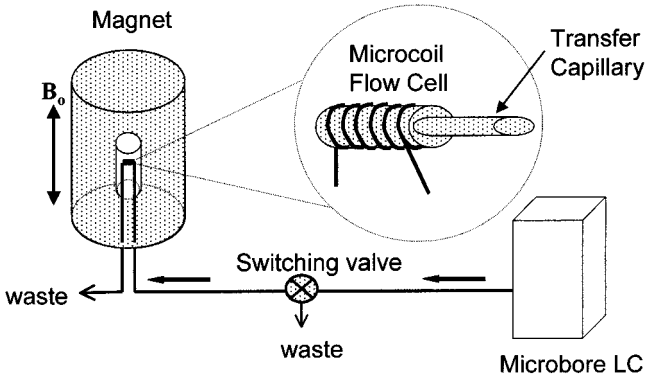

Figure 1. HPLC NMR layout. A HPLC system with a 0.5 × 150 mm C_{18} column is interfaced to a solenoidal microcoil probe. The transfer capillary is connected to the NMR flow cell with a polyimide resin.

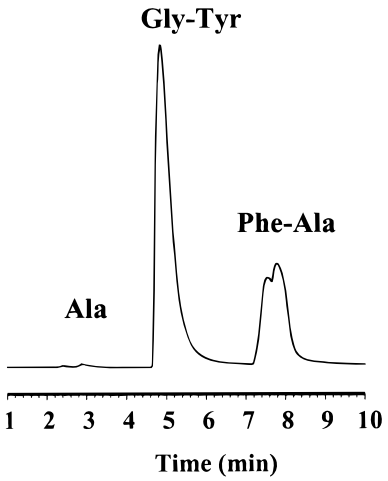

Figure 2. UV chromatogram of Ala, Gly-Tyr, and Phe-Ala detected at 254 nm. Ala is not observed as it has a low molar absorptivity at this wavelength. D_2O is used as the mobile phase with a flow rate of 5 μL/min.

The experimental setup for the HPLC NMR detection is shown in Figure 1. The distance from the HPLC system to the NMR detection coil was ~330 cm, resulting in a 27-μL transfer line volume. The assembly is modular and can be interchanged with ease. A mixture of an amino acid and two peptides was used to characterize the system. The separation was carried out in D_2O with a 500-nL injection volume at a 5 μL/min flow, producing a separation in less than 10 min with Ala eluting first followed by Gly-Tyr and Phe-Ala. The UV chromatogram of the HPLC separation of the amino acid and peptides is depicted in Figure 2, with the Ala not observable because of its low molar absorptivity at 254 nm. For the experiments shown in Figures 3A and 4, the injection contained 2.3 (26 nmol), 4.8 (20 nmol), and 4.8 μg (21 nmol) of Ala, Gly-Tyr, and Phe-Ala, respectively. Figure 3B involves lower injected amounts—750 ng of Ala, 2 μg of Gly-Tyr, and 2 μg of Phe-Ala.

Interestingly, the chromatographic "splitting" of the Phe-Ala peak shown in Figure 2 is observed when the analyte is dissolved and separated in a mobile phase containing D_2O but never when dissolved and separated in H_2O, with all other conditions being equal. The splitting of this peak is reproducible ($n = 9$) over all concentrations of injected analyte concentrations. Possible causes of the splitting of the Phe-Ala peak may be density differences

