PROTON-DEUTERON EXCHANGE IN PROTEIN AND NUCLEOPROTEIN MOLECULES SURROUNDED BY HEAVY WATER

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INTRODUCTION

Considerable progress has been made in recent years in the interpretation of the complex X-ray diffraction patterns given by protein crystals and by oriented molecules of desoxyribonucleic acid (DNA). There is evidence for a fold or spiral of the peptide chains in the proteins, with a 5 A repeat along the chain direction, stabilized by hydrogen bonds between the N–H and C=O groups of the peptide backbone, and for a double spiral of nucleotide chains in DNA stabilized by hydrogen bonds between purine and pyrimidine rings[1,2,3]. In biological systems proteins and nucleoproteins exist for the most part in an aqueous rather than crystalline or paracrystalline environment, so that it is important to determine to what extent the structures shown by X-ray diffracton analysis are maintained in aqueous solution. In the case of the globular proteins diffusion, sedimentation, and other data show a relatively compact molecule in solution, and for the larger protein molecules, in addition to any primary folding of the peptide chains there must also be secondary folding. Heating, or extremes of pH, cause denaturation and a change in the properties of the protein. The molecule must therefore remain in some folded form under sufficiently mild conditions, although it does not follow that this is a rigid structure—there may be partial or complete freedom of movement of some parts of the peptide chains and the structure may change when the composition or concentration of the surrounding salt solution is changed, as, for example, in the contraction of actomyosin fibrils[4].

A possible method of investigating these problems is to dissolve the protein in heavy water and to study the proton–deuteron exchange between the protein and the surrounding D_2O. LINDERSTRØM-LANG[5] has recently found that simple peptides, insulin A-chain, and oxidized ribonuclease show a very rapid exchange of all the protons attached to nitrogen or oxygen atoms. C–H protons show no exchange in molecules of this type. Insulin also shows almost complete exchange of N–H and O–H protons, about 65% of these exchanging rapidly and the rest more slowly. LINDERSTRØM-LANG concludes that a part of the insulin molecule is in a folded configuration between the S–S bonds which are known to link the A- and B-chains[6], while the remainder of each chain has complete freedom of movement in solution.

The present paper is concerned with the study of proton-deuteron exchange in proteins, DNA, nucleoprotamine and tobacco mosaic virus (TMV) as shown by infrared absorption spectra in the range 3600 cm^{-1} to 2100 cm^{-1}, where H-stretching bands

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are separated from D-stretching bands as a result of the difference in mass between proton and deuteron. This approach is inevitably less quantitative than the density method developed by LINDERSTRØM-LANG, but since absorption bands due to different types of hydrogen bond occur at slightly different wavelengths, it offers some hope of identifying the protons which exchange slowly or not at all. Further, the extent of the exchange in a variety of proteins and the dichroism shown by the non-exchanging groups in protein crystals provide additional information on the folding of the peptide chains. The very high absorption of water in the H-(or D-)-stretching region makes it impossible to work with solutions. The materials studied are therefore (a) dry films from D_2O solutions and (b) crystals and fibrils with water content 25% to 50%. Dry films may be viewed in both the H- and D-stretching regions, and wet crystals and fibrils may be viewed in the H-stretching region provided the water present is at least 99% D_2O. PARKER[7] has studied feather keratin in this way and shown considerable proton–deuteron exchange, with increased dichroism at 3300 cm^{-1} in the deuterated material.

EXPERIMENTAL METHODS

Dry films from D_2O solution

The method used to prepare the dry films has already been described[8]. The D_2O solutions were made up in a sealed glass phial with a long drawn-out neck of elliptical cross-section. After a given time at a given temperature a small drop of the solution was transferred, by tipping the phial, to a point between two re-entrant bulges in the neck. The body of the phial was then immersed in a bath at —70° C, the neck remaining at 20° C, until the drop had dried to a film. While the body of the phial was still at —70° C the neck was sealed off, and a spectrum taken on the dry film in the sealed tube. It was essential that the dry film should not be exposed to atmospheric water vapour even momentarily. If, for example, a small crack appeared during sealing-off of the neck, the film would show considerable back-exchange. The dry films had a cracked appearance, like crazy-paving, but were quite suitable for infra-red spectroscopy, provided the optical density was not greater than one.

