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# Molecular Dynamics of Hydrated Proteins [and Discussion]

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*Phil. Trans. R. Soc. Lond. B* 1980 **290**, 639-655
doi: 10.1098/rstb.1980.0123

*Phil. Trans. R. Soc. Lond.* B **290**, 639–655 (1980)
*Printed in Great Britain*



# Molecular dynamics of hydrated proteins

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This contribution presents the first results of an application of high-resolution quasi-elastic neutron scattering to the dynamics of protein hydration. Using two samples (film stacks and 'fluffy' powders) of biosynthetically fully deuterated C-phycocyanin extracted from blue-green algae, we have studied the Doppler-like broadening of the quasi-elastic line as a function of scattering angle $2\theta$ at several sub-monolayer $H_2O$ hydration levels. The backscattering spectrometer IN 10 at the Institut Laue–Langevin (I.L.L.), Grenoble, was employed to measure wet-minus-dry difference broadenings $\Delta E$ of up to $5 \times 10^{-3}$ cm$^{-1}$, or 0.4 μeV, for momentum transfers $k = (4\pi/\lambda_0) \sin\theta$ between 0.15 and 1.7 Å$^{-1}$ (incident wavelength $\lambda_0 = 6$ Å). The results show that $\Delta E(k)$ possesses an oscillatory structure with a first maximum between $k_{max} = 0.4$ and 0.8 Å$^{-1}$. The position of this maximum shifts to higher $k$ with increasing hydration, while its intensity increases and the following minimum at $k_{min} = 0.7$–$1.3$ Å$^{-1}$ becomes progressively more shallow. Structure factor measurements indicate that line narrowing due to structure in $S(k)$ is not the dominant mechanism in determining this oscillatory behaviour of $\Delta E(k)$. A Chudley–Elliott jump diffusion model was adopted as a working hypothesis to extract a characteristic length (water migration distance $a$) and a characteristic time (residence time $\tau_0$ at a hydration site) from the $\Delta E(k)$ data. Values of $a = 6$–$9$ Å and $\tau_0 = 5$–$30$ ns were obtained for the powder sample and are shown to agree well with the average jump distances derived from topographical considerations in conjunction with hydration number estimates.

## 1. Introduction

Neutron inelastic scattering was first used to study the hydration dynamics of biopolymers about 10 years ago (Whittemore 1968; Dahlborg & Rupprecht 1971). It had been realized for some time that radiation scattering techniques employing cold neutrons could contribute significantly to the understanding of hydration processes in complex organic materials, both because of the unique property of neutrons to resolve structural *and* dynamical detail at the molecular level, and because of the large scattering contrast between hydrogen and deuterium. Only during the past few years, with the advent of new high-resolution spectrometers on sources of greatly increased flux, has it become possible to realize some of the early expectations.

In this contribution we present the first results of an application of high-resolution quasi-elastic neutron scattering to the dynamics of protein hydration. Our procedure in this work has been to start at the low-hydration end of the sorption isotherm, i.e. to use moist powders and slightly hydrated film samples rather than proteins in solution. This was done in order first to establish the practice and interpretation of hydration difference experiments from biosynthetically deuterated macromolecules for which there is a large coherent component in the scattering, and also to make better use of the very limited instrument time available to us. A

preliminary account of this work has already been given (Randall *et al.* 1978). An earlier, exploratory experiment with a low-resolution spectrometer was carried out at A.E.R.E. Harwell (J. T. Randall & S. Gilmour, unpublished observations, 1975)

## 2. Protein hydration and dynamics

Water is an important and ubiquitous constituent of all living organisms. Many structural and functional aspects of its interaction with biological molecules have been studied by a variety of techniques ranging from calorimetry to nuclear magnetic resonance (Kuntz & Kauzmann 1974; Berendsen 1975; Packer 1976; Hopfinger 1977; Edsall & McKenzie 1978; Mathur-De Vré 1979). The thermodynamic information on protein hydration processes is provided by measurements of the entropy and enthalpy of sorption. The isopiestic method of Bull (1944; Bull & Breese 1968) is frequently used to measure sorption isotherms, and for proteins and polypeptides these are all of sigmoidal type II (Brunauer *et al.* 1938). At very low relative humidities (r.h.) the water uptake of globular proteins shows a steep rise until at *ca.* 0.40–0.80 g $H_2O$/g protein the primary hydration sites are occupied. Following this there is a 'knee' in the sorption isotherm and further increases in r.h. level lead to a steady but slower increase in water uptake.

While a limited amount of *structural* information on the water of hydration in and around protein molecules can be inferred from isotope exchange studies, infrared spectroscopy and nuclear magnetic resonance, much more detailed information comes from X-ray and neutron diffraction experiments on protein crystals. These have shown, in particular, that a small number of water molecules (3–25) are tightly held in the interior of the protein and that these must be regarded as an essential part of its tertiary and quaternary structure. A larger number (up to several hundred) are less tightly bound but still localizable at or near the molecular surface.

The currently available *dynamical* information on protein hydration at the molecular level comes from a number of spectroscopic techniques sensitive in different regions of the spectrum and to different properties of the water molecule and its immediate environment. These provide measurements mainly of the characteristic times $\tau_r$ for rotational diffusion or reorientational 'tumbling'. The results show, broadly, that there are three classes of water molecules close to a protein in solution: first, a small number bound almost irrotationally ($\tau_r = 10^{-5}$ to $10^{-7}$ s) at specific hydration sites offering very favourable multiple hydrogen-bonding conditions; secondly, a larger number (comparable to monolayer coverage) interacting less strongly with the protein but still sufficiently restricted motionally so that $\tau_r \approx 10^{-9}$ s; thirdly, all water molecules beyond one or two monolayers that possess dynamical properties essentially indistinguishable from bulk water ($\tau_r = 10^{-10}$ to $10^{-11}$ s). Because of the heterogeneity of the protein surface both with respect to topography and binding characteristics (Nedev & Churgin 1975), the distinctions made here are not sharp and depend very much on interpretational details of the techniques used.

Another approach to the study of hydration processes and protein dynamics in general is by numerical simulation. With the increasing availability of large and fast computers, investigations on the static accessibility of a protein to solvent molecules by space-filling techniques (Finney 1977; Lee & Richards 1971) are being extended to full-scale simulations of the dynamics of the water of hydration (Hermans & Rahman 1976; Clementi *et al.* 1977, 1979;




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Hagler & Moult 1978). The internal motions in crystallographically known proteins are being studied on an atomic scale by *ab initio* molecular dynamics simulations in the time domain $10^{-12}$ to $10^{-10}$ s (Levitt 1976; McCammon *et al.* 1977). Further theoretical work of interest in this context is directed towards an understanding of the conformational flexibility and transient accessibility of molecular groups in proteins (Karplus & Weaver 1976; Cooper 1976).

