Diffusion of Bovine Serum Albumin in Amylopectin Gels Measured Using Fourier Transform Infrared Microspectroscopy

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Received November 15, 1993; Revised Manuscript Received February 25, 1994®

ABSTRACT: A new method of characterizing diffusion in gels using Fourier transform infrared microspectroscopy is described. The system studied was bovine serum albumin (BSA) diffusing into amylopectin gels. By following the height of the amide peak of the BSA as a function of position along the sample, it is possible to build up the concentration profile. From this, the diffusion coefficient can be determined as a function of BSA concentration, amylopectin concentration, and added salt concentration. It is shown that charge effects are very important in this system. The dependence of the diffusion coefficient on the amylopectin concentration can be well fitted by a stretched exponential.

Introduction

The diffusion of spherical particles through entangled polymer solutions and gels underlies a wide range of practical problems: it is relevant to food safety (where the particle may be a pathogen), to drug delivery, and to processes involving enzymes. It is also of basic scientific interest, with the mechanisms which govern diffusion through a random collection of obstacles, which is what a gel amounts to, still being incompletely elucidated.

Recently there has been a significant amount of work devoted to understanding diffusion of probe particles through entangled polymer solutions. A picture has emerged which explains how the particle can move through the mesh of the solution, characterized by a mesh size ξ, with little hindrance if the particle is significantly smaller than ξ but with increasing difficulty as the particle size (radius R) becomes comparable with ξ.[1-7] Experimentally it is found that the data can usually be fitted by a stretched exponential dependence on concentration, with additional factors arising if charge effects become important. Additionally, it has been shown that polymer adsorption from the solution to the surface of the particle must be absent or further complications are present.[8,9]

This picture of motion through an entangled polymer solution assumes that the polymer chains are homogeneously distributed through the solvent to give a uniform mesh. This picture need not apply to polymer gels, in which the network may be made of chain aggregates. An early review of the problem of diffusion through gels is given by Muhr and Blanshard.[10] If the gel is heterogeneous, then scaling laws developed for solutions may not apply to gelled systems. There is evidence to show that this is the case in the work of Johansson et al.,[11] who showed that the diffusion of poly(ethylene glycol) was *faster* in a gel of κ-carageenan than a solution of lower concentration. The interpretation of these results was that the gel consisted of thick strands (~5 nm) of aggregated κ-carageenan molecules, whereas the solution comprised molecularly dispersed chains. The effective obstruction of the diffusion of the poly(ethylene glycol) molecules was therefore greater for the solution than for the gel.

In this paper a new methodology for studying probe diffusion through gels based on Fourier transform infrared (FTIR) microspectroscopy is presented. This approach is applied to the problem of bovine serum albumin (BSA) molecules diffusing through amylopectin gels. This system is similar to the one studied by Leloup et al.,[12,13] who used amylose rather than amylopectin as the matrix material, but by comparison the method we describe here is relatively quick and has spatial resolution of better than 100 μm when determining the concentration profile of the BSA.

Experimental Section

Amylopectin from waxy maize, bovine serum albumin (BSA), and deuterium oxide (D_2O) were all supplied by Sigma (catalog nos. A-7780, A-4378, and D-4501, respectively). Sodium chloride (NaCl) was supplied by Aldrich (catalog no. 20,443-9).

Amylopectin was ground with a pestle and mortar for ~20 min to reduce particle size. It was then mixed with 0.1 M NaCl in D_2O to form a slurry of the required concentration. This slurry was placed in an ultrasonic bath for 2 min to break up the amylopectin and then continuously stirred in a water bath at 100 °C for 5 min to form a gel. The grinding and ultrasonication of the amylopectin resulted in a homogeneous gel (as viewed by optical microscopy). Further details of the gels prepared in this way are given in ref 14. The key finding is that the gels, which have a very low modulus of $\sim 10^2$ N m^{-2}, are optically clear and hence cannot contain inhomogeneities on the length scale of light. It is concluded from the separate study of them[14] that gelation is probably occurring due to intermolecular double-helix formation, these helices not then aggregating to give the usual turbid, crystalline gels.[15,16] The addition of NaCl had no effect on the rheological properties of the gels.