Crystals and fibrils

A similar phial was used for crystals and fibrils. The body contained D_2O saturated with NaCl, KBr or KNO_3 to give the desired humidity. The fibrils, or a small drop of crystal suspension, blotted to remove surplus supernatant fluid, were placed on a small piece of glass cover-slip and inserted into the neck of the phial, which was then sealed. It was essential that the NaCl, etc., should be dry and that the amount of H_2O surrounding the crystals or fibrils should be small compared with the volume of D_2O in the phial, so that, at equilibrium, the deuterium concentration remained $\geqslant$ 99%. The phials were left for six days at 20° C and the samples either viewed wet, without sealing off the neck, or dried as described above.

The spectrometer used was a Perkin Elmer 12C modified to give a double beam through a Beck reflecting microscope[9]. The size of specimen required was 20 μ × 200 μ. The insulin used was from Boots, the lysozyme, ribonuclease, β-lactoglobulin, egg albumin, serum albumin and γ-globulin from Armour. Seal myoglobin crystals were very kindly provided by Drs. J.C. KENDREW and HELEN SCOULOUDI, horse methaemoglobin crystals by Dr. M. F. PERUTZ, tobacco mosaic virus by Dr. ROSALIND FRANKLIN, ribonuclease crystals by Dr. C. H. CARLISLE and DNA (Na salt) and trout and herring nucleoprotamine by Drs. M. H. F. WILKINS AND W. E. SEEDS. All the Armour proteins were simply dissolved in D_2O at 2% concentration (pH 6–7) with the addition of NaCl (5%) in the case of β-lactoglobulin and γ-globulin. Insulin was brought to pH 3–4 with DCl, so that the concentration could be brought up to 2%.

EXPERIMENTAL RESULTS

The absorption spectrum of a dry film of ribonuclease from H_2O solution is shown in Fig. 1 and Table I summarizes the correlation between H-groups and the corresponding stretching frequencies[10]. Replacement of a proton by a deuteron lowers O–H and N–H stretching frequencies by a factor $1/\sqrt{1.85}$. The spectrum of the

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ribonuclease film from H_2O solution in Fig. 1 is typical of those given by all proteins in this region[11], and shows that there can be relatively few free O–H and N–H groups, although the variety of these groups and the various strengths of hydrogen bond result in a broad absorption region between 3500 cm^{-1} and 2800 cm^{-1}. The small shoulder at 3070 cm^{-1} could be due to $-\overset{+}{N}H_3$ groups which show an absorption band at this frequency[10], but since a small peak occurs here in the spectra of polypeptides with only unchanged side-chains, it is more probable that the absorption at this frequency is mainly due to a subsidiary peak arising from interaction between the N–H groups of the peptide backbone. The main absorption band in polypeptides due to backbone N–H groups comes at 3295–3305 cm^{-1} [12]. The protein peak at 2950 cm^{-1} is due to C–H groups.

