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# Estimation of deuteration levels in whole cells and cellular proteins by $^1H$ n.m.r. spectroscopy and neutron scattering

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Methods using conventional Fourier transform $^1H$ n.m.r. spectroscopy at 250 MHz for the determination of the overall deuteration levels of cells cultured in media containing $^2H_2O$ or deuterated carbon sources are described. These were developed for *Escherichia coli* as a model, and extended to *Neurospora crassa* hyphae and mouse myeloma cells P815. The results were investigated by $^1H$ n.m.r. and neutron scattering measurements on deuterated proteins that were obtained from *E. coli*. It is concluded that $^1H$ n.m.r. is able to observe the soluble proteins of *E. coli* in certain cases, that deuteration levels can be determined by $^1H$ n.m.r. for small quantities of proteins in their native state, and that glycerol is a more efficient carbon source than glucose for the deuteration of *E. coli* proteins.

Deuteration techniques have been found to be most valuable in the studies of biological systems by both n.m.r. spectroscopy and neutron diffraction studies. In the case of n.m.r., deuteration of the solvent or macromolecule offers the chance to simplify a complicated spectrum (Feeney *et al.*, 1977), to suppress undesired features such as the signals of NH protons or water protons (Campbell & Dobson, 1979), or to allow $^2H$ n.m.r. studies to be made (Brown *et al.*, 1979). For neutron scattering, variation of the ratio of $H_2O$ to $^2H_2O$ in the solvent leads to information on the shape and internal structure of macromolecules. Since deuterated macromolecules have quite different scattering properties from their protonated equivalents, such macromolecules can be studied as a distinct unit in an otherwise protonated multicomponent system (Jacrot, 1976; Kneale *et al.*, 1977; Stuhrmann & Miller, 1978; Koch & Stuhrmann, 1979). The importance of this latter method continues to increase.

Proteins and nucleic acids can be deuterated by the cellular incorporation of $^2H$ from either $^2H_2O$ or deuterated carbon sources that are present in the growth medium (Moore & Engelman, 1976). While cellular techniques achieve some deuteration, chemical methods are more specific for deuterated lipids or detergents (Brown, 1979; Seelig & Seelig, 1974, 1975). With cellular deuteration techniques, it is necessary to follow the incorporation of $^2H$. Procedures may thus be evaluated and optimized, and quantitative estimates of deuteration levels can be provided for prior planning or for data analysis in neutron experiments. High-resolution $^1H$ n.m.r. is the most general technique for this, and has the advantage of being fully independent of the neutron experiment.

Here, it is shown that n.m.r. provides quantitative measures of the deuteration of whole cells, and that in certain cases these results are comparable with the deuteration levels of proteins extracted from these cells. This is of practical relevance where it is needed to optimize the deuteration of cell cultures grown in media containing significant levels of proton impurities, i.e. in 90% $^2H_2O$ media with hydrogenated glycerol, or in ordinary water with deuterated carbon sources. The deuteration of *E. coli* strain MRE600 is used as a model, and the extension to other types of cellular systems is described. The results are confirmed by using comparisons of deuteration levels in proteins from such cultures that are determined both by neutron scattering and by n.m.r. A previous account (Moore, 1977) has described a n.m.r. deuteration assay which was based on large amounts of proteins or RNA (10–60 mg) denatured in trifluoroacetic acid.

## Materials and methods

*E. coli* strain MRE600 was cultured on Anderson's minimal salts medium [in g/l: $NH_4Cl$, 1; $Na_2HSO_4$ (anhydrous), 6; $KH_2PO_4$ (anhydrous), 3;



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$MgSO_4,7H_2O$, 0.27]. The carbon source was usually hydrogenated glycerol (5 g/l) but in several cases glucose was used instead (4 g/l). Cells were grown in media containing 0–90% $^2H_2O$, following the techniques of Moore & Engelman (1976). On harvesting, cells were spun down and stored at −70°C until use. For each deuteration culture, 100 ml of medium is ample for assay.

*N. crassa* was cultured on Vogel's medium (Weiss *et al.*, 1970) [in g/l: trisodium citrate, $2H_2O$, 3; $KH_2PO_4$ (anhydrous), 5; $NH_4NO_3$, 2; $MgSO_4,7H_2O$, 0.2; $CaCl_2,2H_2O$, 0.1; to which has been added biotin and several trace elements]. A carbon source of hydrogenated sucrose was used at 1 g/l in place of the more usual 2 g/l. Deuteration was achieved by the use of media with a $^2H_2O$ content between 0–90%.