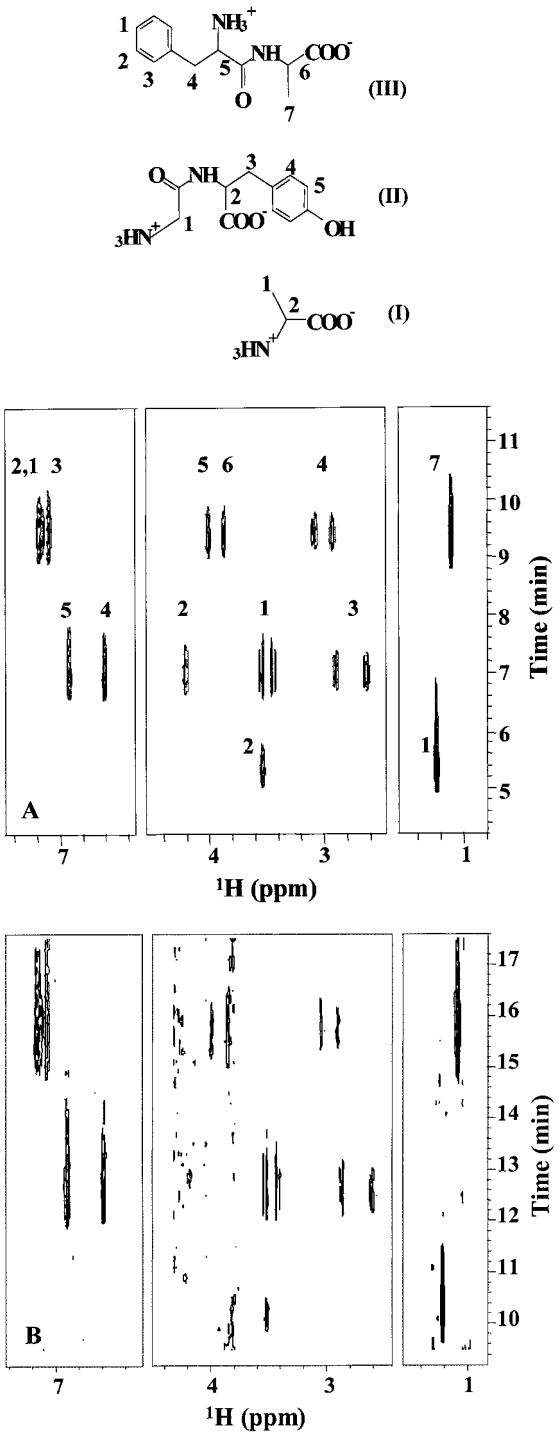

Figure 3. HPLC NMR chromatogram. Each NMR spectrum represents 12 coadded scans acquired in 12 s. No solvent suppression scheme was used, and all spectra were acquired with the spectrometer gain set to maximum. (A) Injected amounts: 2.3 (26 nmol), 4.8 (20 nmol), and 4.8 μg (21 nmol) of Ala, Gly-Tyr, and Phe-Ala (I, II, III), respectively. (B) Injected amounts: ~8 nmol of each component.

between H_2O and D_2O, different partitioning coefficients for the different mobile phases, and even factors related to H−D exchange; further work involves investigating the causes of the peak splitting.

The on-line, continuous-flow NMR spectra are shown as two-dimensional contour plots (NMR chromatograms) in Figure 3. The *x*-axis represents the ^{1}H chemical shift with the *y*-axis

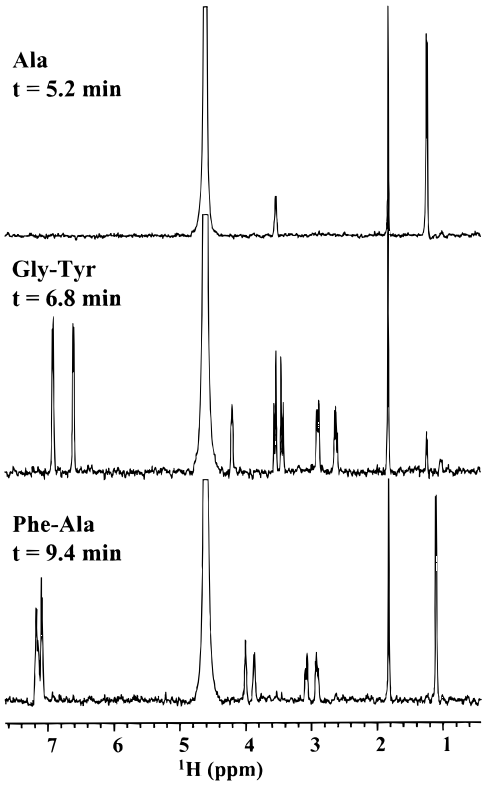

Figure 4. Extracted NMR spectra from the NMR chromatogram shown in Figure 3A. The maximum SNRs with a matched filter (1.4-Hz LB) in the Ala, Gly-Tyr, and Phe-Ala spectra are 167, 45, and 67, respectively.

indicating the elution time. The two chemical shifts (1.83 and 4.60 ppm) corresponding to solvent residences were eliminated from the plot for the sake of clarity. Figure 3B illustrates a NMR chromatogram near the LOD of the combined LC NMR system, with 750 ng of Ala injected onto the column. While the HPLC NMR chromatogram contains several extraneous peaks, the compounds can still be identified at these levels. The time axis in Figure 3B is longer than in Figure 3A as an extended transfer line was used without the switching valve. By examination of the chemical shifts and *J*-splittings, all peaks were readily assigned as indicated.