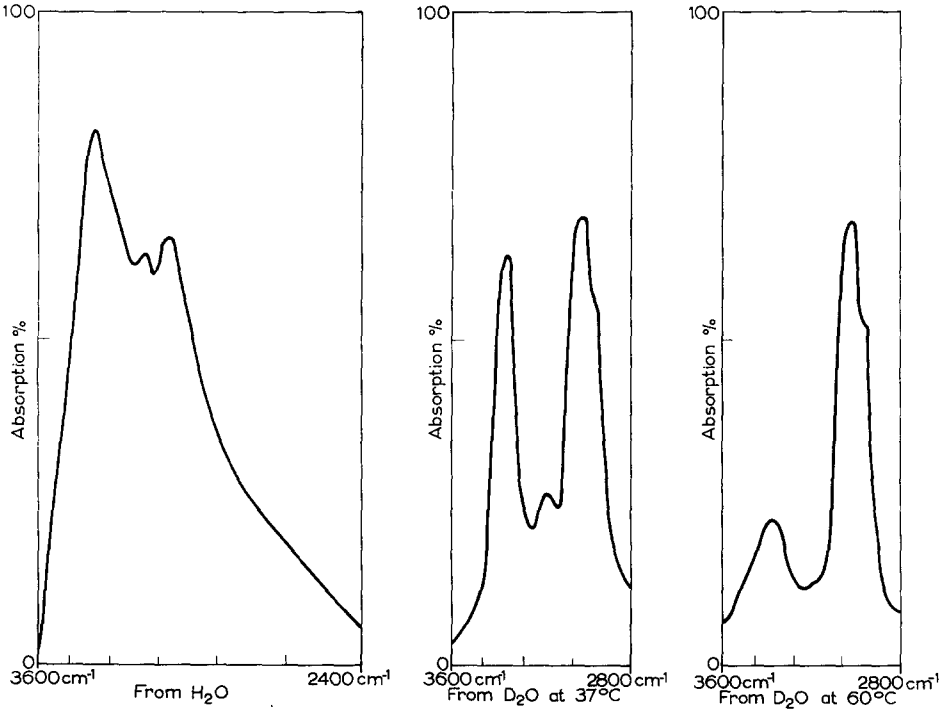

Fig. 1. Ribonuclease from solution (dry film).

TABLE I

FREQUENCIES OF H-STRETCHING VIBRATIONS (BELLAMY[10])

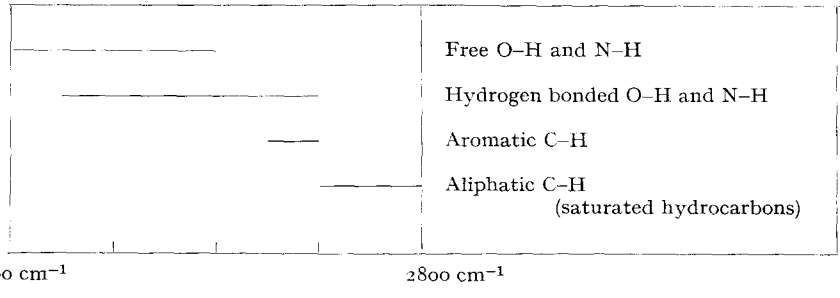

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Fig. 2. Serum albumin from D_2O solution at 20° C (dry film) and haemoglobin crystal at 84% relative humidity (D_2O). ——— electric vector parallel to a-axis; ------- electric vector perpendicular to a-axis.

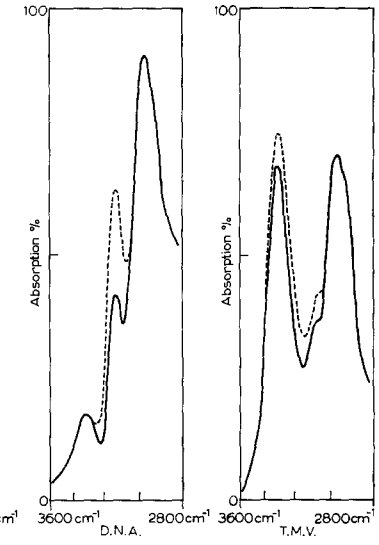

Fig. 3. DNA and TMV. Dry fibrils previously exposed to D_2O at 84% relative humidity. ——— electric vector along the fibril direction; ------- electric vector perpendicular to fibril direction.

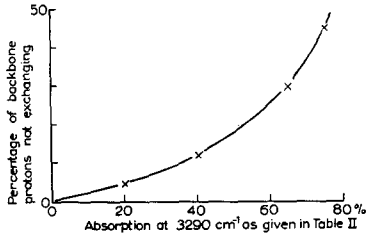

Fig. 4. Relation between the percentage of backbone protons not free to exchange and the absorption at 3290 cm^{-1} as given in Table II.

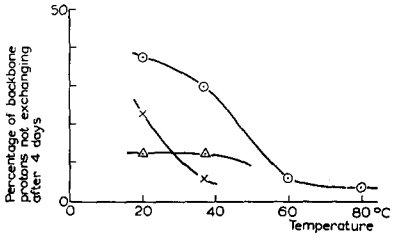

Fig. 5. Graphical representation of the results in Table II interpreted by means of the relation shown in Fig. 4. —×—×— insulin and lysozyme; —△—△— serum albumin; —O—O— ribonuclease, β-lactoglobulin, egg albumin, etc.