For n.m.r., it is necessary both to wash the cells free of protonated growth media and to obtain the relative concentrations of cells from one sample to the next. *E. coli* cells were washed in a 99.9% $^2H_2O$ buffer containing 10 mM-phosphate and 0.9% NaCl, at p$^2$H 7.1 (pH meter readings are given in this text uncorrected for isotope effects). About 50 mg wet wt. of cells was added to a preweighed Eppendorf vial, the cells were suspended in $^2H_2O$ buffer, and they were spun down; three such washes were performed. After removal of supernatant, the vial was reweighed to give the wet wt. of cells, and 0.5 ml of $^2H_2O$ buffer was used to resuspend the cells and transfer them to the n.m.r. tube. For n.m.r. it was most important that the cells remained intact, and this was judged from the appearance of the cell suspension.

Eucaryotic cells of the mouse myeloma strain P815 could be handled by the same washing method. The cells were cultured in Eagle's minimal essential medium, which is a mixture of inorganic salts, glucose (1 g/l), and twelve protonated amino acids (totalling 0.77 g/l). To prevent cell lysis, the $^2H_2O$ buffer had to be iso-osmotic with the cell cytoplasm. By using visual inspection under a light microscope, a buffer containing 0.15 M-NaCl/2 mM-$MgCl_2$/20 mM-$Na_2HPO_4$ in $^2H_2O$ at p$^2$H 7.3 was found to be satisfactory.

Washing and weighing of the *Neurospora* hyphae required a different procedure for satisfactory deuteration assays. About 400 ± 20 mg of hyphae was weighed into an Eppendorf vial. It was washed with $^2H_2O$ buffer three times, cutting the hyphae in the process. The final residue was placed in a small Potter homogenizer, 2 ml of 10 mM-phosphate in $^2H_2O$ at p$^2$H 7.0 was added, and the hyphae were made into a fine suspension. A Pasteur pipette was used to transfer about 1.5 ml of suspension into the n.m.r. tube; the neck of the pipette (which should be slightly wider than usual) avoids the transfer of large masses of hyphae. Solutions were stored at 4°C until the n.m.r. measurement on the same day. The correction factor for concentration was obtained by weighing a dry glass vial, reweighing it after well-draining the n.m.r. tube contents into it (15 min), and reweighing it after drying to constant weight at 90°C.

For n.m.r. work with the deuterated proteins, thorough dialysis was necessary to remove protonated buffers {e.g. acetate, Tris or Hepps [4-(2-hydroxyethyl)-1-piperazinepropane sulphonic acid]} from the protein samples. A buffer of 10 mM $KH_2PO_4$/1 mM-$MgCl_2$ in 99.9% $^2H_2O$ was used, previously adjusted to p$^2$H 7.5 with 1% (v/v) and 8% (v/v) stock solutions of $^2HCl$ and $NaO^2H$. The exchangeable protons of the salts were preexchanged by dissolution in $^2H_2O$ and lyophilization. Very small samples of proteins (>1 mg) were subjected to repeated lyophilization from 99.98% $^2H_2O$ to remove surplus water after this dialysis procedure. Relative protein concentrations in all cases were measured by u.v. absorption at 280 nm in 1 or 2 mm thick quartz Hellma cells.

Ribosomal proteins were separated from ribosomal RNA by using an acidic acetate extraction at 4°C (Spillman & Nierhaus, 1978). To a solution of at least $150A_{260}$ units/ml of 50S *E. coli* ribosomal subunits was added 0.1 vol. of 1 M-magnesium acetate and 2 vol. of acetic acid. This was stirred for 45 min at 0°C, and spun at 10000 rev./min for 30 min to remove RNA. The proteins in the supernatant were dialysed at pH 3 against distilled water to remove acetate, using a Spectrapor dialysis membrane (Spectrum Medical Industries, Los Angeles, CA, U.S.A.) of molecular weight cutoff 2000.

