**POLARIZED NEUTRON SCATTERING BY POLARIZED PROTONS
OF BOVINE SERUM ALBUMIN IN DEUTERATED SOLVENT**

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ABSTRACT

Bovine serum albumin (BSA) was dissolved in a mixture of deuterated glycerol and heavy water. The clusters formed by the 3300 proton spins in each BSA molecule were dynamically polarized up to $P = 40\%$. Spin-contrast variation in small-angle neutron scattering was studied at several target polarizations. Zero contrast, and hence minimum polarized neutron small-angle scattering, is expected at $P_H = 60\%$ from extrapolation of present data. The three basic scattering functions of spin-contrast variation look very similar because the shape of the BSA molecule and its proton distribution are congruent. Neutron small-angle scattering of BSA is similar to X-ray small-angle scattering at room temperature, indicating no deterioration of the molecular structure of BSA on solidification.

Abbreviations: BSA = bovine serum albumin

DNP = dynamic nuclear polarization

NMR = nuclear magnetic resonance

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1. INTRODUCTION

Polarized neutrons are a most sensitive probe for oriented nuclei, and for oriented protons in particular (Abragam and Goldman, 1982). Nuclei with spin I are said to be oriented when the populations $W(m)$ of the magnetic substates with quantum number m are not all equal. The degree of orientation can be described by $2I$ orientation parameters of increasing order $f_1, \dots, f_{2I}$, see, for example, Blin-Stoyle and Grace (1957), Ohlsen (1972). The first parameter is the polarization P . In a high field this can be expressed in terms of the normalized population numbers $W(m)$ as

$$P = f_1 = I^{-1} \sum_m m W(m) . \quad (1a)$$

Here f_1 is the ratio of the average projection of the spins along the static field to its maximum value I ; for $I = 1/2$ we have

$$P = \langle I_z \rangle / I = [n_+ - n_-] / [n_+ + n_-] , \quad (1b)$$

where n_+ and n_- denote the numbers of spins in the states $m = +1/2$ and $m = -1/2$, respectively. In addition, a unit vector $\vec{n}$ along the axis of polarization can be introduced, and a polarization vector $\vec{P}$ defined by

$$\vec{P} = \vec{n} P . \quad (2)$$

For spin $1/2$ particles, e.g. neutrons, protons, electrons, etc., the spin ensemble is completely described by the polarization vector $\vec{P}$. The second parameter f_2 , the 'alignment', is required to define the magnetic level populations for $I = 1$, for example with deuterons.

The possible impact of nuclear spin-dependent neutron scattering on structure research has been summarized by Abragam et al. (1982). Diffraction of polarized neutrons by polarized proton spins was first used by Hayter et al. (1974). Its importance for the precise location of hydrogen atoms in complicated structures has been demonstrated in the case of lanthanum magnesium nitrate hydrate (LMN) by Leslie et al. (1980). Proton spin polarization was achieved by dynamic nuclear polarization (DNP) in the presence of a small amount of neodymium occupying randomly 0.1% of the lanthanum sites.

In the seventies, high-energy physicists developed polarized targets with higher hydrogen content. The advent of organic target materials (such as alcohols and diols) with very high polarization was a big step forward, not only for high-energy physics experiments (de Boer and Niinikoski, 1974) but also for macromolecular structure research, when it turned out that proton spins of globular enzymes in deuterated solvents could be polarized to more than 70% (Stuhrmann et al., 1986).

Another important prerequisite for the present work was the advent of stable paramagnetic organometallic compounds of Cr(V) (Krumpolc and Rocek, 1979), which are soluble in many alcohols, diols, and glycols. These compounds can also be synthesized in deuterated form, which is beneficial both to DNP and to background small-angle scattering (Stuhrmann et al., 1986). In this work, DNP was achieved in the presence of 2% by weight of sodium bis[2-ethyl-2-deuteroxybutyrate]-oxochromate(V) monohydrate, $\text{Na}[\text{C}_{12}\text{D}_{20}\text{O}_7\text{Cr(V)}] \cdot \text{D}_2\text{O}$, abbreviated EHBA-Cr(V) (Krumpolc and Stuhrmann, 1988).

First studies on polarized proton spin clusters of various proteins in mixtures of deuterated glycerol and heavy water, using polarized neutrons, confirm the predicted spin-dependent changes of small-angle scattering (Knop et al., 1986). These experiments were done using a CERN

polarized-target installation, which was moved to the reactor FRG1 of the GKSS Research Centre at Geesthacht.

Another polarized target has been put into operation at the National Laboratory of High-Energy Physics (KEK) in Tsukuba, Japan. Macromolecules of smaller size, such as dicyclohexyl-18-crown-6, $C_{20}H_{32}O_6$, were studied using unpolarized neutron small-angle scattering (Kohgi et al., 1987). With unpolarized neutrons, however, the influence of proton polarization on the scattering cross-section is less pronounced (Kohgi et al., 1987).

This paper is the first detailed report on the studies of polarized neutron scattering of dynamically polarized materials, performed at the GKSS Research Centre from October 1986 to March 1987. Three classes of biological macromolecules were investigated: i) proteins, including lysozyme, myoglobin, urease, ferritin, and bovine serum albumin (BSA); ii) ribonucleic acids (transfer-RNA and ribosomal RNA); and iii) the large subunit of *E. coli* ribosomes consisting of rRNA and 34 ribosomal proteins.

Among the proteins, BSA was the one investigated most extensively. Its high solubility and easy availability made it an ideal test material. It has a molecular weight of 68 000. Solutions of BSA have previously been studied using X-ray small-angle scattering (Luzzati, Witz and Nicolaieff, 1961; Stuhrmann, 1964). From these measurements, the radius of gyration $R = 30 \text{ \AA}$ has been determined. This is slightly more than is expected from a quasi-spherical structure of the same molecular weight, such as hemoglobin with $R = 25 \text{ \AA}$.

