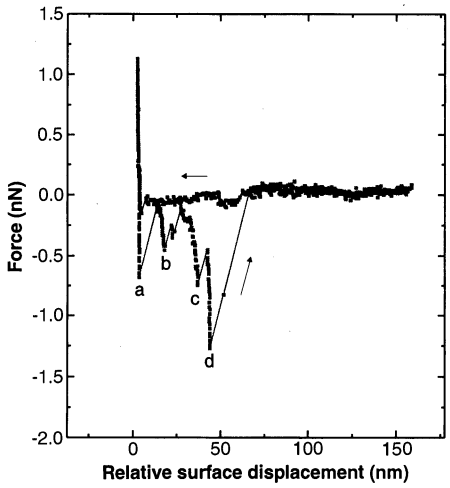

Fig. 4. Force versus relative surface displacement measured between a C_{20} surface functionalized with poly(I) and a C_{20} probe under standard conditions. Rupture forces, periods, and lengths for the individual polymers are as follows: a, 0.6 nN, 0.63 s, 0 nm; b, 0.4 nN, 0.57 s, 14 nm; c, 0.7 nN, 0.86 s, 33 nm; and d, 1.2 nN, 0.92 s, 40 nm.

as illustrated by the force curve shown in Fig. 4. Striking features of this force curve are the multiple rupture points with varying rupture forces. The differing rupture lengths are the result of the wide distribution length of poly(I) and the curvature of the spherical probe. The fact that poly(I)–C_{20} base pairing is not unique but varies from 20 base pairs down to the minimum number of bases that are thermodynamically stable is responsible for the variation in rupture force. The multiple discrete rupture points within a given force curve illustrate the monomolecular nature of these measurements.

These results, the quantitative measurement of the force necessary to rupture the DNA double helix conformation and the elasticity of single-stranded DNA, demonstrate that the AFM can be used to study both the inter- and the intramolecular interactions of complex biological and synthetic macromolecules with atomic force and displacement resolution. Furthermore, this study demonstrates that the AFM has the analytical capacity to detect the presence and relative position of specific base sequences with angstrom resolution.

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12. A slow loss of activity over several hours was observed and is attributed to noncovalently adsorbed oligonucleotides that are transferred from surface to probe [L. A. Chrisey *et al.*, *Mater. Res. Soc. Symp. Proc.* **330**, 179 (1994)].
13. The melting temperature (T_m) of the oligonucleotides may be estimated with the approach of L. A. Marky and K. J. Breslauer [*Biopolymers* **26**, 1601 (1987)]. The T_m of the 12- and 8-mer in 0.1 N NaCl at an oligonucleotide concentration of 10^{-4} M is 48° and 21°C, respectively. Stable double helices will rapidly start to form at temperatures below T_m; therefore, we expect to observe the 12-mer but not the 8-mer interchain interaction under the experimental conditions (27°C). The oligonucleotide concentration was estimated to be $\approx 10^{-4}$ M assuming that (i) 10 active molecules are immobilized within the ≈900-nm² probe contact area (*6*) and (ii) a 100-nm distance should be used to calculate the volume element of the active molecules.
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Use of Taylor-Aris Dispersion for Measurement of a Solute Diffusion Coefficient in Thin Capillaries

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A method for the fast measurement of the diffusion coefficients of both small and large molecules in thin capillaries is reported. The method relies on Taylor-Aris dispersion theory and uses standard instrumentation for capillary zone electrophoresis. With this equipment, which consists of thin capillaries (50 to 100 micrometers in inner diameter), an injection system, detector ports, and computer data acquisition, a sample plug is pumped through the capillary at known velocity and the peak dispersion coefficient (D^*) is measured. With the experimentally measured values of D^* and flow velocity, and knowledge of the inner diameter of the capillary, the molecular diffusion coefficient (D) can be rapidly derived. For example, for ovalbumin a D value of 0.759×10^{-6} square centimeter per second is found versus a tabulated value of 0.776×10^{-6} square centimeter per second (error, 2 percent). For hemoglobin a D value of 0.676×10^{-6} square centimeter per second is obtained versus a literature value of 0.690×10^{-6} square centimeter per second (error, 1.5 percent).