Membrane proteins which have had to be solubilized with detergents can be separated from the detergent and kept in solution by dialysis against 6 M-guanidinium chloride, 0.4 ml of which was added to the lyophilized sample, and the sample was dialysed with four changes against the denaturant. The solvent was replaced with 99·98% $^2H_2O$ by repeated lyophilization. A similar approach is advisable for multisubunit proteins which otherwise may or may not be in the same condition of association in the protonated and deuterated samples. This may affect the n.m.r. assay.

$^1$H n.m.r. spectra were recorded on a Cameca 250 spectrometer at 250 MHz in the Fourier transform mode. Measurements were at 37°C. The times between 90° pulses was at least 5.4 s to allow all proton signals to attain magnetic equilibrium between pulses (i.e. five spin-lattice relaxation times). Spectra were obtained in 10–30 min except for the most dilute protein samples, where times of up to 180 min could be involved. No solvent suppression techniques were used to avoid errors of integration that can arise from spin diffusion or transfer of

saturation effects. The envelope of proton signals in the window of interest was integrated either electronically on the spectrometer computer or more reliably by weighing the area under the signal envelope. This window was taken to be the spectrum upfield of the water signal such that the effect of the water signal on the integration is avoided. Spectra were normalized after Fourier transformation by checking the noise level at the extrema of the spectra and by checking the number of times the intensity had been divided by 2 during Fourier transformation. In the case of cells, all measurements were preferably duplicated on at least two different samples, in particular for the protonated blank (which has been grown under the same conditions as the deuterated cultures), and the n.m.r. tube was inverted several times before measurement to avoid settling.

Small angle neutron scattering measurements were made of the D11 instrument at the Institut Laue–Langevin (Ibel, 1976). The deuterated elongation factor Tu sample used here was studied with a sample–detector distance of 1.79m and 1.18nm wavelength (8% $\Delta\lambda/\lambda$ wavelength spread). Guinier plots were obtained of the intensity $I$ as a function of momentum transfer $Q$ (where $Q = 4\pi \sin\theta/\lambda$, where $2\theta$ is the scattering angle) according to:

$$\ln I_{(Q)} = \ln I_{(0)} - R_g{}^2Q^2/3$$

where $R_g$ is the radius of gyration. The value of $I_{(0)}$, the forward scattering at zero $Q$, can be used to confirm the deuteration level of the macromolecule, either by plotting $\sqrt{I_{(0)}/c}$ as a function of the contrast (where $c$ is the concentration), or by calculation on the basis of the value of $I_{(0)}/c$ measured in $H_2O$ solvent.

In the first case, deuteration levels may be obtained from the relationships shown in Fig. 1. These were calculated by using the average amino acid composition for a protein given by Dayhoff (1978), which is based on 314 known protein sequences, and the amino acid volumes presented by Chothia (1975) and Zamyatnin (1972). Electrostriction is not considered (Cohn & Edsall, 1943). The scattering densities of $H_2O$ and $^2H_2O$ were taken to be $-0.562 \times 10^{-10}\,cm^{-2}$ and $6.404 \times 10^{-10}\,cm^{-2}$ respectively. The scattering lengths of $^1H$, $^2H$, $^{12}C$, $^{14}N$, $^{16}O$, and $^{32}S$ in units of $10^{-12}$cm were taken to be $^1H$, −0.3742; $^2H$, 0.6671; $^{12}C$, 0.6651; $^{14}N$, 0.940; $^{16}O$, 0.5804; $^{32}S$, 0.2847 (Stuhrmann & Miller, 1978). The two lowest traces shown, which are those based on the volumes given by Chothia (1975) and assuming 10% or 20% exchange of the mainchain NH protons (one per residue except for proline) gives better agreement with experiments, but those based on the volumes given by Zamyatnin (1972) are given for comparison. The figures for exchangeable NH protons correspond to 5% and 11% exchange of all exchangeable protons in the Dayhoff (1978) average composition.