The spectrum of a dry film of serum albumin from D_2O solution is shown in Fig. 2. There has been a considerable exchange of N–H and O–H protons leaving a sharp –H peak at 3290 $\pm$ 5 cm^{-1}, which is the frequency of the main backbone N–H absorption in polypeptides. C–H protons show no exchange. Ribonuclease shows rather less exchange than serum albumin at 20° C or 37° C, but a high exchange if the solution is kept at 60° C for 24 hours (Fig. 1). The intensity of this non-exchanging –H peak is tabulated for a variety of proteins and conditions in Table II, referred to a standard thickness of film or crystal, etc., which gives 70% absorption at 2950 cm^{-1}. The peak is always very sharp and always at 3290 cm^{-1}, and it seems very probable that it is in fact mainly due to backbone protons. Amide groups would be expected to give absorption bands at 3350 cm^{-1} and 3180 cm^{-1}, bands due to charged $\overset{+}{N}$–H groups would be expected at frequencies lower than 3290 cm^{-1}. If the peak were due to a mixture of different groups it would be expected to be broader,

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TABLE II

% ABSORPTION AT 3290 cm^{-1} (± 5%) FOR A SPECIMEN THICKNESS WHICH GIVES 70% ABSORPTION AT 2950 cm^{-1}

	20° C			37° C	60° C	80° C
	24 hours	*4 days*	*crystal*	*2 days*	*24 hours*	*24 hours*
Insulin film from solution	70	60	—	20	—	—
Lysozyme film from solution	—	55	—	35	—	—
Ribonuclease film from solution	—	70*	75*	65*	25	15
β-Lactoglobulin film from solution	—	70*	—	65*	20	—
Egg albumin film from solution	—	70*	—	65*	35	—
Haemoglobin crystal	—	—	80 / 70**	—	25	—
Serum albumin film from solution	65	40	—	40	—	15
γ-Globulin film from solution	—	65*	—	60*	—	—
TMV fibrils	—	70	—	—	—	—

* Small peak at 3070 cm^{-1} as in ribonuclease at 37° C (Fig. 1).
** Dichroism with maximum absorption when electric vector is parallel to *a*-axis.

as in films from H_2O solution, and to show a frequency shift with increasing exchange, since some groups would probably exchange more readily than others.

Liquid HDO gives an absorption band near 3400 cm^{-1}, and a shoulder appears at this frequency in films which have not been adequately dried. Although water bound to protein groups might give rise to an absorption band at a rather lower frequency it seems unlikely that this could account for the observed band at 3290 cm^{-1}, unless the water is such an integral part of the protein structure that it does not come to equilibrium with the surrounding solution. Films which have not been very well dried show strong absorption in the –D region, but the absorption in this region shown, for example, by the serum albumin film in Fig. 2. is no greater than the absorption at 3300 cm^{-1} for films of comparable thickness from H_2O solution, which have been thoroughly dried by heating to 60° C, or 100° C, for some hours. Also, films from D_2O solution, when very dry, may be heated to 60° C for some hours, before sealing off the neck of the phial, without any change in the spectrum.

The 3070 cm^{-1} peak may be due to protons in $\overset{+}{N}$–H groups. In egg albumin and β-lactoglobulin all lysine and arginine groups are titratable[13,14] and so carry positive change at pH ~7, but these protons are not necessarily all free to exchange. Alternatively this may be the subsidiary peak associated with backbone N–H groups. In the latter case there must be some reason for its absence in the spectra of films from insulin and serum albumin after 24 hours in D_2O and haemoglobin crystals, when the main N–H peak is as intense as in the other spectra.

Assuming that the main peak at 3290 cm^{-1} is due to backbone N–H groups, it is possible to estimate semi-quantitatively the percentage of these which are not free to exchange at a given temperature, by plotting optical densities against wave-number and integrating the area under the peak. This has been done for serum albumin after different times of exchange and gives the relation shown in Fig. 4*.

* It was assumed that the extinction coefficients for H- and D-stretching vibrations were the same. On this assumption a film from D_2O solution which gives 70% absorption at 2950 cm^{-1}, due to C–H groups, is taken to be the same thickness as a film from H_2O solution giving 85% absorption at 2950 cm^{-1}—the extra 15% being attributed to the tails of the N–H and O–H absorption bands. It was further assumed that the average extinction coefficient for all protein O–H and N–H groups was that of the backbone N–H groups.