The idea of using the dispersion of a solute plug in a laminar Poiseuille flow for measurements of the diffusion coefficient of solute molecules was presented in a classic paper of Taylor (*1*). This method is attractive as it offers a possibility for a fast evaluation of diffusion coefficients and thus estimation of the effective dimensions of the particles in a solution (the Stokes radius). However, this method has not been brought into everyday practice. This report concentrates on a practical application of Taylor's method for measuring diffusion coefficients in a wide range of soluble substances, from ionic solutions to proteins.

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Thin fused-silica capillaries 50 to 100 μm in inner diameter were used to obtain fast (within minutes) evaluation of the diffusion coefficients. No special precautions to avoid convective flows or to maintain constant temperature during the run were necessary to obtain agreement with literature results.

The velocity of the fluid flowing slowly through a tube (laminar flow) varies over the tube cross section. For a cylindrical tube with a circular cross section, the velocity is a parabolic function of the radius, reaching its maximum at the tube axis and approaching zero at the tube wall (*2*). Solute particles introduced into the flow move with the fluid along the tube axis with different velocities corresponding to their positions at the tube cross section. Moreover, molecular diffusion redistributes them over both the cross section and the tube axis. Interaction of the nonuniformity of the fluid velocity and molecular diffusion leads to a specific mechanism of dispersion called "Taylor's dispersion." The approximate analysis of Taylor (*1*) led to a conclusion that, under certain conditions (*3*), the mass flux along the tube axis may be represented as a sum of a convective part and a diffusion part given by Fick's law with an apparent diffusion coefficient D^*

$$Q = UC_m + D^*\frac{\partial C_m}{\partial x} \quad (1)$$

where Q is the mass flux along the tube axis, U is the mean velocity, C_m is the mean analyte concentration over the tube cross section, and x is the distance along the axis.

The apparent diffusion coefficient D^*, which will later be called the "dispersion coefficient," is related to the tube radius, the mean velocity of the fluid, and the coefficient of the molecular diffusion. Aris (*4*) extended the theory of Taylor (*1*) and found the following expression for D^*

$$D^* = D + \frac{R^2U^2}{48D} \quad (2)$$

where D is the coefficient of molecular diffusion and R is the tube radius. Equation 2 provides a basis for obtaining the molecular diffusion coefficient of a solute molecule through an experimental measurement of its dispersion coefficient D^* in a fluid flow of known mean velocity flowing in a tube of known radius.

A limitation imposed by Taylor on the validity of his theory (*1*, *3*) and not overcome by Aris's treatment was that the migration time of the solute through the longitudinal region where the mean concentration changes substantially should be longer than the characteristic diffusion time (*5*). That is, relatively narrow bore capillaries and slow flows should be used for precise measurements.

The theory of Taylor was experimentally verified by himself (*1*) and by other workers (*6*–*8*). Taylor measured the dispersion of potassium permanganate solutions in water flowing through a tube. His experimental results qualitatively confirmed the theory, but quantitative agreement between his measured diffusion coefficient and that found in the literature was not achieved. Further experimental work showed good agreement between measured dispersion and theoretical predictions even for relatively high fluid flow velocities. Good agreement between the theoretically predicted dispersion coefficient and experimentally measured values was found for nitrogen-ethylene mixtures (*8*). The experimentally found coefficient of molecular diffusion differed by only 0.7% from that obtained from viscosity measurements. However, the agreement for other mixtures was not so close as a result of limitations associated with the detector used in this study. Subsequently, the theory of Taylor and Aris was generalized (*9*, *10*), and the time dependence of the dispersion coefficient was shown to be negligible for times longer than $0.5R^2/D$ (*10*).

Thus, work done by the early 1970s showed, both theoretically and experimentally, that Taylor's theory might be successfully applied to molecular diffusion measurements. However, the method has not been generally applied, probably because of difficulties with detection and a need for rather narrow bore capillaries (*11*).

In our experiments we used commercially available narrow bore fused silica capillaries and a standard instrument for capillary electrophoresis, equipped with a pump, an injection system, and an ultraviolet (UV) detector connected to a computer for data storage and processing. We used this system for measurements of the dispersion coefficients D^* of both small and large molecules (potassium hydroxide, citric acid, DL-phenylalanine, DL-tryptophan, hemoglobin, and ovalbumin). We determined the inner diameters of the capillaries, necessary for calculation of D (Eq. 2), by weighing a drop of mercury filling a certain length of the capillary.