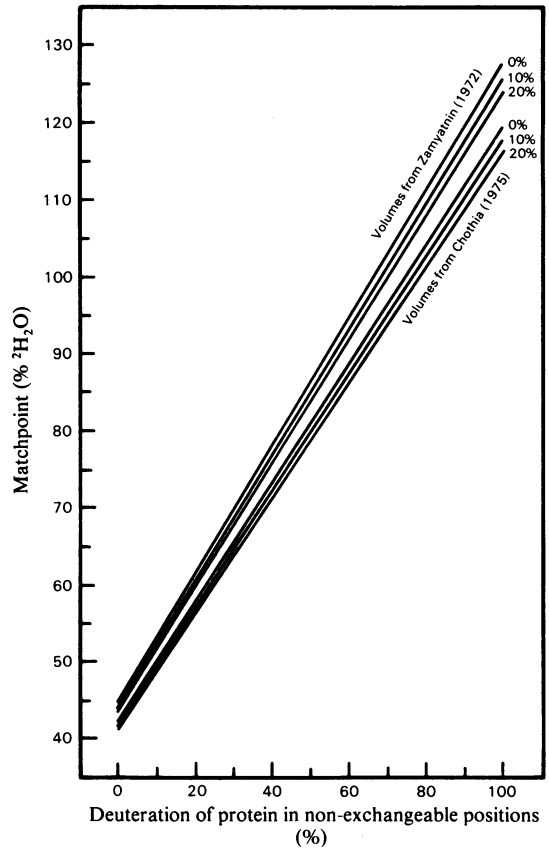


Fig. 1. *Graphical relationships between the deuteration level of the CH protons of the 'Dayhoff average protein' and the percentage $^2H_2O$ matchpoint of the protein*

The details of calculations are given in the Materials and methods section. For each of the Chothia (1975) and Zamyatnin (1972) volumes, lines are drawn to depict 0%, 10% and 20% non-exchange of the mainchain NH protons from the top downwards. The remaining exchangeable protons are fully exchangeable with solvent protons.

The value of $I_{(0)}/c$ measured in $H_2O$ solvent is dependent on the atomic composition of the macromolecule, according to the expression for molecular weights given by Jacrot & Zaccai (1981). This is used to derive the percentage deuteration of the macromolecule on the basis of chemical composition and the amount of deuterated exchangeable protons estimated by n.m.r. (Perkins *et al.*, 1981).

## Results

### *Control spectra*

Control n.m.r. experiments were made with hen

egg white lysozyme (EC 3.2.1.17) to confirm the main assumption of the n.m.r. method, namely that under a range of solution conditions for a given sample, the signal intensity is dependent only on the concentration of the sample. As found also by Moore (1977) for bovine serum albumin denatured in deuterotrifluoroacetic acid, the signal intensity was directly proportional to concentration in a range between 0 and 120mg of lysozyme/ml. It was further found that variation of ionic strengths between 0 and 1M-NaCl did not affect the assumption unless the sample had precipitated. A minimum sample volume of 0.4ml was found to be necessary to position adequately the sample within the probe, but larger volumes had no effect on signal areas.

*Proton spectra of cells and cell extracts*

The general appearance of n.m.r. spectra of *E. coli* grown on protonated glycerol suggest that the chief contribution to the spectrum is from the protein contents of the cell (Fig. 2 versus Fig. 4). Typical nucleic acid spectra have predominant signals from the ribose or deoxyribose sugar protons between 4.7 and 3.9p.p.m. and from the base protons and H-1 protons between 8.5 and 6.0p.p.m. (Davies & Danyluk, 1974; Schimmel & Redfield, 1980). Typical neutral and acid pyranose sugars have proton signals from the ring protons between 4.2 and 3.3p.p.m. and from the acetamido methyl group at 2p.p.m. (Perkins *et al.*, 1977; Welti *et al.*, 1979). Typical spectra of lipid vesicles in $^2H_2O$ are broad-lined, with some sharp features, all upfield of the water signal, and for the most part are not expected to feature in the *E. coli* spectra since the membrane lipids are immobilized on the n.m.r. timescale (Dwek, 1973) in comparison with the soluble cellular proteins of *E. coli*. What differences that are seen between Figs. 2(*a*) and 4 are attributed to minor contributions from sugar proton signals, lipid proton signals, and the highly flexible portions of sidechains of membrane-bound components (i.e. proteins and glycolipids). This lineshape comparison shows that these additional contributions are minor and amount to about 15% of the spectrum upfield of the $HO^2H$ signal relative to that in Fig. 4. When glucose was used as the protonated carbon source, the general appearance of the spectra changed to emphasize the sugar component at the expense of the protein component.