The NMR chromatogram appears similar to the UV trace acquired by the integrated absorbance detector, with a separation efficiency of 4000 theoretical plates (calculated by using the aliphatic proton signal from Phe-Ala). While many classes of biological compounds can be detected by UV absorbance, some compounds absorb poorly or not at all. NMR can detect species that contain spin $^1/_2$ nuclei such as protons and ^{13}C; thus, it is truly a universal detector for organic compounds. Because Ala absorbs poorly at 254 nm, a wavelength more suitable for aromatic species, it was not detected using absorbance detection but was easily detected using NMR. Incorporating UV absorbance in addition to the NMR detector allows an early indication of separation efficiency and concentration. This detector pair will prove useful in the assay of unknown low-molecular-weight compounds and is helpful in optimization of the system.

Individual NMR spectra were extracted from the HPLC NMR chromatogram in Figure 3A and plotted in Figure 4. The peak at 4.6 ppm resulted from residual water in the mobile phase and

the peak at 1.83 ppm was from acetonitrile, a remnant from previous use of the HPLC system. Interestingly, even after careful and extended drying of the solvent reservoirs and purging the LC system with D_2O (running solvent through the LC column and detector cell for more than 24 h), residual acetontrile resonances are observed. This carry-over indicates one problem with many commercial LC systems in terms of the ability to completely change solvents without leaving any residual traces of prior solvents. In addition to solvent carry-over by the gradient system, we also observe a chromatographic degradation of the most intense peaks, likely due to mixing at the capillary NMR−flow cell interface, so that current research is focused on reducing this effect.

With a flow rate of 5 μL/min, the average residence time for each molecule in the flow cell is ∼12 s. Hence, the broadening of the NMR resonances caused by the flow rate[34] is negligible; the resolution is limited by the 1-Hz digital resolution and the 1.4-Hz line width of this particular probe. The maximum SNR (with a matched filter) for the Ala, Gly-Tyr, and Phe-Ala spectra were 167, 45, and 67, respectively for their most intense peaks.

By observing the beginning of the Phe-Ala band using on-line NMR detection, the chromatographic flow through the microcoil was stopped by diverting the flow around the NMR flow cell using the switching valve (shown in Figure 1); this stops the analyte band in the observation zone of the NMR probe. The flow line from the NMR probe to the NMR outlet was then blocked with an outlet stop valve to completely trap the analyte in the probe. Using this arrangement, the COSY data shown in Figure 5 were acquired in 3.5 h. All the expected cross correlations are present. On the basis of the width of the Phe-Ala peak obtained using the UV−visible detector, less than 1 μg of the Phe-Ala was in the NMR detection region during this measurement. Previous multidimensional experiments have been carried out in stopped-flow mode in 4−15-h data acquisition times[18,24,25] using larger amounts of analytes, again demonstrating the sensitivity benefit of the high filling factor size-matched NMR probe used here.

A new microcoil-based HPLC NMR probe has been characterized for interfacing to 0.3−1.0-mm-diameter HPLC columns. For on-line HPLC NMR, spectral line widths of 1.4 Hz are obtained and injection masses as small as 750 ng are detectable, so that structures can be elucidated on submicrogram quantities. Two-dimensional COSY spectra in stopped-flow mode are obtained in shorter acquisition periods than previously reported studies using larger amounts of samples. The system setup is flexible, can be interchanged with other commercial HPLC systems easily, and can be optimized for smaller elution volumes by changing the volume and dimensions of the NMR flow cell.

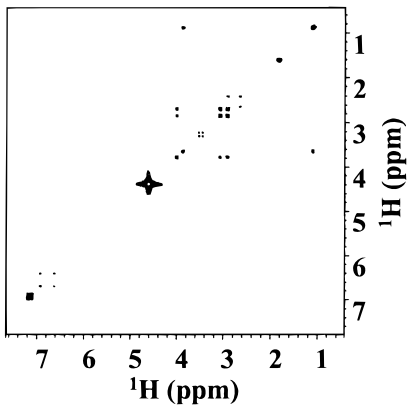

Figure 5. Stopped-flow Phe-Ala COSY spectrum. A 64 transient/spectrum, 1024 × 128 two-dimensional data set was acquired in 3.5 h for the separation shown in Figure 3A. On the basis of the UV peak widths, less than 1 μg of the analyte is present in the V_{obs}.

ACKNOWLEDGMENT

We appreciate the assistance and technical support of Michael E. Lacey, Dr. Paul Molitor, and Billy McNeill. The work was supported by a grant from the National Institutes of Health (PHS 1 R01 GM53030-01). Spectra were obtained using facilities provided by the Varian Oxford Instruments Center for excellence in NMR (VOICE) laboratory at the School of Chemical Sciences at UIUC.

Received for review August 4, 1999. Accepted September 23, 1999.

AC990876S

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