A capillary was initially filled with distilled water or a buffer solution, and then we injected the initial sample plug by inserting the edge of the capillary into the vessel containing the sample and lifting this edge, thus allowing the solute to enter the capillary during some time. Thereafter, the edge of the capillary was returned to the vessel containing the pure solvent and a pressure drop was applied to the ends of the capillary, and the fluid slowly flowed through the capillary. A UV detector was positioned at a distance ℓ from the injection point. The UV detector monitored the UV absorbance signal, giving an elution profile $C(t)$, where C is the amplitude of the signal and t is time. The elution profile in a digital form was stored on a computer hard disk. The length of the initial plug, controlled by the time of the sample injection, was small in comparison with the distance ℓ.

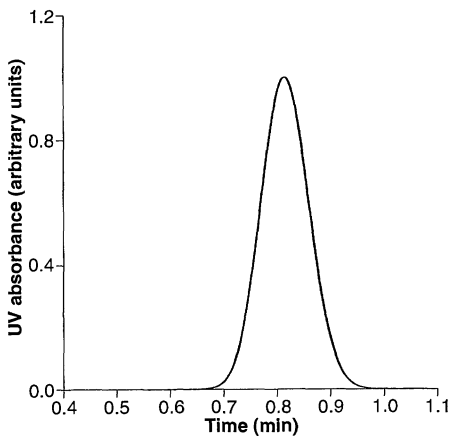

Fig. 1. Five superimposed elution profiles of 0.25% DL-phenylalanine obtained in five consecutive runs. Between runs the capillary was rinsed with distilled water for 2.5 min. The capillary was 98.8 μm in diameter, the length of the capillary was 1 m, and the distance between the injection point and the UV detector was 0.927 m. A Waters Quanta 4000 (Millipore; Milford, Massachusetts) capillary zone electrophoresis unit was used throughout. We injected all samples by lifting the edge of the capillary by 9.8 cm during 30 s. The liquid was pumped through the capillary by a pressure drop of 5.53×10^4 N/m^2 in all experiments. The wavelength used was 214 nm.

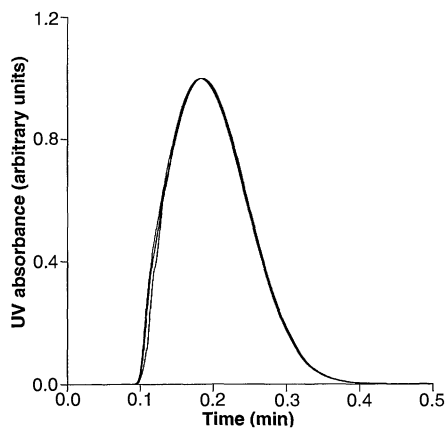

Fig. 2. Five superimposed elution profiles of 1% ovalbumin (hen's egg) solution in a 100 mM tris-borate buffer, pH = 8. The inner diameter of the coated capillary was 75 μm, the total length was 0.398 m, and the distance between the end of the capillary and the UV detector was 0.328 m. The wavelength used was 214 nm.

We numerically filtered the elution profile by a moving-bar nine-point filter; thereafter, the zero moment (M_0) and normalized first (m_1) and second (m_2) moments were calculated according to

$$M_0 = \int_0^\infty C(t)dt$$

$$m_1 = \frac{1}{M_0}\int_0^\infty C(t)t\,dt$$

$$m_2 = \frac{1}{M_0}\int_0^\infty C(t)(t - m_1)^2 dt \qquad (3)$$

According to the Taylor-Aris theory, the distribution of the mean concentration over the capillary axis becomes Gaussian after a relatively short time. We assume that this occurred in our case. For a Gaussian profile, the time moments (Eq. 3) are related to the dispersion coefficient as follows (*12*)

$$m_1 = \frac{\ell}{U} + \frac{2D^*}{U^2}$$

$$m_2 = \frac{2D^*}{U^3}\ell + \frac{8D^{*2}}{U^4} \qquad (4)$$

These equations allow for determination of both ℓ/U and D^* as

$$\frac{\ell}{U} = \frac{m_1}{2}\left(3 - \sqrt{1 + \frac{4m_2}{m_1^2}}\right) \qquad (5)$$

$$D^* = \frac{1}{2}U^2\left(m_1 - \frac{\ell}{U}\right) \qquad (6)$$

Finally, Eq. 2 is applied to calculate the coefficient of molecular diffusion. To compare our diffusion coefficients with those given in the literature for a certain temperature, we read the temperature in the box containing the capillary from the instrument display. It was assumed that the coefficient of molecular diffusion obeys Einstein's relation and thus is proportional to the absolute temperature and inversely proportional to the solution viscosity (*13*).