The use of very large spectral windows of the order of $10^4$Hz together with slow spectral ac-

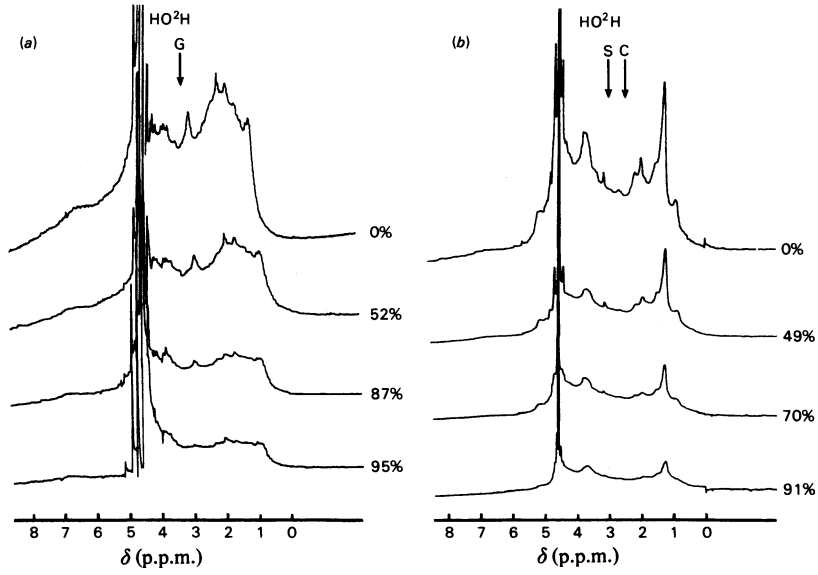


Fig. 2. *250MHz $^1H$ n.m.r. spectra of deuterated E. coli cells and Neurospora extracts*
(*a*) *E. coli* was cultured in Anderson's minimal salts medium with 5g of glycerol/l as the protonated carbon source, and was washed in deuterated phosphate/saline buffer in 99.9% $^2H_2O$ prior to measurement (see the Materials and methods section). The spectra are plotted on the same scale, and are approximately based on the same cell concentrations. The arrow denoted G shows the chemical shift of the proton signal of glycerol. (*b*) *Neurospora* extracts were prepared as described in the Materials and methods section. The arrows denoted S and C show where the n.m.r. signals of sucrose and citrate respectively are centred. In (*a*) and (*b*), the numbers to the right correspond to the percentage of $^2H_2O$ present in the growth medium.

cumulation rates showed that there was a very broad component upon which the above spectra are superimposed. This broad component is assigned to these immobilized cellular contents.

N.m.r. spectra of *E. coli* grown on glycerol are shown in Fig. 2(*a*). With increase of $^2H_2O$ in the growth medium, the signal envelope decreases in area. As expected, a linear relationship is observed between the observed deuteration (referenced to the protonated sample) and the amount of $^2H_2O$ in the medium (Fig. 3), once the integrated spectral areas had been normalized for concentrations. Fig. 3 thus shows that the technique is viable, in particular in the method of determining cellular concentrations, and also indicates the reproducibility of the method. With fast accumulation rates of the spectra, the apparent deuteration of the cells increases slightly. The times of accumulation were about 20 min.

$^1H$ n.m.r. spectra of P815 mouse myeloma cells were recorded. While sufficient amounts of deuterated cells were not available for detailed study, the general appearance of the spectra were similar to those of *E. coli* grown on glycerol. The additional contribution of non-protein lineshape is about one-third less than that of the *E. coli*/glycerol system. This result implies that the soluble proteins