The aim of the present studies on BSA is to show how contrast in small-angle scattering varies with the polarization of the nuclear and neutron spins. In order to increase the intensity of neutron small-angle scattering, in two thirds of the experiments the full thermal neutron spectrum with wavelengths from 2 to 7 $\text{\AA}$ was used. The data obtained with the 'white' spectrum will be compared with those from the quasi-monochromatic neutron beam.

2. METHODS

2.1 Nuclear spin-dependent amplitudes

We shall now discuss the coherent small-angle scattering of polarized neutrons on large hydrogen-rich polarized molecules in the framework of the Fermi pseudopotential model. Denoting the spin operator of the neutron by s and that of the nucleus j by I_j , we may write the Fermi scattering-length operator (scattering-amplitude operator) for a free nucleus j (Abragam and Goldman, 1982):

$$A = b_j + 2B_j I_j \cdot s, \quad (3)$$

and define the two scattering amplitudes

$$U(Q) = \sum_{j=1}^M b_j \exp [i(Q \cdot r_j)],$$

$$V(Q) = \sum_{j=1}^N P_j I_j B_j \exp [i(Q \cdot r_j)], \quad (4)$$

where $U(Q)$ is the invariant and $V(Q)$ is the polarization-dependent amplitude, Q is the momentum transfer, and r_j is the position of the nucleus j . There are M atoms per (macro)molecule or unit cell of a crystal. N out of the M atoms have a nuclear spin-dependent scattering amplitude operator. The corresponding parts of the structure $\varrho(r)$ are

$$\begin{aligned} \varrho_U(\mathbf{r}) &= \sum_{j=1}^M b_j \delta(\mathbf{r} - \mathbf{r}_j) , \\ \varrho_V(\mathbf{r}) &= \sum_{j=1}^N b_j \delta(\mathbf{r} - \mathbf{r}_j) . \end{aligned} \tag{5}$$

The elastic coherent cross-section is given by

$$S = (d\sigma/d\Omega)_{\text{coh.}} = UU^* + P_n(UV^* + VU^*) + VV^* , \tag{6}$$

where the polarization P_n of the incident neutron spins is along the same common axis as that of the target nuclear spins I_j , i.e. along the static magnetic field. Under the assumption that only one species of nuclei is contributing to $V(\mathbf{Q})$ of Eq. (3), the coherent scattering can be expressed as

$$S(\mathbf{Q}) = S_U(\mathbf{Q}) + P_n P S_{UV}(\mathbf{Q}) + P^2 S_V(\mathbf{Q}) , \tag{7}$$

where S_U , S_{UV} , and S_V are the basic scattering functions of spin-contrast variation; $S_U(\mathbf{Q})$ is the scattering function of the unpolarized target, i.e. of $\varrho_U(\mathbf{r})$. The polarized nuclei described by $\varrho_V(\mathbf{r})$ give rise to $S_V(\mathbf{Q})$. The cross-term $S_{UV}(\mathbf{Q})$ is due to the convolution of $\varrho_U(\mathbf{r})$ with $\varrho_V(\mathbf{r})$.

The determination of the basic scattering functions starts from a series of measurements of $S(\mathbf{Q})$ at different P and P_n ; $S_U(\mathbf{Q})$ is observed at $P = 0$. At a given target nuclear polarization P , two directions of the neutron spin polarization, P_n and $-P_n$, can be used most easily in an alternating way, giving rise to

$$\begin{aligned} S(\uparrow\uparrow) &= S_U + P_n P S_{UV} + P^2 S_V , \\ S(\downarrow\uparrow) &= S_U - P_n P S_{UV} + P^2 S_V . \end{aligned} \tag{8}$$

From the difference $S(\uparrow\uparrow) - S(\downarrow\uparrow)$, one obtains

$$S_{UV} = [S(\uparrow\uparrow) - S(\downarrow\uparrow)] / (2P_n P) . \tag{9}$$

The sum of $S(\uparrow\uparrow)$ and $S(\downarrow\uparrow)$ yields

$$S(\uparrow\uparrow) + S(\downarrow\uparrow) = 2S_U + 2P^2 S_V , \tag{10}$$

from where S_V is easily extracted as S_U is known from measurements with the unpolarized target; S_V might also be obtained from experiments using unpolarized neutrons (Kohgi et al., 1987).

2.2 Nuclear spin-dependent small-angle scattering

Dilute solutions of macromolecules give rise to intense neutron small-angle scattering. The overall dimensions of the dissolved particles, of some ten to some hundred ångströms, are large compared with the wavelength of the incident neutrons, and they are small compared with the interparticle distances. The latter fact minimizes interparticle correlations in coherent neutron scattering, i.e. we can treat neutron small-angle scattering as a superposition of one-particle scattering functions. The dissolved particles are randomly oriented. This is taken into account by averaging $S(\mathbf{Q})$ over all orientations of the particle with respect to the incident momentum:

$$\begin{aligned}
\hat{s}(Q) &= 1/4\pi \int_{\Omega} S(\mathbf{Q}) d\Omega \\
&= \sum_i^M \sum_j^M b_i b_j \sin Q|\mathbf{r}_i - \mathbf{r}_j|/Q|\mathbf{r}_i - \mathbf{r}_j| \\
&\quad + 2P_n \text{PIB} \sum_i^M \sum_j^N b_i \sin Q|\mathbf{r}_i - \mathbf{r}_j|/Q|\mathbf{r}_i - \mathbf{r}_j| \\
&\quad + P^2 I^2 B^2 \sum_i^N \sum_j^N \sin Q|\mathbf{r}_i - \mathbf{r}_j|/Q|\mathbf{r}_i - \mathbf{r}_j|.
\end{aligned} \tag{11}$$

This is the averaged scattering function of one particle in the gaseous state. Macromolecules in solution exhibit a different scattering cross-section. The amplitude of the hole in the solvent created by the dissolved macromolecule has to be subtracted from U and V in Eq. (4) first, before the coherent cross-section is calculated according to Eq. (6).