An example of a low molecular mass substance having a well-known diffusion coefficient is DL-phenylalanine. The fused silica capillary used had a nominal inner diameter of 100 μm. Five consecutive runs performed with DL-phenylalanine diluted in water are shown in Fig. 1. All five peaks coincide, thus demonstrating excellent reproducibility. The experimentally found value of the molecular diffusion coefficient in nine runs with a 98% confidence interval is $D_{exp} = (0.708 \pm 0.012) \times 10^{-5}$ cm^2/s, whereas the literature value is $D_{tab} = 0.705 \times 10^{-5}$ cm^2/s (*14*). Therefore, the relative difference between the value found by Taylor's approach and the known value is $\epsilon = 0.43\%$, where $\epsilon = |D_{exp} - D_{tab}|/D_{tab}$.

Another example of a well-characterized macromolecule is ovalbumin. In this case, it was necessary to use a coated capillary (*15*) to avoid interaction of the protein with the capillary surface. The results of a series of five diffusion measurements with a 1% ovalbumin solution on a 100 mM tris-borate buffer, pH = 8, are shown in Fig. 2. In this case also, the peaks almost coincide, demonstrating the very high reproducibility of the method. The peak shape in this case differs from Gaussian, which is characteristic of Taylor-Aris dispersion at the earlier stage of the peak elution (*10*). The experimentally found diffusion coefficient of ovalbumin is $D_{exp} = (0.759 \pm 0.014) \times 10^{-6}$ cm^2/s, whereas the previously found value is $D_{tab} = 0.776 \times 10^{-6}$ cm^2/s (*16*). The relative difference between experimental and tabulated value is $\epsilon = 2\%$.

Results of diffusion measurements = for various substances in capillaries of different inner diameters are presented in Table 1. It can be seen from these data that, whereas the agreement between experimental and tabulated values for KOH is good, the relative difference ϵ for citric acid is rather high. This illustrates an essential limitation of the method: it cannot be applied to a substance having a diffusion coefficient that depends strongly on concentration. In fact, citric acid has three degrees of dissociation, and, as the diffusion coefficient depends on the degree of dissociation, it strongly depends on the concentration of the solution.

Another effect seen from the analysis of the data in Table 1 is the interaction of the solute molecule with the capillary wall in bare, fused silica capillaries. The lower the capillary inner diameter, the higher should be the effects of the wall-molecule interaction. The difference between measured and tabulated values is low for DL-phenylalanine obtained in capillaries of 75- and 100-μm inner diameter, whereas it increases 10-fold in a capillary of 50-μm inner diameter. The data for DL-tryptophan give an additional illustration of the analyte-wall interaction. In this case, the use of a capillary with 100-μm inner diameter is important for obtaining correct results.

Knowledge of the diffusion coefficient of macromolecules is very important in biochemical and biophysical analysis because this quantity is related to the mass and shape of these substances. In the 1950s, Stokes (*17*) proposed a method based on a diffusion cell having a porous disk separating the pure solvent from the solution. The method gave only relative, not absolute,

Table 1. Experimental measurements of the diffusion coefficients based on the use of Taylor-Aris dispersion; ID, inner diameter of the capillary; SD, standard deviation for the diffusion coefficient, found in nine runs. Values D_{tab} have an accuracy of 0.1%.

Sample	ID (μm)	Solvent	D_{exp} (10^{-6} cm^2/s)	SD (10^{-6} cm^2/s)	D_{tab} (10^{-6} cm^2/s)	ϵ (%)
KOH, 1.05 M*	75	Water	27.1	0.8	27.2	0.55
Citric acid, 0.1 M	75	Water	6.8	0.1	6.6	3.2
DL-Phenylalanine, 0.25% w/w	75	Water	7.08	0.1	7.05	0.44
DL-Phenylalanine, 0.25% w/w	98.83	Water	7.08	0.1	7.05	0.43
DL-Phenylalanine, 0.25% w/w	50	Water	6.77	0.24	7.05	3.88
DL-Tryptophan, 0.23% w/w	75	Water	6.31	0.043	6.59	4.26
DL-Tryptophan, 0.23% w/w	98.83	Water	6.51	0.033	6.59	1.3
Hemoglobin†, 0.5%	75 (coated)	Tris-borate (pH = 8)	0.676	0.007	0.69	1.5
Ovalbumin‡, 1%	75 (coated)	Tris-borate (pH = 8)	0.759	0.014	0.776	2

*The profile of KOH was detected by means of the refractive index gradient. †From a normal human adult. ‡From hen's egg.

data. Another method much in vogue then was the use of the analytical ultracentrifuge, by which, on the same time photograms used for measuring the sedimentation coefficient (by monitoring the movement of a boundary through Schlieren optics), one could also assess the diffusion coefficient (*18*).