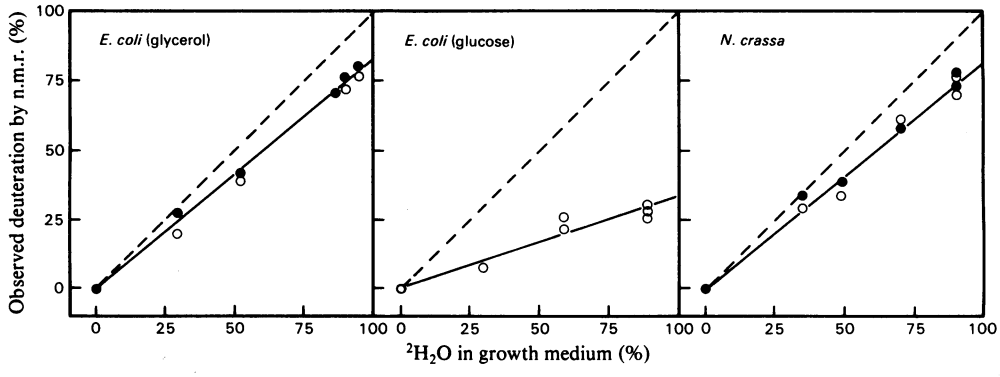


Fig. 3. *Observed percentage deuteration in the cells versus percentage $^2H_2O$ in the growth medium*
The results for *E. coli* and *N. crassa* were normalized for relative concentration (see the Materials and methods section). ●, Rapid accumulation; ○, slow accumulation. The dashed line represents a slope of unity which corresponds to complete incorporation of deuterium from the medium. The window of integration was taken to be 0–3.5 p.p.m. in the spectra of Fig. 2.

Table 1. *Comparisons of deuteration estimates by $^1H$ n.m.r. and by neutron scattering*
All the proteins were extracted from *E. coli*.

| Carbon source | Material | $^2H_2O$ in the growth medium (%) | $^1H$ n.m.r. estimate of deuteration (%) | Neutron scattering estimate of deuteration (%) |
|---|---|---|---|---|
| Glycerol* | | | | |
| | Intact *E. coli* | 90 | 75 | — |
| | 50S ribosomal proteins | 90 | 72 | — |
| | Elongation factor Tu (Wittinghofer & Leberman, 1976) | 90 | 74 | 74 |
| Glucose† | | | | |
| | Intact *E. coli* | 90 | 30 | — |
| | Matrix protein (Rosenbusch, 1974) | 90 | 67 | 64‡ |
| | Phenylalanine-tRNA synthetase (Fayat *et al.*, 1974) | 96 | 66 | 69§ |
| | Intact *E. coli* | 100 | 33 | — |
| | Aspartate transcarbamoylase-rich protein extract | 100 | 71 | — |

* 5 g/l.
† 4 g/l except for synthetase (2 g/l).
‡ M. Zulauf & J. P. Rosenbusch, personal communication.
§ P. Dessen & A. Ducruix, personal communication.

again dominate the spectrum, even though n.m.r. spectra of the Eagle's medium reflect the predominant glucose signal.

The spectra of *Neurospora* extracts are shown in Fig. 2(*b*) and are of different appearance to those of the *E. coli* spectra in Fig. 2(*a*). The most prominent component at 1.3 p.p.m. most probably originates from saturated lipids which have been dispersed in solution (Campbell & Dobson, 1979). The spectra are thus a composite of proteins, carbohydrates and lipids. Fig. 3 shows again that a linear relationship is obtained between observed deuteration and amount of $^2H_2O$ in the medium. This shows that the three main cellular components have undergone deuteration. Here, and for the *E. coli* spectra, the washing procedures were seen to be effective, since no large signals corresponding to the hydrogenated carbon source were observed.

*N.m.r. and neutron studies of deuterated proteins*

The above results were compared with the estimates of deuteration levels in proteins extracted from *E. coli* grown in deuterated media containing either protonated glycerol or glucose (Table 1), for which typical spectroscopic results are shown in Fig. 4 for elongation factor Tu, and the matrix protein of the *E. coli* outer membrane.

For proteins that were deuterated on the basis of a glycerol carbon source, excellent correspondences were found between the deuteration levels observed for the whole cells, two independent protein fractions, and the neutron scattering result for one of these. The neutron results are shown in Fig. 5 for elongation factor Tu, although the available instrument time on D11 for the deuterated sample was limited. A matchpoint of 95% $^2H_2O$ can be estimated for the deuterated material in place of 42% $^2H_2O$ for the protonated material. Calculations were based on the amino acid composition given by Arai *et al.* (1980), the amino acid volumes from Chothia (1975), and estimates of 19% and 10% for the non-exchanged mainchain protons (i.e. 11% and 6% of all exchangeable protons) that are buried within the protonated and deuterated proteins respectively. The latter were determined also by n.m.r. methods (Fig. 4; Perkins *et al.*, 1981). This matchpoint estimate can be translated into a deuteration level of 74% by using the procedure described in the Materials and methods section. From the value of $I_{(0)}/c$ in $H_2O$ from neutron scattering, a deuteration of 74% was also calculated.