The gel was placed in the refrigerator at 4 °C for 15 min to cool and then injected into a 100-μm-diameter glass capillary tube sealed at one end with paraffin wax. The tube was then suspended in a solution of BSA in NaCl in D_2O, with the exposed end of amylopectin gel immersed in the BSA solution (Figure 1). The molarity of the NaCl was 0.1 M for most of the experiments but was altered for those studying the effect of charge. The bottle containing the suspended tube was then sealed to prevent evaporation and placed in a water bath at 23 °C for a known length of time between 48 and 140 h to allow diffusion to take place. BSA concentrations from 0.4 to 3.5% were used, and amylopectin concentrations from 3 to 10.5 wt/wt %.

Once diffusion had been allowed to occur, the tube of gel was removed from the BSA solution and the exposed end sealed with silicone grease. Silicone grease forms a less permanent seal than paraffin wax (used to seal the other end of the tube before diffusion), since it flows more easily at room temperature. It was, however, possible to use it to seal the tubes for long enough to perform the required measurements. In practical terms it is easier to minimize disruption of the gel when sealing the tube with silicone grease than it is with paraffin wax.

The sealed tube was mounted in the microscope of the FTIR and adjusted until the tube was level and therefore in focus along its entire length. The FTIR instrument was a Mattson 40-20

® Abstract published in *Advance ACS Abstracts*, April 1, 1994.

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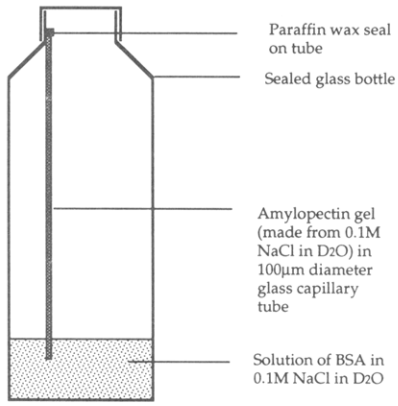

Figure 1. Schematic figure of the apparatus used for the diffusion experiment.

Galaxy FTIR spectrometer with a Spectra Tech IR-PLAN microscope attached. Detection of the IR radiation was made using a narrow-band MCT detector. Apertures were placed above and below the sample and the microscope condenser position optimized for maximum signal. Using the apertures in this way, the sample was screened from view apart from a rectangular section of dimensions 56 μm × 80 μm. The sample was aligned with this aperture, so that its axis bisected the short dimension of the aperture, thus revealing 80 μm of the length of the tube. Data on the BSA concentration were therefore obtained only from this 80-μm region. The microscope was then placed in the infrared mode and a spectrum taken of the revealed portion of sample. The range of frequencies scanned was 1300–4000 cm^{-1} and the resolution was 8 cm^{-1}. Once scanned, the sample was moved along the axis of the tube to reveal a new section of gel, and the scan repeated. In this way, the IR spectra of the gel as a function of position was obtained, and hence the diffusion profile.

FTIR Data Analysis

The IR spectra of an amylopectin gel and a BSA solution (both in a 0.1 M solution of NaCl in D_2O) are shown in Figure 2. Deuterated water is used so that the regions of interest in the IR spectrum in the vicinity of the amide bands are not swamped by the large OH absorption of normal water. In the spectrum of a mixed system of amylopectin gel and BSA in D_2O, it is possible to resolve the amide peak of the BSA. The size of the peak (Figure 2) is seen to depend on the BSA concentration. If Beer's law is valid, the height of the peak is proportional to the amount of BSA present in the mixture. The validity of this is confirmed in Figure 3, which shows the normalized height of the peaks seen in Figure 2 plotted against concentration. A linear relationship between peak height and BSA concentration is seen.