To clarify this point, we consider the case of zero-angle scattering. From Eq. (11) we obtain

$$\hat{s}(0) = \left[\sum_i^M b_i \right]^2 + 2P_n \text{PIB} N \sum_i^M b_i + P^2 I^2 B^2 N^2. \tag{12}$$

With $P_n = \pm 1$ (completely polarized neutron beam), Eq. (12) can be expressed as the square of the sum of scattering lengths:

$$\hat{s}(0) = \left[\sum_i^M b_i \pm \text{PIB} N \right]^2. \tag{13}$$

At this point it is more convenient to express the sum of all scattering lengths in the particle volume v as the product $v\varrho$, where ϱ is the scattering density defined as the sum of scattering lengths in a unit volume:

$$\hat{s}(0) = v^2 [\varrho_U \pm P \varrho_V]^2 \equiv v^2 \varrho^2. \tag{14}$$

Zero-angle scattering of a dissolved particle then is

$$\begin{aligned}
\hat{s}(0) &= v^2 [\varrho(\text{solute}) - \varrho(\text{solvent})]^2 \\
&\equiv v^2 [\delta_U \pm P \delta_V]^2 \equiv v^2 \delta^2,
\end{aligned} \tag{15}$$

δ is the contrast (the indices U and V refer to the invariant and spin-dependent parts of δ , respectively); δ_V is called (nuclear) spin contrast.

Slightly off $Q = 0$, the amplitudes of the scattering centres in Eq. (4) are getting more and more out of phase. Small-angle scattering starts to decrease, reflecting the overall dimension of the particle as described by its radius of gyration R :

$$\hat{s}(Q) = \delta^2 v^2 (1 - R^2 Q^2/3). \tag{16}$$

The variation of R with the contrast is sensitive to differences between ϱ_U and ϱ_V (Stuhrmann and Kirste, 1967; Serdyuk and Grenader, 1975; Ibel and Stuhrmann, 1975).

2.3 On the use of spin-contrast variation

Spin-contrast variation is a non-destructive labelling method. The three basic scattering functions are determined from one sample only. Isotopic replacement methods require the preparation of at least three samples. Spin-contrast variation covers the known possibilities of isotopic-contrast variation. This is demonstrated in the case of a protein solution.

As an example, we take an aqueous solution of lysozyme. Using the scattering densities given in Appendix 1, the contrast (in units of 10^{10} cm^{-2}) is obtained as

$$\begin{aligned}\delta(\text{lysozyme in H}_2\text{O}) &= 1.94 - (-0.56) \pm (8.05 - 9.66)P_H \\ &= 2.50 \pm (-1.61)P_H,\end{aligned}\tag{17}$$

$$\begin{aligned}\delta(\text{lysozyme in D}_2\text{O}) &= 3.10 - 6.40 \pm [6.53 - 9.66/(24.9 - 9.2P_H^2)]P_H \\ &= -3.30 \pm [6.14 - 0.14P_H^2]P_H.\end{aligned}\tag{18}$$

Here the non-linear increase of deuteron spin polarization in the solvent with P_H (see Appendix 2) has been included. The contrast of lysozyme in heavy water varies with proton spin polarization by $12 \times 10^{10} \text{ cm}^{-2}$. This is two times more than can be achieved by isotopic substitution of H by D in water. In the latter case, one makes use of the difference in contrast of the unpolarized targets, i.e. $[2.57 - (-3.21)] \times 10^{10} \text{ cm}^{-2} = 5.78 \times 10^{10} \text{ cm}^{-2}$. Spin-contrast variation also works in undeuterated media: the effect is due to the difference between the proton densities of the protein and that of the solvent. The proton spin-contrast δ_v is then four times weaker than that observed when heavy water is used as a solvent.

When does the scattering density of the protein equal that of the solvent? From neutron scattering in $\text{H}_2\text{O}/\text{D}_2\text{O}$ mixtures as a solvent, it is known that small-angle scattering of proteins vanishes when the D_2O content reaches 0.4. In spin-contrast variation experiments, polarized neutron small-angle scattering of proteins in heavy water will vanish at $P_H = 0.55$. In H_2O as a solvent, the spin contrast ρ_v of the protein is too small to invert the contrast in $-1 < P_H < 1$.

From this it is evident that promising results can be expected from proton spin-contrast variation in highly deuterated media. The spin-dependent amplitude of a small cluster of 10 protons ($P_H = \pm 0.9$) compares with that of a uranium atom label in X-ray diffraction. The size, and hence the power, of the proton-rich label can be tailored to the requirements of the structural studies of biomolecules and of hydrogenous material in general.

2.4 Incoherent scattering

The variation of coherent scattering with the nuclear spin polarization as described in Eq. (6) is accompanied by a change of the incoherent cross-section of neutron scattering:

$$(d\sigma/d\Omega)_{\text{inc}} = 4\pi \sum B_j^2 [I_j(I_j + 1) - P_n P_j I_j - P_j^2 I_j^2].\tag{19}$$

The spin quantum numbers I and the coefficients b and B of some elements are given in Table 1.

Protons show the most pronounced changes of scattering cross-section with the polarization P_H . According to Eq. (19), their incoherent cross-section is, in units of 10^{-24} cm^2 ,

$$\sigma_{\text{inc}} = 106.5(3/4 - 1/2 P_n P_H - 1/4 P_H^2).\tag{20}$$

For deuterons, incoherent scattering is nearly two orders of magnitude smaller:

$$\sigma_{\text{inc}} = 1.02(2 - P_n P_D - P_D^2) . \quad (21)$$

The incoherent cross-section is zero for $P_n P_H = 1$ (or $P_n P_D = 1$). In this case, only coherent scattering is observed and no spin-flip occurs. At opposite polarization directions of neutron spins and nuclear spins, i.e. $P_n P = -1$, a maximum of incoherent scattering is observed, but coherent scattering may be strong as well [see Eqs. (17) and (18)].