It is apparent from many of these studies that there is a relative lack of experimental information on the energy states of proteins in the nanosecond to picosecond region (Williams 1978). Neutron spectroscopic methods are in principle capable of bridging this region by overlapping with nuclear magnetic resonance techniques on the low-frequency side, and with optical techniques on the high-frequency side.

## 3. Basic aspects of neutron scattering

Quasi-elastic neutron scattering explores the low-frequency region of the spectrum characterized by energy transfers of the order of, or smaller than, 1 $cm^{-1}$†. In this region, monochromatic neutron pulses scattered by a sample at ordinary temperatures undergo Doppler-like broadening by non-quantized diffusive motions of various kinds and by a quasi-continuum of slow rotational modes that merges into or overlaps with the inelastic spectrum proper. Because of the large incoherent cross section of protons compared with all other nuclei (Bacon 1962), the scattering from *natural* biological samples is almost wholly incoherent and the hydrogen atoms therefore act as probes of the molecular dynamics. This changes drastically on deuteration, and in the limiting case of a completely deuterated sample the scattering will be predominantly coherent. The technique of H–D contrast variation, already well established in neutron diffraction (Worcester 1976; Jacrot 1976; Kneale *et al.* 1977; Stuhrmann & Miller 1978), is of equal importance in quasi-elastic and inelastic scattering as it provides a means to weight the cross section of a heterogeneous system in favour of a particular component (Egelstaff 1975; White 1975).

In a quasi-elastic or inelastic scattering experiment the double differential cross section, $d^2\delta/d\Omega dE$, is measured with respect to solid angle $\Omega$ and energy $E$ of scattered neutrons. Momentum transfer $\hbar\boldsymbol{k}$ and energy transfer $\hbar\omega$ of a neutron deflected upon scattering by an angle $2\theta$ are obtained from the conservation equations

$$\hbar\boldsymbol{k} = \hbar(\boldsymbol{s}-\boldsymbol{s}_0); \quad \cos 2\theta = \boldsymbol{s}\cdot\boldsymbol{s}_0/ss_0; \tag{3.1}$$

$$\hbar\omega = E - E_0 = \tfrac{1}{2}m(v^2 - v_0^2); \tag{3.2}$$

where $\boldsymbol{s}$, $|\boldsymbol{s}| = s = 2\pi/\lambda$, $v$ and $E$ are the wavevector, wavenumber, wavelength, velocity and energy of the scattered neutron, respectively, and subscript zero denotes incident beam quantities ($m$ is the neutron mass, $\hbar = h/2\pi$). For $\omega = 0$ (no energy exchange), these equations reduce to the familiar condition $k = (4\pi/\lambda_0)\sin\theta$ for elastic scattering. In quasi-elastic scattering, the energy change of a neutron upon scattering is small compared with the incident energy $E_0$. For $\lambda_0 \gtrsim 5$ Å, the wavelengths normally used in studies of this kind, $E_0$ is smaller than the thermal energy $k_B T$ by a factor of at least 8. The parameter range of interest here is thus characterized by the inequalities

$$|\hbar\omega| \ll E_0 \ll k_B T. \tag{3.3}$$

† 1 $cm^{-1} \equiv 124$ μeV.




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The connection between $d^2\delta/d\Omega dE$ and the molecular dynamics of the sample is made by correlation functions describing the evolution of the probability distribution of its nuclei in space and time (Glauber 1962; Egelstaff 1967). The differential cross section can always be written as the sum of an incoherent and a coherent part; for an assembly of $N$ nuclei of the same species this is

$$\frac{d^2\delta}{d\Omega dE} = \frac{N}{\hbar}\frac{s}{s_0}[b_{inc}^2 S_{inc}(\boldsymbol{k}, \omega) + b_{coh}^2 S_{coh}(\boldsymbol{k}, \omega)], \tag{3.4}$$

where

$$S_{inc}(\boldsymbol{k}, \omega) = \frac{1}{2\pi}\iint G_s(\boldsymbol{r}, t)\, e^{-i(\boldsymbol{k}\cdot\boldsymbol{r}-\omega t)}\, d\boldsymbol{r}\, dt$$

and

$$S_{coh}(\boldsymbol{k}, \omega) = \frac{1}{2\pi}\iint G(\boldsymbol{r}, t)\, e^{-i(\boldsymbol{k}\cdot\boldsymbol{r}-\omega t)}\, d\boldsymbol{r}\, dt.$$

Here $G_s$ and $G$ are van Hove's self-correlation and pair correlation function, respectively, and $b_{inc}$, $b_{coh}$ are the incoherent and coherent scattering lengths. The factorization of the two parts in (3.4) into a cross section $\propto b^2$ and a scattering law $S(\boldsymbol{k}, \omega)$ is possible only for a simple system. For a heterogeneous molecular sample, the incoherent part of (3.4) must be replaced by appropriate sums over atomic species or groups and their particular scattering laws, but the formulation of this is still relatively straightforward. The coherent part, however, because it describes the interference effects, assumes a much more complex form. The theory of inelastic neutron scattering from molecular systems has been developed by Steele & Pecora (1965), Larsson (1971) and Egelstaff *et al.* (1975).

## 4. Samples

Samples of the biosynthetically deuterated protein used in this study, C-phycocyanin, were initially provided by D. S. Berns, Albany Medical College, New York, and subsequently by H. L. Crespi, Argonne National Laboratory, Illinois. Dr Berns's material had been extracted from the blue-green alga *Plectonema calothricoides* and that of Dr Crespi from *Synechococcus lividus* grown in 99.7% pure $D_2O$ (Taecker *et al.* 1971; Crespi 1977). Blue-green algae contain large amounts (up to 25%) of phycocyanin and are capable of synthesizing it in extreme environmental conditions. Phycocyanin is a multimeric chromoprotein (molecular mass 190000) located in the stroma region between the thylakoid membranes, and its function is that of a light-harvesting protein involved in photosynthesis as part of photosystem II (O'Carra & O'hEocha 1976; Rüdiger 1980). Although its crystallographic structure is not known, its biophysical and biochemical properties have been studied since the days of Svedberg. Current interest in protio- and deuterio-phycocyanin relates primarily to its photochemical properties and to the opportunities it offers as a model system for studying the inter- and intramolecular forces involved in protein folding and aggregation processes (Berns 1971).

The phycocyanin samples were prepared in two forms. One consisted of glassy protein films cast on 9 μm pure Al foils (surface density *ca.* 5 mg $cm^{-2}$); this was used mainly in our initial experiments on IN 10 (see below). The other was in the form of fluffy aggregates of wisp-like particles obtained by drying from aqueous solutions, placed loosely on a stack of $4 \times 5 \times 40$ mm trays made from 9 μm Al foil. This method of preparation was used in all low-hydration experiments on IN 10 and IN 5. It made it possible, because of the greatly increased surface area, to re-equilibrate the sample to a new, sufficiently close hydration level during a reasonable




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time interval, without disturbing its geometry. A cylindrically symmetrical version of this sample holder was used for the powder diffraction experiment on D1B; here the stack of circular trays was made from 25 μm V foil. The total amount of protein in the beam was between 300 and 500 mg.