Spectra of the different samples were analyzed using the "curvefit" options in the FIRST software with which the Mattson instrument is supplied. The peaks surrounding the amide peak at 1648 cm^{-1} in the region 1300–1700 cm^{-1} were fitted to a series of Lorentzian peaks of variable height and width, but fixed position. The amide peak was fitted to a Lorentzian of fixed position and width, but variable height. A variable baseline was also included in the fit to each spectrum. The composite amide peak at 1648 cm^{-1} studied here is neither purely Lorentzian nor purely Gaussian. In theory, a combination of peaks could be used to build up the overall peak shape.[17] However, given the precision of the experiment, this analysis would result in the addition of unjustifiable extra variables in an already complex fit. Thus, a single peak which approximately fits the peak shape was used for both this peak and the surrounding multiple peaks. In practice, Lorentzian peaks served this purpose best. The same

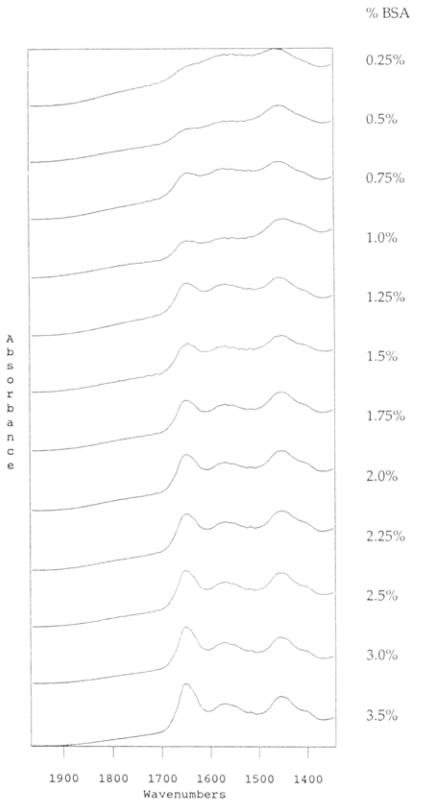

Figure 2. Respresentative spectra from a series of samples with different levels of BSA in the amylopectin gel.

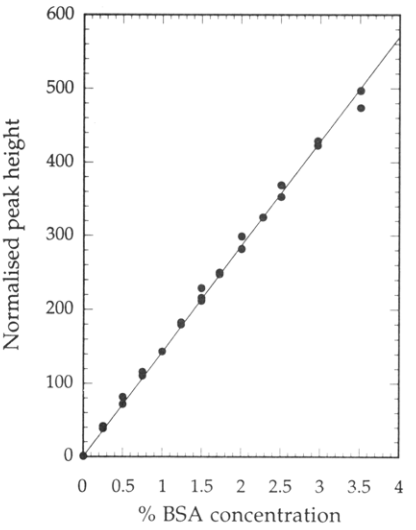

Figure 3. Normalized peak height of the BSA peak versus BSA concentration, using the spectra shown in Figure 2. The line shown is the best fit through the data points.

fitting procedure was applied to the D_2O harmonic peak at 3800 cm^{-1}. This is not a composite peak, and thus a single Gaussian peak would be expected to fit the data well, as indeed it does. The approximations inherent in this analysis are justified in the light of the calibration curve (Figure 3), which shows a linear relationship between normalized amide peak height and BSA concentration.

Since the tube diameter was not the same for each tube, a normalization was required. This was performed by dividing the height obtained for the amide peak by the height of the D_2O peak and multiplying by the concentration of D_2O. For the diffusion experiments, each tube was assumed to be of uniform diameter, and a spectrum was taken at a position well beyond the point at which no amide peak could be discerned. At this point it was

assumed that the gel was unchanged by the diffusion and that the concentration of D_2O was the same as in the original gel. The normalization factor calculated from this spectrum was used throughout the length of the tube.

The normalized height of the amide peak is proportional to the amount of BSA in the portion of the sample viewed. Thus from a plot of the amount of BSA as a function of position along the tube, diffusion profiles of BSA in amylopectin gels were obtained. The experimental conditions described correspond to diffusion into a semi-infinite medium, and the diffusion profile can therefore be described by the equation

$$\frac{c(x)}{c(0)} = \mathrm{erfc}\left(\frac{x}{2(Dt)^{1/2}}\right) \quad (1)$$

where $c(x)$ is the concentration of BSA at position x along the tube and $c(0)$ is the concentration in the bulk solution and is therefore taken as the concentration at position $x = 0$. From this, the diffusion constant of the sample, D, was calculated.