The incoherent cross-section largely determines the attenuation of the neutrons by proton-rich samples. Polarized targets of hydrogenous material are therefore used as neutron spin filters or neutron polarizers (Kohgi et al., 1987). The variation of the transmission of thermal neutrons by highly deuterated samples is due to comparable amounts of both incoherent and coherent scattering (Table 1). Only a minor part of coherent scattering is observed as small-angle scattering.

3. EXPERIMENTAL

3.1 Sample

A sample of 0.4 g BSA (fraction V, batch No. 717 ZA7095118 from Merck) and one of 0.05 g perdeuterated EHBA-Cr(V)-d₂₂ were dissolved in a mixture of heavy water (2.06 g) and perdeuterated glycerol (2.21 g). Glycerol from MSD Isotopes (lot No. 2565-J) was 98.6% deuterated. The Cr(V) compound acts as a paramagnetic centre required for DNP (see below).

The samples were prepared in the form of trapezoidal slabs of 20 mm length, 6 mm depth (along the neutron flight path), and 2.8 mm front width, increasing to 4.2 mm at the rear side of the sample facing the detector. This was done by letting droplets of the solution freeze in a copper mould held at liquid-nitrogen temperature. Four of these slabs were mounted vertically between two quartz spacers inside the mixing chamber of a fast-access dilution refrigerator (Niinikoski and Rieubland, 1978). This sample geometry was chosen so as to exclude ³He in the flight path of neutrons. On the other hand, sufficient surface has to be offered for cooling by a ³He/⁴He mixture (Fig. 1). One of the quartz spacers was cut diagonally in order to use it as a wedge for tighter mounting of the sample blocks. The sum of residual gaps filled with a ³He/⁴He mixture still amounts to some tenths of a millimetre. The transmission of neutrons at $\lambda = 5 \text{ \AA}$ is

$$\frac{\Phi_{\text{out}}}{\Phi_{\text{in}}} = e^{-n\sigma(\lambda)d} = 0.65 , \quad (22)$$

where n is the number density of ³He atoms in a 6% dilute solution, $d = 0.03 \text{ cm}$ is the neutron flight path in the dilute solution, and the neutron absorption cross-section of ³He increases with wavelength as $\sigma(\lambda) = 3050 \lambda/(1 \text{ \AA}) \text{ barn}$. In our experiments the transmission is described by $1/\exp(0.4\lambda)$ (see Section 4). As the neutrons with longer wavelengths are absorbed preferably by ³He, the mean wavelength of the transmitted spectrum gets shifted to smaller values (Fig. 2). The observed transmission of 5 Å neutrons is only 0.14. This indicates a higher ³He content of the mixture (20 to 30% ³He), which is not unexpected at the bath temperature—0.4 to 0.5 K—of the refrigerator.

The choice of the depth of the cell is guided by the atomic composition of biological samples. For the sake of argument, we assume that these are well represented by water. The scattering cross-section of the unpolarized water target at $T < 1 \text{ K}$ is $\sigma = 163 \text{ b}$ and it hardly depends on the wavelength of thermal neutrons (Scherm and Guckelsberger, 1985). This gives an attenuation coefficient $\mu = 0.54 \text{ mm}^{-1}$. The optimum sample thickness for scattering experiments is $1/\mu = 1.8 \text{ mm}$. For perdeuterated samples we have $\sigma = 19.4 \text{ barn}$, which yields the absorption coefficient

$\mu = 0.065 \text{ mm}^{-1}$. Maximum neutron scattering intensity would be obtained from samples of 15 mm thickness. The choice of 6 mm for the sample thickness is a compromise favouring deuterated samples.

3.2 Polarization of target nuclei

Dynamic nuclear polarization (Abragam and Goldman, 1982) was performed at 2.5 T field and 0.4–0.5 K helium bath temperature in a CERN target set-up previously used in high-energy physics experiments. The magnetic field was created in a normal-conducting C-shaped electromagnet with circular cobalt-iron pole pieces shimmed to reach a field homogeneity of about 10^{-4} in a volume of 5 cm diameter and 2 cm height.

The 70 GHz microwave power, needed to saturate the paramagnetic resonance of the EHBA-Cr(V) spins, was generated by a carcinotron source.

The heat generated by microwave losses in the target and surrounding materials was absorbed in a fast-loading dilution refrigerator (Niinikoski and Rieubland, 1982). The target area was modified so that the dilute $^3\text{He}/^4\text{He}$ solution could be excluded, as much as possible, from the neutron beam; this was achieved by filling the beam path with fused quartz and aluminium pieces.

The polarization of the target protons was determined by measuring the integrated continuous-wave nuclear magnetic resonance (NMR) absorption signals using a series-tuned Q-meter circuit (Niinikoski and Rijllart, 1982). The NMR polarization measurement was calibrated by making a comparison with integrated signals arising from the target in thermal equilibrium with the helium bath at about 1 K temperature, measured using calibrated resistance thermometers.

The absolute accuracy of the polarization measurement was about 4%, mainly determined by the uncertainty of the temperature measurement. The integrated signal measurement has a statistical accuracy of better than 1% for the calibration, and of about 0.1% for the polarized signals.

The dynamic polarization of BSA solutions shows the same general characteristics as those of lysozyme solutions (Stuhrmann et al., 1986).

3.3 Polarization of neutrons

The neutron small-angle scattering instrument which we used at the GKSS Research Centre is shown in Fig. 3. Neutrons released from the uranium fuel element of the 5 MW reactor FRG1 are moderated in light water and reflected by a bent neutron guide-tube of 14.7 m length and a radius of curvature of 1250 m. In this way, gamma-rays and neutrons with wavelengths below 2.8 Å are discarded. After further monochromatization by a mechanical velocity selector, the full width at half maximum (FWHM) of the wavelength spectrum $\Delta\lambda/\lambda$ is reduced to 0.2.

Polarization of the neutron beam is achieved by total reflection from the magnetized surfaces of a set of supermirrors (Schärpf, 1982). The polarization was better than 0.98. The divergence of the neutron beam was further decreased by a collimator of 3 m length and apertures of 15 mm at both ends. With the velocity selector running at 60 Hz, only 2500 polarized neutrons per second with wavelengths around 4.8 Å were observed with a BF_3 counter at the sample position. Removal of the velocity selector led to an increase of the neutron intensity to 38000 n/s and to a flux of 17000 n/s cm^{-2} .