The samples were hydrated inside a temperature and humidity controlled sample changer equipped with a movable axial rod carrying an Al frame with the sample holder, a thermocouple and a solid-state humidity sensor. Dry helium of 99.9% purity was passed through wash bottles containing distilled $H_2O$ or saturated salt solutions in $H_2O$; these bottles were kept at a controlled temperature different from that of the sample changer and connected with the sample changer by a heatable steel pipe. The humidity level as measured by an M.C.M. Model 700L hygrometer was recorded continuously, together with the total count rate of scattered neutrons. The humidity control system described here was gradually implemented during four experiments; our earlier measurements were less quantitative but these are currently being recalibrated. The 'dry' reference state of a sample was defined by purging with dry helium for 10–20 h until the lag between neutron count rate and hygrometer reading disappeared and both traces had reached an essentially flat asymptote. Temperatures were controlled to within ± 0.5 °C.

## 5. Instrumentation

Three neutron scattering instruments at the Institut Laue–Langevin, Grenoble, were used in this study: The backscattering spectrometer IN 10 (Birr *et al.* 1971), the multi-chopper time-of-flight spectrometer IN 5 (Lechner *et al.* 1973), and the powder diffractometer D1B (Convert 1975).

The results discussed in this paper are mainly from experiments performed on IN 10. The backscattering technique achieves a quasi-elastic resolution of 0.003 to 0.01 $cm^{-1}$ (f.w.h.m.) as follows. A spatially collimated beam of neutrons from the cold source in the reactor (wavelength distribution 2–12 Å†) is reflected from a silicon monochromator crystal, which gives a highly monochromatic line of neutrons with $\lambda_0 = 6.2708$ Å. By imparting a periodic translational motion of a few hertz to the monochromator, the neutrons selected by it are Doppler-shifted with respect to the fixed sample and continuously scan a spectral window of 0.045 Å in wavelength or 0.24 $cm^{-1}$ in energy centred on $\lambda_0$. On scattering from the nuclei in the sample, these neutrons suffer small energy changes characteristic of thermal and collective motions. The scattered neutrons are energy-analysed by 180° reflexion from a fixed array of silicon crystals positioned 1.5 m from the sample at the scattering angle $2\theta$ observed. The crystals forming this array reflect neutrons back on to a detector located close to the sample. Five to seven detectors are arranged on a semicircle of 5 cm radius concentric with the sample changer axis, opposite the array of analyser crystals. A large cylindrical segment of the sample changer in this region consists of 12 μm pure Al foil and is transparent to cold neutrons. The total Al thickness traversed by the primary beam or the scattered neutrons nowhere exceeds 60 μm. The detectors were distributed over scattering angles from $2\theta = 8.6$ to 114°, corresponding to $k = 0.15$ to 1.68 $Å^{-1}$.

On IN 5, a conventional time-of-flight spectrometer of high resolution, the incident wavelength was 10 Å and eight detector banks were distributed over the scattering angle range $2\theta = 10$ to 130° ($k = 0.11$ to 1.14 $Å^{-1}$). The sample changer used in this experiment was

† 1 Å = $10^{-10}$ m = $10^{-1}$ nm.




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identical in design except for a larger diameter of 12 cm. On the powder diffractometer D1B, the incident wavelength was 2.52 Å and the multi-detector consisted of 400 cells covering the range $2\theta = 4$ to 84° ($k = 0.17$ to 3.33 Å$^{-1}$). Here the sample container was a 2.5 cm diameter V cylinder. The standard I.L.L. user's programs available for each instrument were used to monitor-normalize the raw data and to apply corrections for detector efficiency.

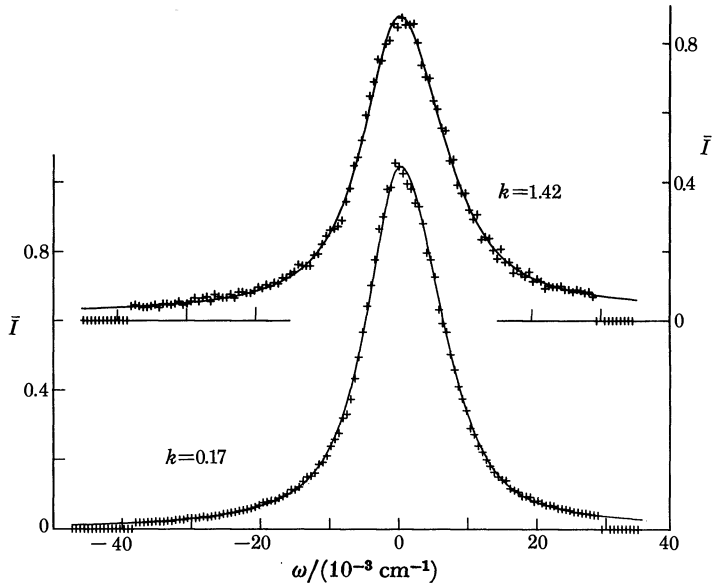


FIGURE 1. Examples of quasi-elastic spectra measured (+) for d-phycocyanin film stacks hydrated at relative humidity r.h. $\lesssim$ 90% $H_2O$ (temperature 24 °C). $I$ = neutron counts corrected for detector efficiency (arbitrary units); $\omega$ = energy transfer. Momentum transfer $k$ (Å$^{-1}$) corresponds to scattering angles $2\theta = 9.8$ and 90°; resolution widths $W_r$ (dry sample) were 0.0129 and 0.0143 cm$^{-1}$, respectively. Solid lines are analytical fits obtained by using asymmetric Peyre–Principi profiles. The term d-phycocyanin will henceforward be used to indicate the fully deuterated protein.

## 6. EXPERIMENTAL RESULTS

### (a) *IN* 10

In our first experiment we used an exchange-protonated film stack sample of d-phycocyanin and recorded sets of quasi-elastic spectra for a sequence of $H_2O$ hydration runs up to approximately 90% relative humidity (r.h.), starting with an almost dry sample (spectral window 0.072 cm$^{-1}$, six detectors). Two examples for the line shapes thus obtained are shown in figure 1. Conventional line broadening analysis (single-Lorentzian fitting) with respect to the reference or resolution widths measured for the dry sample and a Perspex scattering standard gave small broadenings $\Delta E$ of up to $3.2 \times 10^{-3}$ cm$^{-1}$ (f.w.h.m.), or 25% of the resolution width. The unexpected finding was that these broadenings showed a maximum at the position of the 4th detector ($k = 0.85$ Å$^{-1}$) together with the hint of an oscillatory structure appearing at higher angles. Subsequent experiments designed to check this result and guard against possible geometrical or instrumental effects have confirmed unambiguously that $\Delta E(k)$ indeed possesses an oscillatory structure in the hydration range investigated so far. This was done by changing the orientation of the sample with respect to the incident beam (45, 68 and 90°), by employing a different sample holder (perforated Al foil pouch instead of tray stack), changing the spectral window (background test) and interchanging polished and unpolished analyser crystals (different resolution).