It is important to consider what kind of diffusion coefficient is being measured. In these experiments we do not have conditions leading to the measurement of a tracer or a self-diffusion coefficient; mutual diffusion is being measured. Furthermore, the system is a three-component system (even leaving aside the presence of the salt which is of the same concentration both inside and outside the gel), so that the diffusion coefficient determined can be affected by swelling of the gel as well as diffusion of the BSA. Nevertheless, despite these complications, the diffusion coefficient we are measuring is that which is practically important, i.e., the rate at which an externally applied corporeal entity (such as a pathogen) may penetrate a gel matrix. Thus the coefficient being measured is a useful quantity, even if it does not describe the simplest physical process.

Results

Figure 4 shows the spectra obtained from a 4.5% gel of amylopectin into which BSA had been diffusing from a 2.5% solution containing 0.1 M NaCl solution for 95 h. Figure 5 shows the diffusion profile obtained from analysis of the spectra and the fit to an equation of the form of eq 1. Figure 6 shows a compilation of values of D obtained in this way as a function of amylopectin concentration using the same BSA solution. Figure 7 shows the variation of D with BSA concentration when the BSA (in 0.1 M NaCl) is diffusing into gels of 4.5% amylopectin concentration.

It proved difficult, practically, to study diffusion in the absence of amylopectin. This was because without the added viscosity due to the polymer, the material tended to sink in the tube under the pressure exerted by its height and escape into the source solution. This proved a particular problem if the paraffin wax seal also sank in the tube, creating added pressure. In addition, again because of its lower viscosity, the sample in the tube was more susceptible to disruption when sealing the exposed end with silicone grease. The result was that for samples not containing polymer the reproducibility of diffusion coefficients was poor and they are not reported. Values from the literature are quoted below where necessary.

Discussion

The straight line obtained from the calibration of different concentration BSA solutions (Figure 3) validates the data analysis of the curve fitting and normalization.

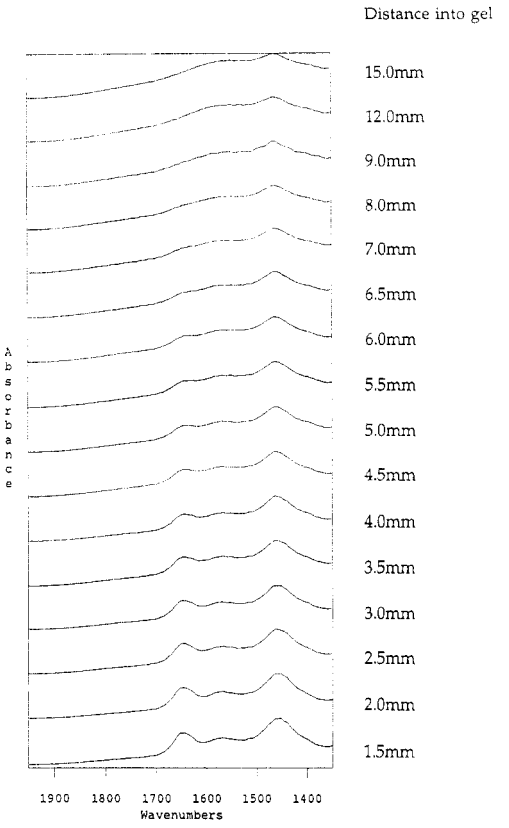

Figure 4. Spectra obtained from different positions x along the capillary containing a 4.5% gel of amylopectin into which BSA had been diffusing from a 2.5% solution for 95 h.