The polarization of the neutron beam is maintained by a magnetic guide-field from a concentric solenoid coil on the outer surface of the cylindrical collimation tube (see, for example, Schärpf, 1982). A flat coil in the centre of the tube allows rotation of the neutron spin direction. The angle of rotation ϕ depends on the current and on the width d of the flat coil. Slow neutrons are more affected by the magnetic field H of the flat coil than by fast neutrons. This is summarized by the following formula (Schärpf, 1982):

$$\phi = \frac{m\gamma}{h} H d \lambda . \quad (23)$$

Flat coils are used as neutron spin flippers. The current is chosen in such a way that the spin direction is inverted, i.e. P_n in Eqs. (4) and (11) changes its sign. In small-angle neutron scattering, a wavelength spread of $\Delta\lambda/\lambda = 0.2$ was admitted and hence the effect of the flat-coil spin flipper led to a distribution of neutron spin directions of the same width, i.e. $180^\circ \pm 18^\circ$. The polarization of the neutron beam defined by the projection of the neutron spins onto the direction of the external magnetic field is then decreased by a factor of $\eta = 0.96$.

This factor has to be taken into account when the efficiency of the neutron polarizers is determined in the following set-up: neutron velocity selector, first neutron polarizer, flat-coil spin flipper, second neutron polarizer acting as a spin analyser, counter. After adjustment of the polarizers to maximum transmission, the flat coil is fed by a current that minimizes transmission of the neutrons by the analyser. We observed a decrease in transmission by a factor of $F = 23$, i.e. $1 - F^{-1} = 95.7\% = n_+ / (n_+ + n_-)$ of the neutron spins did flip. The polarization of the beam is then $1 - 2F^{-1} = 91.3\%$. Assuming the calculated efficiency of the flipper coil to be $\eta = 0.96$, the difference of $0.96 - 0.913 = 4.7\%$ in polarization has to be attributed to the neutron polarizers. A monochromatic neutron beam reflected by the polarizer would have a polarization of about 97.7%. This value of the neutron polarization would be deduced from our data if the efficiency of the flat-coil spin flipper reached the expected value.

The variation of the flipping efficiency with the wavelength of the neutrons is more apparent without velocity selection (Fig. 2). The polarization P_n of the neutrons leaving the active flat-coil spin flipper depends on the wavelength. Assuming that 4.8 Å neutrons are flipped completely ($P_n \equiv -1$), the 2.4 Å neutrons will appear unpolarized ($P_n = 0$) (Fig. 2).

3.4 Data acquisition

Neutron scattering was measured by a ^3He -filled position-sensitive area counter, which has been developed and built by Tauffenbach and Pauls at the GKSS Research Centre at Geesthacht. The sensitive area of the multiwire chamber amounts to 550 mm $\times$ 550 mm. The event of neutron capture by a ^3He nucleus with its decay products ^1H and ^3H is located by charge division at the ends of two orthogonal delay lines, digitized, and stored in an array of 64 $\times$ 64 elements, with a depth of 24 bits, of a multichannel analyser (MCA). The 4 kbytes memory meets the number of pixels resulting from 7 mm spatial resolution of the detector. The MCA also controls the state of the spin flipper.

The measurement of a sample may last from several hours to one day. Every 2 min the state of the spin flipper was changed, i.e. the current of the flat coil was switched on/off, and the data were accumulated in two separate bins of the MCA memory. Every 30 min the contents of the MCA memory were stored on magnetic tape. The data were also transferred to an IBM PC XT for further analysis.

Owing to the preliminary nature of the set-up, only the distance of 3.5 m between sample and detector was used (Fig. 3).

4. RESULTS

Polarized neutron small-angle scattering from frozen BSA solutions was measured at proton spin polarizations of $P_H = -0.4, -0.21, 0.005$, and 0.28 . At a given target polarization, the neutron spin direction was inverted every 30 min. The total measuring time was 10 h at wavelengths around 4.8 Å, and 4 h when no velocity selector was used. Measurements at $P_H = -0.6$ were performed earlier at lower transmission of the sample cell (Knop et al., 1986).

4.1 Measurements with 4.8 Å neutrons

Neutron small-angle scattering of frozen solutions of BSA compares well with the results obtained with X-rays from solutions at room temperature (Luzzati, Witz and Nicolaieff, 1961; Stuhmann, 1964). The radius of gyration of the BSA molecule, $R = 30 \text{ Å}$, is found in both cases (Fig. 4).

Polarized neutron small-angle scattering at various polarizations P_H of the target is shown in Fig. 5 for positive neutron polarization P_n . There is a strong increase of small-angle scattering with negative target polarization P_H . The small-angle scattering curves look very similar at different P_H , as do their basic scattering functions $S_U(Q)$, $S_{UV}(Q)$, and $S_V(Q)$ (Fig. 6). This is expected since the non-hydrogen atoms of the BSA molecule appear to have the same spatial distribution at low resolution. As the solvent molecules are completely deuterated, the polarization of the proton spins allows the BSA molecule, as a whole, to appear darker or lighter with respect to the background in the 'light' of polarized neutrons.

The square root of forward scattering is proportional to the contrast between the dissolved protein and the solvent [Eq. (15)]. Figure 7 shows that the contrast of BSA will vanish when the target polarization reaches 60%. At this point, the scattering density ρ of the solvent equals that of the dissolved BSA molecules. This observation leads to the assumption of a slightly decreased specific volume of the dissolved protein, $0.7 \text{ cm}^3/\text{g BSA}$, compared with values quoted for room-temperature measurements.

For the calculation of the scattering-length density, the mass density of the frozen solvent had been determined at liquid-nitrogen temperature. It is about 3% higher than at room temperature. The scattering-length density of the deuterated solvent at $P_H = 0.6$ thus amounts to $\rho(\text{solvent}) = 7.3 \times 10^{10} \text{ cm}^{-2}$. Figure 8 shows the scattering-length density of the solvent and of BSA molecules as a function of proton spin polarization of the sample.