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In order to study in greater detail the low-hydration régime and to create more uniform hydration conditions by increasing the effective surface area, the 'fluffy' powder samples described in §4 were used in all further experiments. Also, to obtain reliable values for quasi-elastic broadenings that could be ascribed to changes in hydration alone, the spectra recorded for a given hydration level of the sample were analysed not with respect to the instrumental resolution width $W_v$ of the quasi-elastic line (as measured for a purely incoherent scattering standard), but differentially with respect to the width $W_r$ obtained for the identical, dry or very slightly hydrated sample. Under conditions of undisturbed sample and scattering geometry during a series of hydration runs, and sufficiently good counting statistics (more than a few thousand counts integrated over the channels within the half-width points), it was possible to determine relative broadenings *for a given detector* with an estimated error of $\pm$ (1.5–2) $\times 10^{-4}$ cm$^{-1}$. Because of the large differences in resolution between individual detectors, and line shape distortions due to the impossibility of achieving uniform backscattering conditions for all six or seven detectors, the error in $\Delta E$ with respect to scattering angle (at a fixed hydration level) was larger by a factor of perhaps 2.

Considerable care needed to be exercised also in the data analysis. Standard single-Lorentzian fitting routines were found to be inadequate because of the smallness of the broadenings and appreciable non-Lorentzian intensity increases in the wings of the lines. Least-mean-square fits by superpositions or convolutions of Gaussian and Lorentzian functions were used instead. In one method, two parabolic sections meeting at the peak were first fitted to the data points in the upper 25–40 % of a line (depending on the number of points and their scatter) to define a centroid. Asymmetric Peyre–Principi profiles (Middendorf 1974) with a flat background were then fitted separately to the two halves of the line. The functional form used for these was

$$I_p(\omega) \approx a_0 + pL(\omega/w_0) + (1-p)\, G(\omega/w_0), \tag{6.1}$$

where

$$L(x) = 1/(1+x^2); \quad G(x) = \exp(-x^2 \ln 2) \tag{6.2}$$

and $w_0$ is the half width at half maximum when $a_0 = 0$ (no background). Similar fits with the use of the convolution product $L(\omega/w_l) \otimes G(\omega/w_g)$ instead of (6.1) gave analytical approximations that were essentially indistinguishable from the Peyre–Principi (P.P.) profiles, and the latter were therefore employed in most of the data analysis. Two such profiles are shown in figure 1. These analytical representations were then used to calculate contour level widths in steps of 5 % between 5 % and 80 % of the peak intensity with respect to $a_0$ as the baseline. In addition to the 50 % widths thus obtained (denoted $\Delta E$), an attempt was made to define a measure of the broadening (denoted $\Delta E^*$) based on an integral rather than a local property of the quasi-elastic line and therefore more sensitive to non-Lorentzian line shape changes. The peak-normalized lines were integrated over $\omega$ within the spectral window of the instrument and corrected for fractional wing intensities outside the window by using the fitted P.P. profiles, to give corrected total intensities $\bar{A}_r$ and $\bar{A}_s$ for the reference line (dry sample) and the broadened line (wet sample), respectively. Then $\Delta E^* = \bar{A}_s - \bar{A}_r$ and this is related to the analytical approximation (6.1) by

$$\Delta E^* = f(p)\, w_{s_0} - f(q)\, w_{r_0}; \quad f(x) = \pi x + (1-x)\, (\pi/\ln 2)^{\frac{1}{2}}, \tag{6.3}$$

where $w_{s_0}$, $w_{r_0}$ and $p$, $q$ are the widths (h.w.h.m.) and P.P. parameters of sample and reference lines. For purely Lorentzian profiles, $\Delta E^* = \frac{1}{2}\pi \Delta E_{\text{Lorentz.}}$ Comparison with Lorentzian-fitted lines gave values between 1.7 and 2.0 for the proportionality factor appearing here,

instead of $\frac{1}{2}\pi$. Background corrections were determined from those measurements for which the wider spectral window of the instrument was employed; these ranged from 0 to 4.5% of the peak intensity. The principal conclusions about $\Delta E(k)$ and its dependence on hydration proved to be unaffected by background corrections of this kind.

For the 'fluffy' sample configuration at low hydration levels, the variation of the quasi-elastic broadening with scattering angle was found to be more pronounced and the maximum occurred at a lower angle. In the first experiment a proton-exchanged sample was hydrated with $H_2O$ continuously from the dry state at an extremely low rate for 40.53 h (constant inlet flow of very slightly humidified helium). The uptake of water by the sample as reflected in the total detector count rate is shown in figure 2. The first set of spectra was taken after 5.45 h; the initial steep rise in the count rate is due to the rapid hydration of primary surface sites. The broadenings $\Delta E^*$ from this experiment, as evaluated from spectra accumulated over two intervals of several hours, are shown in figure 3 and compared with those obtained for the film stack sample described above.

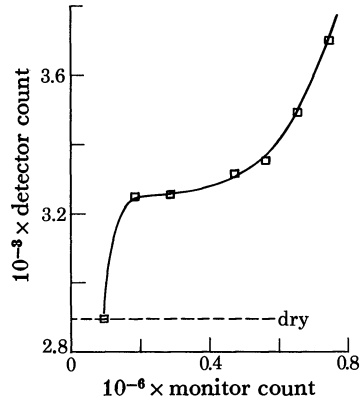


FIGURE 2. Total count rate of scattered neutrons plotted against direct beam monitor count (a count of 18600 represents 1 h) for 'fluffy' powder sample of d-phycocyanin under conditions of continuous hydration (instrument IN 10; $T = 25$ °C).

Whereas this experiment was deliberately performed under slowly changing hydration conditions, an attempt was made in subsequent experiments with the use of 'fluffy' powder samples, following improvements in hygrometry instrumentation, to achieve a reasonable degree of quasi-equilibrium during extended periods of stepwise hydration. The results are shown in figure 4. It is seen that there is a systematic shift in the position of the maximum in $\Delta E(k)$ with increasing hydration, while the minimum at $k = 0.7$ to 1.1 becomes progressively more shallow.