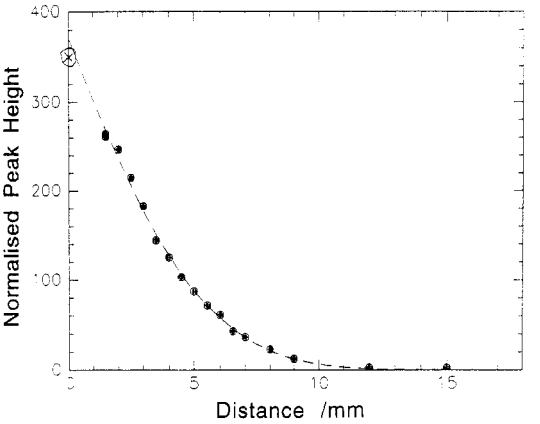

Figure 5. Diffusion profile obtained from analysis of the spectra in Figure 4. The dashed line shows the best fit to an equation of the form of eq 1, yielding a diffusion coefficient D of 2.62×10^{-11} m^2 s^{-1}.

The normalized heights of the amide peaks can therefore be taken as a true indication of the concentration of the BSA.

Looking first at the effect of BSA concentration, it is apparent that there is an effect of BSA concentration on D. At the highest values D tends to a plateau, but as the concentration drops so does the diffusion coefficient. Because of this effect of BSA concentration, the equation used for the diffusion profile (eq 1) is not strictly correct. However, since the changes in D due to BSA concentration are relatively small and since the data fit an error function remarkably well, this form of equation may be used as a first approximation to obtain a diffusion coefficient.

One possible explanation for the effect of BSA concentration is that the charge on the BSA molecules is affecting the diffusion so that mutual charge interactions (which will become more important as the concentration

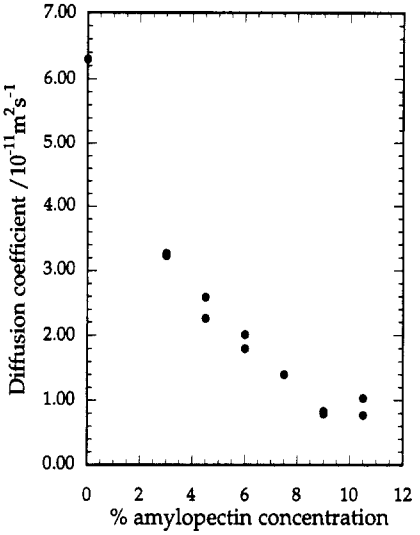

Figure 6. Dependence of D on amylopectin concentration, using 2.5% BSA in 0.1 M NaCl.

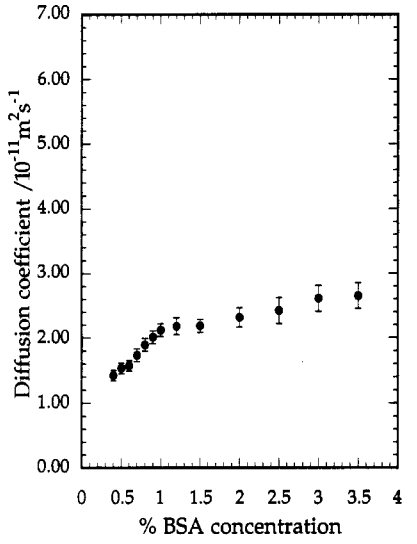

Figure 7. Dependence of D on BSA concentration (in 0.1 M NaCl) when diffusing into gels of 4.5% amylopectin concentration.

of protein increases) enhance the diffusion coefficient. To test this hypothesis the effect of changing the salt concentration in solution was explored. The results for these experiments are shown in Figure 8, where it is seen that there is a strong dependence of D on the salt concentration: D is largest when there is no screening of charge on the BSA molecules due to NaCl in solution. In this case it can be assumed that the inherent charge interactions between two BSA molecules are maximized, so that the mutual diffusion coefficient is enhanced. As ions are introduced into the solution, since no buffering of the ionic strength is present, screening of the charge interactions between BSA particles reduces the charge effect and the diffusion coefficient drops. This effect is seen in Figure 8. Now looking at the data in Figure 7, which is at a fixed ionic strength, when the BSA concentration is high (so that the average distance between protein molecules is small) the interactions are strong and the value of D is substantially larger than when the BSA concentration is lower. The rate of increase in D is seen to flatten off at around 1.5% BSA, suggesting that the effect of charge in enhancing diffusion is beginning to saturate. Although it has not been explored, it seems likely that the BSA concentration at which this change in slope is reached will depend on the ionic strength used (or equivalently pH, which has not been used as an explicit parameter in these