From Fig. 7 it can be seen that forward scattering of the BSA solution may remain finite at any target polarization. In fact, this is expected from the limited number of protons per BSA molecule. On a microscopic scale, the proton spin polarization will differ from one volume element to the other. At a mean polarization of $P_H = 0.6$ as measured by NMR, out of the $n = 3300$ proton spins of one BSA molecule there will be, on the average, $n_+ = (n/2)(1 + P_H) = 2640$ proton spins 'up' and $n_- = (n/2)(1 - P_H) = 660$ proton spins 'down'. The deviations from these most probable numbers are described by a binomial distribution. Their variance is $\sigma^2 = n_+ n_- / n = 528$. The FWHM of polarization distribution is $2\sigma/n = 1.4\%$. Our data are not good enough to show this small effect. Even considerably larger fluctuations of the polarizations of different protein molecules would not readily be seen, since data near the matching point are missing. The broken line in Fig. 7 would correspond to fluctuations of between 50% and 70%. The present data exclude broader distributions of the polarizations of BSA molecules.

We note that the polarization of our sample may have macroscopic inhomogeneities owing to the inhomogeneity of the magnetic and the microwave fields, and of the material and coolant temperature. Experience with larger targets indicates maximum variations of the order of $\pm 5\%$; targets of the size of ours should have much less inhomogeneity.

4.2 Absolute measurement

Zero-angle scattering is proportional to the number N of dissolved particles, their scattering cross-section σ , and the solid angle of observation:

$$\mathfrak{S}(0)/W = N\sigma\Delta\Omega = 2.3 \times 10^{-5}. \quad (24)$$

Here W is the total number of incident neutrons per unit time, and

$$N = cN_L d/M = 4.4 \times 10^{17} \text{ cm}^{-3} ,$$

where $c = 0.083$ is the particle concentration by weight, N_L is the Loschmidt number, $d = 0.6 \text{ g/cm}^2$ is the density, and $M = 6.8 \times 10^4 \text{ g/mol}$ is the molecular weight of the dissolved particles (macromolecules); the cross-section is

$$\sigma = 4\pi(\delta v)^2 = 4\pi \times 0.86 \times 10^{-17} \text{ cm}^{-2} ,$$

where δ is the contrast, as defined by Eq. (15), of the BSA molecule and the deuterated solvent, and v is the volume of the BSA molecule. The solid angle of observation is

$$\Delta\Omega = \frac{A_{\text{pix}}}{4\pi\ell^2} = 6 \times 10^{-6}/4\pi ,$$

where $A_{\text{pix}} = (0.86 \text{ cm})^2$ is the pixel area in the counter, and $\ell = 350 \text{ cm}$ is the distance of the counter from the target. The incident intensity on the cross-section of the sample is attenuated by ^3He in the dilution refrigerator in two ways:

- i) 30% of the neutrons hit the ^3He between the slabs where they are absorbed (Fig. 1);
- ii) the others penetrate thin ^3He -filled gaps. The neutrons with $4.8 \text{ \AA}$ wavelength have a 16% transmission probability (see Section 3).

The total transmission is $0.7 \times 0.16 = 0.112$. Of 2500 incident polarized neutrons, only 280 have a chance of being scattered by the sample. As this is an inconveniently small number, we normalize data to 1 h measuring time. The effective incident flux, integrated over the target section, is $E = 1 \times 10^6$ neutrons per hour. Zero-angle scattering corrected for interparticle scattering (Fig. 4) amounts to 27 counts per hour. The ratio $\hat{s}(0)/W = 2.7 \times 10^{-5}$ is in satisfactory agreement with the predicted value of 2.3×10^{-5} .

4.3 On the use of the ‘white’ thermal neutron spectrum

The cross-terms $\vec{P}_n \cdot \vec{P}_H S_{UV}(2\theta)$, as obtained by using different wavelength spectra, are shown in Fig. 9. These terms are affected not only by the wavelength dependence of the absorption of thermal neutrons by ^3He in the sample cell, but also by the spectral efficiency of the flat-coil neutron spin flipper. To summarize this, we start from the wavelength integral (Guinier and Fournet, 1955; Glatter and Kratky, 1982)

$$S(2\theta) = \int_{\lambda} \omega(\lambda) [S_U(2\theta/\lambda') + P_n(\lambda)P_H S_{UV}(2\theta/\lambda') + P_H^2 S_V(2\theta/\lambda')] d\lambda , \quad (25)$$

where $\lambda' = \lambda/\bar{\lambda}$ ($\bar{\lambda}$ = mean wavelength). The wavelength distribution $\omega(\lambda)$ shown in Fig. 2 has been determined from time-of-flight measurements. In the sample chamber, ^3He cuts down the thermal neutron spectrum on the long-wavelength side according to $1/\exp(c\lambda)$; c is proportional to the known absorption coefficient of ^3He at $\lambda = 1 \text{ \AA}$ and to the number of ^3He atoms in the flight path of thermal neutrons. The latter could not be determined directly, as neither the width of the gaps between the sample and its spacers (see Fig. 1) nor the ^3He content in the cooling medium could be estimated with sufficient precision. However, comparison of small-angle scattering as obtained from a monochromatic neutron beam with measurements using the whole thermal neutron spectrum yields the coefficient c in the above exponent. The wavelength spectrum leaving the sample cell is given by $W(\lambda) = W_0(\lambda)/\exp(0.4\lambda)$, with λ in $\text{\AA}$.

The use of a flat-coil spin flipper leads to a spectral distribution of polarizations $P_n(\lambda) = \cos(\pi\lambda/\lambda_0)$, where λ_0 is the wavelength (here $\lambda_0 = 4.8 \text{ \AA}$) at which the magnetic field of the flat-coil flipper rotates the direction of the neutron spin by π . The $P_n(\lambda)$ is close to 1 when the flipper is switched off. Only the cross-term linear in P_H [second term on the right-hand side of Eq. (11)] is sensitive to neutron spin polarization.