### (*b*) *IN 5*

Fully instrumented experiments performed in parallel with those on IN 10 and covering the 0.1–500 $cm^{-1}$ energy transfer region have only recently begun. The quantity of immediate interest in connection with the interpretation of IN 10 data is the elastic incoherent structure factor (Springer 1972; Volino & Dianoux 1978) which, in favourable circumstances, enables the translational and rotational contributions to the quasi-elastic intensity to be separated. Three examples of the spectra obtained for an $H_2O$-hydrated powder sample at $2\theta = 54$, 96 and 133° are shown in figure 5. Also shown is the spectrum of the dry sample at $2\theta = 54°$;




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all dry sample spectra, apart from small effects in the wings, were similar to this and gave no detectable broadening, i.e. closely reproduced the instrumental resolution function. The main qualitative result for the hydrated sample is that the central elastic peak is essentially unbroadened (at a resolution of 0.17 $cm^{-1}$) throughout the $k$ range covered so far, and that it is superimposed on a relatively broad Lorentzian-like feature the intensity of which increases with $k$, its width being of the order of 1 $cm^{-1}$. The separation of these two features does not present any difficulty, and we expect to be able to determine the elastic incoherent structure factor from current and future IN 5 experiments as required for a full interpretation of the quasi-elastic scattering.

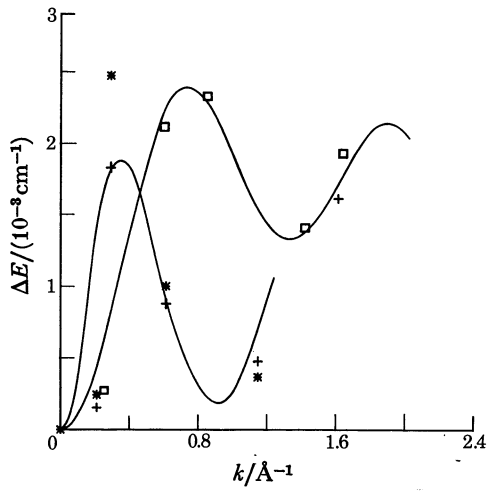


FIGURE 3. Quasi-elastic broadenings $\Delta E$ as a function of momentum transfer $k$ for film stack sample (□) (hydration conditions as in figure 1), 'fluffy' sample at low (+) and intermediate (*) hydration levels (r.h. = 10–30 % and 30–50 %, respectively).

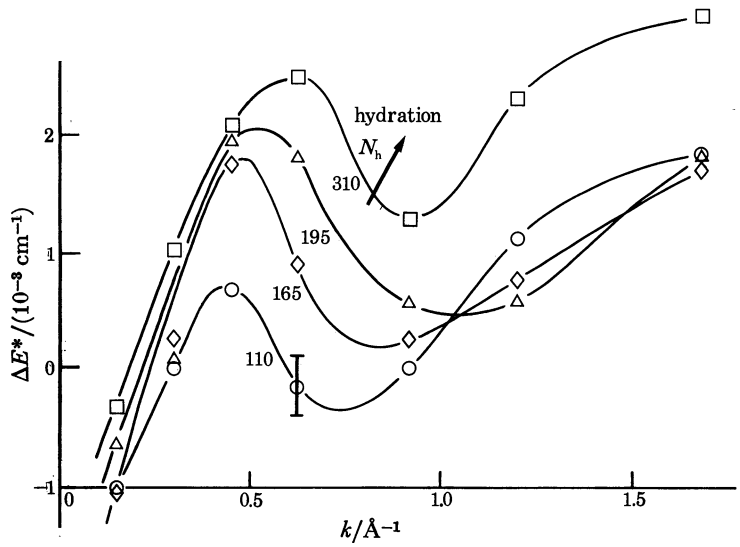


FIGURE 4. Hydration difference broadenings $\Delta E(k)$ for 'fluffy' powder sample at four hydration levels corresponding to r.h. conditions defined by saturated solutions of LiCl (r.h. *ca.* 15 %), NaCl (*ca.* 30 %), $NaHSO_4$ (*ca.* 52 %) and $(NH_4)_2SO_4$ (*ca.* 81 %) in $H_2O$ at 24 °C. Solid lines drawn in to guide the eye.

### (*c*) *D1B*

The purpose of this diffraction experiment was twofold: to determine the structure factor $S(k)$ for the dry powder sample of phycocyanin and for several hydration levels under conditions essentially identical with those on IN 10 and IN 5, and to look for possible conformational changes associated with the transition from the dry to a very slightly hydrated state, and further to the first two or three hydration levels studied on IN 10 and IN 5. Only the first of these objectives will be considered in connection with the interpretation of $\Delta E(k)$ discussed in §7.

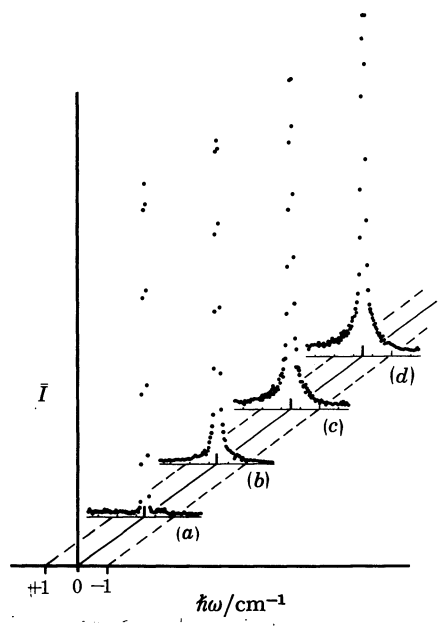


FIGURE 5. Examples of quasi-elastic spectra obtained on IN 5 for (*a*) dry d-phycocyanin sample at $2\theta = 54°$; (*b*), (*c*), (*d*) hydrated sample (r.h. *ca.* 30 %, NaCl in $H_2O$) for $2\theta = 54$, 96 and 133°, respectively. Normalized to give approximately equal peak heights.

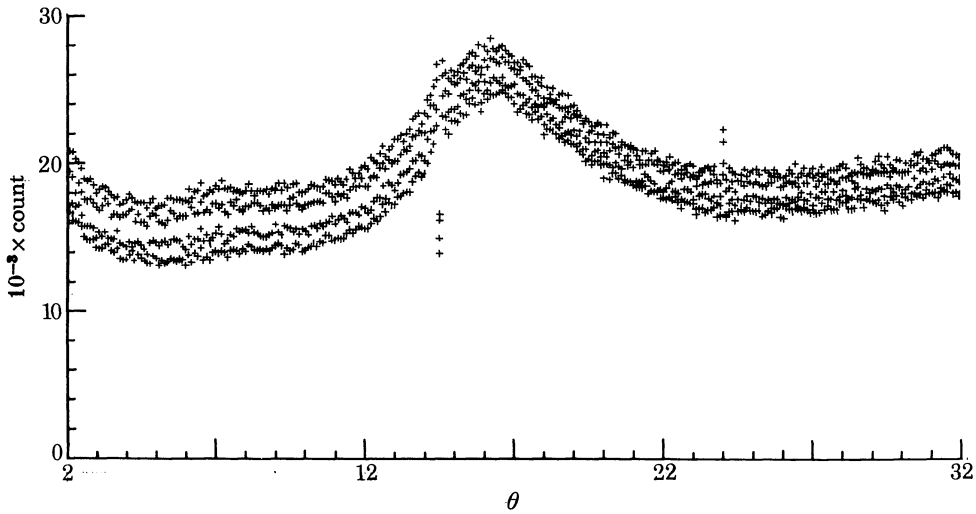


FIGURE 6. Structure factor data obtained by using powder diffractometer D1B for dry d-phycocyanin and for three hydration levels essentially identical with those used on IN 10. The traces shown are the result of subtracting the pattern obtained for a blank container run from those recorded for sample runs.