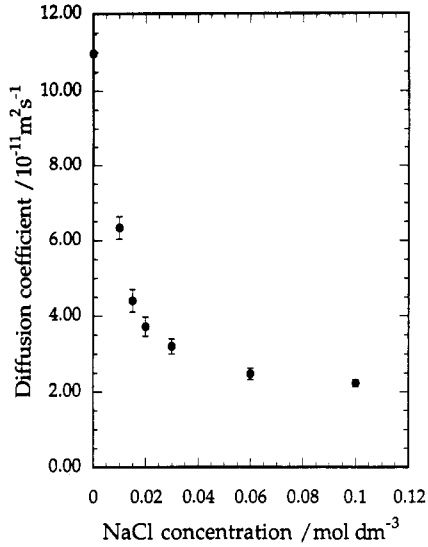

Figure 8. Dependence of D on NaCl concentration: 2.5% BSA solutions were allowed to diffuse into gels of 4.5% amylopectin concentration.

experiments). A similar increase in D with BSA concentration has been previously observed in water (BSA studied in 0.5 M KCl at pH 5 and 1 °C)[18] but, working at rather higher BSA concentrations, Keller et al.[19] observed a falloff in D in water containing 0.1 M acetate buffer at pH 4.7 and 25 °C.

For the remainder of the paper, only data based on 2.5% BSA solutions in 0.1 M NaCl will be discussed. This is in the region where D is becoming less dependent on BSA concentration, so that (since amylopectin itself is uncharged so that no additional charge effects will occur as *its* concentration is increased) changes in D will reflect changes in the ease with which the protein molecule can diffuse through the changing amylopectin matrix.

The published literature contains many studies of the variation of diffusion coefficient with gel concentration. It has been proposed from theoretical calculations[1,2] that the variation obeys an equation of the form

$$\frac{D}{D_0} = \exp(-bc^k) \qquad (2)$$

where D_0 is the diffusion coefficient in a gel of zero concentration, b is a constant which depends on the radius of the probe molecules and the molecular weight of the polymer, and c is the concentration of the gel/solution. k is a constant expected to be in the range 0.5–1.0 and whose exact value depends largely on the polymer species. Gels of rodlike polymers should have a value of k of 0.5.[2] No single value has been predicted for flexible chains, the quality of the solvent and the assumptions made in the treatment affecting the result; the experimental data for semidilute polymer solutions also show a range of values.[3,5,20,21]

The applicability of eq 2 for the data shown in Figure 6 can be tested by plotting the data on a log–log plot. A value for D_0 of 6.3×10^{11} m s^{-2} was taken from the literature, based on the value given by Wagner and Scheraga[18] and corrected for a temperature of 23 °C. A value of k for the BSA–amylopectin system is found from Figure 9 to be 0.93, a value well within the predicted range of 0.5–1.0.

The only reported values in the literature for this system consider the gels when they have been fully retrograded.[12] Gels of amylopectin were cast in open-ended syringes and were then stored at 1 °C for 21 days to allow crystallization to occur to the fullest extent for the system. The gels were then "preequilibrated" in 0.223 M acetate buffer for

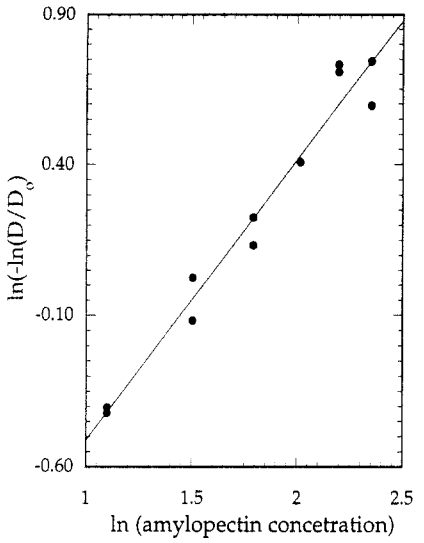

Figure 9. log–log plot of D/D_0 (for samples of 2.5% BSA in 0.1 M NaCl) against amylopectin concentration.