The deuteration levels of proteins from *E. coli* cells grown on glucose are uniformly lower (Table 1). This prompted a fresh n.m.r. study of *E. coli* cells grown on a glucose source. Fig. 3 shows that rather lower overall deuteration levels for the whole cells were measured than for the proteins extracted from such cells. This reflects the incorporation of protonated glucose into the cellular fabric, and could be identified as such from the appearance of the n.m.r. spectra. Moore's (1977) estimates of protein deuteration from cells cultured on a glucose source

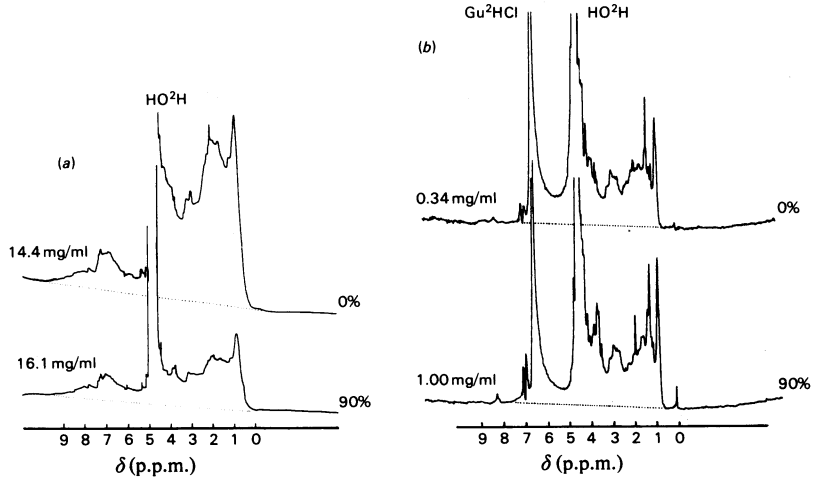


Fig. 4. *N.m.r. spectra of proteins from E. coli*

(*a*) 250 MHz $^1H$ n.m.r. spectra of elongation factor Tu from *E. coli*. The samples were dialysed against 20 mM-sodium phosphate/2 mM-$MgCl_2$ buffer at p$^2$H 7.5 in 99.9% $^2H_2O$. The numbers to the right correspond to the percentage of $^2H_2O$ in the growth medium of *E. coli* and the concentrations are to the left. (*b*) N.m.r. spectra of matrix protein from *E. coli* in 6 M-guanidium chloride. The samples had been repeatedly freeze-dried from solutions in 99.98% $^2H_2O$. Abbreviation used: Gu$^2$HCl, deuterated guanidinium chloride.

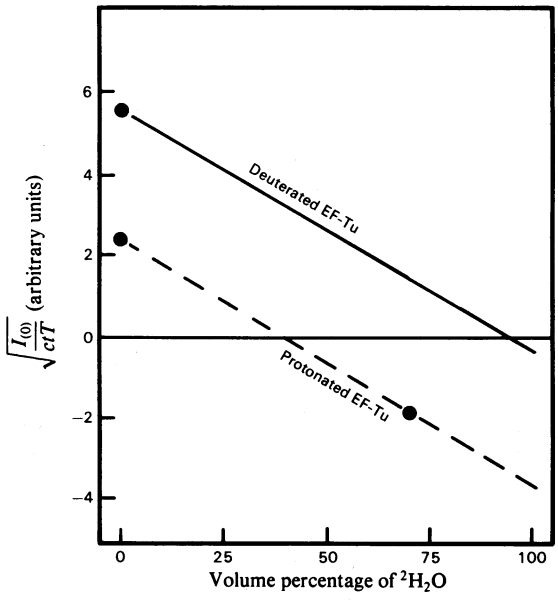


Fig. 5. *Plot of the square root of the intensity of the forward scattering of neutrons at zero angle* $I_{(0)}$ *(normalized for concentration c, sample thickness t and sample neutron transmission T) against the volume fraction of* $^2H_2O$ *in the sample buffer*

The sample is the deuterated elongation factor Tu of Fig. 4, and the dashed line corresponds to the plot obtained for the protonated protein. Since the protonated and deuterated proteins are of the same volume, the slopes are equal (S. J. Perkins, A. Miller, G. A. Bentley, A. Wittinghofer & R. Leberman, unpublished work). The two results for protonated material corresponds to the mean of eight independently measured values.

are 84–90% and are higher than the present values. Those values for proteins from buoyant density methods were however obtained indirectly from combination of the values found for intact ribosomes and ribosomal RNA, and can therefore be open to large systematic errors. The values determined in the present study have been directly checked also by neutron scattering methods and would appear to be satisfactory.