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The results for a proton-exchanged sample hydrated in steps according to the procedure described before are shown in figure 6.

## 7. Interpretation of $\Delta E(k)$

The quantities of primary interest in quasi-elastic scattering studies are the total intensity $S(k)$ and the half-width $\Delta E(k)$ of the quasi-elastic peak. The dependence of these quantities on H–D contrast and temperature can give important clues to the nature of the process observed. Further information may be extracted from a detailed line shape analysis of the quasi-elastic scattering as a function of $k$. In the present paper we shall discuss only $\Delta E(k)$ and, to a limited extent, $S(k)$.

The significance of $k$ is that the motional properties of the sample are probed over a scale length of about $2\pi/k$ (Å). Thus, in incoherent scattering, the macroscopic limit of classical diffusion is observed as $k \to 0$, and the behaviour of $\Delta E_{\mathrm{inc}}(k)$ for $k \gtrsim 1$ reflects the medium and short-range motions over molecular and atomic distances. The detailed form of $\Delta E_{\mathrm{inc}}(k)$ has been investigated both theoretically and experimentally for a variety of dynamic processes (Vineyard 1958; Egelstaff 1967; Springer 1972). The simplest case is that of continuous Fick's law diffusion for which $\Delta E_{\mathrm{inc}} = 2\hbar Dk^2$, where $D$ is the self-diffusion coefficient. In hydrogenous molecular liquids, rotational effects cause departures from Fick's law behaviour such that $\Delta E_{\mathrm{inc}}(k^2)$ for $k \to 1$ falls below the straight line defined by the $k \to 0$ asymptote but always increases monotonically with $k$. Oscillatory behaviour of $\Delta E_{\mathrm{inc}}(k)$, on the other hand, is characteristic of a class of jump diffusion processes discussed widely in the context of neutron scattering from protons diffusing on interstitial lattice sites in metal hydrides (Sköld & Nelin 1967; Gissler & Rother 1970; Rowe *et al.* 1971). For an ensemble of incoherently scattering particles migrating on a two-dimensional or three-dimensional lattice of sites, model calculations indicate that line-width changes similar to those shown in figures 3 and 4 occur whenever the residence time $\tau_0$ of a particle at a site is large compared with the time during which it moves to a neighbouring site. Because of the difficulty of formulating the space and time dependent pair correlation function, $G(\boldsymbol{r}, t)$, for a coherently scattering molecular system of complex structure (compare §3), measurements of the line broadening $\Delta E_{\mathrm{coh}}(k)$ have been analysed in detail only for a few cases. Those on polymers (Allen & Higgins 1973; Maconnachie & Richards 1978) are of some relevance here. Fundamental relations between the coherent and incoherent parts of the scattering law and their moments with respect to $\omega$ (de Gennes 1959; Sköld 1967) indicate that one should expect a line narrowing, i.e. a dip in $\Delta E(k)$, in those regions of $k$ where the structure factor $S(k)$ possesses a maximum, and vice versa. In the classical limit, all moments up to the second are satisfied by an expression known as the effective mass approximation (e.m.a.) and given by

$$S_{\mathrm{coh}}(k, \omega) = S(k)\, S_{\mathrm{inc}}[k/\sqrt{\{S(k)\}}, \omega]. \tag{7.1}$$

This model leads to

$$\Delta E_{\mathrm{coh}}(k) = \Delta E_{\mathrm{inc}}[k/\sqrt{\{S(k)\}}]. \tag{7.2}$$

Turning now to the interpretation of the data presented in §6, we note, first of all, that in hydration *difference* experiments we expect the coherent scattering from the bulk of the deuterated protein to subtract out. However, two points need to be examined very critically. The first relates to the fact that in the low-hydration region the coherent scattering predominates; thus, for the small broadenings observed in this work, the possibility of residual contributions from




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line narrowing effects according to (7.1) and (7.2) arises (accepting the validity of the e.m.a. for present purposes). The other has to do with genuine changes in the coherent scattering as the result of a gradual 'loosening up' of the mantle of the protein molecule with increasing hydration. This is an effect of considerable interest in its own right. It is therefore important to examine the behaviour of the structure factor $S(k)$, which is proportional to the total intensity $I(k)$ measured in the powder diffraction experiment and shown in figure 6. Although it would perhaps be more appropriate here to use total intensity data derived directly from the corresponding IN 10 or IN 5 measurements, the D1B data are chosen in this semi-quantitative discussion because of their better angular resolution and uniform coverage of a large $k$ region. The maxima in $\Delta E(k)$ according to figures 3 and 4 are characterized by values of $k_{\max}$ between 0.35 and 1.0 Å$^{-1}$, and this region corresponds to the $\theta = 4°$ to 12° interval in figure 6. The total intensity $I(k)$ increases very slightly in this region but is obviously devoid of any significant structure. The difference hydration levels, moreover, appear as essentially parallel tram-like traces narrowing almost imperceptibly (because of the Debye–Waller factor) with increasing $k$. We conclude that effects due to coherent scattering are unlikely to contribute more than perhaps 10% to the hydration difference broadenings observed in this $k$ region. We can be less sure of this at higher $k$ because of the broad feature in $I(k)$ centred on $2\theta = 16°$ or $k = 1.4$ Å$^{-1}$. The film-stack broadenings shown in figure 3 and the two highest hydration levels in figure 4 possess minima that partly overlap this feature, and it is possible that there is a larger coherent contribution here. The difference broadenings $\Delta E(k)$ obtained for the hydration sequence in figure 4 show both a systematic shift in their maxima from $k_{\max} = 0.45$ to 0.7 Å$^{-1}$ and increases in peak intensity with increased hydration (for a discussion of the hydration numbers, see §8 below). Although several aspects of the $\Delta E(k)$ data obtained so far need to be examined in greater detail by further experiments, the results considered as a whole suggest an interpretation in terms of water molecule motions between discrete sites separated by distances of the order of $1/k_{\max}$. This broad conclusion will therefore be adopted as a working hypothesis in the remainder of this paper.