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Since the ratio of backbone protons to C–H protons is approximately constant for all globular proteins this relation may be applied to the other proteins in Table II and the results summarized as in Fig. 5. Dissolving a protein in D_2O would not be expected to alter its molecular structure, the optical rotation of egg albumin, for example, is unchanged[15], but there is always the possibility of denaturation and additional exchange during the drying of the films. The high exchange in wet haemoglobin and ribonuclease crystals cannot be due to denaturation, and suggests that the exchange observed for the dry films does in fact represent the exchange in solution. Drying at 20° C is a relatively mild treatment for proteins of this type, and films of all the Armour proteins used can be readily redissolved in distilled water.

DISCUSSION

Extent of proton–deuteron exchange

The present results for insulin are in good agreement with those of HVIDT AND LINDERSTRØM-LANG[16], and confirm their suggestion that the slowly exchanging protons are those of the backbone N–H groups, but for ribonuclease the density method[17] shows complete exchange at 37° C, whereas the present results show only 70 ± 5% exchange at 37° C with 90 ± 3% exchange at 60° C (Figs. 1 and 4). Although there is the possibility of additional exchange during drying, the dry films cannot show *less* exchange than actually takes place in solution. The only ways in which there can be complete exchange in solution but a marked –H peak near 3300 cm^{-1} in the dry films are: (a) incomplete drying of the film, (b) low D:H ratio in the solution and (c) back exchange due to exposure of the film to atmospheric water vapour. The drying of the films has already been discussed, and (b) and (c) are ruled out by the 60° C results, since the solutions and films were prepared in exactly the same way in the two cases and the results have been repeated several times. It seems reasonably certain therefore that a proportion of the protons do not exchange at 37° C, although the actual percentage could be rather lower than the estimated 25–35% if there is interaction between the non-exchanging protons to give a relatively high extinction coefficient per proton, or if the films from D_2O solution are actually rather thicker than has been assumed (see footnote on p. 498). In HVIDT AND LINDERSTRØM-LANG's method the protein is brought up to 60° C before it is completely dry and this might give additional exchange, or it may be that a procedure which is adequate for drying insulin does not remove all the water from ribonuclease. Although the pH of the solutions has not been accurately measured in the present work and accurate comparison is not possible, the present estimate of the exchange in insulin after 24 h at pH 3–4 is in fair quantitative agreement with HVIDT AND LINDERSTRØM-LANG's results. The exchange in ribonuclease crystals is almost as great as the exchange in solution.

β-Lactoglobulin, egg albumin, haemoglobin, γ-globulin and TMV show exchange as great as ribonuclease, which is surprising in view of the large size of γ-globulin (molecular weight ±150,000) and TMV (94% protein, 6% ribonucleic acid, molecular weight ~50 million). There is evidence that haemoglobin is made up of two or four sub-units, and that in TMV sub-units of molecular weight ~18,000 are arranged in a helical ridge round the nucleic acid core[18]. It would seem from the present results that water molecules can penetrate to a large extent between the sub-units in proteins

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of this type. The uniformity of the exchange in this group suggests that it may represent some fundamental arrangement of structure in the globular proteins, and since the exchange is almost as great in haemoglobin and ribonuclease crystals as in films from solution, the high exchange is presumably not due to any considerable freedom of movement of the chains. Insulin and lysozyme in solution show a greater exchange than ribonuclease, and serum albumin shows exceptionally high exchange at 20° C for a molecule of weight ~69,000. RITLAND *et al.*[19] have estimated, from the small-angle X-ray scattering of solutions, that the internal hydration of globular proteins may be as high as 0.2–0.4 g water/g protein. Lysozyme and serum albumin show a higher internal hydration than egg albumin and β-lactoglobulin.