More recent methods for measuring diffusion coefficients of macromolecules are based on dynamic laser light scattering (*19*). Clearly, all these methods require dedicated equipment and are not easily available to scientists in nonspecialized laboratories. The present method is based on commonly available equipment (an instrument for capillary zone electrophoresis or just a capillary, pump, and UV detector), now standard in most biochemical laboratories, and allows an easy and reproducible determination of D values for both small analytes and macromolecules. However, a relatively large difference between D_{exp} and D_{tab} for macromolecules indicates that slower flows should be used in such cases.

The present method allows for a quick and precise estimation of the molecular diffusion coefficient and, thus, of the radius of a molecule in a wide range of molecular mass values and might be useful in a larger number of chemical and biochemical laboratories than in the past. An additional advantage of this application of Taylor's approach is that only small volumes of solution (fractions of nanoliters) are required.

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5. The characteristic diffusion time for a solute in a tube is, by order of magnitude, the time necessary for a solute particle to diffuse over the capillary cross section given by R^2/D. In (*1*) it was defined as the time necessary for a radial nonuniformity to reduce its amplitude e times and was found to be given by $R^2/3.8^2 D$.
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13. This allowed for recalculation of the found coefficients to another temperature, provided the dependence of the viscosity on temperature is known. As diluted water solutions or distilled water was used, the viscosity dependence on temperature was assumed to be that of water in all the cases [*CRC Handbook of Chemistry and Physics* (CRC Press, Boca Raton, FL, 1987), p. F-37].
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Synthesis of Proteins by Native Chemical Ligation

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A simple technique has been devised that allows the direct synthesis of native backbone proteins of moderate size. Chemoselective reaction of two unprotected peptide segments gives an initial thioester-linked species. Spontaneous rearrangement of this transient intermediate yields a full-length product with a native peptide bond at the ligation site. The utility of native chemical ligation was demonstrated by the one-step preparation of a cytokine containing multiple disulfides. The polypeptide ligation product was folded and oxidized to form the native disulfide-containing protein molecule. Native chemical ligation is an important step toward the general application of chemistry to proteins.

Proteins owe their diverse properties to the precisely folded three-dimensional structures of their polypeptide chains. This is the defining feature of a protein, rather than size or molecular mass per se. Merely describing the three-dimensional structure of a protein is insufficient to fully explain its biological properties. A better understanding of how structure dictates the biological properties of a protein would be achieved by systematically varying the covalent structure of the molecule and correlating the effects with the folded structure and biological function.

In this report, we describe an important extension of the chemical ligation method (*1*) to allow the preparation of proteins with native backbone structures. The principle of "native chemical ligation" is shown in Fig. 1. The first step is the chemoselective reaction of an unprotected synthetic peptide-α-thioester (*2*, *3*) with another unprotected peptide segment containing an amino-terminal Cys residue to give a thioester-linked intermediate as the initial covalent product. Without change in the reaction conditions, this intermediate undergoes spontaneous, rapid intramolecular reaction to form a native peptide bond at the ligation site. The target full-length polypeptide product is obtained in the desired final form without further manipulation. We believe that general synthetic access of this type will allow almost unlimited variation of the covalent structure of the protein molecule.

Model studies were undertaken with small peptides to investigate the native chemical ligation approach (*4*). These studies were consistent with the mechanism shown in Fig. 1, in which the initial thioester ligation product was not observed as a discrete intermediate because of the rapid rearrangement to form a stable peptide bond. Facile intramolecular reaction results from the favorable geometric arrangement of the α-NH_2 moiety with respect to the thioester formed in the initial chemoselective ligation reaction. The use of such "entropy activation" for peptide bond formation is based on principles enunciated by Brenner (*5*) and more recently adopted by others (*6*).

Study of a variety of model peptides established that native chemical ligation was generally applicable to peptides containing the full range of functional groups

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