The migration of water molecules on or near the surface of a protein cannot be expected to conform to any kind of regular lattice of hydration sites. To extract some characteristic lengths and times from the data, we have nevertheless attempted to fit simple Chudley–Elliott (C.E.) jump diffusion models (Chudley & Elliott 1961) to the $\Delta E(k)$ data shown in figure 3. If it is assumed that a scattering centre (i.e. a water molecule) moves from its site at $\boldsymbol{r}$ to a new site at $\boldsymbol{r}+\boldsymbol{a}_n$ during $\Delta t \ll \tau_0$, and if there is a spatial distribution of $n_s$ such sites accessible to this centre, then the scattering law for nearest-neighbour jumps may be calculated from a rate equation for the probabilities involved. The result is a Lorentzian of width

$$\Delta E(\boldsymbol{k}) = \frac{2\pi}{n_s \tau_0} \sum_{n=1}^{n_s} [1 - \exp(-i\boldsymbol{k}\cdot\boldsymbol{a}_n)], \tag{7.3}$$

which has been evaluated explicitly or numerically for various geometrical arrangements of sites (Gissler & Rother 1970; Springer 1972). Because of the small number of detectors that can be accommodated in backscattering geometry during a single IN 10 experiment, the structure of $\Delta E(k)$ is only seen rather coarsely and any curve fitting is subject to this limitation. Thus the curves shown in figure 3 and the numbers derived from them must at present be regarded as illustrative of the technique rather than definitive. Except at very low $k$, the film-




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stack broadenings agree reasonably well with an isotropic jump model for which $\Delta E \sim 1 - \sin(ka)/ka$. For the uniform jump distance assumed here, we obtain $a = 5.5$ to 6 Å, and for the residence time $\tau_0 = 5$ to 10 ns. The broadenings measured for the 'fluffy' sample, on the other hand, exhibit a pronounced first minimum at $k_{\min} = 0.8$ to 0.9 Å$^{-1}$ that cannot be modelled in any simple way. The lower curve in figure 3 represents a weighted superposition of $\Delta E(k)$ resulting from one-dimensional and two-dimensional C.E. models, the reasoning for this being that at lower hydration levels one might expect the migration of water molecules to proceed preferentially along polypeptide chains at the surface. Here $a = 7$ to 9 Å and $\tau_0 = 15$ to 30 ns. This sample was hydrated continuously from the dry state at a very low rate; the hydration number at the end of the experiment, estimated from the increase in total neutron counts, was $300 \pm 50$ $H_2O$ per subunit.

## 8. Hydration numbers and topography

Owing to limited instrumental accessibility, it was not possible in these first experiments to equilibrate the samples at different r.h. levels as well as we would have liked. It was necessary to adopt an operational rather than a thermodynamic definition of equilibrium, in the sense that the sample was considered equilibrated at a given humidity whenever the total count rate of scattered neutrons appeared to be reasonably close to its asymptotic value. The backscattering spectrometer was programmed to write a set of spectra accumulated during 30, 60 or 90 min into a memory, and the first 2–5 data blocks recorded between two successive r.h. levels were discarded upon inspection of the continuously monitored total count rate.

In the absence of direct gravimetric data, the number $N_h$ of $H_2O$ molecules per 29000 molecular mass subunit of phycocyanin may be determined either by an absolute calibration (with respect to vanadium) of the increase in the total count rate of scattered neutrons relative to the count rate per unit mass of the dry sample, or by relating this increase to a calculated cross section for the protein molecule. The latter approach was chosen here because it proved to be necessary, with a view to a more detailed interpretation of succeeding experiments on deuteron-exchanged samples, to develop a model for the changes in cross section of a deuterated phycocyanin subunit upon H–D exchange. Approximate values for the coherent cross section at high $k$ were obtained by splitting each of the 17 amino acid residues in two or three ways into fragments of 20–40 Å$^3$ (corresponding to $k \approx 2$ Å$^{-1}$), averaging over their coherent scattering lengths $b_{\mathrm{coh,i}}$ and summing the cross-section averages $4\pi\langle b_{\mathrm{coh,i}}\rangle^2$ for the residue composition given by Berns *et al.* (1964). This procedure may be regarded as a crude variant of the 'cube method' described by Federov *et al.* (1972). A set of 16 residues each containing two or three impurity hydrogens bound covalently to carbon were mixed in to simulate an isotopic impurity level of 98%. This gave $\sigma_{\mathrm{coh}} = 100 \pm 15$ kb† for the proton-exchanged subunit; the corresponding incoherent cross section was $\sigma_{\mathrm{inc}} \approx 40$ kb. The total cross section of 140 kb was then used to scale the intensity increases taken from the D1B data at $k = 2$ Å$^{-1}$ (averaged over $\Delta k = 0.3$ Å$^{-1}$; compare figure 6) in terms of water uptake.

The unique advantage of neutron inelastic scattering to allow energy changes to be studied with spatial information deduced from their $k$ dependence may be further exploited by linking the positions of the maxima in figure 4 with some model of the topography of hydration sites. While it is straightforward to obtain reasonably accurate values for the specific water uptake

† 1 barn (b) = $10^{-28}$ m$^2$.




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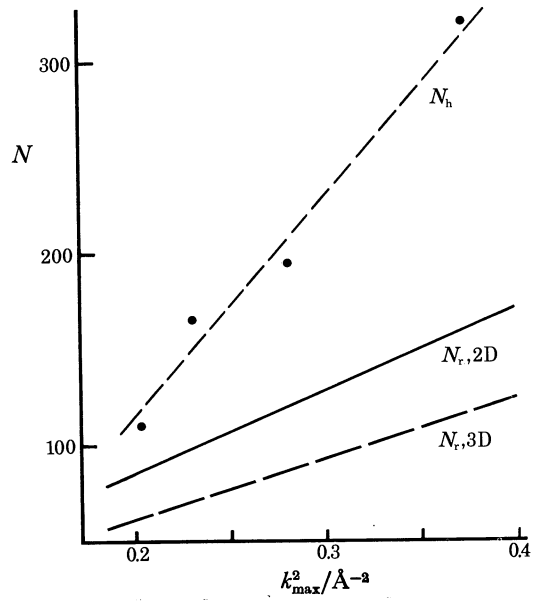
of the sample as a whole and thus to calculate average hydration numbers, it is quite impossible to determine an average value for the area per molecule over which a process of surface diffusion would be effective. This would require a great amount of detailed and largely unknown information about the surface and internal structure of amorphous protein powders of the kind described in §4, the contact of multimeric units, etc. Accepting the interpretation of a surface jump diffusion process advanced in §7, it is possible, however, to argue that there must be a limiting value for the average distance between primary hydration sites that could be




tion numbers $N_h$ and site numbers $N_r$ (for two-dimensional and three-dimensional Chudley–Elliott models) as a function of $k^2_{max}$ for the sample and hydration conditions of figure 4.