48 h at 25 °C and BSA (from a solution of unquoted concentration) was allowed to diffuse into the gel at 25 °C for 72 h. The gel was sectioned, the sections were weighed, and the acetate buffer was added to each slice to allow reverse diffusion. The resulting concentration of BSA in each buffer solution was measured by the Lowry–Hartree test. The value of k obtained was 1.2 ± 0.1 (a value outside the range normally considered acceptable).

The conditions of Leloup et al.'s experiment are different from those used here, which may explain the discrepancy in the results. Their values for the diffusion coefficient are consistently higher, suggesting a lower resistance to the probe diffusion. However, the drop in the coefficient with concentration is faster, which yields the higher value for k of 1.2 rather than the 0.93 obtained here, thus indicating a stronger polymer concentration dependence. The amylopectin gels of this earlier work were fully retrograded and observed in acetate buffer. We were unsuccessful in reproducing the conditions described in ref 12 without forming a highly inhomogeneous gel, both in acetate buffer and in D_2O.

In our experiments the structure of the gel appears to be homogeneous, without the development of optically sized heterogeneities. Indeed at these concentrations at room temperature, previous workers have seen no evidence of crystallization in DSC traces.[22] The difference in gel structure between the earlier work[12] and ours might be expected to result in different diffusion behavior. If a substantial proportion of the amylopectin in Leloup et al.'s gels was associated in crystalline form, a rather inhomogeneous structure is presented to the probe molecules of BSA. In the nonretrograded material the amorphous chains present a uniform obstacle to the BSA, whereas the retrograded material is likely to consist of both impenetrable crystalline material and regions of depleted amylopectin content which are much less restrictive to diffusion. Thus, the diffusion coefficient through the retrograded material is likely to be reduced by less than through the nonretrograded material since the BSA will move in the regions of lower polymer content. This effect would result in higher diffusion coefficients for the retrograded material, as is seen. However, the concentration of amylopectin will have a strong effect on the extent of retrogradation.[23] A greater proportion of the amylopectin will be present in the crystalline form at higher concentrations, and the amount of amylopectin-depleted material will therefore fall off sharply. Thus, compared with the nonretrograded gels used in our experiments, a faster drop in diffusion coefficient with gel concentration would be expected in the retrograded material, resulting in a higher value of k, as is seen. The nonuniform nature of the gel and its changing properties with concentration might also explain why the value for retrograded material lies outside that normally considered acceptable.

Leloup et al.[12] also quote the value of k for aged amylose to be 1.2, the same as for amylopectin. In their later paper,[13] they perform the same experiment on amylose but make a correction for the inaccessible part of the gel by separately measuring accessibilities. (The exact nature of this correction is not clear from the paper, but it appears to contradict the arguments put forward in a paper by Muhr and Blanshard[10] for the correct way of allowing for inaccessible regions of the gel.) This results in yet higher values of the diffusion coefficient and a much slower fall off with concentration, the value of k being quoted at 0.73. Again this can be explained in terms of retrogradation. Their correction means that only the noncrystalline material is considered and the diffusion coefficient for these depleted polymer regions is calculated. The coefficients are naturally higher than those for the amorphous polymers studied by us, since the effective polymer concentration for these regions is lower. They are also higher than those for the whole retrograded gel, since they disregard the effect of the crystalline material blocking the passage of BSA. The concentration dependence is weaker than that for the amorphous gels ($k = 0.73$ rather than 0.93) because the actual concentration of polymer in these depleted regions will not change as fast as the total polymer concentration since at higher concentrations a greater proportion of the material is incorporated into the crystalline material (retrogradation being favored by higher polymer concentration).

Thus the values of the diffusion coefficients observed in our experiments on diffusion of BSA into amorphous amylopectin are not incompatible with the only available published data on this system and the similar system BSA and amylose, if due allowance for changing experimental conditions is made.