The cross-term evaluated from polarization variation of the target with an inactive neutron spin flipper yields S_{UV} , which is very similar to S_U or S_V in Fig. 6. The same procedure, but using data obtained with an active spin flipper, yields a scattering function that clearly reflects a narrower wavelength spectrum. In fact, it is the spectral distribution of polarization $P_n(\lambda)\omega(\lambda)$ shown in Fig. 2 (broken line) that gives rise to the cross-term. The flat-coil spin flipper acts as a poor monochromator of polarized neutrons.

5. CONCLUSIONS

The variation of scattering density of the protein BSA follows the predicted behaviour. At a target polarization of $P_H = 60\%$, the scattering-length density of the BSA molecule is equal to that of the solvent. From this 'matching point', a slightly decreased specific volume of $0.70 \text{ cm}^3/\text{g}$ BSA at $T = 0.5 \text{ K}$ is derived. Usual values are between 0.72 and $0.74 \text{ cm}^3/\text{g}$ at room temperature. Similarly, the density of the frozen solvent at liquid-nitrogen temperature is about 3% higher than at room temperature. Spin-contrast variation provides a direct method for the determination of the density of macromolecules at very low temperatures.

The conservation of the macromolecular structure of BSA in the frozen solvent is not entirely unexpected. Glycerol water mixtures are known to remain in a frozen-liquid, glassy state, a fact that is widely used in biochemical conservation methods and in nature (Douzou, 1977). It is also a necessary prerequisite for successful dynamic nuclear polarization (Niinikoski, 1976). This lucky coincidence is the basis for *in situ* structure determinations of biomolecules.

The quantitative analysis of the polarized neutron scattering on BSA solutions confirms the predicted gain in contrast, compared with isotopic substitution methods. We emphasize that only one sample is needed in spin-contrast variation. This avoids systematic errors, which cannot be excluded in isotopic substitution methods since they start from a new sample for each contrast.

The sample cell needs some improvement. In fact, another dilution refrigerator has been adapted by CERN to the specific requirements of thermal-neutron scattering. The sample is no longer embedded in the strongly neutron-absorbing $^3\text{He}/^4\text{He}$ mixture. The new sample cell is cooled by ^4He , which is thermally coupled to the mixing chamber. Recently, the first test at CERN showed that such a sample cell works properly.

A similar design will soon be ready at CEN-Saclay (Glättli, 1988), and another one, using a ^3He evaporation refrigerator, has been put to work at KEK (Masuda et al., 1988). The latter is specifically designed as a neutron polarizer.

The intensity of 280 neutrons per second, which is available at present, is too low for higher-resolution structure work. For future experiments, the intensity at the sample will be increased by

- a factor of 30, owing to the installation of a cold source in the FRG1 reactor and to some modifications of the reactor core; and

- a factor of 20, owing to the modified dilution refrigerator, excluding ^3He in the sample chamber. The temperature of the new dilution refrigerator reaches 40 mK, and higher nuclear polarizations will be achieved. This means another increase of spin contrast by a factor of 2 to 3 with respect to the results on BSA and on other materials; these data are being analysed.

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Table 1
Parameters of spin-dependent cross-sections of some nuclei
(from Glättli and Goldman, 1987)

Nucleus	I		b (10^{-12} cm)	B (10^{-12} cm)
Hydrogen	^1H	1/2	−0.374	2.912
	^2H	1	0.667	0.285
Carbon	^{12}C	0	0.665	0
	^{13}C	1/2	0.629	−0.06
Nitrogen	^{14}N	1	0.937	0.14
Oxygen	^{16}O	0	0.580	0

APPENDIX 1

NUCLEAR POLARIZATION-DEPENDENT SCATTERING DENSITIES

The scattering density ρ is the sum of coherent scattering lengths in a unit volume:

$$\rho = dN_L(\sum b_i + \sum P_i I_i B_i)/M, \quad (\text{A1.1})$$

where d = density, N_L = Loschmidt number and M = molecular weight.

As an example, we calculate the dependence of the scattering density of water on the proton spin polarization P_H : assuming that the density is 1 g/cm^3 , there are $6 \times 10^{23}/18 = 0.333 \times 10^{23}$ water molecules per cubic centimetre. Each H_2O molecule has a scattering length of

$$[(0.58 - 2 \times 0.374) \pm 2 \times 1.45 P_H] \times 10^{-12} \text{ cm}.$$

Here we have made use of the scattering lengths given in Table 1. The scattering density of water is then

$$\begin{aligned} \{6[(0.58 - 2 \times 0.374) \pm 2 \times 1.45 P_H]/18\} \times 10^{11} \text{ cm/cm}^3 = \\ \rho(\text{H}_2\text{O}) = [-0.56 \pm 9.66 P_H] \times 10^{10} \text{ cm}^{-2}. \end{aligned}$$

Similarly, for heavy water D_2O we obtain

$$\rho(\text{D}_2\text{O}) = [+6.40 \pm 1.50 P_D] \times 10^{10} \text{ cm}^{-2}.$$

For glycerol ($d = 1.26 \text{ g/cm}^3$) and its deuterated form, the corresponding relations are

$$\begin{aligned} \rho(\text{C}_3\text{H}_8\text{O}_3) &= [0.61 \pm 9.56 P_H] \times 10^{10} \text{ cm}^{-2}, \\ \rho(\text{C}_3\text{D}_8\text{O}_3) &= [7.48 \pm 1.48 P_D] \times 10^{10} \text{ cm}^{-2}. \end{aligned}$$