obtained from data of this kind at very low hydration levels. Because of the decreased scattering intensity at low $k$ *and* low $N_h$, a direct measurement of $k_{max}$ closer to its limiting value $k_{max,0}$ does not seem feasible at present with a backscattering spectrometer such as IN 10, although this should be possible with the neutron spin-echo spectrometer IN 11 at Grenoble (Richter *et al.* 1978). By extrapolating the curve connecting the maxima in figure 4 down to the abscissa, we obtain an approximate value for $k_{max,0}$ of 0.35–0.40 Å$^{-1}$. On the basis of simple C.E. models, this would convert into an average jump distance $d_{r_0}$ of 10–12 Å, a quantity characterizing the distribution of primary hydration sites. To obtain an estimate of the number $N_{r_0}$ of such sites, it is necessary to know the fraction of accessible surface area lost on association into an oligomer. This should be about 30–40 % (Teller 1976) and the result for $N_{r_0}$, using $d_{r_0} = 11$ Å, is 32–38. To illustrate these relationships we have, somewhat arbitrarily, scaled the water uptake corresponding to the first *measured* value of $k_{max}$ such that each of these sites is occupied, on average, by three water molecules. Hence $N_h \approx 110$ at $k_{max} = 0.45$ Å$^{-1}$. By using values for the water uptake calculated from D1B data as described above, hydration numbers of 165, 195 and 310 were then determined for the sequence of $\Delta E(k)$ curves shown in figure 4. These $N_h$ values are plotted separately in figure 7 as a function of $k^2_{max}$, together with two lines showing the dependence of $N_r$ evaluated from (7.3) for a two-dimensional or three-dimensional C.E. model relating $d_r$ and $k_{max}$. While the scaling of the reference value is uncertain on the basis

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of the present data, it is seen that the slope of $N_h$ with respect to $k^2_{max}$ differs significantly from that of either of the theoretical curves. This may be interpreted as an increased clustering of water molecules at hydration sites together with a gradual increase in the number of such sites, but may also in part be due to inadequacies in the simple jump diffusion model used. Data from H–D contrast experiments are likely to shed further light on this problem.

We are greatly indebted to all the following: D. S. Berns, Albany Medical College, New York, and H. L. Crespi, Argonne National Laboratory, Illinois, for deuterated C-phycocyanin; S. Gilmour, who took part in exploratory experiments, and A. D. Taylor, Rutherford and Appleton Laboratories, for data analysis on the Harwell results and subsequent participation at the I.L.L.; the Institut Laue–Langevin, Grenoble, for facilities; the I.L.L. staff scientists A. Heidemann, W. S. Howells, G. T. Jenkin (IN 10); A. J. Dianoux, F. Douchin, R. E. Lechner (IN 5); and P. Convert, G. Bomchil and J. L. Buevoz (D1B) for much advice. This work is supported by the Science Research Council.

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## *Discussion*

A. J. Leadbetter (*Department of Chemistry, University of Exeter, U.K.*). The quasi-elastic broadenings that the authors have observed are extremely small, being much less than the instrumental resolution width, and their conclusions about jump motion of water molecules are based on the $Q$-dependence of this very small broadening: $\Delta E(Q)$. Would the authors explain exactly what is the ratio between the incoherent, broadened quasi-elastic signal and the substrate background in their experiments?

Is not this substrate background scattering predominantly coherent with a maximum in $S(Q)$ near the position of the minimum observed in $\Delta E(Q)$? Can the authors therefore be quite sure that the structure observed in $\Delta E(Q)$ is not simply an artefact arising from the very small magnitude of $\Delta E$ and the high background scattering?

Sir John Randall and H. D. Middendorf. As to the magnitude of the effects seen at low levels of hydration, we feel that quasi-elastic broadenings of $0.8–4 \times 10^{-3}$ cm$^{-1}$ (f.w.h.m.), or 0.1–0.5 μeV, cannot be called 'extremely small' if observed in carefully designed and analysed difference experiments (compare, for example, Renouprez *et al.* 1979 (*J. chem. Soc. Faraday Trans.* I **75**, 2473) and Ewen & Richter 1978 (*J. chem. Phys.* **69**, 2954)). We wish to point out, in particular, that the broadenings shown were measured relative to the identical, completely undisturbed sample in successive runs, i.e. *not* relative to a vanadium plate, and that both methods of width analysis gave essentially the same results. We have shown previously (Randall *et al.* 1978, fig. 2) that the stability of IN 10 over uninterrupted run times of the order of 100 h is excellent for this purpose (r.m.s. intensity deviations less than about 2%). We regularly check this by concluding a hydration sequence with a long desorption run to get back to starting conditions. We see no reason why the potential of this and similar instruments for difference spectroscopy should not be exploited more fully in a way familiar to biophysicists and biochemists. The main limiting factors at present are the small number of spectra obtainable




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simultaneously and their non-uniformity as regards line width and shape; these technical drawbacks are being overcome by advanced instruments currently under construction (see contributions by Fender *et al.* and Springer, this symposium). In current neutron spectroscopic work on hydrogeneous biopolymers, the IN 10 line width changes seen on hydration of collagen are comparable to those that we report here, and the inelastic difference spectra observed on IN 5 for lysozyme and DNA are between 6 and 40 % of the 'background' spectrum (see *I.L.L. Annual Report* 1977, annex, pp. 374–377).

With regard to the role played by the coherent scattering, it was not possible because of the time limitation to discuss this adequately in our talk and details may be found in the text. It is important here, as you point out, to consider the variation of $\Delta E(k)$ in conjunction with $S(k)$ and therefore to collect accurate diffraction data in parallel with inelastic experiments. Owing to the difficult beam time situation, this was begun only recently and a full set of structure factor data have not been obtained. It is clear, however, from the limited results shown in figure 6 that the broad feature centred on $k = 1.4$ Å$^{-1}$ neither coincides with the minimum of $\Delta E(k)$ nor shifts on hydration. While it may contribute to the depth and width of the dip in $\Delta E(k)$ at the highest hydration levels studied so far, all the available evidence points to the conclusion that line narrowing due to structure in $S(k)$ is not the dominant mechanism in determining the oscillatory behaviour of $\Delta E$. We wish to emphasize that the results presented here are part of a continuing investigation into the hydration dynamics of *in vivo* deuterated biopolymers, and that phycocyanin is the first protein of this kind to be studied by quasi-elastic and inelastic scattering. Current H–D contrast experiments on 'fading out' the proton signal from the water of hydration and planned experiments on partly deuterated proteins will provide data enabling us to assess more quantitatively the contribution from coherent scattering.




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