Fibrils drawn out from desoxyribonucleic acid gels show complete exchange (98 ± 2%) of N–H and O–H protons after being kept for one day at 84% relative humidity, and dried for 6 hours at 20° C (Fig. 3). The band at 3110 cm^{-1} is almost certainly due to aromatic C–H groups and since this band shows a dichroic ratio (1.80 ± 0.05) of the same order as that of fibres giving good X-ray diffraction patterns[20] an appreciable proportion of the present material must have been in the paracrystalline state[3]. No satisfactory spectra were obtained on wet fibrils and the exchange might have taken place during rearrangement of the chains on drying. Nucleoprotamine fibrils, similar to those used in the X-ray studies of FEUGHELMAN *et al.*[21] also show 98 ± 2% exchange of N–H and O–H protons after 3 days at 20° C. For this material the 3110 cm^{-1} band is less intense than that of DNA and it is less certain that a significant proportion of the fibril was in the paracrystalline state, but the water uptake is less, and spectra showing the same high exchange were also obtained on wet fibrils at 84% relative humidity. If a significant proportion of this material was in fact in the structural form postulated by FEUGHELMAN *et al.* it must be supposed that protons in both the purine-pyrimidine hydrogen bonds and also the arginine N–H links are able to exchange with the surrounding D_2O.

Dichroism of crystals and fibrils

There is good evidence that polybenzylglutamate folds in a PAULING α-helix[22,23,24] and as would be expected both the 4825 cm^{-1} overtone band and the N–H stretching band at 3300 cm^{-1} show high dichroism[12,25]. In view of the X-ray diffraction evidence for a considerable alignment of the chains in methaemoglobin crystals and for the presence of PAULING α-helices in this molecule[1,24] it is therefore surprising that the overtone band[25] and the non-exchanging H-stretching band (Fig. 2) show only a low dichroism (dichroic ratio 1.30 ± 0.05). Measurements on the overtone band were made difficult by the rising water absorption, and other N–H groups could have been masking the dichroism of the backbone groups. In the present work, if the –H peak is due to backbone protons a marked dichroism should be apparent, particularly as the non-exchanging backbone protons would be expected in the more ordered part of the molecule. Controls heated to 60° C showed that the D:H ratio had remained very high, the relative humidity was 84% and the measurements were made on single well formed crystals showing marked dichroism when viewed through an Ilford blue-green filter (No. 603) and extinction between crossed polaroids when the electric vector was parallel to their diagonals. Drying of the crystals showed that the –H peak was not due to water (HDO) or appreciably distorted by the presence of water in both optical paths of the double-beam system. The observed dichroism would be

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produced by about 10% of the backbone N–H groups oriented parallel to the chain direction.

From a study of the intensities of the X-ray diffraction peaks BRAGG *et al.*[26] and CRICK[27] conclude that only one third to half the peptide chains in haemoglobin may be aligned along the direction of the *a*-axis, or else that the chains are aligned in this direction in the whole molecule but pack at a small angle, or with the two parts of the molecule slightly staggered. Tilting of the chains at angles up to 20°, or lateral displacement of the two parts of the molecule would not greatly reduce the infra-red dichroism and there are therefore three possible explanations of the present results:

(1) The non-exchanging –H band is not due to backbone protons, or not the ordered part of the backbone.

(2) The primary fold is not a PAULING α-helix, or any other fold in which the N–H bonds lie closely parallel to the chain axis.

(3) The secondary folding is such that rather less than half the total length of the helices lies in the direction of the *a*-axis, and a high proportion of the remaining length lies approximately perpendicular to this direction and so almost cancels out the dichroism. There is some evidence in the X-ray vector projections that parts of the chains may run parallel to the *b*-axis[1].

Seal myoglobin and ribonuclease crystals have also been studied with the electric vector parallel and perpendicular to the peptide chain direction shown by X-ray diffraction results, but good spectra have been obtained only under conditions where it is known that there is some loss of detail in the X-ray diffraction pattern. The spectra are similar to those of haemoglobin but no dichroism of the non-exchanging H-band has been detected. In each case the condition and appearance of the crystals were such that molecular disorder in the crystal might have obscured a slight but not a marked overall orientation of these N–H groups in the protein molecule. In these smaller molecules there is less possibility of the "corners" in the secondary fold cancelling out the main dichroism, and the present results therefore support other evidence, principally the asymmetry of the "end-on" vector projections of the chains and the absence of a 1.5 A reflection, against the presence of PAULING α-helices in these molecules[2,28].

TMV fibrils show a slight dichroism (dichroic ratio 1.15 ± 0.05) in agreement with other infra-red evidence[29] that the peptide chains are in an α-fold and that the chains run predominantly at right angles to the axis of the molecule (Fig. 3).