As an example of a protein, we take lysozyme, $\text{C}_{613}\text{H}_{949}\text{O}_{186}\text{N}_{192}\text{S}_{10}$ ($M = 14289$, $d = 1.37 \text{ g/cm}^3$). Its scattering density varies with the proton spin polarization as follows:

$$g(\text{lysozyme}) = [1.94 \pm 8.05 P_H] \times 10^{10} \text{ cm}^{-2}.$$

When dissolved in heavy water, the dissociating 194 hydrogens of the lysozyme molecule will be exchanged by deuterium. The chemical composition of lysozyme is then changed into $\text{C}_{613}\text{H}_{755}\text{D}_{194}\text{O}_{186}\text{N}_{192}\text{S}_{10}$. The scattering density of lysozyme in D_2O is

$$\rho(\text{lysozyme in D}_2\text{O}) = [3.10 \pm 6.53 P_H] \times 10^{10} \text{ cm}^{-2}.$$

APPENDIX 2

POLARIZATION OF DEUTERONS AND PROTONS AT THERMAL EQUILIBRIUM

The polarization P is a function of the ratio of the magnetic field B and the temperature T :

$$P_I(\mu B/kT) = (2I + 1)/2I \coth [(2I + 1)\mu B/2IkT] - 1/2I \coth (\mu B/2IkT) . \quad (A2.1)$$

At thermal equilibrium, the static polarization of proton spins ($I = 1/2$, $\mu = 2.793$ nuclear magnetons) differs from that of the deuteron spins ($I = 1$, $\mu = 0.857$ nuclear magnetons). How does the ratio $P_{1/2}(\mu_H B/kT)/P_1(\mu_D B/kT)$ vary with the proton spin polarization P_H ? An empirical formula relating the proton spin polarization P_H to deuteron spin polarization is

$$P_D^* = P_H/[4.88 - 1.8 P_H^2 - P_H^{10}] . \quad (A2.2a)$$

The relative deviation from the exact value of P_D , $(P_D - P_D^*)/P_D$, will not exceed 2% at $P_H \leq 0.96$.

At $P_H \leq 0.75$, a shorter formula meets the same precision:

$$P_D^* = P_H/(4.88 - 1.8 P_H^2) . \quad (A2.2b)$$

The influence of the deuterons on the polarization-dependent part of the amplitude is much smaller than that of the proton spins for two reasons:

- i) the polarization-dependent scattering length of the deuteron is five times smaller than that of the proton (Table 1);
- ii) at thermal equilibrium the deuteron spins are less polarized than the proton spins. This is described by the above relations.

As a result of these two factors, and when $P_n = \pm 1$, the polarization-dependent scattering length of a deuteron in units of 10^{-12} cm is

$$\begin{aligned} b_D &= +0.667 \pm 0.285 P_D \approx 0.667 \pm 0.285 P_D^* \\ &= +0.667 \pm P_H/(17.12 - 6.3 P_H^2 - 3.5 P_H^{10}) . \end{aligned} \quad (A2.3)$$

Compared with the proton with

$$b_H = -0.374 \pm 1.45 P_H , \quad (A2.4)$$

the deuteron contributes less to polarization-dependent scattering length by a factor of between 5 (at $P_D = P_H = \pm 1$) and 25 (at $P_H < 0.3$).

Figure Captions

Fig. 1: Top view of sample cell. Incident neutrons from the right-hand side are scattered by four sample blocks (black area). The $^3\text{He}/^4\text{He}$ mixture is in the cylindrical mixing chamber defined by the open Teflon cylinder closed by an aluminium cap (shaded area). There is some ^3He between the steel walls and the adjacent mixing aluminium. The NMR coil is threaded through the quartz pieces.

Fig. 2: Wavelength distribution of polarized neutrons at the sample position:

- without velocity selector;
- without velocity selector, after sample cell;
- - - without velocity selector, with neutron spin flipper;
- ▲ with velocity selector, after sample cell.

Fig. 3: Neutron small-angle instrument: VS = velocity selector; P = polarizer; SF = spin flipper; EM = electromagnet (2.5 T); C = nose of horizontal dilution refrigerator; μW = microwave generator; F = microwave frequency counter; D1, D2 = detector positions. Only the 3 m position was used. Dots along the collimation path indicate the solenoid providing the magnetic guide-field for polarized neutrons.

Fig. 4: Small-angle scattering of BSA solutions:

- a) X-rays, at room temperature:
 - - - $c = 3.8\%$ by weight of BSA (Stuhrmann, 1964);
 - extrapolated to $c = 0$ from a series of concentrations.
- b) Neutrons, at $T = 0.5$ K:
 - $c = 12\%$ by weight (Knop et al., 1986);
 - $c = 8.3\%$ by weight (this work).

Fig. 5 Polarized neutron small-angle scattering of BSA as a function of the scattering angle 2θ at different target polarizations P . These data were obtained with a 'white' thermal-neutron spectrum.

Fig. 6 The basic scattering functions of BSA as obtained from spin-contrast variation using DNP:
 ● $S_U(Q)$, ▲ $S_{UV}(Q)$, ○ $S_V(Q)$;
 ■ $P S_{UV}(Q)$ from brute force polarization of protons at $T = 0.5$ K in a magnetic field; $H = 2.5$ T; $P = 0.5\%$. The 'white' thermal neutron spectrum was used. Reduction of data in Fig. 5.

Fig. 7 Square root of forward scattering of BSA solutions. The broken line refers to an inhomogeneity of polarizations between different BSA molecules varying between $P = 50\%$ and $P = 70\%$. Since points near the minimum are missing, this can be regarded as an upper limit of polarization inhomogeneities, exceeding the theoretical inhomogeneity presented by the minimum of the full line. Wavelength $\lambda = 4.8 \text{ \AA} \pm 0.9 \text{ \AA}$.

Fig. 8 Scattering densities of the solvent (-----) and the dissolved BSA molecules:

- after two hours, incomplete H/D exchange of dissociating protons (Haas, 1977);
- - - after complete H/D exchange of dissociating protons.

Fig. 9 Cross-term $S_{UV}(2\theta)$ as obtained from

- 'white' neutron spectrum, spin flipper off;
- 'white' neutron spectrum, spin flipper on;
- quasi-monochromatic neutrons at $\lambda = 4.8 \text{ \AA}$.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Fig. 9