Conclusions

A method has been developed to use the technique of FTIR microspectroscopy to study diffusion into polymer gels. The diffusion of BSA into amylopectin gels has been characterized with this method. It is seen that the homogeneity of the gels affects the diffusion coefficient and its scaling behavior. Both BSA concentration and the molarity of the sodium chloride in water solutions are seen to affect the magnitude of the diffusion coefficient. This effect is attributed to the role played by charge interactions in affecting the mutual diffusion of the BSA molecules: the greater the strength of the interactions, because of either a high BSA concentration or a low level of screening by the added salt ions, the faster the diffusion. At a fixed BSA concentration and ionic strength, the diffusion coefficient is seen to follow a stretched exponential law, with the scaling exponent for concentration found to be 0.93.

Acknowledgment. We are grateful to the AFRC for financial support and to Drs. Richard A. L. Jones, David A. Prystupa, and C. Matin Durrani for helpful discussions.

References and Notes

(1) Langevin, D.; Rondelez, F. *Polymer* **1978**, *19*, 875–882.
(2) Cukier, R. *Macromolecules* **1984**, *17*, 252.

(3) Phillies, G. D. J. *Macromolecules* **1987**, *20*, 558.
(4) Ullmann, G. S.; Ullmann, K.; Lindner, R. M.; Phillies, G. D. J. *J. Phys. Chem.* **1985**, *89*, 692–700.
(5) Ullmann, K.; Ullmann, G. S.; Phillies, G. D. J. *J. Colloid Interface Sci.* **1985**, *105*, 315–324.
(6) Brown, W.; Rymden, R. *Macromolecules* **1986**, *19*, 2942–2952.
(7) Brown, W.; Rymden, R. *Macromolecules* **1987**, *20*, 2867.
(8) Cooper, E. C.; Johnson, P.; Donald, A. M. *Macromolecules* **1991**, *24*, 5380–5386.
(9) Cooper, E. C.; Johnson, P.; Donald, A. M. *Polymer* **1991**, *32*, 2815.
(10) Muhr, A. H.; Blanshard, J. M. V. *Polymer* **1982**, *23*, 1012–1026.
(11) Johansson, L.; Skantze, U.; Löfroth, J.-E. *Macromolecules* **1991**, *24*, 6019, 6024.
(12) Leloup, V. G.; Colonna, P.; Ring, S. G. *Food Hydrocolloids* **1987**, *1*, 465–469.
(13) Leloup, V. M.; Colonna, P.; Ring, S. G. *Macromolecules* **1990**, *23*, 862–866.
(14) Cameron, R. E.; Durrani, C. M.; Donald, A. M. *Stärke*, in press.
(15) Ring, S. G.; Colonna, P.; I'Anson, K. J.; Kalichevsky, M. T.; Miles, M. J.; Morris, V. J.; Orford, P. D. *Carbohydr. Res.* **1987**, *162*, 277–293.
(16) Kalichevsky, M. T.; Orford, P. D.; Ring, S. G. *Carbohydr. Res.* **1990**, *198*, 49–55.
(17) de Koeijer, J. A.; Luinge, H. J.; van der Maas, J. H. *Vib. Spectrosc.* **1993**, *4*, 285–299.
(18) Wagner, M. L.; Scheraga, H. A. *J. Phys. Chem.* **1956**, *60*, 1067–1076.
(19) Keller, K. H.; Canales, E. R.; Yum, S. I. *J. Phys. Chem.* **1971**, *75*, 379–387.
(20) Phillies, G. D. J.; Malone, C.; Ullmann, K.; Ullmann, G. S.; Rollings, J.; Yu, L. *Macromolecules* **1987**, *20*, 2280.
(21) Phillies, G. D. J. *Biopolymers* **1985**, *24*, 379–386.
(22) Zeleznak, K. J.; Hoseney, R. C. *Cereal Chem.* **1986**, *63*, 407–411.
(23) German, M. L.; Blumenfeld, A. L.; Guenin, Y. V.; Yuryev, V. P.; Tolstoguzov, V. B. *Carbohydr. Polym.* **1992**, *18*, 27–34.