The primary fold in globular proteins

It has been noted that the main group of proteins studied in the present work including ribonuclease, β-lactoglobulin, egg albumin, haemoglobin, γ-globulin and TMV show closely similar results. Apparently about two thirds of the backbone protons are able to exchange with deuterons at 37° C. Whether or not it is justifiable to derive this semi-quantitative estimate from the infra-red spectra (see footnote on p. 498) there seems little doubt that, for this group of proteins, a considerable and constant proportion of the backbone protons exchange, and in the case of haemoglobin and ribonuclease at least, the exchange takes place in crystals in which there can be little freedom of movement of the peptide chains, and without any possibility of denaturation of the protein molecule (Table II and Fig. 5). A considerable proportion

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of the backbone N–H groups in wet haemoglobin and ribonuclease crystals must be accessible to water, and if the peptide chains throughout most of the molecule are folded in a PAULING α-helix, or other fold in which all the backbone groups form intra-chain hydrogen bonds, the protons in these bonds must be able to exchange without any unfolding of the chain. If, on the other hand, only about one third of the peptide groups are in the fold responsible for the X-ray diffraction patterns given by these crystals, the exchange might take place only in the more amorphous part of the molecule where the folding may be different, or where there may be some freedom of movement of the chains. On either interpretation the present results would indicate that a constant proportion of the molecular volume was in a given folded state, or inaccessible to water, in this group of molecules of varied shapes and sizes, which seems rather surprising.

An alternative possibility is that the peptide chains throughout most of the molecule are in a fold in which only one half or one third of the backbone groups form intra-chain bonds, and that the remaining backbone groups are hydrogen bonded to water molecules and hence able to exchange. For the globular proteins there is really little experimental evidence for or against folds of this type, although such folds are generally considered to be energetically unfavourable, as compared with those in which all backbone groups form intra-chain bonds. In fact in an *aqueous* environment this is not necessarily the case. BRAGG *et al.*[30] discuss three folds in which only one third of the backbone groups form intra-chain bonds, but of these only the $2_{14}\cdot{}^1/_3$ fold could account satisfactorily for the low dichroism of the non-exchanging N–H band in haemoglobin, seal myoglobin and ribonuclease crystals; in the $2_{11}\cdot{}^1/_3$ and $2_{13}\cdot{}^1/_3$ folds, as for PAULING α-helices, the intra-chain bonds must lie closely parallel to the chain axis to give a 5 A repeat. The $2_{14}\cdot{}^1/_3$ fold might actually be energetically more favourable than a PAULING α-helix in an aqueous environment, as a result of the close packing of the side chains, and the fact that C=O groups forming intra-chain bonds can also bond to a water molecule in such a way that the two C=O hydrogen bonds lie at 120°[31]. The X-ray diffraction results for ribonuclease certainly seem to suggest a "flat" fold rather than a helix, and a fold with three residues per 5 A repeat is not incompatible with these results if it is assumed that only some of the chains run the full 48 A length of the molecule. On this view it must be assumed that the group of proteins listed above remain folded to a large extent in solution, but the higher exchange in insulin, lysozyme and serum albumin suggests a partial unfolding of the chains in these proteins.

The foregoing argument must be regarded as tentative until the present estimate of the exchange can be confirmed by other methods, or until further information is available from X-ray diffraction studies. It seems however that the study of deuterium (or tritium) exchange provides a valuable new technique for the study of protein structure, the interaction between proteins and nucleic acids, and other related problems.

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SUMMARY

The extent of proton-deuteron exchange between a variety of proteins and nucleoproteins and surrounding D_2O has been studied from the infra-red absorption spectra of wet crystals and fibrils, and of dry films from D_2O solution. C–H groups do not exchange, nor do a proportion of the backbone N–H groups in the proteins. For ribonuclease and haemoglobin crystals, egg albumin, β-lactoglobulin and γ-globulin solutions, and tobacco mosaic virus fibrils, it is estimated that about one third of the backbone protons fail to exchange after several days at 20° C or 37° C. Only a low dichroism of the non-exchanging N–H band is found for haemoglobin and other crystals in which there is thought to be an overall alignment of the peptide chains. The results are considered to provide evidence against the presence of PAULING α-helices in the globular proteins, and to suggest a fold of the type $2_{14} \cdot {}^1/_3$.

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