## Discussion

$^1H$ n.m.r. methods can be used to assay not only the deuterated cells and the proteins extracted from them, but also the degree of deuteration of the carbon source used for cell culture and that of the other three classes of biomolecules. It can also be used to measure the extent of exchange of the slowly exchanging amide protons in a protein; this is discussed elsewhere (Perkins *et al.*, 1981). This is also important for neutron scattering experiments since the amount of deuterium significantly influences the average scattering density of the protein. Together, n.m.r. is thus a very general technique for measuring deuteration levels at exchangeable and non-exchangeable positions in a macromolecule. This leaves only the ratio of bulk $H_2O$ to $^2H_2O$ in water or buffers which is more readily measured by other means, namely by mass density measurements or by neutron transmission measurements.

In comparisons of the n.m.r. methods in this study with those of Moore (1977), it is emphasized here that n.m.r. can measure deuteration levels with 0.5–5 mg quantities of proteins. This represents the limit to which the n.m.r. method can be taken. These are the same amounts or less as those used for neutron experiments, and this is a convenience of the n.m.r. method. Relative protein concentrations of deuterated and protonated forms are readily obtained by u.v. measurements. N.m.r. has also been shown to be practical with proteins in their native state in aqueous solvent. Figs. 4(*a*) and 4(*b*) show that spectra of the required quality can be obtained from both native and denatured proteins. This implies recovery of active material as circumstances permit. The comparison between protonated and deuterated proteins is complicated by the $HO^2H$ resonance at about 5–4.5 p.p.m. which overlaps with some of the α-proton resonances. In the case of guanidinium chloride the resonance of the denaturant at 6.5 p.p.m. overlaps with the aromatic protons (Fig. 4). This difficulty is minimized by thorough pre-exchange of freely exchangeable protons for deuterons and by working at low temperatures (since the water signal is downfield-shifted with decrease of temperature). However, the relative number of protons involved is low and this loss can be neglected, as is justified by the results shown in Table 1.

Of particular interest is that good estimates of protein deuteration levels were obtained from n.m.r. on whole cells of *E. coli* grown on glycerol. This avoids laborious extractions of the protein from large quantities of cells before a preliminary figure for deuteration is known. In the case of glucose as carbon source, the deuteration of proteins was underestimated because of the enhanced carbohydrate component in the spectrum, and this was self-evident from the spectra.

That different levels of deuteration in proteins is obtained with different protonated carbon sources is already known (Moore *et al.*, 1975). Here, it is shown that the use of glycerol achieves higher deuteration levels for a given percentage of $^2H_2O$ in the growth medium than does glucose. The use of glycerol is thus a cheaper means of attaining a given level of deuteration in a protein. According to circumstances, the lag period before exponential

growth can be decreased correspondingly, since less $^2H_2O$ is required in the medium. Such deuteration levels can be rationalized on the basis of a chart of metabolic pathways. Protonations from the solvent can be seen to be of greater importance in glycerol metabolism than for that of glucose. As before (Moore, 1977), the results shown in Fig. 4 show that aromatic protons are relatively undeuterated whereas the heaviest substitutions are to be found among aliphatic protons.

The precision of the n.m.r. deuteration estimate is to within a few percent of that of the neutron estimate. The accuracy of the n.m.r. method is akin to that of a single measurement of $I_{(0)}/c$ by neutron scattering. Both techniques relate the observed quantity to a reference quantity, which is the protonated sample for n.m.r., and is the incoherent scattering of water for neutron scattering. Both methods thus depend upon instrumental stability. Since the determination of matchpoints by contrast variation gives the desired quantity directly, and is based on values of $I_{(0)}/c$ measured in different contrasts, contrast variation will remain as the method of choice where knowledge of the matchpoint of the deuterated molecule is of importance for analysis. The use of n.m.r. is complementary in the senses that it is an independent technique, it is of use during the preparation of deuterated samples for the neutron experiment, and it measures deuteration levels where these cannot be obtained directly from contrast variation (e.g. in the matrix protein–detergent complex of *E. coli*, the protein can be separated and analysed by n.m.r.).

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